CLINICAL INVESTIGATION

Tailored Assays for Pharmacokinetic and Pharmacodynamic Investigations of Aliskiren and Enalapril in Children: An Application in Serum, Urine, and Saliva

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OBJECTIVES: Drugs that are effectively used to treat hypertension in adults (e.g., enalapril) have not been sufficiently investigated in children. Studies required for pediatric approval require special consideration regarding ethics, study design, and conduct and are also associated with special demands for the bioanalytic method. Pediatric-appropriate assays can overcome these burdens and enable systematic investigations of pharmacokinetics and pharmacodynamic in all pediatric age groups.

METHODS: Tailored assays were developed for pharmacokinetic investigation of a drug in 100 μ L of serum, saliva, and urine. All assays were applied in a proof-of-concept study to 22 healthy volunteers who had been given 300 mg aliskiren hemifumarate or 20 mg enalapril maleate and allowed for dense sampling. Changes in humoral parameters of the renin-angiotensin-aldosterone system were also evaluated with 6 parameters in 2.1 mL blood per time point.

RESULTS: The pharmacokinetic results of aliskiren and enalapril obtained by low-volume assays in serum and urine were comparable to that noted in the literature. The dense sampling enabled very detailed concentration-time profiles that showed high intersubject variability and biphasic absorption behavior of aliskiren. The replacement of invasive sampling by saliva collection appears inappropriate for both drugs because the correlations of drug concentrations in both fluids were low. A low-volume assay was also used to determine values for in the renin-angiotensin-aldosterone system and to compare those results with the published literature.

CONCLUSION: These results support both the use of low-volume assays in pediatric research and the systematic investigation of their use in neonates and infants. Use of this assay methodology will increase information about drug pharmacokinetics and pharmacodynamics in this vulnerable population and might contribute to safe and effective use of pharmacotherapy.

INDEX TERMS: aliskiren, child, enalapril, enalaprilat, pharmacokinetics, renin-angiotensin system, saliva

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INTRODUCTION

According to the European Society of Hypertension, elevated blood pressure (BP) is more common in children than previously assumed.¹ Despite extensive use, the efficacy and safety of most drugs successfully used to treat hypertension in adults are insufficiently investigated in children. The few medications that are specifically licensed for pediatric patients and the frequency of off-label use in children highlight this drawback.^{2–4} Pediatric regulations were initiated in the United States and Europe to increase knowledge about the safety and efficacy

of drugs used in children. The U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) both recommend that clinical investigations involving children should be associated with minimal risk; hence, analytic methods should require the smallest amount of blood possible. Both organizations also recommend alternative, non-invasive sampling procedures, such as urine or saliva collection.^{5,6} Although the FDA has no specific guidelines on the volume that should be drawn in pediatric patients, the EMA recommends a maximum trial-related blood loss of not more than 3% within 4 weeks. For any single sampling event,

the amount of blood withdrawn should not exceed 1% of the total blood volume.⁶ For these reasons, low-volume assays are required for sophisticated investigations of pharmacokinetics and pharmacodynamics in children. Before implementing low-volume assays in pediatric trials, the usefulness of those assays should be demonstrated via a proof-of-concept study in healthy volunteers. Therefore, a study was conducted for aliskiren and enalapril, which are of high interest in pediatric pharmacotherapy and were selected based on information given at the expert group meeting on pediatric heart failure held at the EMA in London on November 29, 2010. Enalapril, an angiotensin-converting enzyme inhibitor (ACEI), is recommended as a first-line treatment for chronic heart failure and hypertension in children.⁷ Because aliskiren is the only available, direct renin inhibitor (DRI), it is listed under investigational drugs.⁸

Aliskiren, the first marketed, orally available, DRI, has been shown to lower BP in humans. Recently, the first pediatric study evaluating the pharmacokinetics of aliskiren in children and adolescents aged 6–17 years was published by Sullivan et al.⁹ For pharmacokinetic determination, 1 mL of blood was drawn per sample, which appears inappropriate for dense sampling in younger children. Because of the high intraindividual and interindividual variability of aliskiren, sparse sampling is not advisable for gathering data in children.¹⁰ Therefore, a tailored method that required only 0.1 mL serum per sample was successfully developed to reliably determine aliskiren concentration.¹¹

The second investigated antihypertensive drug was enalapril, an ACEI. Enalapril is administered as a prodrug with a bioavailability of about 50% to 60% and is subsequently converted by hepatic carboxylesterases (CES1) to its active metabolite, enalaprilat. Enalapril and enalaprilat have been investigated in 3 pediatric pharmacokinetic trials.^{12–14} Although determination by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) appears to be the most suitable assay, given its superior selectivity, each of the above studies determined the concentrations using radioimmunoassays.¹⁵ Therefore, a published HPLC-MS/MS assay¹⁶ was modified for high-throughput requiring 100 μ L serum, which is at least as little as half of the amount necessary in other HPLC-MS/MS setups.^{17–23}

Following regulatory recommendations to elucidate alternative biological fluids for non-invasive sampling,⁵ salivary drug concentrations were determined. Because enalapril penetration into saliva had yet to be studied, we used a validated method for quantification of aliskiren in saliva.²⁴ Preliminary results in 3 subjects suggested that saliva might be an acceptable alternative, but more in-depth investigation on saliva as quantification matrix is needed.

The renin-angiotensin-aldosterone (RAA) system is central in the regulation of BP and a target in current pharmacotherapy. The ability to measure biomarkers in the RAA system (e.g., angiotensin I and II, plasma renin activity [PRA]) is key to a holistic understanding of the effects of pharmacologic intervention and also to investigating potential differences between adults and children. Only sparse data are currently available on aliskiren and enalapril interactions with the RAA system in children. Sullivan et al.⁹ determined the PRA by using 2 mL of blood per sampling point in a pediatric trial, which is the same amount of blood reported in adult studies. In other adult studies, 7 to 9 mL of blood was required to determine concentrations of angiotensin I, angiotensin II, and other RAA biomarkers.^{25,26} Collection of this volume of blood from a neonate or toddler in a single sampling event would be unethical. Even commercial, low-volume assays require 6 mL of blood to determine angiotensin I, angiotensin II, PRA, renin, and prorenin. This clearly indicates the need for child-appropriate analytic approaches for evaluating the pharmacokinetics of administered drugs, the resulting changes in the humoral parameters caused by the drug administration, and consequently, the pharmacodynamics.

In addition, clinical trials in adults would also profit from this low-volume assay approach because dense sampling associated with sophisticated pharmacokinetic and pharmacodynamics drug studies are often connected to trial-related blood loss. The availability of a low-volume assay would make these investigations better tolerated in both pediatric and adults populations. Moreover, the systematic data in a proof-of-concept study are useful for validating physiology-based models and simulations, the latter of which is a recommended tool in (pediatric) study optimization.^{27,28}

A comprehensive, bioanalytic approach for

investigating the pharmacokinetics of aliskiren, enalapril, and enalaprilat, as well as their effects on humoral parameters of the RAA system, was assessed by low-volume assays in blood, saliva, and urine. Conducting this trial in adults was advisable before applying this bioanalytic approach to pediatric studies for both regulatory purposes and to examine the drug exposure and its effect. The primary objective was not to provide new insight on the pharmacokinetics of aliskiren, enalapril, and enalaprilat in adults, but rather to ensure the applicability of the assays and to assess the agreement of the results by comparing them with reported adult data in the literature. If the bioanalytic, low-volume assay proves acceptable for use in pediatric age groups, complementary phase II and III studies to investigate the pharmacokinetics and safety of enalapril and enalaprilat in children will be conducted within the EC-funded LENA project (grant 602295).

METHODS

Study Design and Participants

This study was conducted at the Department of Clinical Pharmacy and Pharmacotherapy, Heinrich-Heine-University (Düsseldorf, Germany), with approval from the university ethics committee (and following the Declaration of Helsinki and Good Clinical Practice recommendations). All subjects provided written, informed consent before participation. Volunteers were enrolled into 2 investigation groups. One group received aliskiren hemifumarate, whereas enalapril maleate was administered to volunteers in the second group.

The study was designed as an open-label, single-sequence, single-dose study in healthy subjects. After collection of a predose sample in the morning of the first investigation day, each participant received 1 tablet of 300-mg aliskiren hemifumarate (Rasilez, Novartis Europharm Limited, Frimley, UK) or 1 tablet of 20-mg enalapril maleate (EnaHEXAL, HEXAL, Holzkirchen, Germany). The participants fasted at least 10 hours before drug administration. The study medication was administered with 250 mL of water. No further fluid intake was allowed for 30 minutes before and 1 hour after dosing with enalapril or 4 hours after administration of aliskiren. The first meal was served 4 hours postdose.

The participants went home 8 hours postdose and returned every 24 hours until the end of the study. Intake of xanthine-containing food or beverages and alcohol were not permitted for at least 48 hours after drug administration.

On day 1 of the study, an indwelling cannula was inserted into a peripheral arm vein for blood sampling. On the following investigation days, blood samples were taken by direct venipuncture. All participants rested in a supine position for at least 30 minutes before sampling to determine humoral parameters.

Drug-Level Sampling Schedule Serum

Blood samples were collected predose. Samples were then collected every 20 minutes over the next 3 hours followed by sampling every 30 minutes until 8 hours postdose. Further samples were taken 24, 48, and 72 hours postdose. Volunteers in the aliskiren group provided additional samples at 96, 120, 144, 168, and 192 hours postdose. Marked, multiple-peak phenomena had previously been noted in patients given aliskiren; hence, an intensive sampling strategy was used to make a precise determination of the multiple-peak behavior per individual. This was important because individual concentration-time profiles showing this phenomenon are lacking. To describe the pharmacokinetic parameters (e.g., T_{max}) as exactly as possible, the frequent sampling was also used for enalapril/enalaprilat.

Despite the dense sampling schedule, total blood volume of only approximately 105 mL per volunteer was taken for full pharmacokinetic determination in serum. This is less than one-quarter of the usual blood-donation volume and, consequently, was ethically acceptable in adults. Drawn samples were kept at room temperature until centrifuged at $2000 \times g$ for 10 minutes at 4°C , and then the supernatant was frozen at -80°C until analysis.

Urine

Urine samples were collected predose. Samples were collected for up to 192 hours thereafter in the aliskiren group and up to 72 hours in the enalapril group. Every spontaneous urination was collected and analyzed as a discrete sample without pooling. Although a correction for creatinine concentration is sometimes used to minimize error in urinary concentrations because of the variability

in fluid composition, this study did not require the correction because the excreted amount of the drug was calculated by multiplying the urine volume by the urinary drug concentration.

Saliva

Saliva was examined as an alternative fluid for obtaining drug concentrations and, in turn, pharmacokinetic determination, which might constitute an advantage in pediatric research. Although some authors²⁹ collect saliva from adults by having them spit the oral fluid into collection bowls, Hiremath et al³⁰ concluded that collection of saliva by oral swabs offers methodological advantages over passive drool in the pediatric population. Preliminary investigations were performed with 2 commercially available devices. One swab was primarily designed to collect buccal cell samples for DNA testing (Omniswab, GE Healthcare, Germany), and the second was a chewing swab on a cotton base (Salivette, Sarstedt, Nuembrecht, Germany). We chose to use salivettes because they are routinely used to determine concentrations in saliva (e.g., cortisol) and have already been used in pediatric research.⁴⁷ The saliva samples were taken without any stimulation every 20 minutes for the first 8 hours after drug administration and then every 24 hours. Immediately after blood collection, the participant was asked to chew slightly on the swab for 1 minute. The swab was stored on ice until centrifuged at 1000 × *g* for 2 minutes at 4°C, then frozen at –20°C or below until analysis.

Sampling Schedule for the RAA System Parameters

All humoral parameters were determined pre-dose and again at 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 hours postdose. On subsequent days, samples were taken every 24 hours. In total, a minimum blood volume of 2.1 mL was required for analysis of all RAA system parameters per timepoint. Although blood withdrawal was performed with substantial excess, allowing for replicate determinations, overall, only 250 mL blood were collected for determination of the pharmacokinetics of drugs and humoral parameters. This is equal to about one-half of the usual blood-donation volume. Collection tubes intended for angiotensin I and II determination were equipped with an inhibitor cocktail consisting of 0.44 mM *o*-phenanthroline, 25 mM ethylenediaminetetraacetic acid (EDTA), 0.12 mM pepstatin A, and 1 mM *p*-hydroxy-

mercuric benzoic acid (absent in angiotensin I tubes). In addition, the pharmacodynamics were characterized by systolic and diastolic BP and heart rate. The corresponding RAA system is illustrated in Figure 1.

Prorenin and Renin

To prevent renin cryoactivation, blood samples for renin and prorenin were collected in EDTA tubes and immediately centrifuged at 2000 × *g* for 10 minutes at room temperature. The supernatant was snap-frozen before being stored at –80°C until assayed. Before the analysis of prorenin by enzyme-linked immunosorbent assay (ELISA), the plasma samples (100 µL) were rapidly thawed. Renin required a minimum of 250 µL of plasma for determination with a chemiluminescent immunoassay based on monoclonal antibodies.

Plasma Renin Activity

Plasma renin activity was determined using angiotensin I antibodies and circulating immunoreactive angiotensin I concentrations because that approach was expected to be more precise.³¹ The chosen ¹²⁵I radioimmunoassay (RIA) has a wide angiotensin I calibration range, even in children who have up to 15-fold greater PRA than adults have.³² Samples were drawn in cooled EDTA tubes without inhibitor. The tubes were then centrifuged at 2000 × *g* for 10 minutes at 4°C and subsequently snap-frozen and stored at –80°C.

Angiotensin I and Angiotensin II

Cooled and inhibitor-spiked EDTA tubes were used to collect samples for angiotensin I and II determination. During blood withdrawal, the tubes were kept on ice and were immediately centrifuged at 2000 × *g* for 10 min at 4°C. Obtained plasma was snap-frozen and stored at –80°C until analysis; 100 µL of plasma was used to obtain the angiotensin I concentration with a commercial ¹²⁵I RIA, whereas 500 µL was necessary for the angiotensin II measurement using a modified ELISA. During the entire analysis, all samples were kept between 2°C and 8°C, unless otherwise described.

Analytical Methods

Serum, Saliva, and Urine Assays for Determination of Aliskiren, Enalapril, and Enalaprilat Concentrations

Before this study, bioanalytic assays for deter-

Figure 1. Scheme of the renin-angiotensin-aldosterone system.

Scheme of renin-angiotensin-aldosterone (RAA) system with aliskiren and enalapril as well as their effects on the RAA system (arrows). Based on its route of action, aliskiren causes an increase in renin and a decrease in plasma renin activity (PRA) and angiotensin I and II. By contrast, enalapril increases renin, PRA, and angiotensin I, but causes a drop in angiotensin-converting enzyme (ACE) activity and angiotensin II. Additionally, the neprilysin pathway is included because it is of interest in new drug entities for controlling blood pressure.

mining aliskiren and enalapril drug concentrations were developed and validated in serum, urine, and saliva.^{11,16,24,33} In brief, sample purification of serum and saliva was conducted by solid-phase extraction (SPE) using Oasis MCX 96-well plates (Waters, Eschborn, Germany). The SPE of urinary samples used Oasis WAX and MCX 96-well plates. The HPLC separation was performed on Xselect C18 CSH columns (Waters), followed by MS/MS detection with an API 2000 (AB Sciex, Concord, Canada). The mass spectrometer operated in positive mode with electrospray ionization to determine the transitions of aliskiren (552.2 $m/z \rightarrow 436.2 m/z$), enalapril (377.3 $m/z \rightarrow 234.2 m/z$), enalaprilat (349.3 $m/z \rightarrow 206.1 m/z$), and the internal standard benazepril (425.3 $m/z \rightarrow 351.2 m/z$). For aliskiren, the calibration range was 0.15 (lower limit of quantification [LLOQ]) to 1200 ng/mL in serum, from 9.4 (LLOQ) to 9600 ng/mL in urine, and from 0.59 (LLOQ) to 1200 ng/mL in saliva. For enalapril and enalaprilat, the LLOQs were 0.78 ng/mL and 0.70 ng/mL, respectively, in

serum, and 0.1 ng/mL in saliva. The calibration curve in urine was from 11.6 (LLOQ) to 12,000 ng/mL for enalapril and 8.8 (LLOQ) to 9000 ng/mL for enalaprilat.

Plasma Assays for Angiotensin I and II, PRA, Prorenin, and Renin

Before analysis of the humoral parameters of interest, the available commercial assays were screened for their suitability in investigations of pediatric patients by assessing the assay's detection range, sensitivity, selectivity, and required blood volume. If necessary, the chosen assays were modified to fit to the enclosed concentration claims or required sample volumes. The selected assays covered the main parameters of the RAA system and enabled a comprehensive description of changes in the humoral parameters after administration of aliskiren, enalapril, and enalaprilat. All assays were validated at minimum for intrarun and interrater accuracy and precision according to EMA guidelines on bioanalytic method validation for ligand-binding

assays and immunoassays.³⁴

Angiotensin I and PRA

Although blood volumes required for reliable determination of angiotensin I by commercial assays range from 20 to 500 µL of plasma, an assay (75 µL) with a broader range, instead of lowest possible volume, was chosen. This property allows the assay to be applied to all age groups because PRA varies significantly between adults and children.³⁵ Angiotensin I and PRA were both determined with a commercial ¹²⁵I RIA (Immunotech, Prague, Czech Republic). The assay's intraday and interday precisions were 10.2% and 8.2% (coefficient of variance [CV]) with an angiotensin I calibration range of 0.2 to 30 ng/mL and were determined by 10 different runs. Calculation of the PRA was performed using equation 1:

$$\text{PRA (ng/mL/h)} = \frac{(\text{Conc of Ang I at } 37^{\circ}\text{C} - \text{Conc of Ang I at } 4^{\circ}\text{C}) \times 2}{\text{Incubation (h)}} \quad (1)$$

where Ang I is angiotensin I, Conc is concentration, and PRA is plasma renin activity.

Prorenin

An ELISA was used to analyze prorenin concentration in 100 µL of plasma within a calibration range of 0.02 to 10 ng/mL. This assay required the lowest sample volume among those evaluated, detected prorenin directly, and had a broad calibration range (Molecular Innovations, Novi, USA). The CV was 8.2%, with an accuracy of 102.5% ± 8.4% (mean ± SD) and was determined by 10 different runs. The intraday precision was evaluated on 9 different days with a CV of 5.2%.

Angiotensin II

By contrast, commercial ELISAs were all inappropriate for precisely determining angiotensin II concentrations in low volumes. Therefore, the assay was optimized by combining SPE (in-house) and a modified ELISA (IBL, Hamburg, Germany). The assay required 500 µL of plasma, whereas common ELISAs required about 1 to 2 mL. The purification was performed on Oasis HLB 96-well plates, which allowed angiotensin II concentrations to be reliably determined with a calibration range of 1 to 125 pg/mL. The LLOQ was as small as possible because a decline in angiotensin II was expected with both drugs. The assay had

an accuracy of 95.5% to 106.4% and an interday precision of 6.7% (CV), which was determined by 7 different runs on 4 different days.

Renin

For renin determination, effort was successfully made to halve the required plasma volume of the chemiluminescence immunoassay (CLIA) used (Diasorin, Saluggia, Italy). Renin was evaluated using 250 µL plasma with a LLOQ of 2.5 micro international units/mL and a calibration range of 2.53 to 500 microunits/mL. The intraday and interday precisions were 2.5% and 3.9% (CV). Both were evaluated by 10 different runs.

Angiotensin-Converting Enzyme Activity

Angiotensin-converting enzyme (ACE) activity was assessed by determining the ratio of plasma angiotensin II to angiotensin I concentrations in blood. The change in activity was a percentage and was calculated according to the equation 2:

$$\text{Change in ACE activity (\%)} = \left[\frac{100}{\frac{\text{Ang II predose (pg mL)}}{\text{Ang I predose (pg mL)}} \times \frac{\text{Ang II } t \text{ predose (pg mL)}}{\text{Ang I } t \text{ predose (pg mL)}}} \right] - 100 \quad (2)$$

where Ang I and Ang II are angiotensin I and II, respectively, and *t* is the timepoint for determining angiotensin concentration.

Calculation of Pharmacokinetic Parameters

The pharmacokinetics of the compounds in serum were characterized by maximum serum concentration (*C*_{max}), time to maximum concentration (*T*_{max}), area under the curve from zero to timepoint *t* (*AUC*_{0-*t*}), AUC from zero to infinity (*AUC*_{0-∞}), half-life (*t*_{1/2}), elimination rate constant (*k*_e), and oral clearance of a drug (*CL*_F). The AUC was calculated by applying the trapezoidal rule with infinity extrapolation. The oral clearance was determined by dividing the administered dose by the *AUC*_{0-∞}. The dose was adopted for enalaprilat according to the molar equivalent of enalapril maleate. Furthermore, the conversion ratio of enalapril and enalaprilat was calculated using the ratio of the *AUC*_{0-∞} of enalaprilat to the *AUC*_{0-∞} of enalapril. The apparent volume of distribution (*Vd*_F) was determined by a non-compartmental analysis as follows: *Vd*_F = *CL*_F/*k*_e, whereas *CL*_F was defined as Dose/*AUC*_{0-∞}. In urine, the unchanged amount of the compounds

Table 1. Baseline Characteristics

	Aliskiren Group (n = 13)*	Enalapril Group (n = 9)*
Age, yr	26 ± 3	23 ± 4
Sex, male (%)	6 (46)	6 (67)
White, %	100	100
Height, cm	176 ± 8	181 ± 10
Body weight, kg	76 ± 19	70 ± 13
BMI, kg/m ²	24.3 ± 4.7	21.2 ± 2.4

BMI, body mass index

*All values are reported as mean ± SD, except T_{max} (median and range)

excreted into this fluid (A_e) and the renal clearance (CL_R) were evaluated. Saliva concentrations were examined for C_{max} , T_{max} , AUC_{0-t} , and the corresponding ratio between serum and saliva. Pharmacokinetic parameters were determined by non-compartmental analysis using a Phoenix 6.3.0.395 (Certara, St. Louis, MO). For humoral parameters, C_{max} and T_{max} were analyzed. Because the primary objective of this study was not to provide new insights into the pharmacokinetics of the 3 drugs in adults, but rather to ensure the appropriateness of the low-volume analytic method and to assess the agreement of the results with those reported in the literature, a non-compartmental analysis, rather than a compartmental analysis, was used.

Safety Assessments

The patient's health status was evaluated in personal interviews at least 3 days before and on day 1 of the study. Based on the interview, only subjects who were 18 years and older with a body weight greater than 50 kg and with no known organ diseases were allowed to participate. In addition, subjects with angioedema, urticaria, known allergies, or low BP values in the past (<90/60 mm Hg) were excluded for safety reasons. The hypotensive value limit was defined according to the recommendations of the National Heart, Lung, and Blood Institute. Before drug administration on day 1, all female subjects were asked to perform a pregnancy test. For the duration of the study, BP and heart rate measurements were conducted regularly. The BP was measured after at least 5 minutes of rest in a supine position using an automatic upper arm oscillometric BP monitor (Omron M5, Omron, Mannheim, Germany). Adverse events were also monitored, their duration was recorded,

and, if necessary, any concomitant drug given was documented.

RESULTS

Study Population

All 22 volunteers enrolled were white and healthy. A group of 13 volunteers (6 males, 7 females), aged 19 to 31 years, were enrolled to the aliskiren group, and 6 men and 3 women (aged 19-30 years) were enrolled to the enalapril group. Baseline characteristics for all participants are shown in Table 1.

Pharmacokinetics of Aliskiren and Enalapril

Aliskiren, enalapril, and enalaprilat were investigated in serum, urine, and saliva by highly dense sampling of the drug concentrations in the corresponding biological fluid for 5 elimination half-lives of the drugs. Data are presented as means ± SD.

Aliskiren in Serum and Urine

Eleven of 13 subjects had double peaks of aliskiren in serum. The extent and timepoint of the occurrence of the double peak differed among subjects. However, the first peak occurred with a median T_{max} of 0.7 hours and reached a lower maximum concentration compared with the second peak. The T_{max} of the second peak ranged between 0.8 and 4.3 hours, with the median of 2.7 hours. Individual concentration-time profiles are arranged in Figure 2. The mean $AUC_{0-\infty}$ of aliskiren was 2076.1 ± 1340.9 ng × hr/mL with a C_{max} of 502.5 ± 503.1 ng/mL. The mean terminal half-life of aliskiren was 75.6 hours. The total amount recovered in urine was $0.7\% \pm 0.4\%$ of the administered dose. The mean calculated CL_R was 1.18 L. Corresponding urinary profiles are ar-

Figure 2. Comparison of measured drug concentrations in serum and saliva after oral administration of 300 mg aliskiren hemifumarate in 13 healthy volunteers (left side) or after oral administration of 20 mg enalapril maleate to five healthy volunteers (right side). Since enalapril is converted to the active metabolite enalaprilat, both substances are mentioned on the right side of the figure. Black line: serum concentration [primary y-axis], grey line: saliva concentration [secondary y-axis].

ranged in Figure 3. Pharmacokinetic parameters obtained in serum and urine are listed in Table 2.

Aliskiren in Saliva

The concentration-time profiles of aliskiren in saliva showed 2 different distribution phases over time with a comparable profile shape up to 5 hours, whereas it changed substantially at later timepoints when compared with the serum concentration. The mean C_{max} for 0 to 9 hours for aliskiren in saliva was 7.1 ± 8.4 ng/mL, whereas the C_{max} for 0 to 192 hours was 7.3 ± 7.1 ng/mL. The AUC for 0 to 4.9 hours was 11.0 ± 10.5 ng $\times$ hr/mL and changed to 352.2 ± 367.4 ng $\times$ hr/mL from 0 to 192 hours. The T_{max} during the investigation period up to 5 hours was 2.7 hours, whereas it changed to 6 hours when the entire study period (192 hours) was considered. The saliva-to-serum ratio of k_e ranged between 0.84 and 1.31 for 0 to 4.9 hours and clearly altered for 0 to 192 hours (0.2-4.4). If compared with serum, the mean C_{max} in saliva—up to first 5 hours after

administration—was about 1.4%, and the AUC reflected 1.5% of the serum concentration. In contrast, the AUC for 0 to 192 hours was about 18.2% of that seen in serum. Comparative individual concentration-time profiles of aliskiren in serum and saliva are presented in Figure 2, and a comparative data set is compiled in Table 3. Table 4 provides individual ratios of AUC, T_{max} , and C_{max} in saliva and serum.

Enalapril and Enalaprilat in Serum and Urine

After administration of 20 mg enalapril maleate, the prodrug was rapidly absorbed (mean T_{max} , 0.9 hours) with maximum concentration of 159.7 ± 67.8 ng/mL. The AUC_{0-∞} was 231.6 ng $\times$ hr/mL; therefore, it was about half that of the enalaprilat. Calculated mean elimination half-life for enalapril was 0.9 hour. In total, 17% of enalapril was found in urine after 72 hours, with a renal clearance of 15.1 ± 3.5 L/hr. The active metabolite enalaprilat reached a mean C_{max} of 52.5 ± 25.3 ng/mL after 4 hours. The

Figure 3. Obtained urine profiles of aliskiren within the proof-of-concept study—Dotted lines represent the individual cumulative amount (left y-axis) excreted into urine after administration of 1 x 300 mg aliskiren hemifumarate to healthy volunteers. Additionally, the black line shows the mean percentage of excreted urinary aliskiren (right y-axis) $n=13$.

observed $T_{\max}$ ranged between 2.8 to 4.4 hours. The mean elimination half-life was 10 hours. The mean AUC_{0-72h} was 491.4 ± 148.0 ng $\times$ hr/mL. The conversion rate of enalapril and enalaprilat, calculated by the corresponding $AUC_{0-\infty}$, ranged from 1.66 to 4.92, with the median of 2.0. The amount of excreted enalaprilat was about 27% of the dose administered. The calculated renal clearance for enalaprilat was 7.5 ± 2.1 L/hr. Figure 4 shows the urinary excretion amount of enalapril and enalaprilat. Pharmacokinetic parameters in serum and urine are listed in Table 2.

Enalapril and Enalaprilat in Saliva

The correlations between serum and saliva concentration of enalapril and enalaprilat were investigated in 7 volunteers. In 5 of 7 volunteers, salivary concentration-time profiles were obtained. One subject was excluded because the mouth was not washed after drug administration and thus detected salivary drug concentrations represented a combination of the residual of the tablet itself and the penetration into saliva. The second subject developed gum bleeding, and the concentrations obtained cannot, therefore, safely be accounted for saliva only. The evaluable concentration-time profiles revealed low concentrations of enalapril and in particular very low concentrations of enalaprilat in saliva. For enalapril, the $C_{\max}$ in sa-

liva was 5.5 ± 6.1 ng/mL and varied between 1% and 14% of that found in serum. The median $T_{\max}$ was at 1 hour in both fluids. The AUC_{0-72hr} of enalapril was 6.6 ± 6.5 ng $\times$ hr/mL, and the individual ratios of saliva to serum for that period ranged between 0.01 and 0.1. The determined AUC_{0-72hr} of enalaprilat was 2.34 ± 1.9 ng $\times$ hr/mL, with a mean maximum concentration of 0.3 ± 0.1 ng/mL at 4 hours ($T_{\max}$). Although visual inspection confirmed a comparable profile shape for the prodrug enalapril in serum and saliva, that cannot be claimed for the active metabolite enalaprilat. The penetration was less than the 1 of that for enalapril

and was occasionally below the LLOQ. The evaluated correlation between saliva and serum concentrations was not good because the shape of the concentration time profiles and the corresponding elimination rates were different in both fluids. All individual ratios of AUC, $T_{\max}$, and $C_{\max}$ are arranged in Table 4. Because of the variable ratios, it is not advisable to apply a matrix factor to the saliva AUC to estimate the serum AUC. The corresponding comparative individual profiles in serum and saliva are compiled in Figure 2. Further comparative pharmacokinetic parameters are arranged in Table 2.

In addition, the closeness of the agreement in the measured concentrations of aliskiren, enalapril, and enalaprilat were assessed with Passing and Bablok regression scatter plots. The corresponding plots for aliskiren, enalapril, and enalaprilat are shown in Figure 5.

Pharmacokinetics of Humoral Parameters Change in Humoral Parameters After Administration of Aliskiren

Based on published results in adults, and as shown in Figure 1, aliskiren directly blocks the enzyme renin and subsequently affects the PRA, which was confirmed by the study results. Mean PRA was maximally reduced 34-fold with a $T_{\max}$ at 1 hour. Moreover, the PRA did not return to

Table 2. Pharmacokinetics of aliskiren, enalapril and enalaprilat in serum and urine

Pharmacokinetic Parameters	Aliskiren*	Enalapril	Enalaprilat
C _{max} (ng/mL), mean ± SD	502.5 ± 503.1	159.7 ± 67.8	52.5 ± 25.3
C _{max} (ng/mL), No. (median)	307 (267–517)	158 (109–200.5)	41.8 (39.1–62.8)
T _{max} (hr), median (25th–75th percentile)	2.67 (0.8–4.3)	1 (0.7–1.0)	4 (2.8–4.4)
AUC _{0–t} (ng × hr/mL), mean ± SD	1946.2 ± 1285.3	230.2 ± 78.2	491.4 ± 148.0
AUC _{0–∞} (ng × hr/mL), mean ± SD	2076.1 ± 1340.9	231.6 ± 78.3	513.5 ± 145.9
AUC _{0–∞} (ng × hr/mL), median (25th–75th percentile)	1678.1 (1292.9–5731.4)	229.3 (178.3–270.5)	477.1 (380.7–601.8)
t _{1/2} (hr), mean ± SD	75.6 ± 20.0	0.9 ± 0.3	10.4 ± 11.3
t _{1/2} (hr), median (25th–75th percentile)	65.9 (62.7–80.5)	0.9 (0.8–1.0)	7.3 (6.1–7.7)
k _e (1/hr), mean ± SD	0.0097 ± 0.002	0.8050 ± 0.247	0.0968 ± 0.036
CL _F (L/hr), mean ± SD	191.5 ± 97.2	97.5 ± 40.3	29.7 ± 7.8
CL _F (L/hr), median (25th–75th percentile)	178.8 (138.7–232.0)	87.2 (73.9–112.2)	28.9 (22.9–36.3)
Vd _F (L), mean ± SD	21,368.3 ± 12,694.2		
A _e at 24 hr† (mg), mean ± SD	1.51 ± 1.0	—	—
A _e last‡ (mg), mean ± SD	2.15 ± 1.36	3.41 ± 1.08	3.78 ± 1.63
A _e last‡ (mg), median (25th–75th percentile)	1.71 (1.26–5.6)	3.44 (2.73–4.26)	3.3 (2.42–6.34)
CL _R (L/hr), mean ± SD	1.18 ± 0.43	15.1 ± 3.5	7.5 ± 2.1
CL _R (L/hr), median (25th–75th percentile)	1.14 (0.92–1.03)	15.3 (12.3–15.8)	7.6 (5.5–11.3)

AUC, area under the serum-time curve; CL_R, renal clearance; CL_F, oral clearance; C_{max}, maximum serum concentration; k_e, elimination rate constant; t_{1/2}, elimination half-life; T_{max}, timepoint of maximum concentration

* Aliskiren showed biphasic absorption, and both C_{max} and T_{max} concentrations were enclosed

† A_e, amount excreted into urine within 24 hrs

‡ A_e, amount excreted into urine within 192 hrs

predose values within the observation period. Because of aliskiren administration, we expected the RAA system biomarkers angiotensin I and angiotensin II to be influenced. However, no noticeable change in angiotensin I was observed. All mean predose values were already borderline with or at the LLOQ, and subsequently, no drop in angiotensin I concentrations was seen as a result of aliskiren administration. Because a more-sensitive assay requiring the same small amount of blood was not available, a reanalysis could not be performed. In contrast, the optimized assay was able to measure concentrations of angiotensin II at all time points. The angiotensin II concentrations evaluated had already decreased 30 minutes after administration of aliskiren and reached their minimum around 2 hours postdose. After 48 hours, at least the pre-

dose values had again been reached, whereas, in the meantime, there was a mean reduction of 70% from the baseline observed. Renin concentration increased approximately 19-fold compared with initial values, with a T_{max} at 5 hours. On day 9, the renin in the aliskiren group was still about 4.7-fold higher than it was for the predose value. Renin concentration was highly variable, as indicated by the SD of 434 microunits/mL for aliskiren at C_{max}. Prorenin was only determined in participants taking aliskiren hemifumarate. The mean predose value for prorenin was 0.71 ng/mL and varied between 0.21 and 1.83 ng/mL. The mean predose prorenin concentrations were about 45-fold higher than the measured renin concentrations. Within the first 8 hours, the concentrations started to increase slightly and reached their maximum mean concentration 48

Table 3. Comparative Data Set of Pharmacokinetics in Saliva and Serum of Aliskiren, Enalapril and Enalaprilat

	Serum	Saliva
Aliskiren (n = 13)		
C_{max} at 0-5 hr (ng/mL), mean $\pm$ SD	502.5 $\pm$ 503.1	7.1 $\pm$ 8.4
C_{max} at 0-192 hr (ng/mL), mean $\pm$ SD	502.5 $\pm$ 503.1	7.3 $\pm$ 7.1
T_{max} at 0-5 hr (hr), median	2.67	2.7
T_{max} at 0-192 hr (hr), median	2.67	6
AUC_{0-5hr} (ng $\times$ hr/mL), mean $\pm$ SD	743.7 $\pm$ 574.7	11.0 $\pm$ 10.5
$AUC_{0-192hr}$ (ng $\times$ hr/mL), mean $\pm$ SD	1946.2 $\pm$ 1285.3	353.2 $\pm$ 367.4
$AUC_{0-\infty}$ (ng $\times$ hr/mL), mean $\pm$ SD	2079.7 $\pm$ 1340.4	516.8 $\pm$ 513.2
Enalapril (n = 5)		
$C_{max0-72hr}$ (ng/mL), mean $\pm$ SD	151.8 $\pm$ 61.2	5.5 $\pm$ 6.1
$T_{max0-72hr}$ (hr), median	1	1
AUC_{0-72hr} (ng $\times$ hr/mL), mean $\pm$ SD	219.5 $\pm$ 46.9	6.6 $\pm$ 6.5
$AUC_{0-\infty}$ (ng $\times$ hr/mL), mean $\pm$ SD	220.7 $\pm$ 47.0	6.7 $\pm$ 5.8
Enalaprilat (n = 5)		
$C_{max0-72h}$ (ng/mL), mean $\pm$ SD	53.4 $\pm$ 31.1	0.3 $\pm$ 0.1
$T_{max0-72hr}$ (hr), median	4.3	4
AUC_{0-72hr} (ng $\times$ hr/mL), mean $\pm$ SD	514.5 $\pm$ 163.7	2.34 $\pm$ 1.9
$AUC_{0-\infty}$ (ng $\times$ hr/mL), mean $\pm$ SD	525.0 $\pm$ 150.3	3.93 $\pm$ 2.5

AUC, area under the serum-time curve; C_{max} maximum serum concentration; T_{max} timepoint of maximum concentration

hours after dosing with aliskiren (156% of mean predose value).

Change in Humoral Parameters After Administration of Enalapril/Enalaprilat

Enalaprilat, the active metabolite of enalapril, binds competitively to the ACE and blocks the conversion of angiotensin I to angiotensin II. The angiotensin I concentrations rose with enalaprilat and reached maximum concentrations in all 9 subjects in 5 to 7 hours after oral administration of the prodrug enalapril. The mean increase was 3.7-fold when compared with baseline concentrations. In contrast, angiotensin II concentrations were reduced and reached a maximum reduction between 3 and 7 hours postdose. The mean inhibition was 64% of the predose value, showing a broad range of 30% to 95% reduction. When comparing aliskiren and enalapril, the inhibition of angiotensin II

synthesis was reduced for 24 to 48 hours with aliskiren, whereas with enalapril treatment, the value was reduced for 8 hours only. The ACE activity was specifically inhibited by enalaprilat and was, therefore, calculated in volunteers taking enalapril only. The activity was maximum reduced by 76% between 3 and 8 hours postdose. The median T_{max} was 6 hours. Within 24 to 48 hours postdose, almost all patients had returned to at least predose values. Renin concentrations increased with enalapril approximately 16-fold from initial values, with a T_{max} of about 6 hours after enalapril administration. The concentrations slowly went back to a 2.7-fold increase by day 3 of the study. The variability in renin concentration is indicated by an SD of 89 microunits/mL (464 microunits/mL if the volunteer with the short-term hypotensive values is included) at the C_{max} . With enalapril, the PRA remained nearly unchanged for the

Table 4. Individual datasets of pharmacokinetics in saliva and serum of Aliskiren, Enalapril and Enalaprilat

	Ratio					
	AUC _{0-last}		T _{max}		C _{max}	
Aliskiren	0-5 hr	0-192 hr	0-5 hr	0-192 hr	0-5 hr	0-192 hr
Volunteer 1	0.0100	0.1777	1.0	1.0	0.0115	0.0115
Volunteer 2	0.0076	0.0581	1.2	1.2	0.0052	0.0052
Volunteer 3	0.0030	0.0131	1.1	1.5	0.0024	0.0063
Volunteer 4	0.0091	0.1590	1.1	1.9	0.0084	0.0132
Volunteer 5	0.0004	0.0009	0.9	1.5	0.0025	0.0047
Volunteer 6	0.0644	0.0279	1.0	2.2	0.0907	0.0065
Volunteer 7	0.0680	1.2895	0.2	3.5	0.0811	0.1304
Volunteer 8	0.0055	0.1363	1.0	2.6	0.0062	0.0081
Volunteer 9	0.0068	0.0899	1.3	1.3	0.0064	0.0064
Volunteer 10	0.0061	0.0644	1.0	2.8	0.0069	0.0093
Volunteer 11	0.0681	1.0215	1.3	8.4	0.0642	0.1960
Volunteer 12	0.0100	0.1915	1.0	1.0	0.0084	0.0084
Volunteer 13	0.0086	0.0966	0.9	1.8	0.0092	0.0216
Enalapril	AUC _{0-last}		T _{max}		C _{max}	
Volunteer 1	0.0116		1.0		0.0083	
Volunteer 2	0.0141		1.0		0.0211	
Volunteer 3	0.1026		1.3		0.1430	
Volunteer 4	0.0169		1.0		0.0221	
Volunteer 5	0.0260		1.7		0.0258	
Enalaprilat						
Volunteer 1	0.0033		1.5		0.0057	
Volunteer 2	0.0028		1.3		0.0088	
Volunteer 3	0.0026		0.9		0.0070	
Volunteer 4	0.0046		*		*	
Volunteer 5	0.0074		1.1		0.0047	

AUC, area under the serum-time curve; C_{max} maximum serum concentration; T_{max} timepoint of maximum concentration

first 3 hours after dosing. The activity started to increase at 3 hours after administration and reached a maximum concentration 5 hours postdose. The mean activity was increased by up to 19-fold compared with the mean predose values. Figure 6 represents the mean pharmacokinetics of aliskiren and enalapril/enalaprilat and the corresponding changes in the humoral parameters within the RAA system. Details on the pharmacokinetic parameters of the RAA are listed in Table 5.

Safety Assessment

Both drugs were generally well tolerated. Three

adverse events of dizziness were reported by subjects taking 20 mg enalapril maleate. Subjects who received 300 mg aliskiren hemifumarate also reported 3 adverse events: 2 complained about headaches, 1 about dizziness. All adverse events were mild or moderate and discontinued within 12 hours after the first incidence. No notable changes in BP values were detected in the healthy volunteers of either group. The ratios of the mean systolic and diastolic BP values to heart rate remained nearly unchanged. Details are summarized in Table 6. Nevertheless, 1 subject taking enalapril experienced approximately 30-minutes of hypotensive BP values of 80/44

Figure 4. Urine profiles of enalapril and enalaprilat.

The dotted lines are the individual cumulative amount excreted into urine after administration of 20 mg enalapril maleate once to healthy volunteers. Additionally, the black lines represent the percentage of excreted enalapril (A) and of enalaprilat (B). The grey line in B shows the total excreted amount of the prodrug and active metabolite combined. $n=9$.

mm Hg RR. The timing of the hypotensive values occurred around the T_{max} of the active metabolite Enalaprilat; however, the volunteer was clinically asymptomatic.

DISCUSSION

In summary, this proof-of-concept study confirmed the applicability of the child-appropriate assays for determining pharmacokinetics of aliskiren, enalapril, and enalaprilat and the corresponding changes in the humoral parameters in the RAA system. These assays reliably quantified blood, urine, and saliva concentrations of both drugs and the corresponding humoral parameters over the course of 5 elimination half-lives.

Tailoring drug assays to determine drug concentrations and biomarkers of the RAA system in small-volume samples fully succeeded with the required blood volumes. Despite the very

dense sampling (in total, 40 pharmacokinetic sampling points for 1 pharmacokinetic profile), the tailored assays applied here allowed us to reliably determine the drug concentrations and the subsequent changes in humoral parameters in only 45 mL of blood per subject. Although the drug concentrations were determined in a maximum of 100 μ L of serum per sampling point, all 5 humoral parameters of the RAA system were analyzed in 2.1 mL blood. Compared with Sullivan et al⁹ who investigated only 1 humoral parameter in children and required 2 mL blood, this optimization in sample volume by developing the low-volume assays should be a huge benefit in pediatric pharmacotherapy. By applying this bioanalytic platform, evaluation of pharmacokinetics and pharmacodynamics is even possible in neonates and infants. This novel option facilitates obtaining

sufficient data points per child and circumvents the population pharmacokinetic approach with its possible imprecisions. The results obtained by the low-volume assays proved their reliability by comparing them with values in the literature. This legitimizes the chosen bioanalytic settings to be applied for pediatric populations and provides systematic data sets for enalapril/enalaprilat. For aliskiren, all bioanalytic assays were suitable for a precise determination, except the angiotensin I assay. Because of the low angiotensin I baseline in the aliskiren group, the expected decrease in concentration was not detectable. Consequently, its suitability for determining those decreases are not yet possible, and further optimization is required for future investigations.

The findings regarding the pharmacokinetics of aliskiren in adults were in line with those in the literature and reflected high interindividual variability in serum concentration and low uri-

Figure 5. Passing and Bablok regression scatter plots of detected aliskiren, enalapril, and enalaprilat concentrations in saliva and serum.

The closeness of agreement between the 2 different pharmacokinetic determination methods in serum and saliva were assessed with a scatter plot. The upper graph represents saliva and serum concentrations of 13 healthy volunteers after administration of aliskiren. The middle and lower graphs show the measured concentrations of enalapril and enalaprilat in 5 healthy volunteers. The solid lines indicate the regression lines. The 95% confidence intervals are shown with dashed lines.

nary excretion. Aliskiren displayed a biphasic absorption behavior in varying degree among all healthy volunteers, that could be precisely described by applying the dense sampling used in this study. Currently, mean concentration-time profiles for Aliskiren are primarily published, which only vaguely suggest this behavior. The reason for this double-peak phenomenon can only be assumed because it has not been previously reported in detail. Comparable behavior with a first small peak followed by a second, much larger peak can be seen with phenazopyridine,³⁶ but the reason for that phenomenon has not been reported either. In the literature, double peaks are generally correlated with enterohepatic recirculation, changes in salt, changes in bile solubility, with the formulation, and with *p*-glycoprotein, among others. For aliskiren, a change in salt, an additional absorption site along the gastrointestinal tract, or precipitation is likely but needs to be investigated further. The mean variability in AUC of 65% in this study is in line with data reported by Vaidyanathan et al⁸ who claimed a range between 40% to 70%. Because of the confirmed high intraindividual and interindividual variability, population pharmacokinetics and sparse sampling are not advisable for aliskiren. Moreover, the elimination half-life obtained by this study differed from those compared in previous publications. In studies with an investigation period of 96 hours, a half-life of 40 hours was claimed,¹⁰ but this study, with its investigation period of 192 hours, revealed a mean elimination half-life of 75.6 hours (median, 65.9 hours). These results should be considered during the planning and conduction of future studies; in particular, the calculation of the wash-out period in crossover studies should be made carefully because aliskiren elimination is much slower than previously assumed. It was possible to determine drug concentrations even 3 weeks after a single oral administration. The other pharmacokinetic parameters, such as C_{max} and T_{max} as determined by the low-volume LC-MS/MS assay, were generally comparable to those found by Vaidyanathan et al.¹⁰ Likewise, the amount recovered in urine (0.7%) was comparable with to the Waldmeier et al³⁷ data of 0.6% aliskiren. The mean CL_R was similar to that reported by Vaidyanathan et al.³⁸ As mentioned, weeks after administration of aliskiren, drug concentrations were still detectable in concentrated urine

Table 5. Dataset of measured humoral parameters in all healthy volunteers after drug administration

		Renin		Plasma renin activity		Angiotensin I		Angiotensin II	
		C _{max} [μIU/mL]	t _{max} (hr)	Activity [ng/mL/hr]	t _{max} (hr)	Max change [%]	t _{max} (hr)	Max neg. change from baseline[%]	t _{max} (hr)
After administration of	300 mg aliskiren (n=13)	Mean	272	5§	0.01	1§	*		2§
		Range	(48 – 1694)	(3 – 7)	0.01	(0.5 – 6)	[0 – (-98)]		(0.5 – 4)
	20 mg enalapril (n=9)	Mean	128 [280†]	6§	9 [14†]	5§	263 [396†]	6	- 64
		Range	(50 – 300) [1499‡]	(5 – 6)	(2 – 17) [51‡]	(5 – 6)	(151 – 400) [1460‡]	(5 – 7)	[(-57) – (-95)]

* Mean predose value was at the LLOQ already. A reliable change could not be determined

† Mean value if subject with hypotensive blood pressure values are included and not marked as outlier

‡ Upper limit of range if subject with hypotensive blood pressure values are included and not marked as outlier

§ t_{max} value were reported as median

samples, which accounts for a more-prolonged elimination phase than previously assumed.

For enalapril, successful implementation of assays to determine serum and urinary concentrations were demonstrated. Mean C_{max} values of 160 ng/mL were obtained for the prodrug enalapril, as well as 53 ng/mL for the active metabolite enalaprilat, which are comparable to those of Najib et al,²³ who reported 124 ng/mL and 54 ng/mL also using LC-MS/MS. Determined elimination half-lives of enalapril (0.9 hour) and enalaprilat (10 hours) were consistent with Najib et al.²³ The bioavailability of enalapril is about 50%, which is primarily excreted renally. This study found a mean total renal excretion of 44% of the administered dose, which differs slightly from data reported by Rippley et al,³⁹ who found 50% excretion, and Noormohamad et al,⁴⁰ who reported 51%. The difference might be explained by the variability in bioavailability of 21% to 61%,⁴⁰ which, consequently, affects the amount excreted into urine. The amount of enalaprilat in urine was 27%, which is comparable to other reported values of 30% to 36%.³⁹⁻⁴¹ The conversion rate of enalapril and enalaprilat (61%) was within the ratios determined by Till et al⁴² (67%) and Rippley et al³⁹ (60%). Observed CL_R of enalapril (15 L/hr) and enalaprilat (8 L/hr) were also in line with the literature.^{43,44} All findings confirmed the suitability of the preparation of urinary samples, avoided extensive degradation, and proved the applicability of the applied low-volume assay.

The replacement of invasive pharmacokinetic sampling using saliva seems to be inappropriate for the drugs investigated. The evaluation by a validated assay did not result in saliva drug profiles comparable to profiles obtained in serum,

which prohibits a meaningful interpretation of the data. The aliskiren concentrations obtained were substantially less than the expected 50% penetration from serum to saliva, as calculated based on physicochemical properties. There was a change in the penetration at 5 hours, which persisted for the next days. The shift in penetration rate was observed in nearly all volunteers taking aliskiren and was evaluated by a posteriori analysis. The subsetting for 0 to 5 hour and 0 to 192 hours was performed after visual inspection of the data and was strengthened by the estimations of pharmacokinetic parameters using a non-compartmental analysis. The persistence points toward additional drug penetration from other compartments as the cause of the change because ingestion resulted in a temporary change only. However, a similar finding has not been reported in literature, and the reason for such a shift requires further research. Regarding enalapril and enalaprilat, the expected low penetration rates, based on theoretical assumptions about the unbound fraction in blood and the pK_a values, were confirmed. Nevertheless, although salivary enalapril concentrations are comparable to concentrations in serum, the pharmacokinetics of the active metabolite enalaprilat was not covered sufficiently by saliva when compared with serum. The reason for the poorer penetration of enalaprilat into saliva might be due to the drug's ability to form a zwitterion, which could contribute to insufficient penetration from serum into saliva.

Because of the low drug concentration observed in saliva, some swabs were belatedly and randomly re-eluted with ammonia in acetonitrile/methanol solution. No additional drug substance was obtained by this approach, which

Figure 6. Mean concentration-time profiles of aliskiren and enalapril/enalaprilat and the measured changes in humoral parameters of the renin-angiotensin-aldosterone (RAA) system after drug administration.

Mean concentration-time profile after administration of 300 mg aliskiren hemifumarate to 13 healthy volunteers (A) and mean concentration-time profile after administration of 20 mg enalapril maleate to 9 healthy volunteers (B). Detected changes in humoral parameters after administration of aliskiren (left column) and enalapril/enalaprilat (right column) are shown. All profiles are represent means $\pm$ SD. Dotted lines indicate the measured baseline of each humoral parameter.

Table 6. Change in blood pressure

	Aliskiren group (n=13)	Enalapril group (n=9)
Systolic blood pressure [mmHg]		
Baseline	125 ± 14	109 ± 11
Average 0-24h	118 ± 12	107 ± 10
Average 0-end	118 ± 12	107 ± 10
Ratio (average 0-24h/baseline)	0.95 ± 0.05	0.98 ± 0.04
Ratio (average 0-end/baseline)	0.95 ± 0.04	0.99 ± 0.04
Minimum value	107 ± 11	97 ± 11
Diastolic blood pressure [mmHg]		
Baseline	70 ± 6	67 ± 6
Average 0-24hr	65 ± 7	64 ± 6
Average 0-end	66 ± 7	64 ± 6
Ratio (average 0-24hr/baseline)	0.93 ± 0.07	0.95 ± 0.04
Ratio (average 0-end/baseline)	0.95 ± 0.06	0.96 ± 0.04
Minimum value	55 ± 7	54 ± 7
Heart rate [beats/minute]		
Baseline	59 ± 10	65 ± 10
Average 0-24h	62 ± 9	69 ± 9
Average 0-end	63 ± 10	69 ± 9
Ratio (average 0-24h/baseline)	1.06 ± 0.07	1.07 ± 0.1
Ratio (average 0-end/baseline)	1.07 ± 0.07	1.07 ± 0.1
Maximum heart rate	77 ± 13	85 ± 15

Baseline, blood pressure value before administration of drug; Data are compiled as mean ± SD

suggests that the low concentrations were based on the drug distribution in the body and not on a bad recovery from the swabs. Furthermore, the low concentration cannot be explained as artificial dilution because of stimulated salivation. Only oral swabs (Salivette) without any stimulants (e.g., citric acid) or other adjuvants were used. Because only the free fraction can penetrate into saliva, a probable reason for the low concentrations found might be that the previously reported protein binding of about 50% of the DRI aliskiren was too low. This assumption can be supported by findings in marmosets, in which the binding was 92%.⁴⁵

The chosen saliva collection device or method was already successfully used to evaluate cortisol and cytokines in all pediatric age groups. Gunnala et al⁴⁶ investigated salivary cortisol concentrations in children between 4 weeks and 18 years and found a correlation in critically ill children. Davis et al⁴⁷ used cotton swabs in combination with the salivette device to collect saliva from 18 premature

infants to determine betamethasone exposure. Glover et al⁴⁸ measured the cortisol concentrations in newborns and infants up to 1 year old with salivettes. Using the same saliva collection device was considered promising for establishing a comparable method for cardiovascular drugs. In summary, we cannot recommend saliva as biological fluid for determining the pharmacokinetics of these 2 drugs, based on our findings.

Both drugs showed marked and sustained inhibition of the RAA system in healthy volunteers. The measured changes in all humoral parameters were in temporal concordance with serum drug concentrations. They confirmed the current knowledge about the RAA system, with the exception of angiotensin I in the aliskiren group, because of the low predose values. Although the first parameters changed within 0.5 to 1 hour after administration of aliskiren, change took about 3 to 4 hours in the enalapril group because of the bioactivation of enalapril to enalaprilat. Although the T_{max} of aliskiren in this

study was about 2.7 hours after administration, most humoral parameters were altered earlier. Aliskiren showed a double peak phenomenon, which suggests that the first peak of the double peak was sufficient to intensively interact with the RAA system. This first peak appeared between 0.3 and 1.5 hours after oral administration. Additionally, this assumption is supported by the aliskiren concentrations of the first peak (162 ng/mL), which exceeds the 50% inhibitory concentration of renin (0.6 nmol/L).⁴⁹ Even on day 9 of the investigation, the evaluated mean drug concentration in serum was 1.2 ng/mL and, therefore, still accounted for at least 50% inhibition of the target structure.

The detected rise in renin concentration after oral administration of aliskiren and enalapril was comparable to the observations of Nussberger et al.²⁵ They mentioned an up to 20-fold dose-dependent increase after administration of 640 mg aliskiren, whereas this study showed a 19-fold rise in renin following a 300-mg aliskiren administration (maximum approved dose). Furthermore, after administration of 20 mg enalapril, a 16-fold up-regulation of renin was confirmed through the child-appropriate assay. The time to reach the highest mean renin concentration under aliskiren treatment differed between Nussberger et al.²⁵ and this study (10 vs. 5 hours). The difference might be explained by the much denser sampling in this study, which allowed a more-precise determination of the T_{max} . Furthermore, by comparing the results obtained by Nussberger et al.²⁵ with those of the current study, the data from Nussberger et al.²⁵ showed a C_{max} of renin with enalapril after 3 hours, whereas, with aliskiren, it was reached 10 hours postdose. With regard to the known pharmacokinetic parameters of both compounds, an earlier C_{max} with aliskiren and a later one with enalapril treatment would be more rational. This study found the C_{max} of renin after administration at about the same time postdose (5 hours aliskiren vs 6 hours enalapril). Finally, an additional benefit for future pediatric studies is the low required volume of 250 μ L plasma.

The alteration of PRA with enalapril/enalaprilat and aliskiren, respectively, was comparable to that found by Nussberger et al.²⁵ Nevertheless, an appreciable improvement was gained by the reduction in required blood volume for a reliable determination. Although Sullivan et al.⁹ required 2 mL of blood per sampling point, only 400 μ L of

blood was necessary for reliable determination of PRA in children in this study.

The low-volume assay developed reliably determined angiotensin II concentrations. Because available commercial assays to determine angiotensin II were less sensitive, a combination of high sample purification and sample concentration by SPE and an ELISA with monoclonal antibodies was established to measure the low concentrations adequately. This enabled detection in 500 μ L plasma, which is a reduction of up to 75% compared with the required volumes of kits available on the market. The decreased angiotensin II concentrations with enalapril were comparable to the ones reported by Brunner et al.⁵⁰ Our study found a decline of 64%, whereas the Brunner et al.⁵⁰ results showed a decline of 58%. The slight deviation in angiotensin II concentrations is best explained by the different analytic methods and the high variability observed. The reduction in angiotensin II results with aliskiren of 70% were comparable to those found by Nussberger et al.,²⁵ who found about a 70% decline with 160 mg aliskiren.

Finally, this comprehensive data set of drug concentrations and their effects on humoral parameters allow us to validate the physiologic-based computer models for pharmacokinetics and pharmacodynamics. These models are of increasing interest for optimal planning and conduction of pediatric clinical trials. The modeling approach is highly recommended by the EMA and the FDA for sophisticated pediatric research.^{27,28} The combination of both tools enables high-quality clinical trials to be conducted in future pediatric populations with a maximum of knowledge gained with a minimum blood volume required. Such an approach is particularly useful for future marketing authorization in the pediatric population.

CONCLUSION

The pharmacokinetics of aliskiren and enalapril and the changes in humoral parameters of the RAA system after a single dose of both drugs were systematically investigated, and the low-volume assays used enabled a comprehensive determination. This proof-of-concept study confirmed the applicability of the low-volume assays for determining drug concentrations and enabled the reliable investigation of pharmacokinetics

and pharmacodynamics after administration of aliskiren hemifumarate and enalapril maleate. This comprehensive data set of drug concentrations and their effects on humoral parameters allow us to validate physiologic-based computer models that are of increasing interest for the optimal planning and conduction of pediatric clinical trials. Saliva seemed inappropriate as a replacement for invasive PKa sampling of aliskiren and enalapril. Moreover, these findings mean low-volume assays can be applied in pediatric research, and their applications will allow researchers to increase the important information required on drug exposure and its effect on the RAA system in children.

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Abbreviations ACE, angiotensin converting enzyme; ACEI, angiotensin converting enzyme inhibitor; A_u , amount excreted into urine; Ang I, angiotensin I; Ang II, angiotensin II; AUC, area under the concentration-time curve; BP, blood pressure; CL_r , oral clearance; $CL_{r,r}$, renal clearance; CLIA, chemiluminescence immunoassay; C_{max} , maximum concentration; CV, coefficient of variation; DRI, direct renin inhibitor; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; EMA, European Medicines Agency; FDA, Food and Drug Administration; g , earth's gravitational acceleration; hr, hour; HPLC, high-performance liquid chromatography; k_e , elimination constant; LLOQ, lower limit of quantification; mM, millimolar; mm Hg, millimeters of mercury; MS/MS, tandem mass spectrometer; m/z , mass-to-charge ratio; PRA, plasma renin activity; RAA, renin-angiotensin-aldosterone; RIA, radio immunoassay; SPE, solid-phase extraction; $t_{1/2}$, half-life; T_{max} , time of maximum concentration; V_d , apparent volume of distribution

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