Low-volume LC–MS/MS method for the pharmacokinetic investigation of carvedilol, enalapril and their metabolites in whole blood and plasma: Application to a paediatric clinical trial

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Abstract
Evidence-based pharmacotherapy with carvedilol and enalapril in children suffering from heart failure is insufficient owing to limited pharmacokinetic data. Although a few data sets regarding enalapril, its metabolite enalaprilat and carvedilol in children have been published, pharmacokinetic data on carvedilol metabolites are missing. However, for both drug substances, their active metabolites contribute substantially to drug efficacy. As data can hardly be derived from adults owing to the unknown impacts of enzymatic maturation and ontogeny during childhood, customised assays are important to facilitate paediatric evidence-based pharmacotherapy. Considering ethical paediatric constraints, a low-volume liquid chromatography coupled to mass spectrometry (LC–MS/MS) assay was developed using whole blood or plasma for the quantification of enalapril, enalaprilat, carvedilol, O-desmethyl carvedilol, 4- and 5-hydroxyphenyl carvedilol as well as 3- and 8-hydroxy carvedilol. To facilitate broader applications in adults, the elderly and children, a wide calibration range—between 0.024/0.049 and 50.000 ng/ml—was achieved with good linearity ($r \geq 0.995$ for all analytes). In compliance with international bioanalytical guidelines, accuracy, precision, sensitivity and internal standard normalised matrix effects were further successfully validated with the exception of those for 3-hydroxy carvedilol, which was therefore assessed semi-quantitatively. Distinct haematocrits did not impact matrix effects or recoveries when analysing whole blood. Blood-to-plasma ratios were determined for all analytes to form the basis for pharmacokinetic modelling. Finally, incurred sample reanalysis of paediatric samples confirmed the reproducibility of the developed low-volume LC–MS/MS method during study sample analysis. The assay facilitates the reliable generation of important data and contributes towards a safe drug therapy in children.
INTRODUCTION

Paediatric heart failure caused by dilated cardiomyopathy is a rare, but deadly disease associated with worst outcomes compared with other paediatric cardiac diseases. Within 5 years of their diagnosis, 50%–60% of the patients die or require heart transplants. To mitigate symptoms and prevent further heart damage, drugs like the angiotensin-converting enzyme (ACE) inhibitor enalapril and the β-blocker carvedilol are used in children alone or in combination. Similarly, these drugs have been applied in children with congenital heart disease and systemic ventricular dysfunction. For β-blockers, a Cochrane review in 2020 concluded potential benefits, but claimed further (pharmacokinetic) investigations to be necessary to provide effective dosing and to improve evidence. Similarly, the evidence for administration of enalapril in paediatric heart failure is limited. Therefore, currently insufficient evidence is available for a safe and rational pharmacotherapy in this vulnerable paediatric population.

Bodyweight-adjusted downscaling of adult dosage regimens to children increases the risk of toxicity or subtherapeutic dosing, because the metabolising capacities of children depend on enzyme ontogeny and maturation. Based on adult data, carvedilol and enalapril are extensively metabolised into active metabolites after administration and absorption. Enalapril undergoes hydrolysis by hepatic carboxylesterase 1 and is converted into the active enalaprilat. Enalapril inhibits the ACE and thus the conversion of Angiotensin I into Angiotensin II, which results in vasodilation, decreased preload, natriuresis and prevention of heart remodelling. Carvedilol nonselectively inhibits α1- and β-receptors, resulting in peripheral vasodilation and negative chronotropic and inotropic effects. Carvedilol is applied as a racemate and enantioselectively metabolised through aromatic ring hydroxylation by cytochrome P enzymes and glucuronidation. Whereas a pharmacokinetic model for the enantioselective disposition of carvedilol in children has been developed, no data are available on the carvedilol metabolites. Five positional isomers are reported, which include next to the inactive 1-hydroxy carvedilol, 3-hydroxy carvedilol and 8-hydroxy carvedilol, the active 4-hydroxyphenyl carvedilol and 5-hydroxyphenyl carvedilol as well as the further active metabolite O-desmethyl carvedilol. The metabolite 4-hydroxyphenyl carvedilol shows a 13-fold increase in β-receptor blocking activity compared with carvedilol and is present in quantities of about 10% of the carvedilol concentration in adults. It is indicated that a faster and altered metabolism of carvedilol is present in children, because significantly shorter half-lives of carvedilol have been reported. Owing to the limited knowledge on the matter, pharmacokinetic investigation of enalapril and carvedilol—along with their metabolites—is necessary and essential for safe pharmacotherapy in children.

However, ethical constraints in studies on children regarding blood sampling (such as fear, discomfort and restricted blood volumes) impede meaningful paediatric clinical trials. To address these concerns, the use of (capillary) whole blood is a reasonable choice for paediatric pharmacokinetic investigations, because it reduces the amount of required blood compared with plasma or serum (no losses due to centrifugation) and is easier to sample than venous blood (finger prick). Difficulties arising from the use of whole blood as the matrix of choice are—amongst others—the effects of haemolysis, the presence of distinct haematocrits and a more complex matrix for sample preparation. Further, differences in drug concentration between plasma and whole blood are to be expected owing to the impact of the haematocrit and the blood-to-plasma ratio of the drugs and metabolites. Because paediatric haematocrit reference values vary widely, between 29% and 50% in infants to adolescents, with even higher values in neonates (44%–70%), these determinants have to be carefully evaluated during method development. Further, the investigation of red blood cell (RBC) partitioning is necessary for adequate pharmacokinetic modelling.

The use of liquid chromatography coupled to mass spectrometry (LC–MS/MS) offers a tool to quantify sensitively and targeted drugs and metabolites. Even if LC–MS/MS methods suitable for the detection of enalapril, enalaprilat and carvedilol have been developed for paediatric demands, to our knowledge, no bioanalytical method is available to simultaneously assess enalapril, carvedilol and their metabolites. However, this is paramount to ensure safe and evidence-based pharmacotherapy in children suffering from heart failure.

Therefore, the aim of this study was to develop an LC–MS/MS method using low volumes of whole blood or plasma facilitating the pharmacokinetic investigation of the drugs carvedilol and enalapril, along with their metabolites (enalaprilat, O-desmethyl carvedilol, 4- and 5-hydroxyphenyl carvedilol and 3- and 8-hydroxy carvedilol) within the framework of a national Dutch paediatric clinical study “CARS II” (CArvedilol Registry Study). This multicentre, prospective observational study included children in the age range of 0–18 years with dilated cardiomyopathy.

MATERIALS AND METHODS

2 Chemicals and consumables

The drug substances enalapril maleate chemical reference standard (CRS), enalapril dihydrate CRS and carvedilol CRS (European Pharmacopoeia standards) were supplied by the European Directorate for the Quality of Medicines & HealthCare (Strasbourg, France). The carvedilol metabolites were purchased as follows: (S)-(−)-O-desmethyl carvedilol (95%, 1H-NMR and MS), (R)-(+) O-desmethyl carvedilol...
(98%, 1H-NMR and MS), 3-hydroxy carvedilol (95%, 1H-NMR and MS), (R)-(+)−4-hydroxyphenyl carvedilol (98%, 1H-NMR and MS), (S)(−)−4-hydroxyphenyl carvedilol (98%, 1H-NMR and MS), 5-hydroxyphenyl carvedilol (98%, 1H-NMR and MS) from Toronto Research Chemicals (Toronto, Canada) and 8-hydroxy carvedilol (98.9%, high-performance liquid chromatography (HPLC)) from Santa Cruz Biotechnology (Dallas, TX, USA). For the internal standards, 5-hydroxyphenyl carvedilol-d5 (95%, 1H-NMR and MS) was obtained from Toronto Research Chemicals (Toronto, Canada), carvedilol-d5 (99.1%, HPLC−MS) from Alschim (Strasbourg, France) and enalapril-(phenyl-d5) maleate (99.0%, HPLC) from Sigma-Aldrich (Saint Louis, MO, USA), MS-grade water, 0.1 M aqueous zinc sulphate solution (Honeywell, Muskegon, MI, USA) and MS-grade methanol (Fisher Chemicals, Fair Lawn, NJ, USA), as well as methyl tert-butyl ether (≥99%, Merck, Darmstadt, Germany) and ammonia (30.9%, VWR Chemicals, Radnor, PA, USA) were further purchased. Formic acid (≥98%), 1,10-phenanthroline monohydrate (≥99.0%), 1-butanol (99.8%), dichloroacetic acid (≥99%) and ethyl acetate (p.a.) were all purchased from Sigma-Aldrich (Saint Louis, MO, USA). HPLC-grade methanol, HPLC-grade water, HPLC-grade acetone, HPLC-grade acetonitrile, trisodium citrate dihydrate (99.6%) and ammonium acetate (99.5%) were bought from Sigma-Aldrich (Saint Louis, MO, USA), as well as methyl tert-butyl ether (≥99%, Merck, Darmstadt, Germany) and ammonia (30.9%, VWR Chemicals, Radnor, PA, USA) were further purchased. Formic acid (≥98%), 1,10-phenanthroline monohydrate (≥99.0%), 1-butanol (99.8%), dichloroacetic acid (≥99%) and ethyl acetate (p.a.) were all purchased from Sigma-Aldrich (Saint Louis, MO, USA). HPLC-grade methanol, HPLC-grade water, HPLC-grade acetone, HPLC-grade acetonitrile, trisodium citrate dihydrate (99.6%) and ammonium acetate (99.5%) were bought from Fisher Chemicals (Fair Lawn, NJ, USA). For the sample clean-up, Oasis® MCX μ-elution 96-well plates by Waters (Milford, MA, Germany) were used.

Blood samples for method development and validation were donated by healthy volunteers and sampled in ethylenediaminetetraacetic acid S-Monovettes® (Sarstedt, Nümbrecht, Germany). Written informed consent was obtained from all participants before enrolment. The approval of the ethics committee of the University of Düsseldorf was granted in May 2019 (study number: 2019-469-KFOgU). Paediatric samples for incurred sample reanalysis (ISR) were obtained from study participants of CARS II, which was approved by the Medical Ethical Committee of the Erasmus MC, Rotterdam, The Netherlands (MEC 2014-062) (Supporting Information).

### 2.2 Preparation of working solutions

Each analyte was dissolved in methanol to obtain stock solutions of approximately 100 μg/ml (depending on the exact weight of drug substance). Using these, one working solution containing exactly 10 μg/ml of all analytes and another one containing 1 μg/ml of the internal standards were prepared by dilution with methanol. The internal standard solution was further diluted with methanol on the day of analysis to obtain a working solution of 20 ng/ml.

### 2.3 Preparation of QCs and calibration curve samples

The quality control (QC) and calibration curve samples were both prepared by spiking whole blood or plasma with the analyte working solution to obtain a concentration of 50.000 ng/ml (upper limit of quantification [ULOQ]).

Calibration curve samples were subsequently serially diluted in a 1:2 ratio to a final concentration of 0.024 ng/ml.

The QCs were prepared independently by serially diluting spiked whole blood or plasma. Five QC concentration levels were investigated during accuracy and precision runs. QC high (37.500 ng/ml) was obtained by mixing equal parts of the 50.000 and 25.000 ng/ml concentration levels. Further QC mid (1.563 ng/ml), two QC low (0.098 and 0.049 ng/ml) and the lower limit of quantification (LLOQ, 0.024 ng/ml) were assessed.

### 2.4 Sample preparation

The combination of protein precipitation prior to mixed-mode strong cation exchange solid-phase extraction (SPE) was used for the sample preparation. A total of 100 μl of whole blood or plasma sample was mixed with 5 μl of the internal standard solution (20.000 ng/ml); subsequently, 5 μl of 2M aqueous trisodium citrate solution was added. Next, samples were precipitated with 300 μl of acetonitrile:acetone (70/30, v/v) and subsequently shaken for 30 min at 800 rpm at room temperature. Subsequently, samples were centrifuged at 16.100×g and 20°C for 10 minutes. Thereafter, 300 μl of the supernatant for whole blood—or 350 μl of the supernatant for plasma—was evaporated under a gentle nitrogen stream and shaking at 400 rpm and 60°C. The residues were dissolved in 100 μl methanol. Evaporation and dissolution after precipitation were required to reduce the organic fraction and preserve the binding of the analytes to the SPE material by simultaneously maintaining a good solubility of all analytes. A customised SPE protocol was applied, where cavities were equilibrated with 200 μl methanol, followed by 200 μl water. Next, the sample was loaded onto the SPE cartridges prefilled with 600 μl of 1% FA in water (v/v). Samples were washed with 200 μl of 1% FA in water (v/v), followed by 200 μl of 5% methanol in water (v/v) and 400 μl of methanol/water 80/20 (v/v). Elution was conducted in two steps, using 50 μl of 5% ammonium acetate in acetonitrile (v/v) first, and 50 μl of 5% ammonia in 0.6M ammonium acetate in methanol (v/v) second. The eluate was evaporated to dryness under a gentle nitrogen stream and shaking at 400 rpm and 80°C. Finally, the residues were reconstituted with 75 μl methanol/water 40/60 (v/v).

### 2.5 LC−MS/MS

Chromatographic separation was performed using an Acquity UPLC system (Waters Corporation, Milford, MA, USA), equipped with a Kinetex® 1.7 μm Biphenyl column (2.1×10.0 mm, Phenomenex, Torrance, CA, USA). For the mobile phase, 1% FA in water (v/v) and 1% FA in methanol (v/v), B) were applied. Gradient separation was used, involving the following steps: 0.0–5.0 min: 5% B, 5.0–5.1 min: 5%–35% B, 5.1–7.0 min: 35%–45% B, 7.0–9.5 min: 45%–50% B, 9.5–10.5 min: 50%–75% B, 10.5–11.0 min: 75%–95% B,
11.0–11.5 min: 95% B, 11.5–13.0 min: 5% B. The injection volume was set to 50 μl, and the column oven was maintained at 60°C.

A Waters Quattro Premier XE (Milford, MA, USA) was used for the mass spectrometric detection. A desolvation gas flow of 900 L/h and a cone gas flow of 50 L/h were applied. Argon was utilised as the collision gas and its flow was set to 0.15 ml/min. The source temperature was maintained at 135°C and the desolvation temperature at 500°C. The capillary voltage was 3.5 kV. Substance-specific parameters are shown in Table 1, and the product ion spectra of all analytes and internal standards are displayed in Figure 1.

MassLynx™ 4.1 was used for LC–MS/MS data acquisition and TargetLynx™ for raw data evaluation (Waters Corporation, Milford, MA, USA).

2.6 | Validation

Validation was carried out based on current bioanalytical method validation guidelines of the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA).26,27 The following validation parameters were assessed: linearity, accuracy, precision, absolute and internal standard normalised matrix effect, carry-over, sensitivity, recovery and dilution integrity.

2.6.1 | Linearity

To confirm linearity, four calibration curves were required. Each curve was assessed in single determination using 12 calibrator levels, ranging from 0.024 to 50.000 ng/ml for all analytes. A standard had to be rejected if its back-calculated concentration (relative error [RE]) deviated by more than ±15% (±20% at the LLOQ) from the nominal concentration. At least 75% of all calibration curve standards had to be compliant according to the regulatory guidelines of the EMA and FDA.26,27

2.6.2 | Accuracy, precision and sensitivity

Within-run and between-run accuracies, as well as precision, were determined in three runs on three different days. In accordance with the bioanalytical guidelines of the EMA and FDA,26,27 five levels in quintuplicates (37.500 [QC high], 1.563 [QC mid], 0.098 [QC low], 0.049 [QC low/LLOQ] and 0.024 ng/ml [LLOQ]) were investigated. Mean accuracy (RE) was allowed to deviate by ±15% (+20% at the LLOQ) from the nominal concentration. Precision (coefficient of variation [CV]) was calculated using one-way ANOVA for within-run (repeatability) and between-run precisions (day-different intermediate precision) and was limited to ≤15% (≤20% at the LLOQ). The signal-to-noise ratio was aimed to exceed 5 at the LLOQ.

2.6.3 | Matrix effect

One source was used for investigating the absolute matrix effect. Thereby, a low (0.098 ng/ml in whole blood and 0.049 ng/ml in plasma), a mid (1.563 ng/ml) and a high (37.500 ng/ml) QC were applied.

Further, seven different human sources were used to assess the internal standard normalised matrix effect. Therefore, the extracted matrix was spiked with a low (0.049 ng/ml in plasma/0.098 ng/ml in whole blood) and a high concentration (37.500 ng/ml) in triplicates and compared with the spiked neat solution. The CV of the internal standard normalised matrix effect across all seven sources was restricted to ≤15%, according to the regulatory guidelines of the EMA.26

| TABLE 1 | Substance specific mass spectrometric parameters |
|--------|---------------------------------|
| Analyte | MRM transition (m/z) | Cone voltage (V) | Collision energy (V) | Dwell time (s) |
| Enalapril | 376.9 — 234.2 | 31 | 21 | 0.075 |
| Enalaprilat | 348.7 — 206.2 | 27 | 18 | 0.075 |
| Carvedilol | 406.8 — 100.0 | 36 | 29 | 0.075 |
| 3-Hydroxy carvedilol | 423.2 — 100.0 | 30 | 32 | 0.060 |
| 4-Hydroxyphenyl carvedilol | 422.8 — 100.1 | 36 | 30 | 0.060 |
| 5-Hydroxyphenyl carvedilol | 422.8 — 100.1 | 36 | 30 | 0.060 |
| 8-Hydroxy carvedilol | 422.8 — 100.0 | 30 | 31 | 0.060 |
| O-Desmethyl carvedilol | 392.9 — 99.9 | 35 | 30 | 0.060 |
| Enalapril-d5 | 381.9 — 239.1 | 31 | 18 | 0.075 |
| Carvedilol-d5 | 411.9 — 105.0 | 35 | 33 | 0.075 |
| 5-Hydroxyphenyl carvedilol-d5 | 427.7 — 104.9 | 38 | 31 | 0.060 |

Abbreviations: MRM, multiple reaction monitoring; m/z, mass-to-charge-ratio; s, seconds; V, volt.
2.6.4 | Recovery

To determine the recovery of the extraction process, low (0.049 ng/ml in plasma and 0.098 ng/ml in whole blood), mid (1.563 ng/ml) and high QCs (37.500 ng/ml) were analysed in triplicates using one source (n = 3). Because the guidelines do not specify target values for the recovery, it was aimed to be constant and maximised during method development.

2.6.5 | Carry-over

Carry-over was evaluated by analysing the ULOQ of the calibration curve and a consecutive blank sample without matrix. This setting was measured five times and the mean response in the blank sample was determined. The carry-over should not exceed the signal of the LLOQ by more than 20% for the analytes and 5% for the internal standards.26,27

2.6.6 | Dilution integrity

Whether concentrations above the ULOQ can be adequately determined was evaluated by diluting a 120.000 ng/ml of whole blood or plasma sample with blank whole blood/plasma (in a 1:4 ratio). This dilution approach was applied to fit the concentration in the calibration curve range. These samples were measured using a threefold approach. The accuracy was allowed to deviate by ±15% (RE) from the nominal concentration, and precision was limited to ±15% (CV).
2.7 Pharmacokinetic investigations and applicability

2.7.1 Evaluation of the influence of the haematocrit

Because distinct haematocrits might influence the matrix effects or recoveries of analytes, haematocrits of 30%, 40%, 50% and 60% were investigated. These values were chosen, as they reflect the reported haematocrit range in children between 2 weeks and 18 years of age. Therefore, blood was separated into plasma and the RBC fraction. Subsequently, RBCs were lysed by vortexing and multiple freeze-thaw cycles. Plasma was then spiked with 30%, 40%, 50% or 60% RBCs. A concentration of 10,000 ng/ml was analysed in triplicates for each haematocrit using prespiked and postspiked samples, along with neat solutions.

2.7.2 Determination of the blood-to-plasma ratio

The blood-to-plasma ratio (\(K_P\)) was determined to investigate the drug and metabolite binding to erythrocytes. Therefore, freshly drawn whole blood was spiked with 10,000 ng/ml of the analytes, cautiously shaken to avoid lysis and left at room temperature. After 30 min, the spiked and unspiked whole blood was centrifuged for 10 min at room temperature and 2,000xg. Plasma and RBC fractions were separated for both samples. The obtained blank plasma and RBCs were also spiked with 10,000 ng/ml of the analyte mix (reference samples). All four obtained plasma and RBC samples were analysed in triplicates, and the blood-to-plasma ratios were calculated (Equation 1). A modified calculation of that used by Hinderling et al. 1997 and Yu et al. 2005 was applied to correct for recovery or possible matrix effects in the distinct matrices.

\[
P = \frac{\text{area ratio RBC fraction}}{\text{area ratio reference RBC}} \times \frac{\text{area ratio plasma fraction}}{\text{area ratio reference plasma}} \times H + (1 - H),
\]

where \(K_P\) is the calculation of the blood to plasma ratio, RBC is the red blood cell, and \(H\) is the haematocrit.

2.7.3 Continued method performance verification

The method was applied to the determination of carvedilol, enalapril and their metabolites in whole blood and plasma samples of the paediatric CARS II study, a nationwide prospective study in children with dilated cardiomyopathy in the Netherlands. More detailed information on this study is given in the Supporting Information. To continuously monitor the reproducibility of the assay during the analysis of study samples, ISR was applied based on previously established in-house bioanalytical QC systems. Following regulatory bioanalytical guidelines, reanalysis of 10% of the first 1,000 study samples with concentrations close to the maximum concentration (\(c_{max}\)) and during the elimination phase is recommended. Two ISR samples accompanied every run. The reanalysed samples were selected randomly, provided that sufficient residual volume was available. The guidelines demand that the original and the repeat value differ by \(\leq \pm 20\%\) for \(\geq 67\%\) of the reanalysed samples. This difference was calculated according to Equation 2.

\[
\text{Difference} [\%] = \frac{\text{Reanalysis concentration} - \text{Initial concentration}}{\text{Mean concentration}} \times 100,
\]

where calculation of percentage difference for the ISR pairs is presented.

3 RESULTS AND DISCUSSION

3.1 Challenges during method development

SPE using diluted whole blood was only possible applying high pressures owing to the clogging of SPE cavities and resulted in low recoveries (Figure 2a). It was found that especially haemolysed samples substantially affected the recoveries of the carvedilol metabolites. Haemolysis can hardly be avoided when using whole blood, as the permanent presence of RBCs can lead to cell lysis because of freeze-thaw cycles or vortexing during sample preparation. As the metabolites were substantial for achieving the aim of the pharmacokinetic investigations, effort was made to increase recovery and was facilitated by a combination of precipitation and SPE (Figure 2b,c,d). Recoveries were further improved by the addition of aqueous trisodium citrate for all analytes (Figure 2e). The finding that the addition of 2M aqueous trisodium citrate and following precipitation with acetonitrile/acetic acid (7/3) in a ratio of 1:3 prior to SPE increased recovery substantially formed the basis for the subsequent validation.

3.2 Method validation

3.2.1 Linearity

Whole blood

The linearity of enalapril, enalaprilit, carvedilol and 4- and 5-hydroxyphenyl carvedilol was shown between 0.024 and 50,000 ng/ml in whole blood, with the exception of O-desmethyl carvedilol (LLOQ 0.049 ng/ml). The correlation coefficients of \(r \geq 0.996\) for all individual runs indicated good fits using a linear correlation with a weighting of \(1/x^2\).

Plasma

In plasma, a linear range from 0.024 to 50,000 ng/ml was found, which included—in addition to the assessed analytes in whole
blood—8-hydroxy carvedilol. A correlation coefficient of at least $r \geq 0.995$ for all individual runs also yielded good fits (weighting $1/x^2$).

For the evaluation of linearity, calibration curves were measured on four different days for plasma and whole blood. Deviations in back-calculated concentrations complied with FDA and EMA criteria (RE $\leq 15\%$, $\leq 20\%$ at the LLOQ).26,27

3.2.2 | Accuracy, precision and sensitivity

Whole blood

In whole blood, the REs at the distinct QC levels ranged between $-14.4\%$ and $+13.7\%$ (within-run accuracies) for enalapril, enalaprilat, carvedilol, O-desmethyl carvedilol and 4- and 5-hydroxyphenyl carvedilol and varied between $-7.1\%$ and $+5.6\%$ for between-run accuracy. Within-run precision was between $3.0\%$ and $11.5\%$ (CV) and between-run precision was between $3.6\%$ and $14.6\%$ (CV). The sensitivity of $0.024$ ng/ml for the assessed analytes was confirmed by the accuracy and precision results at the LLOQ (LLOQ: $0.049$ ng/ml for O-desmethyl carvedilol). These were between $-16.3\%$ and $+15.8\%$ (RE) for the within-run accuracy, between $8.0\%$ and $15.0\%$ (CV) for within-run precision, between $-3.1\%$ to $+6.4\%$ (RE) for between-run accuracy and between $8.0\%$ and $17.1\%$ (CV) for between-run precision. The signal-to-noise ratio at the LLOQ was between 19 (4-hydroxyphenyl carvedilol) and 134 (enalapril).

Plasma

In addition to whole blood, 8-hydroxy carvedilol was successfully included in plasma. Within-run accuracies at the distinct QC levels ranged between $-12.7\%$ and $+14.6\%$ (RE) and between-run accuracies between $-7.0\%$ and $+7.9\%$ (RE). Within-run precision was $1.6\%$–$11.9\%$ (CV), and between-run precision was $2.3\%$–$13.3\%$ (CV). At the LLOQ ($0.024$ ng/ml), within-run accuracy ($-8.3\%$ to $+17.5\%$, RE) and precision ($8.4\%$–$14.0\%$, CV) as well as between-run accuracy ($-2.4\%$ to $+6.4\%$, RE) and precision ($8.8\%$–$14.9\%$, CV) fulfilled guideline requirements.26,27 The signal-to-noise ratio at the LLOQ ranged between 26 (5-hydroxyphenyl carvedilol) and 177 (enalapril).

The accuracy and precision results for the QC levels and the LLOQ complied with international bioanalytical guidelines of the EMA and FDA.26,27 Detailed results for all analytes and both matrices are shown in Table 2. Representative chromatograms for the blank, LLOQ and QC high in whole blood and plasma are displayed in Figure 3.
| Table 2 | Within-run and between-run accuracy and precision (using one-way ANOVA) results for the analytes of the 3-day different runs at the five investigated levels (n = 5) |
|---------|-------------------------------------------------------------------------------------------------|
| **Analytes** | **Nominal concentration (ng/ml)** | **Accuracy** | **Precision** |
|          |                                    | **Whole blood** |             | **Plasma** |             | **Whole blood** |             | **Plasma** |             |
|          |                                    | **Day 1 RE (%)** | **Day 2 RE (%)** | **Day 3 RE (%)** | **Between-run RE (%)** | **Day 1 RE (%)** | **Day 2 RE (%)** | **Day 3 RE (%)** | **Between-run RE (%)** | **Within-run CV (%)** | **Between-run CV (%)** | **Within-run CV (%)** | **Between-run CV (%)** |
| Enalapril | 37.500                              | −0.37 | −3.01 | −3.01 | −0.28 | −0.25 | −0.63 | −4.43 | −1.77 | 3.62 | 3.62 | 1.62 | 2.76 |
|          | 1.563                               | 6.62  | −3.33 | 4.84  | 0.95  | 1.73  | 0.69  | −0.82 | 0.53  | 3.04 | 5.90 | 2.20 | 2.34 |
|          | 0.098                               | 2.45  | −2.04 | 13.67 | 4.69  | 9.80  | 6.53  | −0.82 | 5.17  | 5.57 | 9.20 | 5.29 | 7.01 |
|          | 0.049                               | −2.45 | −6.12 | 11.43 | 2.78  | 7.76  | 11.84 | 4.08  | 7.89  | 4.95 | 10.18 | 7.52 | 7.63 |
|          | 0.024                               | −10.00| 1.67  | 7.50  | −2.13 | 6.83  | 8.33  | 0.00  | 4.72  | 11.00| 13.29 | 8.69 | 8.78 |
| Enalaprilat | 37.500                             | 5.91  | 7.43  | −0.79 | 4.18  | 3.84  | 9.66  | 4.37  | 5.96  | 4.58 | 5.87 | 2.53 | 3.79 |
|          | 1.563                               | 0.72  | −0.22 | 1.84  | 0.78  | −4.68 | −3.65 | −1.91 | −3.41 | 4.05 | 4.05 | 4.38 | 4.38 |
|          | 0.098                               | 0.82  | 0.20  | 5.31  | 2.11  | 1.84  | 0.61  | −1.22 | 0.41  | 8.38 | 8.38 | 9.05 | 9.05 |
|          | 0.049                               | 1.22  | 0.82  | 1.63  | 1.22  | 13.06| −6.63 | 2.04  | 2.86  | 7.01 | 7.01 | 9.85 | 12.99 |
|          | 0.024                               | 0.00  | 4.17  | −0.83 | 1.11  | 17.50| −2.50 | 4.17  | 6.39  | 7.96 | 7.96 | 12.69| 14.85 |
| Carvedilol | 37.500                              | 0.12  | −4.89 | −0.25 | −1.67 | −0.24 | −0.11 | −6.90 | −2.42 | 3.87 | 4.48 | 1.91 | 4.33 |
|          | 1.563                               | 11.54 | −0.17 | 4.27  | 5.22  | −1.93 | 2.51  | 2.94  | 1.17  | 4.79 | 7.06 | 2.56 | 3.51 |
|          | 0.098                               | 7.55  | 1.43  | 7.76  | 5.58  | 8.57  | 7.96  | 0.41  | 5.65  | 3.27 | 4.49 | 5.34 | 6.43 |
|          | 0.049                               | 2.86  | −8.57 | −6.94 | −4.22 | 0.00  | −6.94 | 4.90  | 11.70 | 8.81 | 10.19| 5.88 | 7.97 |
|          | 0.024                               | 2.50  | −13.33| 1.67  | −3.06 | −3.33 | −8.33 | 10.83| −2.42 | 15.05| 16.30| 8.36 | 12.46 |
| O-des    | 37.500                              | −2.32 | −4.53 | −1087 | −5.91 | −3.05 | −7.33 | −8.23 | −6.20 | 10.36| 10.40| 3.55 | 4.33 |
|          | 1.563                               | −5.04 | −9.60 | −2.15 | −5.61 | −7.73 | −7.46 | −1.55 | −5.58 | 6.89 | 7.34 | 5.69 | 6.29 |
|          | 0.098                               | −10.20| −14.39| 3.67  | −7.07 | 5.10  | −8.37 | 3.47  | 0.07  | 11.54| 14.59| 7.39 | 9.88 |
|          | 0.049                               | −0.41 | −16.33| 6.94  | −3.27 | 3.67  | −1.22 | −4.90 | −0.82 | 13.29| 17.10| 7.77 | 8.19 |
|          | 0.024                               | n.a.  | n.a.  | n.a.  | n.a.  | 10.83| −4.17 | 9.17  | 5.28  | n.a. | n.a. | 13.61| 14.47 |
| 4-OH     | 37.500                              | −7.60 | −0.96 | 0.15  | −2.80 | −0.49 | −7.88 | −12.50| −6.96 | 9.42 | 9.46 | 4.23 | 7.53 |
|          | 1.563                               | 9.34  | 0.00  | 3.37  | −0.54 | 14.63| 1.82  | −2.75 | 4.56  | 6.20 | 7.16 | 6.62 | 10.45 |
|          | 0.098                               | 2.65  | 1.02  | 11.02 | 4.9  | 3.27  | 8.57  | −2.65 | 3.06  | 9.86 | 10.20| 5.99 | 7.64 |
|          | 0.049                               | −1.63 | −4.08 | 4.08  | −0.54 | −2.04 | 4.90  | 4.08  | 2.31  | 9.96 | 9.96 | 8.45 | 8.45 |
|          | 0.024                               | 2.50  | −10.00| 11.67 | 1.39  | −1.67 | 16.67 | 3.33  | 6.11  | 11.93| 15.13| 12.72| 14.47 |
| 5-OH     | 37.500                              | −7.68 | −2.25 | 4.24  | −1.90 | 2.55  | −1.41 | −7.15 | −2.00 | 7.05 | 8.76 | 3.52 | 5.89 |
|          | 1.563                               | 6.62  | −6.63 | 1.31  | 0.43  | 9.49  | 0.70  | −5.76 | 1.48  | 5.37 | 8.19 | 4.04 | 8.37 |
|          | 0.098                               | −0.20 | −5.51 | 2.65  | −1.02 | 7.76  | 5.71  | −2.86 | 3.54  | 8.92 | 9.01 | 4.94 | 7.01 |
|          | 0.049                               | 11.43 | −2.86 | 2.86  | 3.81  | 6.53  | 2.04  | 0.82  | 3.13  | 6.75 | 9.19 | 8.12 | 8.12 |
|          | 0.024                               | 15.83 | −0.83 | 4.17  | 6.39  | 14.17| −0.83 | 5.83  | 6.39  | 14.28| 15.09| 13.99| 14.37 |

(Continues)
3.2.3 | Matrix effect

Whole blood

In whole blood, the absolute matrix effect ranged from −19.0% (4-hydroxyphenyl carvedilol) to +11.9% (enalaprilat) at QC low (0.098 ng/ml), from −32.6% (O-desmethyl carvedilol) to +5.9% (enalaprilat) at QC mid (1.563 ng/ml) and from −21.4% (O-desmethyl carvedilol) to +5.9% (enalaprilat) at QC high (37.500 ng/ml). The internal standard normalised matrix effect of seven different human sources in whole blood at QC low (0.098 ng/ml) was 4.7% for enalapril, 2.3% for enalaprilat, 4.8% for carvedilol, 13.1% for O-desmethyl carvedilol, 5.1% for 4-hydroxyphenyl carvedilol and 4.9% for 5-hydroxyphenyl carvedilol. At QC high (37.500 ng/ml), CVs were lower and amounted to 1.2% for enalapril, 3.7% for enalaprilat, 1.5% for carvedilol, 4.5% for O-desmethyl carvedilol, 3.5% for 4-hydroxyphenyl carvedilol and 4.3% for 5-hydroxyphenyl carvedilol.

Plasma

In plasma, the absolute matrix effect at QC low (0.049 ng/ml) was between −24.6% (5-hydroxyphenyl carvedilol) and +17.6% (8-hydroxy carvedilol). Values of −21.9% (5-hydroxyphenyl carvedilol) to +21.3% (carvedilol) at QC mid (1.563 ng/ml) and of −18.8% (5-hydroxyphenyl carvedilol) to +22.0% (carvedilol) at QC high (37.500 ng/ml) were determined. In plasma, the internal standard normalised matrix effect at QC low (0.049 ng/ml) was 2.9% for enalapril, 10.0% for enalaprilat, 2.6% for carvedilol, 13.6% for O-desmethyl carvedilol, 8.3% for 4-hydroxyphenyl carvedilol, 94.7% for 5-hydroxyphenyl carvedilol and 84.4% for 8-hydroxy carvedilol. Detailed results for each level are shown in Table 3.

The obtained results complied with current guideline requirements of the EMA and indicate that different sources and varying haematocrits do not negatively impact reliable quantification.

3.2.4 | Recovery

Whole blood

In whole blood, the mean recoveries of all three QC levels were 96.5% (enalapril), 66.1% (enalaprilat), 83.7% (carvedilol), 77.3% (O-desmethyl carvedilol), 67.7% (4-hydroxyphenyl carvedilol) and 64.0% (5-hydroxyphenyl carvedilol). The recovery showed a tendency to increase with higher concentrations for carvedilol-derived analytes in whole blood (Table 3).

Plasma

In plasma, mean recoveries were higher than those in whole blood, at 90.1% for enalapril, 71.9% for enalaprilat, 87.6% for carvedilol, 91.1% for O-desmethyl carvedilol, 94.7% for 5-hydroxyphenyl carvedilol and 84.4% for 8-hydroxy carvedilol. Detailed results for each level are shown in Table 3.

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**Table 2** (Continued)

| Analytes | Nominal concentration (ng/ml) | Whole blood Day 1 | Whole blood Day 2 | Whole blood Day 3 | Plasma Day 1 | Plasma Day 2 | Plasma Day 3 | Between-run | Within-run | Between-run | Within-run | Between-run | Within-run |
|----------|-------------------------------|-------------------|-------------------|-------------------|-------------|-------------|-------------|--------------|------------|--------------|------------|--------------|------------|
| 8-OH     | 37.500                        | 96.5%             | 66.1%             | 83.7%             | 90.1%       | 71.9%       | 87.6%       | 91.1%        | 94.7%      | 84.4%        | 91.1%      | 84.4%        | 91.1%      |
| 1.563    | 96.5%                         | 66.1%             | 83.7%             | 90.1%             | 71.9%       | 87.6%       | 91.1%       | 94.7%        | 84.4%      | 91.1%        | 84.4%      | 91.1%        | 84.4%      |
| 0.098    | 96.5%                         | 66.1%             | 83.7%             | 90.1%             | 71.9%       | 87.6%       | 91.1%       | 94.7%        | 84.4%      | 91.1%        | 84.4%      | 91.1%        | 84.4%      |

Abbreviations: 4-OH, 4-hydroxyphenyl carvedilol; 5-OH, 5-hydroxyphenyl carvedilol; 8-OH, 8-hydroxy carvedilol; CV, coefficient of variation; n.a., not applicable; O-des, O-desmethyl carvedilol; RE, relative error.
3.2.5 | Carry-over

Whole blood/plasma
The carry-over was below 20% of the LLOQ for the following analytes (whole blood/plasma): enalapril (3.9%/2.5%), enalaprilat (4.2%/5.3%), 4-hydroxyphenyl carvedilol (4.1%/16.6%), 5-hydroxyphenyl carvedilol (7.8%/18.8%) and 8-hydroxy carvedilol (7.5% in plasma). The carry-over exceeded the requirement of ≤20% of the LLOQ for O-desmethyl carvedilol and carvedilol. Despite careful optimisation of gradient, autosampler rinsing and checking of autosampler parts, carry-over was not eliminated for the two most lipophilic compounds. Introduction of a washing method and change of injection mode reduced the carry-over substantially to levels below the LLOQ. Further, carry-over was monitored in all subsequent study sample runs to exclude erroneous results. No carry-over was observed for the internal standards.

3.2.6 | Dilution integrity

Whole blood
Dilution integrity was evaluated for the 1:4 dilution of a 120 ng/ml of sample, and accuracy (RE) was −3.2% for enalapril, −1.0% for enalaprilat, −3.8% for carvedilol, −4.1% for O-desmethyl carvedilol, and −3.2% for 4-OH, 4-hydroxyphenyl carvedilol; −3.8% for 5-OH, 5-hydroxyphenyl carvedilol; −4.1% for 8-OH, 8-hydroxy carvedilol; and −3.2% for O-des, O-desmethyl carvedilol.
+18.3% for 4-hydroxyphenyl carvedilol and +20.1% for 5-hydroxyphenyl carvedilol in whole blood. The precisions (CV) in whole blood amounted to 0.8% for enalapril, 2.3% for enalaprilat, 0.6% for carvedilol, 2.3% for O-desmethyl carvedilol, 4.4% for 4-hydroxyphenyl carvedilol and 2.2% for 5-hydroxyphenyl carvedilol.

Plasma

In plasma, the accuracies (RE) were 0.7% for enalapril, 9.4% for enalaprilat, −1.7% for carvedilol, −5.1% for O-desmethyl carvedilol, −3.1% for 4-hydroxyphenyl carvedilol, +3.3% for 5-hydroxyphenyl carvedilol and +7.2% for 8-hydroxy carvedilol. The precisions (CV) were 1.4% for enalapril, 0.9% for enalaprilat, 3.5% for carvedilol, 5.3% for O-desmethyl carvedilol, 7.8% for 4-hydroxyphenyl carvedilol, 2.2% for 5-hydroxyphenyl carvedilol and 4.6% for 8-hydroxy carvedilol.

Thus, other than the accuracies of 4- and 5-hydroxyphenyl carvedilol in whole blood, dilution integrity was confirmed for all analytes with accuracies (RE) and precisions (CV) of ±15%. Because it is expected that the carvedilol metabolite concentrations appear in much lower concentrations than the parent drug, the missing dilutional linearity has no major impacts on future quantitative measurements. All in all, the successful validation of a low-volume LC−MS/MS method with high sensitivity encompassing a broad calibration curve range was achieved for the detection of enalapril, enalaprilat, carvedilol, O-desmethyl carvedilol and 4- and 5-hydroxyphenyl carvedilol in whole blood. In plasma, 8-hydroxy carvedilol additionally fulfilled all guideline criteria. The method requires a total of 100 μl of whole blood or plasma, which facilitates frequent determination in adults, the elderly and paediatrics without infringing ethical constraints on blood volumes. The collectable blood volume in children is limited to 3% of the total blood volume within 4 weeks according to ethical recommendations in European guidelines. Thus, the blood volume for routine analysis and research together amounts to about 10 ml within 4 weeks for a neonate with 4 kg bodyweight.

Although several LC−MS assays concerning the determination of single drug substances have been published, none of them facilitates the simultaneous quantification of both mother substances enalapril and carvedilol with their coherent metabolites. Applying 200 μl of blood, Patel et al. accomplished quantification limits of 50 pg/ml and one additional metabolite (4-hydroxyphenyl carvedilol). Other LC−MS assays for only 50 μl serum but is characterised by a higher LLOQ of 0.200 ng/ml for enalapril and 0.180 ng/ml for enalaprilat. The required volume of 50 μl serum—assuming a haematocrit of 40%–50%—amounts for 100 μl whole blood. Besides comparable required blood volumes, the lower LLOQs of 0.024 ng/ml in this study allow for the prolonged and more accurate determination of drug levels plus the simultaneous monitoring of metabolism during the elimination phase.

Further, the simultaneous determination of two drugs, which represent a common treatment choice in paediatric heart failure (single or combined), facilitates the reduction of required blood volumes in children, as both drugs and their metabolites can be determined in one sample. A method to determine carvedilol metabolites in the here presented extend by LC−MS/MS was lacking. It allows for the pharmacokinetic investigation with maximised gain of knowledge from one blood sample in compliance with ethical recommendations on paediatric blood sampling.

Moreover, the metabolite 3-hydroxy carvedilol was retained semi-quantitatively in the method although guideline-compliant accuracy and precision could not be established over the entire calibration range. However, 3-hydroxy carvedilol was not reported to be successfully determined in human patient samples prior to this, to the best of our knowledge. Thus, data on this metabolite are completely lacking and, therefore, even the semi-quantitative approach provides substantial useful pharmacokinetic knowledge for estimating the ontogeny in childhood as 3-hydroxy carvedilol is metabolised by cytochrome P enzymes.

Overall, this method facilitates the pharmacokinetic investigation within a wide age- and weight-dependent dosage range and allows for the investigation of the impacts of enzymatic ontogeny and maturation on expected drug and metabolite concentrations in children, adults and the elderly.

3.3 | Pharmacokinetic and applicability investigations

3.3.1 | Influence of the haematocrit

During method development, the influence of the haematocrit on the determination of the analytes from whole blood was evaluated. It was observed that the addition of the internal standards with the precipitation solvent could not balance the effects of distinct haematocrits. However, when spiking the internal standards to whole blood samples before precipitation, analyte and internal standard variations between haematocrits resembled each other. Increasing haematocrits from 30% to 60% led to a decrease in recovery of up to −37%, which was especially pronounced for carvedilol (Figure 4a). However, the normalisation to the internal standard compensated for these losses substantially (Figure 4b). The absolute matrix effects were not influenced by haematocrit variations.

Haematocrit-independent determination of the analytes is important in face of interindividual haematocrit variations and a broader haematocrit range in children. Further, it facilitates conducting the calibration curve in adult whole blood, which is indispensable considering the ethical restrictions regarding paediatric blood sampling.
3.3.2  |  Blood-to-plasma ratio

The distribution of the drugs between the RBC and plasma fraction was evaluated by determining the blood-to-plasma ratio. The mean blood-to-plasma ratios, calculated according to Equation 1 (Section 2.7.2), were 0.74 for enalapril, 0.73 for enalaprilat, 0.76 for carvedilol, 0.78 for O-desmethyl carvedilol, 0.76 for 4-hydroxyphenyl carvedilol, 0.79 for 5-hydroxyphenyl carvedilol and 0.92 for 8-hydroxy carvedilol. Blood-to-plasma ratios of less than 1 indicate a sequestration of analytes in plasma rather than in the RBC fraction. Calculated blood-to-plasma ratios reported in the literature, for example, for carvedilol (0.69 and 0.71), were close to the described values.\textsuperscript{45,46} Values for carvedilol metabolites were not found in literature and seem to be experimentally obtained for the first time within these investigations.

3.3.3  |  Continued method performance verification

As part of in-house established comprehensive QC systems,\textsuperscript{31,32} ISR was conducted for paediatric plasma samples to continuously monitor and verify the method performance after validation. Eight ISR pairs were randomly selected out of the total available plasma samples...
samples within the CARS II study. This accounts for 6.2% of the study samples. Despite random selection based on sufficient residual volume, the measured concentrations of the eight IRS samples lay within a wide range for all analytes. Namely, concentrations of 0–6.288 ng/ml for enalapril, 0.619–44.453 ng/ml for enalaprilat, 0–30.625 ng/ml for carvedilol, 0–3.472 ng/ml for O-desmethyl carvedilol, 0–6.075 for 4-hydroxycarvedilol, 0–3.351 ng/ml for 5-hydroxycarvedilol and 0–0.415 ng/ml for 8-hydroxy carvedilol were detected (Figure 5a). Of these, at least 87.5% met the EMA and FDA guideline requirements of ≤20% deviation from the initial determined value for all analytes (Figure 5b).26,27 Four outliers were observed, a maximum of one per analyte. This finding conforms fully with current guideline criteria (Figure 5a,c). Limitations of the conducted ISR arose owing to the special nature of paediatric pharmacokinetic investigations. It was not feasible to comply with the demanded reanalysis rate of 10%, because typically less than 200 µl sample was collected from the clinical trial sites owing to ethical restrictions. As samples were reanalysed randomly and blinded based on sufficient residual volume, it was further not achievable to select specifically samples during the elimination phase or at the cmax. Additionally, the distinct times of maximum concentration of the analytes (in adults: 1–4 h) would demand for a higher number of reanalysed samples.47,48 However, the unknown impacts of maturation-dependent enzyme ontogeny on drug metabolism might lead to distinct times of maximum concentration in children. Therefore, it was decided to analyse samples blinded. ISR could not be applied to whole blood samples, as residual volumes of paediatric samples were limited with regard to capillary sampling. Nevertheless, the continued method performance-monitoring approach confirmed the reproducibility of the developed LC–MS/MS assay.

4 | CONCLUSION

An LC–MS/MS method for the quantification of enalapril, carvedilol and their metabolites tailored for paediatric demands was validated based on key features of bioanalytical guidelines of the FDA and EMA regarding linearity, accuracy, precision, sensitivity, dilution integrity, matrix effect and recovery in whole blood and plasma. The developed method was used to investigate the influence of different haematocrits as well as the drug and metabolite distribution between whole blood and plasma and proved to be suitable for use within these investigations. Next, the method was applied to the measurement of paediatric study samples, where guideline-conforming ISR results were obtained, which confirmed the reproducibility of the method beyond validation. Thus, the assay facilitates the reliable generation of data, which therefore supports the implication of evidence-based pharmacotherapy in children.

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CONFLICT OF INTEREST

None.

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REFERENCES

1. Webster G, Zhang J, Rosenthal D. Comparison of the epidemiology and co-morbidities of heart failure in the pediatric and adult populations: a retrospective, cross-sectional study. BMC Cardiovasc Disord. 2006;6(23). https://doi.org/10.1186/1471-2261-6-23

2. Lipshultz SE, Law YM, Asante-Korang A, et al. Cardiomyopathy in children: classification and diagnosis: a scientific statement from the American Heart Association. Circulation. 2019;140(1):1090-e68. https://doi.org/10.1161/CIR.0000000000000682

3. Rickers C, Lær S, Diller GP, et al. Leitlinie Pädiatrische Kardiologie: Chronische Herzinsuffizienz: S2k-Leitlinien 023–006: Chronische Herzinsuffizienz. 2015.

4. Masarone D, Valente F, Rubino M, et al. Pediatric heart failure: a practical guide to diagnosis and management. Pediatr Neonatol. 2017;58(4):303-312. https://doi.org/10.1016/j.pedneo.2017.01.001

5. Castro Diez C, Khalil F, Schwender H, et al. Pharmacotherapeutic management of paediatric heart failure and ACE-I use patterns: a European survey. BMJ. 2019;3(1):e000365. https://doi.org/10.1136/bmjpo-2018-000365

6. Shaddy RE, Boucek MM, Hsu DT, et al. Carvedilol for children and adolescents with heart failure. JAMA. 2007;298(10):1171-1179. https://doi.org/10.1001/jama.298.10.1171

7. Alabed S, Sabouni A, Al Dakhoul S, Bdaiwi Y. Beta-blockers for congestive heart failure in children. Cochrane Database Syst Rev. 2020;7:CD007037. https://doi.org/10.1002/14651858.CD007037.pub4

8. Smeets NJL, Schreuder MF, Dalinghaus M, et al. Pharmacology of enalapril in children: a review. Drug Discov Today. 2020. https://doi.org/10.1016/j.drudis.2020.08.005

9. Albers S, Meibohm B, Mir TS, Lær S. Population pharmacokinetics and dose simulation of carvedilol in paediatric patients with congestive heart failure. Br J Clin Pharmacol. 2006;65(4):511-522. https://doi.org/10.1111/j.1365-2125.2007.03046.x

10. Wells T, Rippley R, Hogg R, et al. The pharmacokinetics of Enalapril in children and infants with hypertension. J Clin Pharm. 2001;41(10): 1064-1074. https://doi.org/10.1177/001488640100100106

11. Barker CJS, Standing JF, Kelly LE, et al. Pharmacokinetic studies in children: recommendations for practice and research. Arch Dis Child. 2018;103:695-702. https://doi.org/10.1136/archdischild-2017-314506

12. Johnson TN. The problems in scaling adult drug doses to children. Arch Dis Child. 2008;93(3):207-211. https://doi.org/10.1136/adc. 2006.114835

13. Laizure SC, Herring V, Hu Z, Witbrodt K, Parker RB. The role of human carboxylesterases in drug metabolism: have we overlooked their importance? Pharmacotherapy. 2013;33(2):210-222. https://doi. org/10.1002/phar.1194

14. Demers C, Mody A, Teo KK, Mc Kelvie RS. ACE inhibitors in heart failure: what more do we need to know? Am J Cardiovasc Drugs. 2005;5(6):351-359.

15. Tenero D, Boike S, Boyle D, et al. Steady-state pharmacokinetics of carvedilol and its metabolites in patients with congestive heart failure. J Clin Pharm. 2000;40(8):844-853. https://doi.org/10.1177/001488640002000844
16. Rasool MF, Khalil F, Läer S. Predicting stereoselective disposition of carvedilol in adult and pediatric chronic heart failure patients by incorporating pathophysiological changes in organ blood flows—a physiologically based pharmacokinetic approach. Drug Metab Dispos. 2016;44(7):1103-1115. https://doi.org/10.1124/dmd.115.068858

17. Machida M, Watanabe M, Takechi S, Kakinoki S, Nomura A. Measurement of carvedilol in plasma by high-performance liquid chromatography with electrochemical detection. J Chromatogr B. 2003;798(2):187-191. https://doi.org/10.1016/j.jchromb.2003.09.039

18. Gehr TWB, Tenero DM, Boyle DA, Qian Y, Sica DA, Shusterman NH. The pharmacokinetics of carvedilol and its metabolites after single and multiple dose oral administration in patients with hypertension and renal insufficiency. Eur J Clin Pharmacol. 1999;55(4):269-277. https://doi.org/10.1007/s002280050628

19. Läer S, Mir TS, Behn F, et al. Carvedilol therapy in pediatric patients with congestive heart failure: a study investigating clinical and pharmacokinetic parameters. Am Heart J. 2002;143(5):916-922. https://doi.org/10.1067/mhj.2002.121265

20. Sikma MA, van Maarseveen EM, Hunault CC, et al. Unbound plasma, total plasma, and whole-blood tacrolimus pharmacokinetics early after thoracic organ transplantation. Clin Pharmacokinet. 2019;59(6):771-780. https://doi.org/10.1007/s40262-019-00854-1

21. Emmons G, Rowland M. Pharmacokinetic considerations as to when to use dried blood spot sampling. Bioanalysis. 2010;2(11):1791-1796. https://doi.org/10.4155/bio.10.159

22. Gregory GA, Andropoulos DB. Gregory’s Pediatric Anesthesia. 5th ed. Chichester: John Wiley & Sons; 2011.

23. Burckhardt BB, Laer S. Sample preparation and extraction in small sample volumes suitable for pediatric clinical studies: challenges, advances, and experiences of a bioanalytical HPLC-MS/MS/MS method validation using enalapril and enalaprilat. Int J Anal Chem. 2015;2015:1. 796249-11. https://doi.org/10.1155/2015/796249

24. Butnaru A, Popa D-S, Vlase L, Andreica M, Muntean D, Leucuta S. New high-throughput liquid chromatographic tandem mass spectrometry assay for therapeutic drug monitoring of carvedilol in children with congestive heart failure. Revista Română de Medicină de Laborator. 2009;15(2):7-15.

25. den Boer SL, Baart SJ, van der Meulen MH, et al. Parent reports of health-related quality of life and heart failure severity score independently predict outcome in children with dilated cardiomyopathy. Cardiol Young. 2017;27(6):1194-1202. https://doi.org/10.1017/s1047951116002813

26. European Medicines Agency. Guideline Bioanalytical method validation: EMEA/CHMP/EWP/192217/2009 Rev 1 Corr. 2. 2012.

27. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine. Bioanalytical Method Validation Guidance for Industry. 2018.

28. Pagana KD, Pagana TJ, Pagana TN. Mosby’s Diagnostic and Laboratory Test Reference. St. Louis, Missouri, USA: Elsevier Mosby; 2015.

29. Hinderling PH. Red blood cells: a neglected compartment in pharmacokinetics and pharmacodynamics. Pharmacoel Rev. 1997;49(3):279-295.

30. Yu S, Li S, Yang H, Lee F, Wu J-T, Qian MG. A novel liquid chromatography/tandem mass spectrometry based depletion method for measuring red blood cell partitioning of pharmaceutical compounds in drug discovery. Rapid Commun Mass Spectrom. 2005;19(2):250-254. https://doi.org/10.1002/rcm.1777

31. Suessenbach FK, Makowski N, Feickert M, et al. A quality control system for ligand-binding assay of plasma renin activity; proof-of-concept within a pharmacodynamic study. J Pharm Biomed Anal. 2020;181:113090. https://doi.org/10.1016/j.jpba.2019.113090

32. Ali M, Tins J, Burckhardt BB. On behalf of the LENA consortium. Fit-for-purpose quality control system in continuous bioanalysis during long-term pediatric studies. AAPS J. 2019;21(6):104. https://doi.org/10.1208/s12248-019-0375-1

33. Ethical considerations for the clinical trials on medicinal products conducted with the paediatric population: Recommendations of the ad hoc group for the development of implementing guidelines for Directive 2001/20/EC relating to good clinical practice in the conduct of clinical trials on medicinal products for human use. Eur J Health Law. 2008;15(10):223-250. https://doi.org/10.1163/157180908x333228

34. European Medicines Agency. Guideline on the investigation of medicinal products in the term and preterm neonate.

35. Patel DP, Sharma P, Sanyal M, Singhal P, Shrivastav PS. UPLC-MS/MS assay for the simultaneous quantification of carvedilol and its active metabolite 4′-hydroxyphenyl carvedilol in human plasma to support a bioequivalence study in healthy volunteers. Biomed Chromatogr. 2013;27(8):974-986. https://doi.org/10.1002/bmc.2889

36. Gomes NA, Vaidya VV, Pudage AM, Joshi SS, Parekh SA, Tamhankar AV. Application of a sensitive, rapid and validated LC–MS–MS method for the determination of carvedilol in human plasma. Chromatographia. 2009;69(1–2):19-25. https://doi.org/10.1007/s10337-008-0848-3

37. Janjani KC, Kumar Bimireddy BP. Analysis of carvedilol and its metabolite in human plasma using liquid chromatography coupled with tandem mass spectrometry. IJPT. 2017;10(4):256-264. https://doi.org/10.20902/IJPT.2017.10432

38. Kim S-H, Lee SH, Lee HJ. Rapid and sensitive carvedilol assay in human plasma using a high-performance liquid chromatography with mass/mass spectrometer detection employed for a bioequivalence study. AJAC. 2010;1(3):135-143. https://doi.org/10.4236/ajac.2010.10301

39. Li S, Liu G, Jia J, et al. Simultaneous determination of ten antiarrhythmic drugs and a metabolite in human plasma by liquid chromatography–tandem mass spectrometry. J Chromatogr B. 2007;847(2):174-181. https://doi.org/10.1016/j.jchromb.2006.10.013

40. Hålder D, Dan S, Pal MM, et al. LC–MS/MS assay for quantitation of enalapril and enalaprilat in plasma for bioequivalence study in Indian subjects. Future Science OA. 2017;3(1):FS0165. https://doi.org/10.4155/fsa-2016-0071

41. Gu Q, Chen X, Zhong D, Wang Y. Simultaneous determination of enalapril and enalaprilat in human plasma by liquid chromatography–tandem mass spectrometry. J Chromatogr B. 2004;813(1–2):337-342. https://doi.org/10.1016/j.jchromb.2004.09.031

42. Ghosh C, Jain I, Shinde CP, Chakraborty BS. Rapid and sensitive liquid chromatography/tandem mass spectrometry method for simultaneous determination of enalapril and its major metabolite enalaprilat, in human plasma: application to a bioequivalence study. Drug Test Anal. 2012;4(2):94-103. https://doi.org/10.1002/dta.241

43. Ramusovic S, Thielking G, Läer S. Determination of enalapril and enalaprilat in small human serum quantities for pediatric trials by HPLC–tandem mass spectrometry. Biomed Chromatogr. 2012;26(6):697-702. https://doi.org/10.1002/bmc.1716

44. Logoyda L, Kovalenko S, Gafar M, Abdel-Megied AM, Elbarbry FA. A validated LC–MS/MS method for the quantification of amlodipine, bisoprolol, enalapril and enalaprilat—application to pharmacokinetic study in healthy volunteers. Microchem J. 2020;155:104700. https://doi.org/10.1016/j.micron.2020.104700

45. Fujimaki M, Murakoshi Y, Haksusi H. Assay and disposition of carvedilol enantiomers in humans and monkeys: evidence of stereoselective presystemic metabolism. J Pharm Sci. 1990;79(7):568-572. https://doi.org/10.1002/jps.2600790704

46. Poulin P, Theil F-P. Prediction of pharmacokinetics prior to in vivo studies. II. Generic physiologically based pharmacokinetic models of
drug disposition. *J Pharm Sci*. 2002;91(5):1358-1370. https://doi.org/10.1002/jps.10128

47. Arafat T, Awad R, Hamad M, et al. Pharmacokinetics and pharmacodynamics profiles of enalapril maleate in healthy volunteers following determination of enalapril and enalaprilat by two specific enzyme immunoassays. *J Clin Pharm Ther*. 2005;30(4):319-328. https://doi.org/10.1111/j.1365-2710.2005.00646.x

48. Nardotto GHB, Coelho EB, Marques MP, Lanchote VL. Chiral analysis of carvedilol and its metabolites hydroxyphenyl carvedilol and O-desmethyl carvedilol in human plasma by liquid chromatography-tandem mass spectrometry: application to a clinical pharmacokinetic study. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2016;1015–1016:173-180. https://doi.org/10.1016/j.jchromb.2016.02.028

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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