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Research Article

Fit-for-Purpose Quality Control System in Continuous Bioanalysis During Long-Term Pediatric Studies

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Abstract. Pharmacokinetic studies are key to evidence-based pharmacotherapy. The reliability of pharmacokinetic parameters is closely related to the quality of bioanalytical data. Bioanalytical method validation is fully described by regulatory guidelines; however, it is conducted just once. To ensure reliability and comparability of clinical data, appropriate quality control systems must be enforced to monitor post-validation bioanalytical runs. While single bioanalytical run evaluation is described in international guidelines, somehow, the long-term reproducibility of the bioanalytical method is unattended; it becomes pivotal with the involvement of pediatric population. Therefore, a customized quality control system was developed that addresses regulatory requirements and encompasses the specific demands of pediatric research. It consisted of continuous multi-parameter assessment, including calibration curves, quality control samples, incurred sample reanalysis, and internal standard data. The recommendations provided by the guidelines were combined with the additional Westgard rules, statistical evaluation, and graphical observations. The applicability of the developed quality control system was investigated by using data from three pediatric clinical trials, where the system was able to identify 16% of all analytical runs as invalid. Using a pooled standard deviation provided a better estimate of long-term reproducibility by calculating the %CV, which ranged from 3.6 to 10.3% at all quality control levels. Irrespective of the difficulties encountered owing to vulnerable pediatric populations, the incurred sample reanalysis fulfilled the regulatory requirement of at least 67%. This quality control approach ensured reliable and comparable results over a whole 31-month duration in relation to pediatric studies.

KEY WORDS: incurred sample reanalysis (ISR); in-study bioanalytical method validation; LC-MS/MS; pediatric clinical trial; quality control.

INTRODUCTION

In 1990, the first bioanalytical workshop was held by the American Association of Pharmaceutical Scientists (AAPS)/U.S. Food and Drug Administration (FDA) (1). Later in 2001, the first edition of the Bioanalytical Method Validation (BMV) guidance was issued by the FDA. Over the last decade, a consensus has been reached surrounding numerous specifications and recommendations offered by the guidelines regarding the conduct of method validation and its routine clinical application. BMV is often performed once against the criteria outlined in regulatory guidelines for key performance parameters, including accuracy, precision, reproducibility, and stability (2,3). Once validated, the bioanalytical methods are repeatedly applied to create pharmacokinetic (PK) and pharmacodynamics (PD) data for making decisions during drug development. Therefore, ensuring the highest quality of bioanalytical methods is of utmost importance.

The structured guidance for assessing the data quality of a single analytical run is available in terms of quality control (QC) checks. However, monitoring of associated analytical runs in clinical studies is important to ensure the comparability of study data, even if it comes from different analytical runs over different time spans. This would also facilitate the evaluation of the least-attended aspect of between-run applicability of the bioanalytical method. Additionally, the guidelines suggested incurred sample reanalysis (ISR) as one of the tools to determine the long-term reproducibility of the validated bioanalytical method; however, this area is still under discussion amongst experts (4).

As in clinical bioanalysis, long-term reproducibility and consistent results across closely related multiple studies (e.g., main study plus follow-up study) are required. Assessing the reproducibility across multiple clinical studies makes certain of the reliability and quality of the reported unknown

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concentrations from the study samples for further PK and PD interpretations (5). There is no direct method to assess the integrity of the determined concentrations from unknown samples except to establish the reliability and reproducibility of the accompanying bioanalytical data from the calibration curve (CC), QC checks, ISR, and internal standard (IS) (6–8).

In the pediatric population, comprehensive PK and PD data sets are lacking. Subsequently, clinical studies are highly necessary for evidence-based pharmacotherapy in children (9). Based on, e.g., ethical constraints, comprehensive studies in vulnerable populations are often only performed once. These data sets will form the basis for evidence-based pharmacotherapy. It is therefore of utmost importance that rigorous clinical data generated during pediatric clinical trials must be reliable. Appropriate reliability of clinical data can be ensured by the implementation of a suitable analytical quality control system. While international guideline suggestions for quality control of essential parameters (e.g., CC, QC checks, etc.) are limited to single batches, a reliable clinical data set can only be generated if the quality is monitored across batches or studies. Moreover, the peculiarity of pediatric studies must be reflected within such a quality control system.

An institutionalized multi-parameter quality control system utilizing descriptive statistical and graphical techniques for evaluating the accuracy and reproducibility of accompanied bioanalytical data was applied. The validity of the individual runs was determined immediately after each run, while the between-run performance was performed as a post hoc analysis. The applicability of the established quality control system was investigated by monitoring bioanalysis within the EU-funded, academically driven "Labelling of Enalapril from Neonates up to Adolescents" project (LENA). This project aimed to assess a novel child-appropriate dosage form in children suffering from heart failure comprised of PK and PD bioanalysis of three parallel clinical studies across all pediatric age groups (0–12 years). Besides method validation and system suitability tests, this approach served as an additional tool for making certain the reliable and comparable results of unknown pediatric samples over a whole 31-month duration in three closely related pediatric studies.

METHODS

In-Study Sample Analysis

Bioanalysis of clinical study samples was performed with a fully validated bioanalytical method according to the European Medicine Agency (EMA) and FDA bioanalytical method validation guidelines (2,3). European Pharmacopoeia Reference Standards of the drug, enalapril, and its active metabolite, enalaprilat (European Directorate for the Quality of Medicines, France) were used. Benazepril hydrochloride (Sigma-Aldrich Seelze, Germany) acted as an IS. The stability of the stock solutions in methanol was 6 months as previously established during validation. Solid-phase extraction (Oasis MAX 96-well plates, Waters Eschborn, Germany was implemented for sample preparation. The measurements were conducted with high-performance liquid chromatography (Modular 10-series, Shimadzu Duisburg, Germany) coupled with triple quadrupole tandem mass spectrometry (LC-MS/MS) in positive ionization mode (API 2000, AB Sciex Concord, Canada). A gradient elution was used consisting of methanol and water (both featuring 1% formic acid and 2 mM ammonium formate) as the mobile phase with a total run time of 7 min. An XBridge® BEH C18 3.5 μm column (3.0 mm × 150 mm) was employed for chromatographic separation at a column temperature of 40°C. Following multiple reaction-monitoring (MRM) transitions were set for quantification of enalapril and enalaprilat: 377.2 $m/z \rightarrow 234.2$ m/z and 349.1 $m/z \rightarrow 206.1$ m/z, respectively.

The initial full validation proved accuracy (determined by relative error) within guideline criteria ± 15% (± 20% at LLOQ) for both analytes. The between-run precision ranged from 2.2 to 5.0% for enalapril and from 4.9 to 18.0% for enalaprilat. Additionally, a confirmatory partial validation was performed for accuracy and precision using four different runs in 2017 while making no change to assay. The obtained mean accuracy values of enalapril ranged from − 3.9 to 8.4% and for enalaprilat from − 12.0 to 6.4% (relative error) at all levels. Within-run precision varied from 4.7 to 7.5% for enalapril and from 2.6 to 10.3% for enalaprilat. Between-run precision was as followed: 5.0 to 9.5% for enalapril and 4.3 to 13.4% for enalaprilat. Moreover, internal standard normalized relative matrix effect was evaluated using seven donors (age = 29–86 years) of both gender at lower (enalapril 0.39 ng/mL, enalaprilat: 0.35 ng/mL) and high concentration (enalpril 200 ng/mL, enalaprilat 180 ng/mL). The coefficient of variation (%CV) between donors ranged from 1.87 to 12.56% for both analytes at both levels. The absolute matrix effect for IS (benazepril) varied by − 7.2%CV. Other parameters including selectivity, recovery and stability were also evaluated (10).

Good Clinical Laboratory Practices

Good clinical laboratory practice (GCLP) compliant environment facilitates the generation of data in high quality within clinical trials. In the academia-driven LENA project, such a GCLP-compliant environment was established. This included GCLP trainings for laboratory as well as medical personnel (11), establishment of a standard operating procedure (SOP) system, traceable raw data generation, computerized system validation, and guideline compliant reporting to make clinical outcomes reliable, reproducible, and auditable. A tailored laboratory information management system (LIMS) was also implemented as part of GCLP for receiving, processing, storage, specimen detail, and traceability of the data. The whole GCLP system was successfully audited by an external auditor.

At a glance, the conduct of sample collection and bioanalysis was conducted as follows: At first, ready-to-use pouches with unique pre-labeled consumables (collection tubes, etc.) were sent to the respective clinical sites for sample collection. After sample collection and documented on-ward sample preparation by trained staff, samples were shipped back to the bioanalytical laboratory under tracked temperature conditions (− 80°C) according to Good Distribution Practice. The received samples were kept at − 80°C before and after the analysis using appropriate monitoring systems. All bioanalytical determinations were conducted and

documented in four-eye principle. Once a measured batch was declared valid by the analysts, an additional double check was performed by using a validated in-house software that confirmed the compliance with all quality specifications on CC, QCs, blank, etc. The overall final release of the data was in the responsibility of the head of bioanalytical team.

Established Quality Control System for Bioanalysis

An owned and customized institutionalized quality control system was enrolled. This established internal quality control system was comprised of evaluating CC data, QC samples, and IS response data from each analytical batch. Moreover, the ISR evaluation of randomly selected study samples to further establish the quality of the data reported from unknown concentrations was also included. Bracketing the specifications from the EMA and FDA bioanalytical guideline approaches within the established bioanalytical quality control systems and addressing the specific demands in pediatric research constituted the fit-for-purpose control system. The system suitability and performance qualification utilized before the study analysis is conducted to assure whether the system is suitable for the purpose required. This quality control system lies within the study sample analysis and evaluates the post-validation analysis which in turn ensures the data quality. The control system was applied within the bioanalysis of the three pediatric studies of the LENA project ("Labelling enalapril from neonates up to adolescents") with the trial registration numbers: EudraCT 2015-002335-17, EudraCT 2015-002396-18, EudraCT 2015-002397-21. Written informed consent from parent(s)/legal representative and assent from the patient according to national legislation and as far as achievable from the child was obtained. The subsequent sections deal with the quality control of the individual parameter part of the quality control system.

Calibration Curve

CC consistency and accuracy were considered the first component of the adapted quality control process.

All calibration standards were purified and determined as described in the "In-Study Sample Analysis" section. Based on a previously conducted validation, a linearity range from 0.195 to 200 ng/mL and 0.175 to 188 ng/mL was established for enalapril and enalaprilat, respectively. Eleven calibration standards were used to construct a CC by plotting x_i *vs.* y_i, where x_i represents the analyte concentration ratio (analyte concentration/internal standard concentration) and y_i value represents an instrumental response ratio (peak area analyte/peak area internal standard). Three replicates ($n = 3$) were measured for each calibration level. Linear regression ($y = b + mx$) with a weighting factor $\left(w_i = \frac{1}{x_i^2}\right)$ was applied to determine regression parameters. Additionally, the variability in terms of coefficient of variation (%CV) in regression parameters (slope and intercept) was calculated for enalapril and enalaprilat from each run over the whole bioanalytical period. The coefficient of correlation (r value) was employed to express the dependence of two variables having a linear relationship. The CC with an r value ≥ 0.995 were considered linear (12). The validity of the CC was defined as given in the international EMA and FDA bioanalytical guidelines (2,3). The deviation was assessed by calculating the relative error (%RE) using the following expression (13):

$$\%\mathrm{RE} = \frac{\mathrm{Con_{found}} - \mathrm{Con_{actual}}}{\mathrm{Con_{actual}}} \times 100 \quad (1)$$

where $\mathrm{Con_{actual}}$ is the nominal concentration and $\mathrm{Con_{found}}$ is the predicted concentration or back-calculated concentration from the CC.

Quality Control Samples

The CC is commonly measured once during routine clinical sample analysis; however, QC samples with known concentration are distributed equally as a measure for assay performance within routine clinical sample analysis.

Freshly prepared QC samples were implemented to cover the entire expected concentration range of the unknown samples for both analytes of interest. EMA guidelines suggest to include QC samples at three levels in duplicate or 5% of the study sample, or whichever number is high. However, stricter criteria were implemented by using more levels and amount of the QC samples to monitor accuracy and precision in equidistance manner across the whole calibration range. In the applied 96-well approach, at least 10 single QCs, whereby five different QC levels (upper limit of quantification (ULOQ) [200 ng/mL enalapril, 188 ng/mL enalaprilat], high [100 ng/mL enalapril, 94 ng/mL enalaprilat], medium [25 ng/mL enalapril, 23.5 ng/mL enalaprilat], and low [3.13 ng/mL enalapril, 2.93 ng/mL enalaprilat], roughly three times the lower limit of quantification ($3 \times$ LLOQ) [0.78 ng/mL enalapril, 0.73 ng/mL enalaprilat]), were determined in duplicate. Passed criteria for all QC levels were followed as stated in the international guidelines (2,3). The accuracy of the QCs was expressed as %RE of their back-calculated concentrations. Please refer to Eq. 1 for further details.

The long-term reproducibility was determined in terms of the %CV. The mean pooled standard deviation ($\mathrm{S_{pooled}}$) was calculated for each QC level for both analytes to establish the %CV by using the following expression (14):

$$\mathrm{S_{pooled}} = \sqrt{\frac{(n_1-1){s_1}^2 + (n_2-1){s_2}^2 + \ldots + (n_k-1){s_k}^2}{n_1 + n_2 + \ldots + n_k - K}} \quad (2)$$

where $n_k - K$ is the degree of freedom and s_k is the standard deviation of the measured concentration at each QC level for K_{th} time within 6 months for both analytes.

Additionally, modified Westgard rules as multiple statistical rules were combined with Shewhart control charts (5,15). Westgard rules can detect any systematic and random variation in contrast to the "4–6–X" rule used for batch-wise acceptance, which refers to at least 67% of the QC samples should be within ± X% (where $X = \pm 15\%$). However, the strict application of Westgard rules may lead to the rejection of acceptable data as per current guideline recommendations. Therefore, the following decision rules derived from the Westgard rules were applied: (1) Consecutive two points

exceeds the action limit (mean ± 3sd); (2) consecutive four points are outside warning limit (mean ± 2sd); and (3) consecutive 11 points are on the same side of the mean to evaluate the control charts (5). The mean value was obtained from the observed concentration over 6-month intervals depending upon the established stability of the stock solution.

Incurred Sample Reanalysis (ISR)

The third component of the established quality control system was the evaluation of the ISR. The non-pooled samples from the dosed subjects (incurred samples) were used to demonstrate the reproducibility of the bioanalytical method on a different occasion in addition to QC samples. The ISR represents an important measure of accuracy and comparability within pediatric studies as the pediatric serum was not considered during method validation owing to ethical constraints, e.g., matrix effect was investigated with the human serum of adults. In particular, the ongoing maturation in childhood would have necessitated many pediatric matrix samples of several pediatric age groups to assess the possible effect of the difference in protein-binding, concomitant medication, matrix composition, and changing metabolic behavior.

Incurred sample analysis was performed blinded and all samples were selected on a random basis. As such, the goal was to reanalyze 7.5% of the total samples within ISR. The %difference of the reanalyzed incurred samples was calculated by using expression given in regulatory guidelines (2,3). The %difference of at least 66.7% of the total reanalyzed incurred samples within ± 20% was considered as acceptable to establish method reproducibility. The following expression was employed to calculate the %cumulative ISR (16):

$$\%\text{Cumulative ISR} = \frac{\text{Number of ISR pairs with absolute \%difference} \le 20\%}{\text{Total number of ISR pairs}} \times 100 \quad (3)$$

Internal Standard Response

An IS is normally employed during the analysis to compensate any discrepancies that arise during sample preparation, actual injection volumes, and instrument performance owing to the matrix effect, specifically in LC-MS/MS (7). Both the FDA and EMA guidelines suggest monitoring the IS response variation (4).

The IS response check was adapted as an additional parameter in the quality assessment system to establish the reliability of the results of unknown samples from the LENA trials. Benazepril hydrochloride was used as IS at a concentration of 80 ng/mL, which is a structural analogue to enalapril and enalaprilat. As deuterated IS was not commercially available at a time point of method validation, the structurally related compound benazepril was applied. The IS was added to all known calibration standards, QCs, and study samples at equal concentration prior to the sample extraction process. Based on the obtained mean IS response of the known standards (CC and QCs), the detected IS response of each samples should vary between ± 3sd of the mean of known standards (CC and QCs). Deviations from this rule were only acceptable in case of justified reasons (15). Additionally, %CV from the first to the last injection within each analytical run for IS response was calculated for information.

Integration of the Studied Parameters

Beside single observation per parameter, the data was monitored for any trend in observed outliers of CC, QCs, unmatched ISR, and deviation in IS. Therefore, all invalid runs were plotted using scattered matrix plots to identify any impact of trend amongst studied parameters.

RESULTS

Calibration Curve

Thirty-eight CCs were constructed per analyte of interest (enalapril and enalaprilat) during the whole duration of bioanalysis from February 2016 to August 2018. For all valid CCs, a total of 939 and 919 calibration standards were measured for enalapril and enalaprilat, respectively. Within those measured calibration standards, 30 and 67 outliers were detected against the outlined criteria for enalapril and enalaprilat, respectively, resulting in 97% and 93% of total calibration standards within the guideline specific criteria for both analytes. This low number of outliers confirmed the goodness of fit of the weighted $\left(w_i = \frac{1}{x_i^2}\right)$ linear regression model. Further, only two and six CCs were reconstructed with a narrowed linearity range for enalapril and enalaprilat, respectively, owing to outliers at the LLOQ and ULOQ. Out of total 38 analytical runs for each analyte, four runs for enalapril and five runs for enalaprilat were considered invalid owing to the inaccuracy of more than 50% of calibration standards. Amongst these invalid runs, different standard levels were effected that subsequently does not allow to identify any trend. The overall pass rate for valid analytical runs was 84% with not more than two consecutive invalid runs. The slope varied from 1.0841 to 4.3641 with 22.69%CV for enalapril and 0.1035 to 0.7581 for enalaprilat with 39.93%CV during 31 months. Shapiro Wilk test showed slope values were normally distributed for enalapril ($p = 0.127$) and enalaprilat ($p = 0.156$). More variation was observed in intercept value between the runs. However, this variation caused no impact on the linearity of the CC (8). Slope and intercept data is represented in Appendix.

Quality Control Samples

Investigation of QC samples resulted in the exclusion of an additional two enalapril run. In both runs, 50% of lower QC (3 × LLOQ = 0.76 ng/mL) failed to pass. One invalid run for enalaprilat was found to be associated with only 58% QC passed (67% required according to the guideline). Here, also the QC level at 3 × LLOQ caused the invalidity (less than 50% of standards within the accuracy limits). Within 32 valid analytical runs, based upon the CC and the QC sample

acceptance criteria, on average, 94% of enalapril QC samples were within guideline limits [(ULOQ, 200 ng/mL = 94%), (high, 100 ng/mL = 90%), (medium, 25 ng/mL = 94%), (low, 3.13 ng/mL = 96%), (3 × LLOQ, 0.78 ng/mL = 95%)]. Similarly, 89% of all enalaprilat QC samples were within the limits with individual success rates: [(ULOQ, 180 ng/mL = 92%), (high, 90 ng/mL = 92%), (medium, 22.5 ng/mL = 92%), (low, 2.81 ng/mL = 91%), (3 × LLOQ, 0.70 ng/mL = 78%)]. These results exhibited strong agreement with the guidelines recommended in terms of acceptance criteria of at least 67% passing QC checks. The distribution of the observed back-calculated concentrations for the QC samples at all levels for both analytes was observed using marginal histograms (Fig. 1). The upper specification limit (USL) and lower specification limit (LSL) correspond to the target range recommended by the guidelines (± 15% of the nominal concentration). The gray-shaded area represents the exact number of 67% + QC control samples. At all levels, this gray area did not surpass the USL or LSL, thereby indicating guideline compliance. The box plots were employed to observe the variation in %RE for back-calculated concentration at all QC levels from the valid runs (Fig. 2). The box plot showed that the mean and median was centered at all QC levels with equal variation in the upper and lower quantiles (except for the LLOQ of enalaprilat with a slightly higher variability). The %CV was employed to observe the long-term reproducibility and it ranged from 3.6 to 10.6% for enalapril and 5.7 to 10.4% for enalaprilat (Table I). New stock solution was regularly prepared every 6 months due to stability reasons and the resulting nominal concentration adopted according to the exact weighting of drug analytes. Depending upon the total number of valid and invalid analytical runs within these 6-month periods, the number of analysis differed as only valid runs were considered. Figure 3 depicted the trend analysis charts for enalapril and enalaprilat. The plotted graphs were investigated against the pre-defined derived Westgard rules and it was observed that no violation of these rules was found, hence providing more confidence in terms of the method applicability over the entire duration of the bioanalysis.

Incurred Sample Reanalysis

Based upon the pre-calculated estimated sample size for the LENA trials in accordance with the study protocols, almost 7.5% of incurred samples were reanalyzed for enalapril and enalaprilat. This calculation took into account that not all scheduled samples might become available because of the missing sample point and inappropriate sample volume.

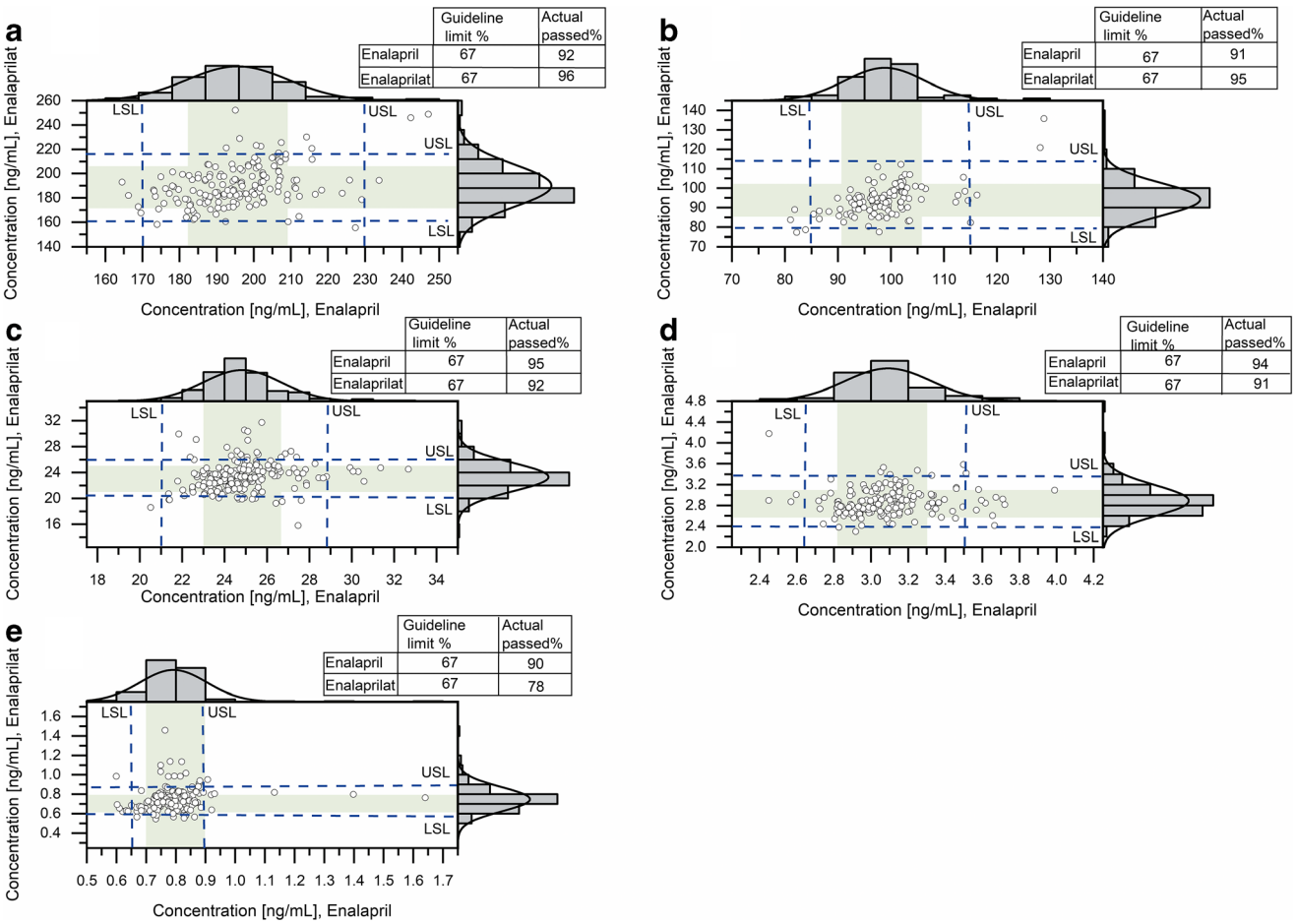

Fig. 1. Marginal histograms for all QC levels for enalapril and enalaprilat. LSL, lower specification limit (− 15% of the nominal concentration); USL, upper specification limit (+ 15% of the nominal concentration); gray-shaded area, one standard deviation (1SD) of the mean of the observed concentration. Circle indicate the back calculated concentrations. Dotted lines represent the specification limits of ±15%.

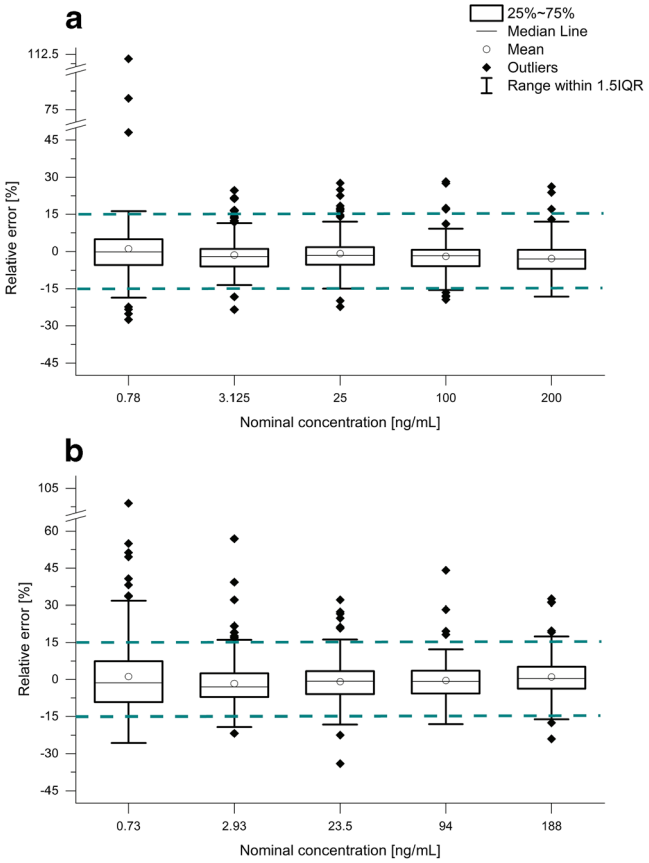

Fig. 2. Box plots for %RE of the back-calculated concentration of the QC samples for enalpril and enalaprilat from all valid analytical runs. Any observation outside the range Q1–1.5 × IQR and Q3 + 1.5 × IQR was considered as an outlier; **a** enalapril, **b** enalaprilat

In total, 93 and 103 randomly selected incurred samples were reanalyzed for enalapril and enalaprilat, respectively. Enalapril (71%) and enalaprilat (67%) were within predefined guideline criteria of ± 20% for at least 66.7% of the total reanalyzed samples showing the reproducibility of the applied method. The Bland-Altman plot revealed that randomly selected sample concentrations ranged between 0 ng/mL (below LLOQ values reported as zero) to 37 ng/mL for enalapril and 0–45 ng/mL for enalaprilat, respectively. The contribution of each ISR pair towards the overall performance of at least 67% ISR was shown by using %cumulative graphs (Fig. 4).

Internal Standard Response

Internal standard (IS) response variation through the analytical run can be used to assess the validity of the results and hence the acceptability of the unknown results. High robustness of the internal standard response was observed during the whole study period of February 2016 to August 2018 indicated by acceptable %CV values of 3.0 to 20.9 for all analytical runs. Only for two runs, the %CV values of 63.5 and 44.1 were detected. However, as an adequate signal to noise ratio was maintained for all samples; therefore, a negative impact on reliable quantification of unknown samples was excluded (15). Subsequently, only a few individuals IS response per analytical

Table I. Long-Term Reproducibility of all Conducted Bioanalytical Runs (February 2016 until August 2018) in Terms of %CV (Coefficient of Variation) of Enalapril and Enalaprilat

	Solution 1	Solution 2	Solution 3	Solution 4	Solution 5
Enalapril (ng/mL)					
200	187.32	183.33	172.15	192.27	204.76
	194.38	195.18	188.68	199.79	202.53
	194.06	208.89	200.20	183.50	206.49
	199.30	189.27	187.96	205.92	196.11
	189.19	220.66	205.28	181.79	186.89
				197.99	188.40
				201.36	209.21
S_{pooled}			10.30		
Mean			195.46		
%CV			5.27		
100	104.36	86.92	95.08	99.65	
	99.29	92.87	97.18	100.91	
	97.95	99.74	98.68	101.73	
	95.41	96.60	103.47	100.65	
		97.70	96.59	99.87	
		101.32	97.74		
S_{pooled}			3.58		
Mean			98.43		
%CV			3.64		
25	25.14	24.13	24.03	23.40	28.12
	23.98	25.84	24.53	24.39	25.23
	23.88	25.16	25.01	25.56	25.46
	24.76	24.74	24.33	25.75	24.97
	25.23	24.57	24.60	25.52	23.20
	23.74		23.31	23.62	24.72
				23.61	26.60
					25.33
S_{pooled}			0.99		
Mean			24.81		
%CV			3.99		
3.13	3.44	2.98	3.07	2.86	3.22
	3.12	3.33	3.01	3.02	3.07
	2.94	3.12	3.09	3.20	3.20
	3.04	3.08	2.96	3.18	3.12
	3.35	2.96	3.19	2.82	3.02
	3.13		2.99	3.27	3.22
				3.02	3.25
					3.14
S_{pooled}			0.14		
Mean			3.10		
%CV			4.49		
0.78	0.84	1.15*	0.76	0.87	
	0.82	0.82	0.79	0.75	
	0.76	0.73	0.80	0.81	
	0.85	0.75	0.82	0.77	
		0.77	0.77	0.81	
		0.74	0.75	0.81	
			0.73	0.78	
				0.77	
S_{pooled}			0.09		
Mean			0.80		
%CV			10.63		
Enalaprilat (ng/mL)					
188	167.63	182.24	171.75	187.68	207.36
	177.19	179.18	205.51	194.20	208.94
	180.11	197.66	186.91	180.17	187.18
	183.84	219.11*	185.10	192.33	187.51
	181.76		182.05	178.55	193.11

Table I. (continued)

	Solution 1	Solution 2	Solution 3	Solution 4	Solution 5
	171.46		199.40	188.67	221.54
				196.84	219.27
S_{pooled}			11.71		
Mean			190.57		
%CV			6.14		
94	87.83	83.43	90.94	98.58	
	108.24*	86.97	92.39	99.96	
	88.06	97.10	95.81	91.53	
		87.61	95.08	94.88	
		90.77	94.75	97.95	
		96.64	91.33		
		96.86	97.34		
S_{pooled}			5.49		
Mean			93.82		
%CV			5.86		
23.5	21.17	22.10	21.62	22.04	27.43
	23.35	22.16	23.19	23.03	24.49
	20.99	26.30	23.79	24.13	23.09
	22.28	22.46	24.72	22.89	23.96
	22.66		22.13	23.03	22.92
	22.59		23.31	21.62	25.78
			23.31	22.43	26.93
					23.08
S_{pooled}			1.35		
Mean			23.27		
%CV			5.81		
2.87	2.67	2.74	2.87	2.66	3.08
	3.00	2.72	2.68	2.83	2.99
	2.73	2.85	3.00	2.91	2.99
	2.72	2.81	2.95	2.80	3.27
	2.88		2.63	2.62	2.81
	2.77		2.96	3.14	3.17
			2.77	2.85	3.10
					3.55*
S_{pooled}			0.16		
Mean			2.89		
%CV			5.70		
0.73	0.75	0.93*	0.80	0.78	
	0.69	0.80	0.72	0.75	
	0.92*	0.74	0.73	0.66	
		0.82	0.66	0.77	
		0.68	0.70	0.83	
		0.73	0.78	0.79	
		0.66	0.62	0.79	
				0.84	
S_{pooled}			0.08		
Mean			0.75		
%CV			10.41		

SD, standard deviation; *Spooled*, pooled standard deviation. Number of enclosed runs differed within each 6-month period as only valid analytical run were enclosed. *Single values outside the acceptance limit (± 15%)

run that exceeded the maximum limit of ± 3sd of the run-specific mean were found indicating random errors.

Integration of the Studied Parameters

On the basis of the available data from six invalid runs, the plotted data adumbrated that runs with more outliers in CC were associated with decrease passed QC (%). Increase in slope might be associated with increased peak area ratio of the calibration curve. A substantial alteration in IS response was found to be indirectly related to peak area ratio and slope. However, the small number of invalid runs constrained any reliable conclusion on definitive inferences while evaluating this data set.

DISCUSSION

A fit-for-purpose quality control system in pediatric research was successfully developed. It addresses current bioanalytical requirements advised in international guidelines (EMA, FDA) and also encompasses the specific situations in pediatric research current insufficiently reflected by guidelines. This quality control system analyzed a multi-parameter approach and their relationship to each other during three pediatric clinical studies within the EU-funded LENA project. The customized system monitored and ensured reliable assay performance over the whole period of bioanalysis. This developed in-house quality control system applies primarily for analysis using LC-MS/MS. For immunoassay or bioassay, different criteria are defined in current bioanalytical guidelines and should be implemented into separate quality control systems accordingly.

As PK data obtained in adults can only be very moderately extrapolated to the vulnerable pediatric population, it became obvious that clinical studies in the pediatric population are highly necessary for rational and safe drug therapy. However, these studies are often lacking or being discontinued (9). Unfortunately, clinical studies are commonly only conducted once in the pediatric population owing to, e.g., ethical constraints. It is therefore of utmost importance to generate high-quality data in the limited amount of conducted pediatric studies as this data forms the bases for evidence-based pharmacotherapy in this population. From a bioanalytical point of view, comprehensive method validation is a regulatory prerequisite for reliable data generation, and subsequently for method application in clinical studies. Nevertheless, such validations are often performed only once and do not automatically ensure reliability over the entire period of a clinical study. Additionally, blood samples of healthy or diseased pediatrics, especially at very young ages, are often unavailable for validation based on ethical constraints and can be taken into consideration during the earliest analytical runs. Although EMA and FDA outlined the specifications for individual bioanalytical runs, monitoring comparability of study data in continuous bioanalysis is unattended. The developed control system was meant to overcome these current hurdles, especially in pediatric research where "en bloc" bioanalysis is often impossible because of the long recruitment periods.

Well-established regulatory requirements regarding the suitability of CC and within-run QCs were directly incorporated in the present control system. However, long-term comparability indicates reliability in the case of the bioanalytical data. Therefore, the control system was amended concerning between-run QCs as well as the long-term performance of IS and ISR.

With regard to the monitoring of between-run quality control performance and comparability, multiple statistical rules, known as Westgard rules, were implemented. These have sought to assess any

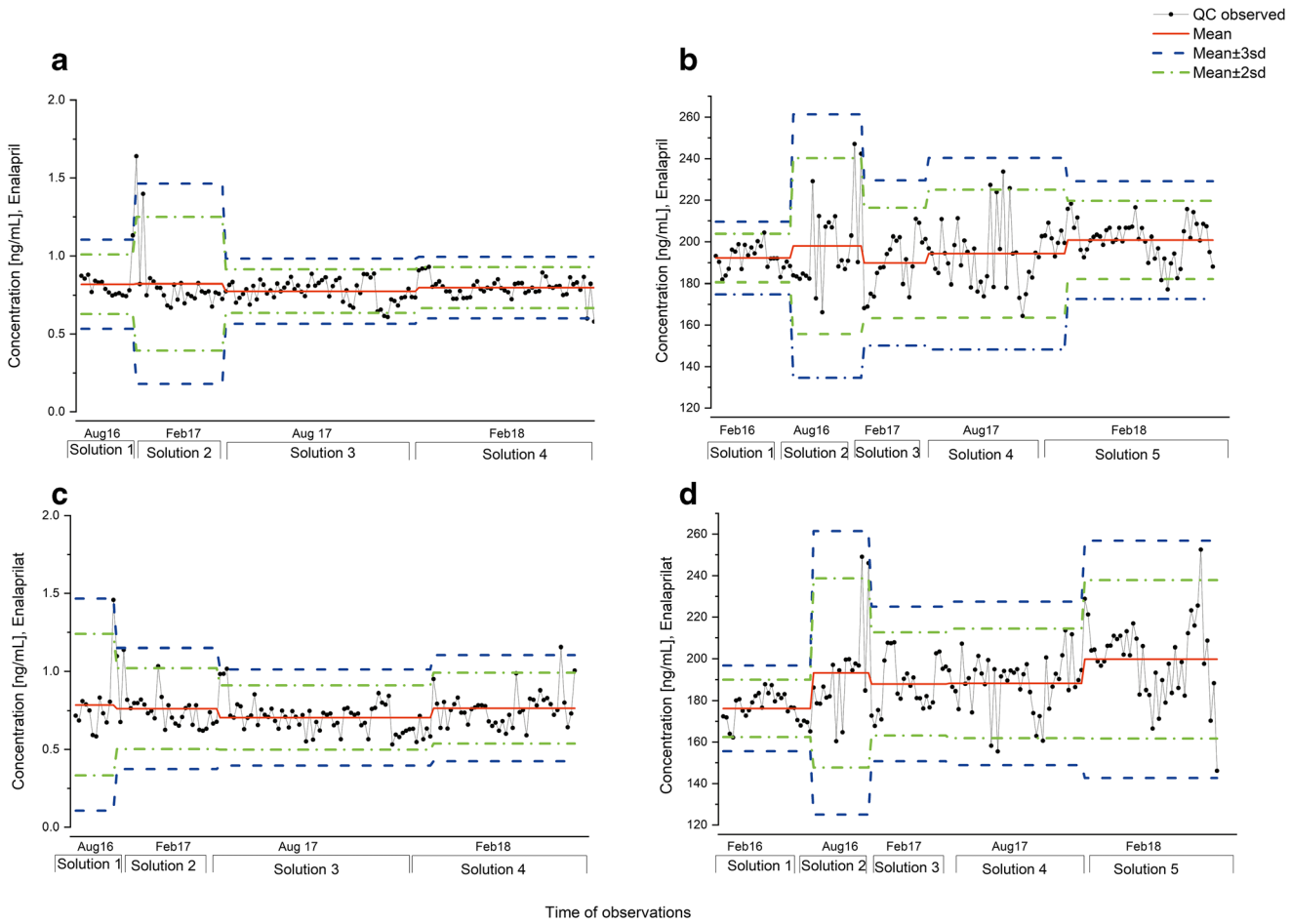

Fig. 3. Process control charts for QC samples for enalpril and enalaprilat at 3 × LLOQ and ULOQ. **a** Enalapril (3 × LLOQ): 0.78 ng/mL; **b** enalapril (ULOQ): 200 ng/mL); **c** enalaprilat 3 × LLOQ = 0.70 ng/mL; **d** enalaprilat (ULOQ): 188 ng/mL

systematic and random variation between-run performance in contrast to the guideline approach by the "4–6–*X* rule" (at least 67% of the data should be within ± *X*%) focusing on single analytical runs. However, the strict application of all Westgard rules may lead to the rejection of the acceptable data as per current guideline recommendations. Therefore, modified rules based on suggestions from Bruijnsvoort *et al.* were applied (5). No violation of the modified Westgard rules was observed across any bioanalytical runs of the LENA project, thereby indicating no systemic pattern or random variation in the applied bioanalytical method.

In pediatric research, many clinical studies last for several years owing to, e.g., poor recruitment. Therefore, long-term reproducibility of the method is important as the stability of analytes of interest is often limited and needing continuous bioanalysis. The reliable long-term performance was assessed via the concept of pooled standard deviation, which provided a strong estimate of variation along with the whole duration of the analysis characterized by coefficients of variation (%CV) ranging between 3.6 to 10.6% for enalapril and 5.7 to 10.4% for enalaprilat. Moreover, no remarkable variation amongst the different QC levels was observed by comparing the relative error over time and level. The latter proved that no QC check differed substantially or level tended towards inaccuracy over time. Overall, evaluation of within and between-run QC checks supported reliable bioanalysis and confidence for the measured unknown concentrations over a long period of time.

The actual matrix of unknown samples could substantially impact precise and accurate determination. Usually, the impact of the sample matrix is investigated during method validation. However, ethical constraints impede the investigation of a pediatric sample matrix as it may potentially differ from adults. Nevertheless, the maturation of the pediatric organism, unknown metabolites, concomitant medications, and changing protein-binding reflects certain reasons for possible imprecisions during bioanalysis within clinical studies (17,18). The use of ISR of pediatric samples was therefore implemented into the bioanalytical quality control system to evaluate the impact of the actual matrix on reproducibility and subsequently on accuracy and precision. Sample volume restrictions and preferable measurement of the PK primary endpoint and secondary PD endpoints using the same sample volume restricted the reanalysis of the incurred samples. Almost 7.5% of the incurred sample was reanalyzed for both analytes. Although the FDA asks for 10% of reanalyzed samples for the first 1000 samples, it reached 7.5% of ISR with a total of 1250 unknown samples within these pediatric trials, thereby appearing sufficient bearing in mind ethical constraints for the sample volume involving vulnerable pediatric population. Rudzki *et al.* (19) have demonstrated that reproducibility of the assay is not exclusively dependent on ISR fixed-rate (e.g., 10% for 1000 samples and 5% for subsequent samples) as currently recommended in regulatory

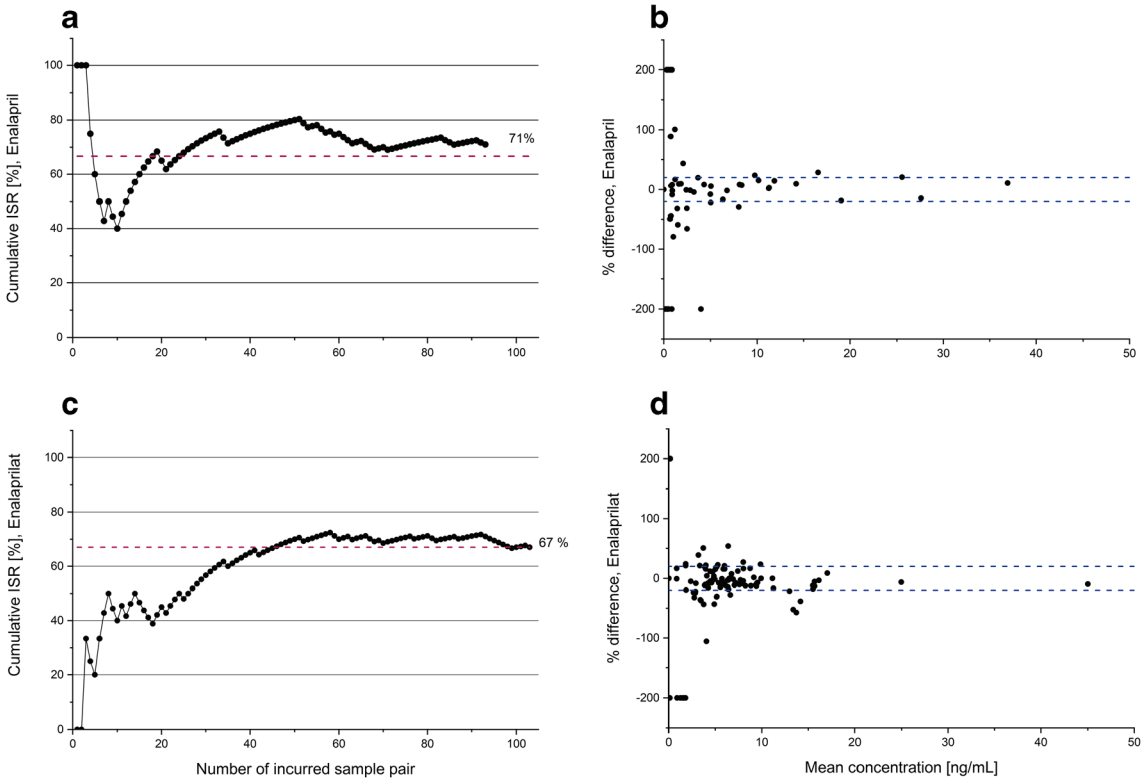

Fig. 4. %Cumulative plot and %difference plot for enalapril and enalaprilat incurred sample reanalysis. **a** %cumulative plot for enalapril; **b** %difference plot for enalapril; **c** %cumulative plot for enalaprilat; **d** %difference plot for enalapril. Blue-dashed upper and lower lines: ± 15% limit; gray-dashed line: 66.7%; calculation of %difference: Repeat − Original/Mean × 100. The different total number of valid enalapril and enalaprilat ISR pairs is due to the fact that the validity of the two substances per analytical run was independently determined. Thus, for each analytical run, a valid evaluation could possibly only be made for one substance, so that only these ISR pairs were included in the evaluation. The ISR pair are plotted according sequence of reanalysis. Additionally, each pair does not reflect the same sample for enalapril and enalaprilat

guidelines. They have proposed to use fixed numbers of ISR pairs (e.g., 30) regardless of total sample size as it sufficiently allows to check the reproducibility and non-reproducibility of the assay. Bridging both current suggestions together, here evaluated 100 ISR pairs (7.5%) sufficiently allowed for appropriate assessment of reproducibility.

The FDA recommendations on ISR suggested using samples at/close to the maximum concentration (C_{max}) and near the end of the elimination phase (20). This condition was difficult to comply with during the pediatric LENA project for several reasons. First, bioanalysis of enalapril and enalaprilat was conducted blinded and randomized. Second, the pro-drug, enalapril, and its active metabolite, enalaprilat, are characterized by different PK parameters (in adults: t_{max} at roughly 1 h *vs.* approximately 4 h) and subsequently would necessitate more samples to be analyzed to address the regulatory conditions appropriately.

In addition to above restrictions, the bioanalysis was performed completely independent of PK evaluation. The latter was started after all study samples had been successfully analyzed. Thus, there was no feedback from the PK analysis to identify samples at the C_{max} or elimination phase of each patient. Therefore, recommendations to select only samples in the range of three times LLOQ and 80% of ULOQ could not be realized. Thus, performing this sample selection for reanalysis was a worst case scenario. Furthermore, it should be kept in mind that the study population is very heterogeneous due to the maturation of the organism and that no uniform C_{max} and elimination concentrations, such as these might be expected from adult studies, could be derived. There is no detailed information available about the C_{max} and concentration at the elimination phase of enalapril and its active metabolite in pediatrics. Different studies revealed enalaprilat serum concentration varying between 0.8 and 12.7 ng/mL (using immunoassay) in congestive heart failure patients (age < 12 months) and 2–25 ng/mL in children (age 2 months–15 years) at a dose 0.07 to 0.14 mg/kg with hypertension, respectively (21,22). Within the here presented quality control system, concentrations between 0-37 ng/mL (enalapril) and 0-45 ng/mL (enalaprilat) were determined (see Fig. 4). Therefore, based upon age range of studied population from neonates to 12 years, it was anticipated that randomly selected incurred samples appropriately covered possible C_{max} and concentration values around the elimination phase for different age range. Moreover, it was assumed that reanalyzed incurred sample covered the lower, middle, and the higher concentration ranges across pediatric age and assay range appropriately.

The possible reasons for close agreement of the ISR (enalapril = 71%; enalaprilat = 67%) to the guideline acceptance limit (66.7%) were found to be the variations associated with peak area ratio, slope, and IS response especially in two runs. As these runs covered a higher number of ISR samples, they contributed towards the close agreement of acceptance

limit. Subsequently, it is suggested to ensure that number of incurred samples is equally distributed amongst the analytical runs. The latter avoids biasing the reproducibility of the assay based on ISR results obtained in borderline runs (e.g., high variability in IS response). However, there is still much consensus to be achieved in this area regarding the selection, number, and assurance criteria of the ISR (4).

The international bioanalytical guidelines preferred to implement labeled IS like deuterated, C^{13} or N^{15}, which may compensate more efficiently for matrix effect during LC-MS/MS analysis. The current method utilized non-labeled IS because deuterated IS was unavailable commercially at the time of method validation. During validation, IS-normalized matrix effect was within guidelines for both analytes. Nevertheless, application of labeled IS should be prioritized whenever applicable. If a labeled IS is not applicable for any reason, it is advisable to monitor in parallel for specific mass transition of phospholipids (e.g., *m*/*z* 524.0/184.0) in future research (23). Mostly phospholipids (glycerophosphocholines and lysophospholipids) are associated with ion suppression or ion enhancement (24). Therefore, their monitoring allows observing possible matrix effect that non-labeled IS might not compensate sufficiently.

The investigator-driven LENA project collected unique data on the treatment of children aged 0–12 years with heart failure with ACE therapy. The data collected should met the high-quality requirements known for clinical studies in a regulated environment. Therefore, a GCLP-compliant environment was created for the collection of bioanalytical data that could be successfully accomplished despite the limited personal and financial situation of the academic project. The results showed that such a quality ensuring approach like the here presented quality control system is worthwhile and achievable from an academic point of view. It ensured the optimal monitoring and evaluation of bioanalytical data. Invalid data, which otherwise would not be detected if only the validity of the particular batch would had been monitored, were identified and contributed to the increase in quality. This outcome showed that this undertaking was feasible and should encourage other research groups in, e.g., academia to establish comparable system aiming to contribute towards better data quality.

CONCLUSION

A fit-for-purpose quality control system pertinent to pediatric research was successfully developed. It addresses current bioanalytical requirements of international guidelines (EMA, FDA) but also encompasses specific situations in pediatric research. Descriptive statistical and graphical representations allowed for monitoring bioanalytical data quality of three pediatric studies.

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APPENDIX

Table II. Slope and Intercept Values for Enalapril and Enalaprilat from Valid Analytical Runs

Number of valid analytical run (2016 to 2018)	Enalapril		Enalaprilat	
	Slope	Intercept	Slope	Intercept
1	3.8620	0.0164	0.5405	0.0013
2	4.3758	0.0089	0.4184	0.0008
3	2.8060	0.0019	0.3409	0.0004
4	3.0682	0.0046	0.3911	0.0002
5	3.4733	0.0067	0.4367	0.0004
6	3.4346	0.0030	0.4574	0.0006
7	2.9138	0.0094	0.4139	0.0004
8	3.0281	0.0019	0.4282	0.0008
9	2.4828	0.0012	0.1587	0.0008
10	3.3020	0.0041	0.4360	0.0011
11	2.9920	0.0024	0.2741	0.0010
12	2.4958	0.0526	0.4540	0.0006
13	1.0841	0.0000	0.2735	0.0020
14	2.4374	0.0176	0.1923	0.0001
15	2.5350	0.0014	0.4147	0.0002
16	1.8817	0.0009	0.3044	0.0002
17	2.6824	0.0033	0.5265	0.0010
18	2.7958	0.0015	0.4689	0.0005
19	2.3532	0.0008	0.4290	0.0003
20	2.5349	0.0003	0.3969	0.0001
21	2.4509	−0.0002	0.5039	0.0007
22	2.6068	0.0020	0.7581	0.0007
23	2.5828	0.0069	0.6755	0.0004
24	2.1048	0.0017	0.5480	0.0007
25	2.5507	0.0046	0.4306	0.0007
26	2.7781	0.0012	0.3599	0.0002
27	2.7896	0.0011	0.4186	0.0006
28	4.1120	0.0014	0.4050	0.0006
29	3.2384	0.0213	0.7055	0.0007
30	2.5860	0.0064	0.1872	0.0001
31	2.4090	0.0031	0.1133	0.0003
32	3.4986	0.0051	0.1035	0.0003
Mean	2.8202	0.0060	0.4011	0.0006
SD	0.6400	0.0100	0.1602	0.0004
%CV	22.6944	164.8058	39.9318	66.3023

SD, standard deviation; *%CV*, coefficient of variation

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