Validated low-volume aldosterone immunoassay tailored to GCLP-compliant investigations in small sample volumes

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A B S T R A C T

Introduction: Heart failure is well investigated in adults, but data in children is lacking. To overcome this shortage of reliable data, appropriate bioanalytical assays are required.
Objectives: Development and validation of a bioanalytical assay for the determination of aldosterone concentrations in small sample volumes applicable to clinical studies under Good Clinical Laboratory Practice.
Methods: An immunoassay was developed based on a commercially available enzyme-linked immunosorbent assay and validated according to current bioanalytical guidelines of the European Medicines Agency (EMA) and U.S. Food and Drug Administration (FDA).
Results: The assay (range 31.3–1000 pg/mL [86.9–2775 pmol/L]) is characterized by a between-run accuracy from − 3.8% to − 0.8% and a between-run imprecision ranging from 4.9% to 8.9% (coefficient of variation). For within-run accuracy, the relative error was between − 11.1% and + 9.0%, while within-run imprecision ranged from 1.2% to 11.8% (CV). For parallelism and dilutional linearity, the relative error of back-calculated concentrations varied from − 14.1% to + 8.4% and from − 7.4% to + 10.5%, respectively.
Conclusions: The immunoassay is compliant with the bioanalytical guidelines of the EMA and FDA and allows accurate and precise aldosterone determinations. As the assay can run low-volume samples of 40 μL, it is especially valuable for pediatric investigations.

1. Introduction

Heart failure is a severe cardiovascular disease in adults and children and is characterized by structural or functional abnormalities of the heart. Heart failure leads to an inappropriate blood circulation and subsequently to an undersupply of metabolizing tissues with oxygen and nutrients. Clinical symptoms like edema, respiratory distress, growth failure, and exercise intolerance are as common as neuro-hormonal alterations.

The humoral parameter aldosterone is part of the renin-angiotensin-aldosterone system (RAAS), which plays a major role in the body's salt and water balance. In clinical settings, serum aldosterone is recommended as a screening and diagnosis parameter for primary aldosteronism in high-risk patients. However, aldosterone levels are also altered in cardiovascular diseases like heart failure and seem to have a major impact on remodeling processes. While its effects are well investigated in adults suffering from heart failure, little is known about pediatric patients, because reliable data is lacking. One reason for this is the shortage of child-appropriate bioanalytical assays. Suitable assays should require preferably low blood volumes and follow international guidelines.

As blood volume is limited especially in neonates and varies between 80 and 90 mL/kg body weight, the European Medicines

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Agency (EMA) recommends that the trial-related blood loss should not exceed 3% of the total blood volume (1.2–1.4 mL serum or plasma) during a period of four weeks [1].

However, most of the currently available aldosterone assays either require high sample volumes which are inappropriate for pediatric applications or do not show to be compliant with recommendations for bioanalytical method validation by the EMA and U.S. Food and Drug Administration (FDA) [2,3]. Widely established automated chemiluminescent assays (e.g., LIAISON® by DiaSorin Inc., iSYS by IDS Ltd.) are FDA approved, but require sample volumes of at least 250 μL serum/plasma [4,5]. As two or three sampling points are required for collecting meaningful research data in a new population and the blood volume available is also used for monitoring safety parameters, the use of these assays would exceed the recommended maximum blood loss during a period of four weeks [1]. Furthermore, Fortunato et al. [6] showed that platforms like iSYS by IDS Ltd. and LIAISON® by DiaSorin are characterized by a significant proportional bias. Even though these platforms might have the advantage of better reproducibility, a shorter laboratory turnaround time, and a reduced requirement of 'hands-on labor' in contrast to the usually applied EIA and RIA methods, Fortunato et al. suggest that appropriate definitions of reference ranges for each method are mandatory for translating aldosterone concentrations into clinical information.

While most of the commercially available enzyme-linked immunosorbent assays (ELISAs) are characterized by lower blood volume requirements (approximately 50 μL serum/plasma) [7,8], validation according to bioanalytical guidelines of EMA and FDA has not been reported [2,3]. Therefore, the latter cannot be used for data generation in clinical studies under Good Clinical Laboratory Practice (GCLP). Reported liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods require a volume of 30–500 μL serum/plasma [9–11]. Although some of these specific methods require low sample volumes, gas chromatography or high-performance liquid chromatography in combination with mass spectrometry are not currently used by the majority of clinical services [12].

The need of investigations in pediatric patients has been repeatedly addressed by the European Commission [13–15]. Against this background, an aldosterone immunoassay for low-volume applications was developed and validated according to current bioanalytical guidelines.

2. Materials and methods

2.1. Materials

Microtiter wells, lyophilized aldosterone standard (1000 pg/mL), enzyme conjugate (aldosterone conjugated to horseradish peroxidase), substrate (tetramethylbenzidine, TMB) solution, stop solution (0.5 M sulfuric acid), and wash solution were components of the commercially available aldosterone ELISA kit EIA-5298 by DRG Instruments GmbH (Marburg, Germany) [16]. Additional aldosterone standard 1000 pg/mL (lyophilized), artificial blank matrix, assay buffer, and aldosterone stock solution 100 ng/mL were also obtained from this vendor. All standards used are intended to mimic the matrix of study samples and contain treated human serum and preservatives. Double-distilled water was purchased from Carl Roth GmbH and Co. KG (Karlsruhe, Germany) and serum samples were supplied by healthy volunteers.

2.2. Preparation of calibration standards and Quality Control samples

The 1000 pg/mL lyophilized aldosterone standard was reconstituted with double-distilled water. The prepared solution corresponds to the highest calibration standard, the upper limit of quantification (ULOQ). The remaining calibration standards 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, and 31.3 pg/mL were obtained by serial dilution of the ULOQ (1000 pg/mL) with artificial blank matrix.

For preparation of Quality Control (QC) samples, a separate lyophilized aldosterone standard 1000 pg/mL was reconstituted with double-distilled water and diluted with artificial blank matrix to obtain following concentration levels: 500 pg/mL, 250 pg/mL, 125 pg/mL, 83.3 pg/mL.

Prior to the conduct of an analytical run, calibration standards and QC samples were frozen at − 80 °C to mimic the treatment of study samples. Immediately before analysis, standards and QC samples were thawed together with unknown samples using a water bath at 23 ± 3 °C.

2.3. Serum samples

Serum samples were donated by healthy volunteers who provided written, informed consent (study no. 5392 at local Ethics Committee at Heinrich-Heine-University Duesseldorf). Samples were collected using S-Monovettes® by Sarstedt AG & Co. (Nümbrecht, Germany).

2.4. Assay procedure

All standards, QC samples, and unknown samples had to reach room temperature (water bath at 23 ± 3 °C) before use. After dispensing 40 μL assay buffer into each well, the microtiter plate was incubated for 5 min at room temperature. 40 μL of each standard, QC sample, unknown sample, and blank were pipetted into the respective wells and mixed for 1 min at 500 rpm in the dark, followed by an incubation at 300 rpm for 30 min in the dark. After adding 150 μL enzyme conjugate (aldosterone conjugated to horseradish peroxidase), the microplate was incubated for 7 min at 300 rpm, for 1 min at 700 rpm and for 29 min at 300 rpm in the

dark. Afterwards, the contents of the wells were shaken out and wells were rinsed with 300 μL wash buffer 8 times. Between all washing steps, the liquid was removed thoroughly. 200 μL TMB solution were added to each well, followed by an incubation for 30 min in the dark (300 rpm). Then, the microtiter plate was incubated for additional 5 min in daylight without shaking. The enzyme reaction was stopped by pipetting 100 μL 0.5 M sulfuric acid into each well. After mixing for 1 min at 300 rpm, the optical density (OD) was determined at 450 nm. To guarantee constant conditions, all incubation steps were performed using a ThermoMixer® by Eppendorf AG (Hamburg, Germany) controlled at 23 °C.

2.5. Calculation of results

The optical density was measured using the absorbance reader infinite®F50 by Tecan (Männedorf, Switzerland). Calculations of concentrations and the statistical evaluation were carried out with the corresponding Magellan™ software Tracker V 7.0 (Tecan). All values are given in pg/mL and the corresponding value in pmol/L is given in parentheses. 1 pg/mL aldosterone is equivalent to 2.775 pmol/L [17].

2.6. Bioanalytical method validation according to EMA and FDA guidelines

The validation covered accuracy, precision, parallelism, dilutional linearity, matrix effect, short-term stability, freeze-thaw stability, long-term stability, processed sample stability, reproducibility, and impact of hemolyzed blood samples.

The parameter requirements applied in the following sections comply with the current bioanalytical guidelines of EMA and FDA [2,3]. In case of a discrepancy between the guideline criteria of EMA and FDA, the stricter criteria were applied.

Analytical selectivity and specificity had been investigated by the vendor previously. Specificity, being affected mostly by the antibodies chosen, had been studied for steroids and their metabolites [16]. Both validation parameters were regarded as sufficiently investigated and were excluded from the validation as no modifications to the antibody were made.

2.6.1. Calibration curve and QC levels

The calibration curve consisting of six non-zero calibration standards in duplicate covered the range from 31.3 to 1000 pg/mL [86.9–2775 pmol/L] aldosterone in serum. Each assay run included four QC levels, one within three times the LLOQ (83.3 pg/mL [231.2 pmol/L]), two in the medium range of the assay (125 pg/mL [347 pmol/L] and 250 pg/mL [694 pmol/L]) and one with a high concentration (500 pg/mL [1388 pmol/L]), all measured in duplicate.

To evaluate the regression model of the calibration curve and the suitability of QC levels, six independent assay runs over three days were performed. In each assay run, all six calibration standards and four QC levels were determined in duplicate. Within-run and between-run accuracy of calibration standards and QC levels were calculated. For calibration standards, values had to be within the limits of ± 20% of the nominal values (± 25% at the lower limit of quantification, LLOQ). In case of the QC levels, mean values of the replicates must be within the limit of ± 20% of the nominal values for at least three out of four quality control levels. Moreover, at least one value of each duplicate per concentration level had to be within the limits of ± 20% of the nominal value.

2.6.2. Accuracy and precision

Five concentration levels (1000 pg/mL [2775 pmol/L], 500 pg/mL [1388 pmol/L], 125 pg/mL [347 pmol/L], 62.5 pg/mL [173.4 pmol/L], 31.3 pg/mL [86.9 pmol/L]) were determined in six independent assay runs over three different days in order to evaluate accuracy and precision of the assay. Each concentration level was determined with five replicates per assay run.

The mean values of within-run and between-run accuracy had to be within ± 20% (± 25% at LLOQ) of the nominal values. Within-run and between-run imprecision, reported as coefficient of variation (CV), were regarded as acceptable provided if the limit of 20% (25% at LLOQ) was not exceeded. Time-different intermediate precision (between-run) was evaluated by one-way ANOVA. The total error of the method was calculated as the sum of the absolute value of the relative error (%) and the CV (%) and had to be less than the limit of 30%. The latter is recommended by the EMA guideline [2].

2.6.3. Parallelism

Three different sources were spiked at high concentrations within the assay range. Afterwards, a serial dilution of spiked samples with artificial blank matrix was performed. Native and spiked samples as well as dilutions of spiked samples were determined, with three replicates each. The different sources were analyzed in separate assay runs over two days. Back-calculated values of diluted samples were compared to values determined for spiked samples. The maximum deviation accepted was ± 20%. Imprecision between serially diluted samples was reported as CV and had to be ≤ 30%.

2.6.4. Dilutional linearity

In contrast to parallelism, samples of three different sources were spiked at concentrations exceeding the ULOQ. A serial dilution of spiked samples with artificial blank matrix was performed and dilutions as well as native samples were determined in three assay runs over two days. Native samples and dilutions of spiked samples were determined with three replicates each. The values obtained were back-calculated and compared to theoretically calculated values of the spiked samples. The percentage deviation had to be less than ± 20% of the calculated value and imprecision between diluted samples was not allowed to exceed 20% (CV).

2.6.5. Matrix effect

As aldosterone is inherently present in native samples, the possible effect of the matrix on accurate determinations was investigated by the determination of spiked biological samples.

Native samples of ten sources with different inherent aldosterone concentration levels were spiked using one aldosterone solution with a lower concentration level and one with a higher concentration level. The volume of the spiking solution added to serum was ≤ 10% of the total sample volume to minimize matrix dilution. Native and spiked samples were determined with three replicates each. Considering the inherent aldosterone concentrations in native samples, target values of the spiked samples were theoretically calculated and compared to the actually determined values. The percentage deviation of actually determined value from calculated value (nominal value) had to be within ± 20% for at least 80% of the matrices evaluated.

2.6.6. Stability

Short-term stability, freeze-thaw stability, long-term stability, and processed sample stability were assessed. Native samples were used to assess low concentration samples, while spiked samples were used for the evaluation of higher concentration levels. Samples of all concentrations were determined with six replicates each on the day of blood collection (day 0). The obtained mean values served as reference values for investigations further on. While international recommendations on the total allowable error for aldosterone determinations in serum vary between 15% and 36.7% [18], the stability limit in this investigation was defined as ± 20% of the reference value (mean value at day 0). This acceptance criterion complies with the accuracy limits given by EMA/FDA bioanalytical guidelines. Since it was shown (by other validation parameters) that substantial inter-source variability in the assay's performance was absent and good precision results from different sources were obtained, stability was evaluated on one single human source.

For evaluation of short-term stability, aliquots of two serum samples with different concentration levels were placed on the laboratory bench (room temperature) on day 0. The following day, samples were determined with six replicates each and the percentage deviation of mean values from the reference values was calculated and must not exceed ± 20%. In case of freeze-thaw stability, aliquots of two serum samples with different concentration levels were stored at − 80 °C for 44 days after blood collection and passed through three freeze and thaw cycles (all freezing steps overnight) over the last three days of this period. The storage period of at least one month prior to analysis was chosen to imitate the usual treatment of study samples. After the last cycle, values were determined (six replicates per sample) and mean values were compared to reference values (maximum permitted deviation ± 20%). For long-term stability, aliquots of three serum samples with different concentration levels were stored at − 80 °C and determined in regular intervals over an extended period of time (six replicates per sample). For eight weeks, determinations were performed weekly and subsequently every couple of weeks. Mean values had to be within ± 20% of the reference values. All freezing steps mentioned above were performed as snap freezing to guarantee the same treatment as for study samples.

To assess processed sample stability, replicate readings of the microplate were performed at regular intervals over two hours at the end of an analytical run. During the first hour, readings were performed every five to ten minutes and subsequently at intervals of thirty minutes. Mean values for calibration standards and QC levels were not allowed to deviate by more than ± 20% (± 25% at LLOQ) from the nominal values.

2.6.7. Reproducibility

For an assessment of reproducibility, samples of three different human sources were analyzed in five repetitive assay runs on different days. The sources were selected randomly to cover various concentration levels in the low and medium concentration range of the assay. The exact concentration levels per source were determined by three replicates at the first run. Values of the subsequent assay runs had to be within ± 20% of their mean and imprecision, reported as coefficient of variation, must not exceed 20%

2.6.8. Impact of hemolyzed blood samples on accurate sample determination

To assess the impact of hemolysis on accurate sample determination, blood of three different human sources was hemolyzed by use of an ultrasonic bath. The impact of hemolysis on the accurate determination of aldosterone levels was regarded neglectable, when the percentage deviation of hemolyzed to non-hemolyzed reference sample of the same source was ≤20%. All samples were determined with three replicates each.

2.7. External quality assurance

For an external verification of accuracy, the assay took part in two ring tests, which are the common quality assurance demanded by the German Medical Association for quality assurance of laboratory medicine (Rili-BÄK) in Germany. Samples with two unknown concentration levels were analyzed in duplicate each in 2016 and 2017. Mean values were reported to the Reference Institute for Bioanalytics (Bonn, Germany) and compared to LC-MS/MS, which served as the reference method.

2.8. 12-h profile

Aldosterone concentrations of a 30-year-old healthy volunteer (female) were determined over 12 h. The first sampling was performed after night's sleep, while the subject was in supine position for at least 30 min prior to all other sampling points. Samples were taken every hour or every two hours, respectively. A snack was served between 11:00 a.m. and 11:30 a.m. Samples were analyzed in duplicate and mean concentrations were calculated for each time point. Mean concentrations were plotted against the corresponding time to obtain the aldosterone concentration-time profile over 12 h.
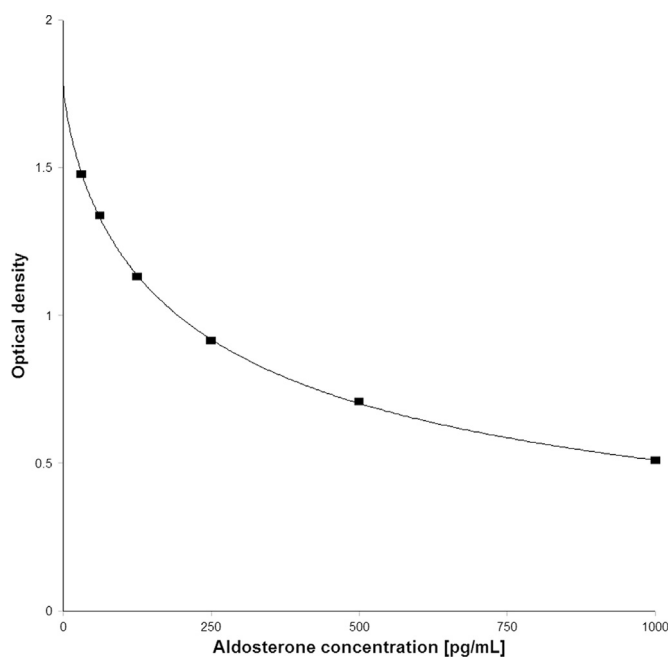
Fig. 1. Aldosterone standard curve. The calibration curve consists of six non-zero calibration standards covering the range from 31.3 pg/mL to 1000 pg/mL [from 86.9 pmol/L to 2775 pmol/L].

3. Results

3.1. Bioanalytical method validation according to EMA and FDA guidelines

3.1.1. Calibration curve and quality control levels

The regression model of the calibration curve and the suitability of QC levels were assessed in six assay runs. The best curve fitting was obtained by applying the 4-parameter Marquardt regression model (extrapolation 1.5). With respect to between-run accuracy, the relative error for calibration standards ranged from − 2.1% and + 1.5%, while the relative error for QC levels was between − 2.3% and + 1.1%. All determinations for calibration standards and QC levels were within the required limits of ± 20% (± 25% at LLOQ). A typical standard curve is shown in Fig. 1.

3.1.2. Accuracy and precision

In six independent assay runs, within-run accuracy over all five different concentration levels (31.3 pg/mL [86.9 pmol/L], 62.5 pg/mL [173.4 pmol/L], 125 pg/mL [347 pmol/L], 500 pg/mL [1388 pmol/L], 1000 pg/mL [2775 pmol/L]) showed a relative error between − 11.1% and + 9.0%. For between-run accuracy, the relative error ranged from − 3.8% to − 0.8%. All results were within ± 20% (± 25% at LLOQ) of the nominal values and thus, complied with guideline requirements.

Within-run imprecision over all five concentration levels was between 1.2% and 11.8% (CV), while between-run imprecision (one-way ANOVA) ranged from 4.9% to 8.9% (CV) across the five different concentration levels tested. All results did not exceed the guideline limits of 20% (25% at LLOQ). Results of all concentration levels in the six assay runs are shown in Fig. 2.

At the LLOQ (31.3 pg/mL), relative error for within-run accuracy ranged from − 9.6% to + 3.5%, while between-run accuracy showed a relative error of − 3.8%. Within-run imprecision was between 3.7% and 11.8% (CV) and between-run imprecision (one-way ANOVA), reported as CV, was 8.8%. At the ULOQ (1000 pg/mL), the relative error for within-run accuracy ranged between − 7.5% and + 1.2%, while the relative error for between-run accuracy was − 3.3%. Within-run imprecision varied from 2.0% to 4.3% and between-run imprecision (one-way ANOVA) was 4.9%. All values were within the required guideline limits.

The total error of the method ranged between 8.0% (concentration level 1000 pg/mL) and 12.5% (concentration level 31.3 pg/mL) and thus also complied with guideline requirements.

The assessment of accuracy was performed using Quality Control samples based on an artificial matrix made from treated human samples with preservatives. This matrix is intended to mimic the biological samples. The risk of underestimating a possible matrix effect by utilizing the artificial matrix instead of pooled serum samples with low aldosterone was carefully investigated. During the validation process, tests on parallelism were performed to assess the accuracy of the assay utilizing the intended dilution procedure. Native biological samples, diluted with analyte-free artificial matrix, were compared to a calibration curve. Despite this dilution with artificial matrix, the results of the diluted samples were within ± 20% of the expected nominal value after correction for dilution. As the dilutions of samples lay parallel to the calibration curve, no negative impact of the artificial matrix was observed. Thereby, it was shown that the artificial matrix sufficiently mimics the biological sample matrix.
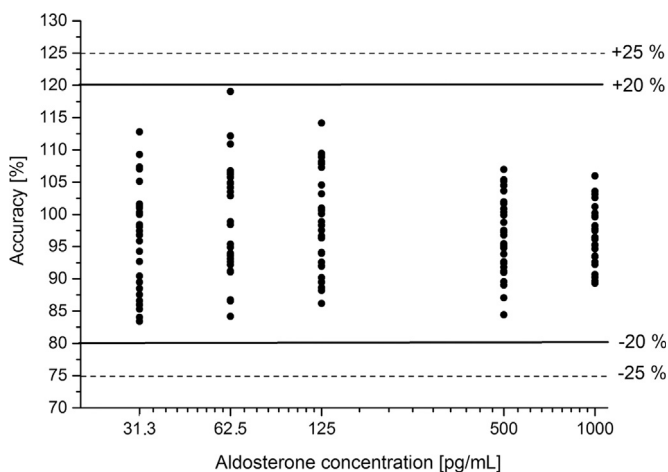
Fig. 2. Accuracy of aldosterone assay. Five concentration levels (31.3 pg/mL [86.9 pmol/L], 62.5 pg/mL [173.4 pmol/L], 125 pg/mL [347 pmol/L], 500 pg/mL [1388 pmol/L], and 1000 pg/mL [2775 pmol/L]) were determined in six independent assay runs. For each concentration level, values obtained for five replicates in six assay runs are shown (n = 30 determinations per concentration level). Dashed black lines indicate the ± 25% limit for the LLOQ, while the continuous black lines highlight the accuracy limits for all other concentration levels.

3.1.3. Parallelism

After spiking, aldosterone concentrations in three different sources ranged from 600.0 pg/mL [1665.0 pmol/L] to 634.1 pg/mL [1759.6 pmol/L]. All back-calculated concentrations of diluted samples were within ± 20% of concentrations determined for the corresponding spiked sample. Imprecision between serially diluted samples ranged from 3.6% to 5.6% (CV) over all three assay runs and thus complied with guideline requirements. Results of the parallelism studies are shown in Table 1.

3.1.4. Dilutional linearity

For three different sources, the relative error ranged from − 7.4% to + 10.5% and was within the acceptance limits of ± 20%. Details are shown in Table 2. Over all three assay runs, imprecision ranged from 2.9% to 7.4% (CV).

Table 1
Parallelism of aldosterone assay. Values of spiked samples and back-calculated values of samples in dilution series. For back-calculated values, percentage deviation from spiked samples was calculated.

	1st source	2nd source	3rd source
Concentration of spiked sample	634.1 pg/mL	611.7 pg/mL	600.0 pg/mL
Dilution factor	Back-calculated concentration [pg/mL] (deviation from spiked sample [%])		
2	635.2 (+ 0.2)	646.9 (+ 5.8)	571.0 (− 4.8)
4	616.9 (− 2.7)	633.8 (+ 3.6)	556.4 (− 7.3)
8	555.8 (− 12.3)	609.4 (− 0.4)	515.3 (− 14.1)
16	605.4 (− 4.5)	663.2 (+ 8.4)	-EXT
CV [%]	5.6	3.6	5.3

-EXT: value not reported, because the obtained optical density was outside the assay range; CV: coefficient of variation; 1 pg/mL aldosterone is equivalent to 2.775 pmol/L.

Table 2
Dilutional linearity of aldosterone assay. Values of spiked samples were theoretically calculated and values of serially diluted samples were back-calculated. Percentage deviations of back-calculated from theoretically calculated values are shown in parentheses.

	1st source	2nd source	3rd source
Concentration of spiked sample	2663.4 pg/mL[a]	3190.3 pg/mL[a]	3404.8 pg/mL[a]
Dilution factor	Back-calculated concentration [pg/mL] (deviation from calculated value of spiked sample [%])		
2	-EXT	-EXT	-EXT
4	2778.5 (+ 4.3)	2954.1 (− 7.4)	3404.1 (± 0.0)
8	2943.5 (+ 10.5)	3360.7 (+ 5.3)	3425.6 (+ 0.6)
16	2860.0 (+ 7.4)	3377.2 (+ 5.9)	3592.5 (+ 5.5)
CV [%]	2.9	7.4	3.0

-EXT: value not reported, because the obtained optical density was outside the assay range.
[a] Theoretically calculated values.

3.1.5. Matrix effect

Ten sources were assessed for matrix effect. As native samples differ in their inherent aldosterone levels, aldosterone concentrations of the assessed ten human sources varied after treatment with spiking solutions. Samples that had been spiked with the low concentration aldosterone solution ranged from 85.4 pg/mL [237.0 pmol/L] to 212.1 pg/mL [588.6 pmol/L], while samples spiked with the high concentration solution showed concentrations between 516.6 pg/mL [1433.6 pmol/L] and 844.7 pg/mL [2344.0 pmol/L].

For nine samples spiked at lower concentrations, the aldosterone concentration ranged from − 8.7% to + 12.5% of the nominal value. One hemolyzed sample showed a deviation of +32.7%. For all ten samples spiked at higher concentrations, the aldosterone concentration was between − 9.9% and + 8.8% of the nominal value. For 90% of the matrices tested, the concentration was within the required limit of ± 20% of the nominal value. This complies with EMA requirements, which specify that this limit must be met by at least 80% of all matrices assessed. Results are shown in Table 3.

Table 3
Matrix effect in ten human sources. The percentage deviation of values obtained by spiked samples from nominal values is shown for 10 sources spiked at a low and a high concentration level each.

		Percentage deviation [%] Concentration level	
		Low	**High**
♀	Source 1	+ 1.0	− 0.7
♂	Source 2	− 1.2	+ 8.8
♀	Source 3	+ 0.6	+ 1.4
♂	Source 4	+ 2.3	+ 0.1
♀	Source 5	− 8.7	− 8.9
♀	Source 6	+ 1.5	− 9.9
♀	Source 7 (hemolyzed)	+ 32.7	− 2.0
♂	Source 8	+ 12.5	+ 5.3
♂	Source 9	− 5.0[a]	− 6.1[a]
♀	Source 10	+ 3.9	− 8.7

♀: female, ♂: male.
[a] Calculations of native values were based on one instead of three replicates as the optical density of the other replicates were outside the assay range; 1 pg/mL aldosterone is equivalent to 2.775 pmol/L.

3.1.6. Stability

Mean values ± standard deviation, determined on the day of blood collection (day 0), were 85.1 ± 9.9 pg/mL [236.2 ± 27.5 pmol/L] for the lower concentration sample and 391.0 ± 13.1 pg/mL [1085.0 ± 36.4 pmol/L] for the higher concentration sample, and these were used as reference values subsequently.

After one day on the laboratory bench, the mean value for the short-term stability sample at a lower concentration level showed a deviation of − 10.7% from the reference value, while the mean value for the serum sample at a higher concentration level deviated by + 7.3%. Thus, short-term stability over one day for serum samples was proven, because the results did not exceed the accuracy limit of ± 20% from the reference values.

For freeze-thaw stability, the same reference samples as for short-term stability were applied. After storage of samples at − 80 °C for 44 days and three freeze and thaw cycles over the last three days of this period, percentage deviation of the mean values for the stressed samples from the reference value were − 16.3% for the lower concentration sample and − 9.1% for the higher concentration level. Because the values did not deviate by more than the limit of ± 20% from reference values, it was proven that three freeze and thaw cycles do not affect accurate sample analysis.

Results of assay runs evaluating long-term stability are shown in Table 4. All mean values determined over a period of 70 days, were within ± 20% of values determined on the day of blood collection (day 0). Thus, long-term stability for aldosterone over 10 weeks was proven.

With regard to processed sample stability, all mean values obtained for calibration standards and QC levels were within ± 20% of the nominal values over the total time period of 120 min after stopping the enzyme reaction. However, it became obvious that the assay's discrimination ability decreased over the time due to changes in the slope of the regression curve. Therefore, a maximum period of 30 min between termination of the enzyme reaction and determination should not be exceeded.

3.1.7. Reproducibility

Three different human sources were determined with five replicates each. Mean values of the five replicates per source were 64.0 pg/mL [177.6 pmol/L], 66.4 pg/mL [184.3 pmol/L], and 174.5 pg/mL [484.2 pmol/L]. The deviation of the replicates from their mean ranged from − 7.2% to + 13.7%, while imprecision was 8.6%, 4.7%, and 4.0% (CV), respectively. All values were within the required limits.

Table 4

Long-term stability of aldosterone. Values were determined at different time points after first analysis on day 0. Percentage deviation from reference value (day 0) was calculated (reported in parentheses).

Days after blood collection	Long-term stability Mean concentration [pg/mL] (deviation from reference value [%])		
	1st conc. level	2nd conc. level	3rd conc. level
0	85.1	391.0	468.5
7	82.3 (− 3.3)	400.7 (+ 2.5)	507.6 (+ 8.3)
14	100.9 (+ 18.6)	377.6 (− 3.4)	513.6 (+ 9.6)
21	75.9 (− 10.8)	368.7 (− 5.7)	511.9 (+ 9.3)
28	79.4 (− 6.7)	390.9 (± 0.0)	519.4 (+ 10.9)
35	93.6 (+ 10.0)	385.1 (− 1.5)	499.0 (+ 6.5)
42	83.2 (− 2.2)	389.2 (− 0.5)	489.7 (+ 4.5)
49	78.5 (− 7.8)	397.2 (+ 1.6)	482.7 (+ 3.0)
56	87.2 (+ 2.5)	387.9 (− 0.8)	501.9 (+ 7.1)
70	79.7 (− 6.3)	376.7 (− 3.7)	520.8 (+ 11.2)

Conc. level: concentration level; 1 pg/mL aldosterone is equivalent to 2.775 pmol/L.

3.1.8. Impact of hemolyzed blood samples on accurate sample determination

The effect was investigated in three human sources. No impact of hemolysis on accurate sample determinations was observed. Percentage deviations of hemolyzed samples compared to non-hemolyzed samples were − 1.6%, − 6.8%, and − 13.6%. However, one hemolyzed sample of a different source showed results out of specification when assessed for matrix effect (see Section 3.1.5).

3.2. External quality assurance

The Reference Institute for Bioanalytics (Bonn, Germany) certified that accuracy requirements were met in two ring tests. The unknown samples were accurately determined utilizing the low-volume assay when compared to the reference method value obtained by LC-MS/MS. The target value for sample A and B were as follows: 440 pg/mL and 243 pg/mL (2016) as well as 263 pg/mL and 154 pg/mL (2017). Fig. 3 shows the test results of all participants of the ring test in 2016. The reported concentrations during the ring test in 2017 were 223 pg/mL and 139 pg/mL showing a relative error of 15.2% and 9.7%, respectively,
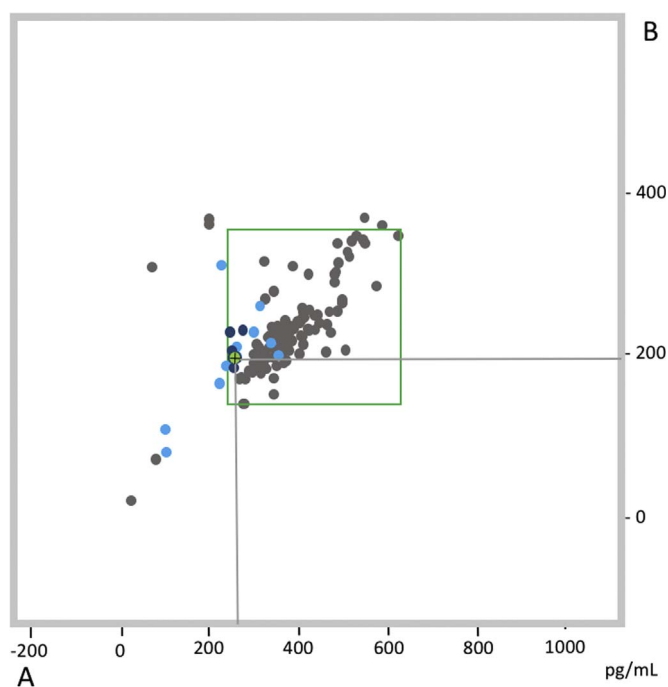

Fig. 3. Youden plot showing the results of the aldosterone ring test conducted in April 2016, republished with permission from the Reference Institute for Bioanalytics (Bonn, Germany). Further details are accessible on the following website: www.rfb.bio. The marked circle shows the location of the pair of values for sample A and sample B determined in the ring test. Values reported by other participants of the same collective (same method and manufacturer combination) are colored dark blue and those of participants using the same method (methods with photometric measurement) are shown in light blue. Grey circles indicate other determination methods (e.g., radioimmunoassays or liquid chromatography-mass spectrometry). Most of the methods applied in the ring test were immunoassays. The green rectangle highlights the valid limits for the applied method.
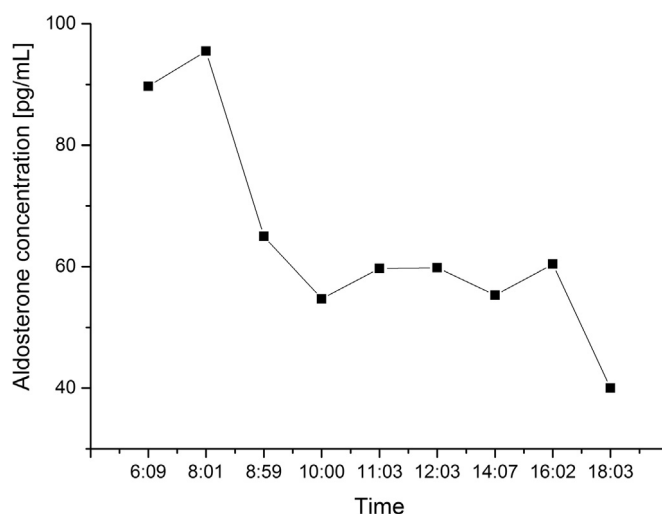
Fig. 4. 12-h aldosterone profile. Aldosterone concentrations of a supine healthy female (30 years of age) over 12 h. The first sampling at 6:09 a.m. was performed after night rest, while the subject was in the supine position for at least 30 min prior to all other sampling points.

when compared to the target value.

3.3. 12-h profile

Aldosterone concentrations in a 30-year-old healthy female were determined over a period of 12 h (Fig. 4).

The obtained concentration-time profile reflects the known circadian rhythm of aldosterone. All determined concentrations were within the expected reference values of healthy adults in a supine position (from 17.6 pg/mL [48.8 pmol/L] to 232 pg/mL [644 pmol/L] [19]) and support the applicability of the assay. Together with the successful validation according to EMA and FDA bioanalytical guidelines described above, and the successful participation in ring tests, the assay's applicability to clinical routine and research to obtain reliable data sets of aldosterone was proven.

4. Discussion

An ELISA for the determination of aldosterone concentrations in low-volume samples (40 μL serum) was developed and validated according to current bioanalytical guidelines of EMA and FDA [2,3]. The developed low-volume immunoassay is a contribution to alleviate the shortage of high-quality data in children suffering from heart failure. The modified assay has advantages when compared to the original CE-marked assay of the manufacturer.

First, the assay was optimized in required blood volume which was reduced by 20% if compared to the DRG ELISA. Although the absolute volume reduction appears small if compared to the unmodified DRG ELISA, lowering blood volume is of high benefit especially in pediatric patients. According to the EMA guideline [1] on total blood loss for clinical routine and research (e.g., clinical trial), the blood loss should not exceed 1% at a single determination. The latter results in a maximum collectable whole blood volume of 2.2 mL in a 3 kg neonate. As most assays require either serum or plasma, about 1.1 mL can subsequently be used for clinical routine plus research. Any reduction in required sample volume for reliable determination might enable a determination in residual blood volume which was previously impossible. Second, this low-volume assay was successfully validated according to EMA and FDA bioanalytical guidance. This allows for an application of this assay e.g. in pivotal studies during drug development as well as in clinical routine. The applied validation is regarded as at least equal to the performed evaluation of the CE marked DRG ELISA. Third, the applicability and reliability of the assay was supported by two EQA participations in 2016 and 2017.

Using this assay, the collection of reliable data on aldosterone levels in children with heart failure becomes feasible and this will broaden the knowledge of the underlying pathophysiology and support optimization of the corresponding pharmacotherapy. Owing to the low sample volume required and the conducted validation, the assay is especially valuable for investigations in pediatric patients under GCLP conditions (e.g., in pivotal studies).

Compared to the established automated immunoassays (e.g., LIAISON® by DiaSorin Inc. or iSYS by IDS Ltd.), the required sample volume was markedly reduced. Reported minimum volumes of serum or plasma required by these automated systems vary between 250 to 300 μL serum/plasma (sample volume plus dead volume) [4,5]. As regular blood collections in clinical routine are required, further investigations are restricted to the blood volume available after safety parameters have been determined. Therefore, the sample volume required by these assays exceeds the maximum blood loss in pediatric patients recommended by the EMA, that is 3% of the total blood volume over four weeks [1]. The sample volume required by these methods could be reduced by a pre-dilution step before analysis. A 6-fold dilution for example would reduce the required volume to about 42 μL serum in case of the Liaison assay, which would be similar to the sample volume required for the here presented ELISA. However, dilution steps imply an aldosterone

concentration high enough so that the optical density of the diluted sample will remain within the assay range. With a LLOQ of 30 pg/mL [83 pmol/L], the minimum aldosterone concentrations in a 6-fold dilution must to be about 180 pg/mL [500 pmol/L]. Yet, this cannot be assumed for all pediatric patients - especially not those on drugs acting on the RAAS (e.g., ACE inhibitors). Moreover, a pre-dilution step might affect the matrix and therefore should not form part of the standard procedure.

The ELISA presented requires 40 μL serum for reliable determinations. This small sample volume allows for investigations even in the youngest infants without infringing the ethically recommended maximum daily blood loss during clinical routine and research. However, the low-volume assay can also be beneficial to the analysis of samples from adults and the elderly. Under challenging sampling conditions (e.g., adrenal vein sampling in primary aldosteronism for subtype differentiation) or in severely ill multimorbid patients, in whom additional blood sampling stresses the recovery process of patients, substantially reduced blood volumes for reliable determinations are advantageous.

Low-volume assays will also contribute to better research in adults, particularly in multiple drug studies with a cross-over design, because high total blood volumes are currently necessary in those investigations.

Although the blood volume was reduced, the calibration range is still comparable to the commercial automated immunoassays listed above. While automated aldosterone assays cover a measuring range from 30 pg/mL to 1000 pg/mL [from 83 pmol/L to 2775 pmol/L] [4] or from 37 pg/mL to 1320 pg/mL [from 103 pmol/L to 3663 pmol/L] [5] respectively, the developed aldosterone assay shows a similar measuring range from 31.3 pg/ml to 1000 pg/mL [from 86.9 pmol/L to 2775 pmol/L].

Although most of the available ELISA methods require only about 50 μL serum/plasma [6,7], a validation according to the bioanalytical guidelines of EMA and FDA [2,3] has not been demonstrated. For the low-volume assay reported here, validation according to EMA and FDA criteria is fulfilled.

Liquid chromatography-mass spectrometry methods, that are highly specific and sensitive, require sample volumes of 30–500 μL serum or plasma [8–10,20]. Although gas chromatography or high-performance liquid chromatography in combination with mass spectrometry are known as methods of high specificity, they are not used by the majority of the clinical services [11]. In contrast to these reference methods, immunoassays usually do not employ sample extraction steps, have shorter analytical run times and are generally less costly.

In summary, the developed assay covers a similar assay range to other available methods, but uses a markedly lower sample volume. Since it has been validated in accordance with EMA and FDA requirements, the assay is appropriate for the generation of reliable data in a GCLP-compliant setting (e.g., in pivotal studies). Since all modifications of the assay were validated according to international guidelines and comply with corresponding requirements of the IVD directive, the assay's applicability and reliability were proven. Therefore, the assay can also be fully applied by users in clinical routine testing.

The validated assay was externally cross-checked by participation in ring tests. The obtained deviation from the median of the corresponding subgroup (same analytical technique (photometric determination [ELISA, EIA etc.]) and same manufacturer) was 0.49% (low level) and − 2.71% (high level) in 2016, while it deviated by 3.1% (low level) and − 9.7% (high level) in 2017. In addition to the enclosed results of the ring test in 2016 (see Fig. 3), the recently received results for the ring test in 2017 show a deviation of − 9.7% and − 15.2% from the nominal concentration by direct comparison to LC-MS/MS. Although the determined deviations were within assay variability of ligand-binding assays, the low number of investigated samples are a limitation of this work.

Currently, the assay is used in the pediatric studies of the LENA project, investigating a new drug formulation of the ACE inhibitor enalapril in children suffering from heart failure. This will help to obtain missing data in this vulnerable population, which allows for a deeper understanding of the underlying pathophysiology. As a result, an optimization of the pharmacotherapy of heart failure in this young population, currently done by extrapolation from adults, becomes feasible.

5. Conclusion

The present immunoassay is compliant with current bioanalytical guidelines of EMA and FDA [2,3] and allows accurate and precise aldosterone determinations in 40 μL serum. As the assay runs with low-volume samples, it is especially valuable for pediatric investigations - but is not limited to this population.

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Declaration of interest

The authors declare that they have no conflict of interest.

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Glossary

ACE: Angiotensin-converting enzyme
ANOVA: Analysis of variance
CV: Coefficient of variation
ELISA: Enzyme-linked immunosorbent assay
EMA: European Medicines Agency
FDA: U.S. Food and Drug Administration
GCLP: Good Clinical Laboratory Practice
LC-MS/MS: Liquid chromatography-tandem mass spectrometry
LENA: Labeling of Enalapril from Neonates up to Adolescents
LLOQ: Lower limit of quantification
OD: Optical density
QC: Quality Control
RAAS: Renin-angiotensin-aldosterone system
Rpm: Revolutions per minute
TMB: Tetramethylbenzidine
ULOQ: Upper limit of quantification