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Analytical validation of an IFCC-endorsed mass spectrometry-based semi-automated reference measurement procedure for global standardization of serum apolipoprotein(a)

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Abstract

Objectives: An LC-MRM-MS-based Reference Measurement Procedure (RMP) with manual sample preparation for standardization of commercial Lp(a)/apo(a) test kits in molar units was previously developed by three calibration laboratories of the IFCC Working Group on Apolipoprotein Standardization. Semi-automation of the sample preparation was introduced in the Leiden Apolipoprotein Reference Laboratory to improve the precision of the RMP.

Methods: The RMP was adapted for semi-automation on an Agilent BRAVO Automated Liquid Handling Platform using VWorks software. Measurement of apo(a) is based on three proteotypic peptides and five serum calibrators traceable to the former WHO-IFCC reference material SRM2B, and is independent of the apo(a) size polymorphism. Concentration-dependent performance criteria were established according to the EFLM Biological Variation Database, and precision was assessed per CLSI EP05-A3.

Results: Compared to manual sample preparation, semi-automation improved analytical precision, resulting in within-run imprecision estimates for quantifying peptide LFLEPTQADIALLK of 5.0, 4.7, and 4.5 % at 146.8, 197.4 and 238.7 nmol/L, respectively. Furthermore, compared to the concentration-dependent maximum allowable uncertainty, and following correction for repeated measurements, the measurement uncertainty of the RMP was deemed acceptable over the entire measurement range of apo(a) (3.8–456.0 nmol/L).

Conclusions: Semi-automation improved the analytical precision of the RMP for the quantitation of apo(a), meeting concentration-dependent analytical performance criteria and facilitating longitudinal accuracy of Lp(a)/apo(a) test results produced by CE-IVD Lp(a)/apo(a) kits. With semi-automation of the sample preparation, a more precise RMP has been developed, making it easier to meet the maximum allowable measurement uncertainty in the Lp(a)/apo(a) calibration hierarchy.

Keywords: cardiovascular risk assessment; analytical validation; apolipoprotein; reference measurement procedure; automation

Introduction

The renaissance of Lipoprotein(a) (Lp(a)) and its characteristic protein apolipoprotein(a) (apo(a)) as an independent genetic risk factor for cardiovascular disease (CVD), and the development of novel Lp(a)/apo(a)-targeting therapeutics require accurate medical tests. However, standardization of apo(a) tests faces multiple hurdles, including the characteristic Kringle-IV type 2 (K-IV_2) size-polymorphism, a large physiological range and differing units of measurement [1]. In the late 1990's and early 2000's, the first WHO-IFCC endorsed Reference Measurement System (RMS) for apo(a) was established [2], but its Reference Measurement Procedure (RMP) is no longer operational, and reference material SRM2B is no longer available. Consequently, there is need for a new, next-generation apo(a) RMS.

Recently, on behalf of the IFCC Working Group Apolipoproteins by Mass Spectrometry (IFCC WG-APO MS), Ruhaak et al. described the analytical validation of a successor RMP for the molar quantification of apo(a) in serum, in multiplex with six other apolipoproteins [3]. Apo(a) quantification by this RMP is based on bottom-up proteomics, in which apo(a) is

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digested into several proteotypic peptides, separated using liquid-chromatography and quantified via multiple-reaction-monitoring mass-spectrometry (LC-MRM-MS) [3]. Through selection of peptides outside the K-IV$_2$ region, the quantification of apo(a) is size-polymorphism independent. This RMP will play a key role in the new RMS for apo(a). The RMS allows manufacturers of *in vitro* diagnostic (IVD) medical devices to standardize their apo(a) test kits, facilitates the transition to measurement of apo(a) in molar units, and enables the value assignment of trueness verifiers, such as those used in external quality assessment (EQA) schemes. As suitable higher-order matrix-based reference materials for apo(a) are no longer available, the MS-based RMP is used in conjunction with a value assigned secondary reference material traceable to former reference material SRM2B.

International Organization for Standardization (ISO) 15193:2009 specifies the key elements for the analytical validation of an RMP intended for the standardization of CE-marked IVD medical tests [4]. The analytical merits of the manual RMP for apo(a), as previously described, were in line with required performance [3]. Linearity was assessed according to Clinical and Laboratory Standards Institute (CLSI) EP06 [5], and using quantifying peptide LFLEPTQADIALLK a linear range of 3.8–456.0 nmol/L with an allowable deviation of linearity (ADL) of 12 % was defined. Analytical selectivity was shown through interpeptide correlations (Pearson's *r*) of 0.987 and 0.984, and the %CVs of the ion ratios were <20 % for quantifying apo(a) peptide LFLEPTQADIALLK, suggesting the RMP is not prone to interferences. Carryover was <1 % for all apo(a) peptides and the limit of quantitation (LoQ), determined according to CLSI EP17-A2, was 3.8 nmol/L. A method comparison between the manual MS-based RMP and the 2nd generation Roche Tina-quant LPA2 immunoturbidimetric assay (ITA), performed according to EP09-A3 [6], reported a slope of 0.977 and a bias of −2.57 % for LFLEPTQADIALLK, indicating concordance between both methods and good performance of the manual MS-based RMP. More recently, transferability and reproducibility of the RMP for apo(a) were assessed by comparing the measurement results of 12 clinical samples over three laboratories. The concentration-dependent reproducibility, or between-laboratory precision (CV_{bl}), may be described using the formula: $CV_{bl}=206.32x^{-0.899}$ [7].

To reduce the imprecision of the RMP, the sample preparation of the MS-based RMP has now been semi-automated, justifying its position in the apo(a) calibration hierarchy as an RMP [8]. Importantly, analytical performance specifications (APS), based on biological variation, were established to determine whether the RMP has acceptable analytical precision. As the biological variation of apo(a) is concentration-dependent [9], a concentration-dependent expanded maximum allowable uncertainty (MAU) criterion was defined.

Building upon the original analytical validation of the manual RMP [3], we here present the analytical validation of the semi-automated RMP for apo(a). To complement the aspects of analytical validation already accomplished, a concentration-dependent precision profile was established, including an estimation of measurement uncertainty (MU), as well as values for the limit of blank (LoB) and limit of detection (LoD). Moreover, various matrices were investigated, as well as SRM2B recovery. Last, a method comparison was conducted between the semi-automated MS-based RMP and the 2nd generation Roche Tina-quant LPA2 ITA.

Materials and methods

A detailed description of the materials and methods of the semi-automated RMP and its analytical validation is provided as Supplementary Material (Supplement 1). A summary of the materials and methods is provided below.

Semi-automated sample preparation

Sample preparation has been previously described [3], and is detailed in Supplement 1. With the exception of the addition of Lys-C, centrifugation and solid phase extraction (SPE), all steps were programmed and automated on a customized G5409/A BRAVO Automated Liquid-Handling Platform (Agilent Technologies, Santa Clara, CA, USA). To provide deck position flexibility, the sample preparation procedure was divided into three protocols for semi-automation. All steps of the procedure were programmed in VWorks Automation Control Software, version 12-1-0-1295 (Agilent Technologies, Santa Clara, CA, USA). All pipetting steps were optimized for draw speed, sampling volume and ejection speed, to ensure accurate pipetting of various substances. Prior to and following sample transfer, samples were mixed by pipetting up and down. During incubation steps, plates were placed on the Teleshake 95, and closed by the heated lid. Rotation speed during incubations was 700 rpm.

Calibration and IQC

Calibration was performed through five single donor native human serum calibrators prepared according to CLSI C37-A and stored at −80 °C [9]. Apo(a) concentrations ranged from 10.1 to 275.4 nmol/L. Given the absence of suitable higher-order reference materials, traceability to former WHO-IFCC

reference material SRM2B was preferred. As such, value assignment of the calibrators was performed according to ISO 17511:2020 [8], clause 5.5, with traceability to SRM2B to maintain its accuracy base. Calibrator isoform sizes, ranged from 17 to 36 K-IV$_2$ repeats. Two native human serum samples, also prepared according to CLSI C37-A and stored at −80 °C [9], were used for internal quality control (IQC). Results were compared to established target values derived from long-term precision data using modified Westgard rules [10].

Data evaluation

LC-MS/MS data was processed using Mass Hunter Workstation Quantitative/Qualitative Analysis software Version 10.0 (Agilent Technologies, Santa Clara, CA, USA) and exported as comma-separated values (CSV) files. Further data analysis and statistical evaluation was performed using R (version 4.4.0) in RStudio (RStudio Inc., Boston, MA, USA).

Analytical performance specifications

The APS of the RMP for the quantitation of apo(a) were based on the maximum allowable uncertainty at minimum performance criteria. The MAU of apo(a) was based on the within-subject variation (CV_i), which itself may be estimated using the formula: $CV_i=40.7x^{-0.31}$, in which x is the concentration of apo(a) in nmol/L [11]. To reflect the MAU at minimum performance criteria, the CV_i was multiplied by 0.75. In addition, to reflect a 95 % CI, the MAU was expanded with coverage factor k=2. Last, half of the MAU was reserved for the RMP. This resulted in the final formula: $MAU_{RMP}=30.5x^{-0.31}$.

Analytical validation

The analytical performance was evaluated for the following characteristics: recovery, intermediate precision and MU, LoB and LoD, as well as the influence of pre-analytical variation according to CLSI protocols. Last, a method comparison between the semi-automated MS-based RMP and the Roche Tina-quant LPA2 ITA (Roche, Mannheim, Germany) was performed.

Measurement precision

Precision profiles were established and evaluated for all apo(a) peptides, according to CLSI EP05-A3 [12]. In total, 18 samples were collected, with apo(a) concentrations ranging from 6.5 to 377.3 nmol/L. Samples were each quantified in 20 runs (1 run per day) in duplicate, alongside two IQC samples in triplicate, resulting in a 20 × 2 (or 20 × 3 for IQCs) design. Precision profiles were established for each peptide based on the CVs, and a power function-based model was fitted to the data.

Measurement uncertainty

MU was determined based on Joint Committee for Guides in Metrology (JCGM) 100:2008 [13], using a bottom-up approach. In the absence of suitable higher-order reference materials for apo(a), ISO 17511:2020 was used as functional framework [8]. The MU of the RMP for the quantification of apo(a) was calculated as follows: $MU=\%rU_{rw}=k*CV_r$; where $\%rU_{rw}$ denotes the relative percent uncertainty of the RMP, which is based on long-term precision under repeatability conditions, and in which k is a coverage factor and CV_r is the repeatability (within-run precision) of the RMP [8]. Furthermore, to improve the accuracy of value assignment, clinical samples may be measured in replicate. Hence, the formula for MU may be adjusted using the correction factor $\sqrt{n}$ to: $MU_{adj} = \%rU_{rw} = k*\frac{CV_r}{\sqrt{n}}$; in which n represents the number of repeated measurements of the clinical samples.

Method comparison

To establish whether the semi-automated MS-based RMP is suitable for the global standardization of immunoassays, a method comparison between the semi-automated MS-based RMP and the 2nd generation Roche Tina-quant LPA2 ITA (Roche, Mannheim, Germany) was performed according to CLSI EP09-A3 [6]. A total of 125 patient samples were included in duplicate, reflecting the analytical measurement range of apo(a). Ordinary least squares (OLS) regressions were performed to investigate the concordance of both methods for each peptide.

Evaluation of the pre-analytical phase

The effects of pre-analytical variation were assessed in two studies. First, to assess the effects of sample collection matrices on the results of the RMP for apo(a), samples from a previous study were analyzed retrospectively [14]. For 42 donors, six matrices were analyzed in duplicate (N=504). Absolute and percent bias of the alternative matrices, relative to the serum gel matrix, was calculated for all peptides.

A repeated-measures ANOVA was performed, followed by a post-hoc Wilcoxon signed-rank test with Benjamini-Hochberg correction to identify significant differences between the matrices. Second, peptide stability was assessed largely based on recommendations provided by Gomez-Rioja et al. [15]. In total, 10 clinical serum samples (apo(a): 22.2–218.0 nmol/L) were used. Storage conditions included 48 h at RT, 7 days at 4 °C, or direct freezing at −80 °C. Stability conditions included 1 freeze/thaw (F/T) cycle, 3 F/T cycles, 5 F/T cycles, and 7 F/T cycles. During a F/T cycle, samples were thawed at RT for 2 h, mixed by vortex, and subsequently refrozen at −80 °C for at least 22 h. Percent bias relative to the 1x F/T cycle was calculated and a repeated-measures ANOVA with Bonferroni correction was performed to determine the significance of the differences in concentration between F/T cycles and storage conditions.

Recovery

To assess recovery, WHO-IFCC reference material SRM2B was measured in duplicate, across 7 experiments (n=14). For each peptide measured, the mean concentration and standard error of the mean (SEM) were calculated, and a percent recovery was determined relative to the SRM2B target concentration of 107.1±8.6 nmol/L [2].

LoB and LoD

LoB was determined according to CLSI EP17-A2 using two types of matrices [16]. First, a delipidated serum, and second, two surrogate matrices comprising either a protein matrix containing 40 g/L albumin and 1.5 g/L IgG, prepared in PBS (n=30), or a commercial Albuman preparation containing 40 g/L albumin (n=30) (Prothya Biosolutions, Amsterdam, Netherlands). Using non-parametric statistics, based on a total of *N*=60 measurements, the LoB was calculated at the 95th percentile as the mean concentration at the 57th and 58th ranked positions. The LoD was also determined according to CLSI EP17-A2 [16], using five samples with a low apo(a) concentration. The LoD was determined using a parametric statistical approach by adding the LoB to the critical value multiplier for the 95th percentile of a normal distribution, multiplied by the pooled SD

Results

Results for quantifying apo(a) peptide LFLEPTQADIALLK are detailed in text, and shown in Table 1 and Figure 1. Results for qualifying peptides GISSTVTGR and TPENYPNAGLTR are shown in Supplementary Figure 1 and 2, respectively.

Measurement precision

Concentration-dependent precision profiles were established for each apo(a) peptide according to CLSI EP05-A3 [12]. For quantifying peptide LFLEPTQADIALLK, repeatability (within-run precision) may be estimated as $CV_r=14.522x^{-0.214}$, between-run precision as $CV_{br}=16.987x^{-0.267}$, and within-laboratory precision as $CV_{wl}=23.722x^{-0.252}$, in which x is the apo(a) concentration in nmol/L (Figure 1A–C). The dynamic MAU_{RMP} is based on the formula: $MAU_{RMP}=30.5x^{-0.31}$, and is visualized as a red dot-dashed line in Figure 1A–C. The dynamic MAU_{RMP} lies within the 95 % CI of the within-laboratory precision estimate for LFLEPTQADIALLK (Figure 1C). As such, the RMP has acceptable analytical performance for the quantitation of apo(a) by quantifying peptide LFLEPTQADIALLK. Table 2 shows the precision estimates of the RMP at various apo(a) population percentiles [17].

Measurement uncertainty

Based on the precision study, the repeatability (within-run precision) of the RMP for quantifying peptide LFLEPTQADIALLK is defined as: $CV_r=14.522x^{-0.214}$ (Figure 1A). Combining the formulas for CV_r and MU yields the formulas: $MU=\%rU_{rw}=29.044x^{-0.214}$ and $MU_{adj} = \%rU_{rw} = \frac{29.044x^{-0.214}}{\sqrt{n}}$. To conclude whether the RMP meets its predefined APS (the MAU_{RMP}) across the analytical measurement range of apo(a), the MU, MU_{adj} and MAU_{RMP} are plotted in Figure 2. Without correction for multiple testing, the MU of the RMP lies above the MAU_{RMP} at minimum performance criteria. However, following correction by $\sqrt{2}$ for duplicate testing, the MU_{adj} of the RMP lies below the MAU_{RMP} at concentrations below the clinical cut-off, and following correction by $\sqrt{3}$ for triplicate testing, the MU_{adj} of the RMP would theoretically lie below the MAU_{RMP} at concentrations below 500 nmol/L.

Method comparison

A method comparison was performed between the semi-automated MS-based RMP and the 2nd generation Roche Tina-quant LPA2 ITA (Roche, Mannheim, Germany). Both methods express apo(a) in nmol/L. Interpeptide comparisons between quantifying peptide LFLEPTQADIALLK and qualifying peptides GISSTVTGR and TPENYPNAGLTR yielded slopes of 1.066

Table 1: Summary of the results of the analytical validation of the MS-based RMP for the quantitation of apo(a).

Analytical performance characteristic	Methods, materials & conditions	Desired performance criterion	Results
Measurement precision	CLSI EP05-A3	CV_{wl} below the MAU_{RMP}	For LFLEPTQADIALLK: within-laboratory precision may be estimated using: $CV_{wl}=23.722x^{-0.252}$. The dynamic MAU_{RMP} falls inside of the 95 % CI of the CV_{wl} of LFLEPTQADIALLK.
Measurement uncertainty	JCGM 100:2008	Below the MAU_{RMP}	For LFLEPTQADIALLK: an adjusted MU may be estimated using: $MU_{adj} = \%rU_{rw} = \frac{29.044x^{-0.214}}{\sqrt{n}}$.
Method comparison	CLSI EP09-A3	r>0.975	For LFLEPTQADIALLK: MS against 2nd generation Roche LPA2 ITA resulted in slope=1.185 and Pearson's r=0.985, with a mean bias of 6.0 %.
Sample collection matrix evaluation	ANOVA with post-hoc testing	No significant matrix-related bias	LFLEPTQADIALLK concentration is significantly underestimated in sodium citrate matrix (3B) and EDTA + PIC matrix (P100).
Sample stability	Controlled storage conditions	No significant stability loss	LFLEPTQADIALLK is significantly affected by F/T cycles, and is not stable during storage at RT or 4 °C.
Recovery	SRM2B-based recovery	Mean recovery of 100 %	For quantifying peptide LFLEPTQADIALLK: Recovery of 100.0 % compared to the certified SRM2B value of 107.1±8.6 nmol/L
LoB	CLSI EP17-A2	95th percentile of blank samples	The LoB of LFLEPTQADIALLK is 0.73 nmol/L in the Albuman group.
LoD	CLSI EP17-A2	LoB + 1.645 * pooled SD	The LoD of LFLEPTQADIALLK is 1.7 nmol/L.

CLSI, Clinical and Laboratory Standards Institute; CV_{wl}, within-laboratory precision; MAU, maximum allowable uncertainty; CI, confidence interval; JCGM, Joint Committee for Guides in Metrology; MU, measurement uncertainty; MU_{adj}, adjusted measurement uncertainty; F/T, freeze/thaw; RT, room temperature; LoB, limit of blank; LoD, limit of detection.

and 1.028 and Pearson's r's of 0.991 and 0.979, respectively, indicating good analytical selectivity (Figure 1G–J). Furthermore, when comparing the RMP and the ITA methods for quantifying apo(a) peptide LFLEPTQADIALLK, a slope of 1.185 and a Pearson's r of 0.985 were found (mean bias=6.0 %) (Figure 1D and E). As visualized in Figure 1D, immunoturbidimetric tests using polyclonal antibodies show a slight deviation when compared to MS. With this lot, negative bias is observed at lower concentrations, while positive bias is observed at higher concentrations (Figure 1E). Furthermore, considerable scatter is observed over the entire measurement range of apo(a).

Evaluation of the pre-analytical phase (sample collection matrix)

A repeated-measures ANOVA, followed by a post-hoc Wilcoxon signed-rank test confirmed significant matrix effects for all peptides. For quantifying peptide LFLEPTQADIALLK, a significant ($p<0.001$) negative bias of 13.2 % was observed in the sodium citrate matrix (3B), relative to the gold standard serum gel matrix (5B) (Figure 3A, D). This originates from the dilution effect of the liquid-phase citrate additive. Additionally, a significant ($p<0.05$) negative bias of 2.9 % was observed in the EDTA + PIC matrix (P100), while the other matrices showed performances similar to the gold standard serum gel matrix (5B) with non-significant biases of ± 1.2 % (Figure 3A and D). Consequently, the RMP for the quantitation of apo(a) is analytically validated for serum matrices, either with or without gel. Comparable results may be obtained for the LiHe (6B) and EDTA (7B) plasma matrices, but these results cannot be guaranteed.

Evaluation of the pre-analytical phase (sample stability)

Protein stability was assessed based on 10 clinical serum samples, each subjected to either 1, 3, 5, or 7 F/T cycles, and storage at RT, 4 °C, or −80 °C. Mean concentrations of peptides LFLEPTQADIALLK and GISSTVTGR decreased significantly ($p<0.05$) following additional F/T cycles of fresh frozen samples (Figure 3B). Peptide TPENYPNAGLTR showed a similar, but non-significant decrease between F/T cycles. With regard to the various storage conditions, only quantifying peptide LFLEPTQADIALLK showed a significant ($p<0.05$) difference in mean concentrations between frozen samples and samples stored at RT or 4 °C (Figure 3C).

Recovery

The values assigned to the five native human serum calibrators are traceable to WHO-IFCC secondary reference material SRM2B. Analyzing SRM2B using quantifying

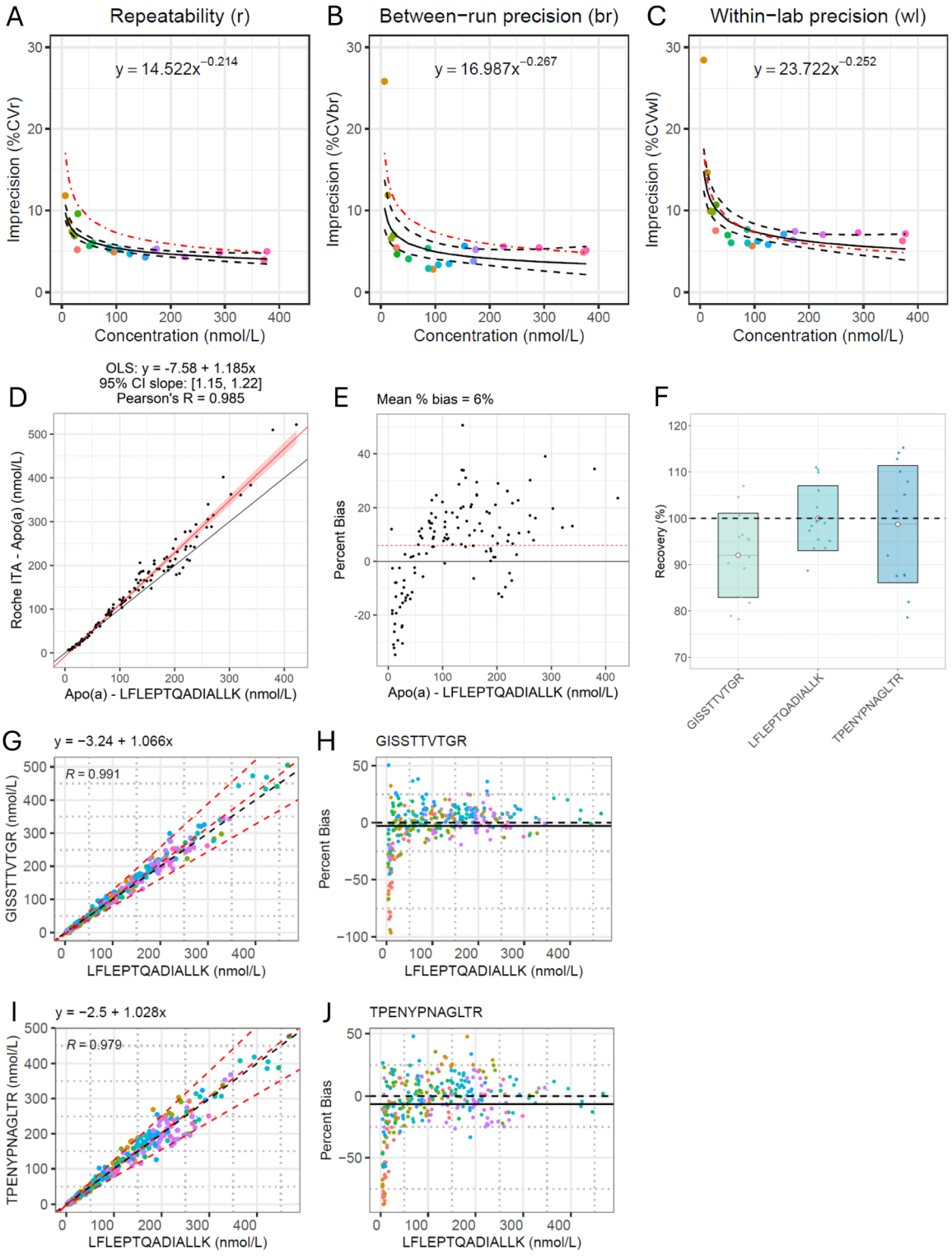


Figure 1: Analytical validation of the MS-based RMP for the quantitation of apo(a). (A–C) Precision profiles for quantifying peptide LFLEPTQADIALLK, showing its repeatability (A), between-run (B), and within-laboratory precision (C). All precision types may be estimated over the entire analytical range using their respective dynamic power functions. The dynamic power functions are illustrated as a black line with dotted lines representing their 95 % CI. Last, the dynamic MAU_{RMP} is illustrated with a red dot-dashed line. (D–E) Method comparison, illustrated by correlation and percent bias, between the MS-based RMP and the gold standard Roche Tina-quant LPA2 ITA (Roche, Mannheim, Germany). OLS regression with Pearson correlation (D), and corresponding Bland-Altman plots (E) in which the mean percent bias is indicated by a red dotted line. (F) Percent recovery of all peptides used for the quantitation of apo(a) in serum. The dashed line indicates 100 % recovery. (G–J) Interpeptide agreement of quantifying peptide LFLEPTQADIALLK vs. qualifying peptides GISSTTVTGR (G–H) and TPENYPNAGLTR (I–J), illustrated by linear regression (G and I) and percent bias (H and J). OLS, ordinary least squares; CI, confidence interval; ITA, immunoturbidimetric assay.

Table 2: Within-run precision (CV_r), between-run precision (CV_{br}) and within-laboratory precision (CV_{wl}) of the RMP for quantifying apo(a) peptide LFLEPTQADIALLK at various population percentiles.

Unit	[a]Percentiles									
	2.5th	5th	10th	25th	50th	75th	90th	95th	97.5th	100th
nmol/L	1.7	2.0	3.3	8.7	19.6	61.2	146.8	197.4	238.7	438.4
$\%CV_r$	13.0	12.5	11.2	9.1	7.7	6.0	5.0	4.7	4.5	4.0
$\%CV_{br}$	14.7	14.1	12.4	9.5	7.7	5.7	4.5	4.1	3.9	3.3
$\%CV_{wl}$	20.7	19.9	17.6	13.8	11.2	8.4	6.7	6.3	6.0	5.1

[a]Percentiles are based on Reijnders et al. [17], as measured in our laboratory using the Designated Comparison Method [18]. CV_r, within-run precision; CV_{br}, between-run precision; CV_{wl}, within-laboratory precision.

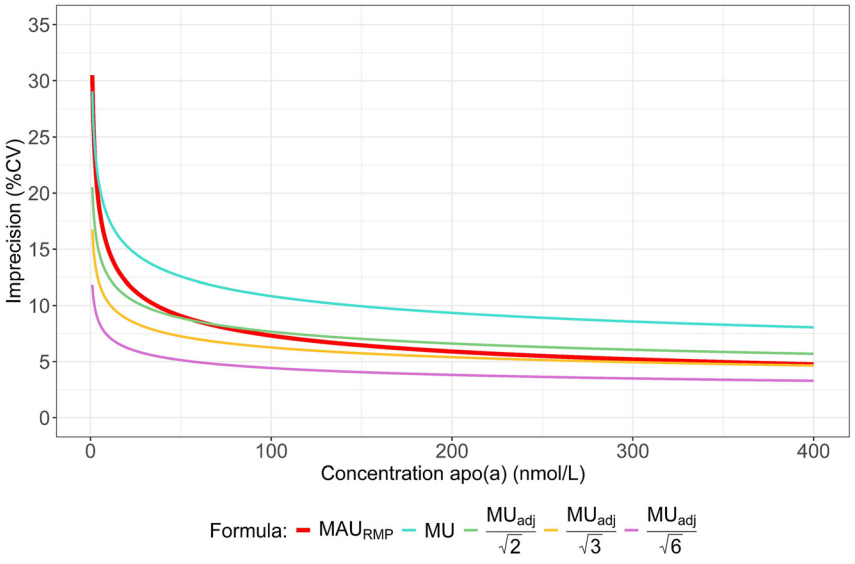


Figure 2: Maximum allowable uncertainty of the RMP (MAU_{RMP}; red), the measurement uncertainty of the RMP (MU; blue), and corrections that may be applied to the measurement uncertainty of the RMP in case of multiple testing (MU_{adj}; green ($\sqrt{2}$), yellow ($\sqrt{3}$) and purple ($\sqrt{6}$)). MAU_{RMP}, maximum allowable uncertainty of the reference measurement procedure; MU, measurement uncertainty; MU_{adj}, adjusted measurement uncertainty.

peptide LFLEPTQADIALLK showed a (mean±SEM) result of 107.1±2.0 nmol/L. Compared to the certified SRM2B target concentration of 107.1±8.6 nmol/L, this translates into a recovery of 100.0 % (Figure 1F). As such, there is no significant deviation from the target concentration, indicating adequate value transfer to the native human serum calibrators.

LoB and LoD

The LoB of apo(a) was calculated for various matrices. Quantifying peptide LFLEPTQADIALLK was determined to have an LoB of 0.73 nmol/L in the Albuman group, and an LoB of 1.07 nmol/L in the delipidated serum matrix. The delipidated serum matrix may have a higher LoB due to incomplete depletion of endogenous apo(a). Therefore, the Albuman matrix was considered to be the most suitable matrix for LoB evaluation. Last, the LoD of apo(a) was determined based on 51 replicate analyses of five samples with a low apo(a) concentration. For quantifying peptide LFLEPTQADIALLK, this resulted in an LoD of 1.7 nmol/L.

Discussion

With the inoperability of the 1st generation ELISA-based apo(a) RMP and the unavailability of its associated reference materials, there is a need for a new, next-generation apo(a) RMS. In this context, we previously described the analytical validation of a manual IFCC-endorsed MS-based RMP for the molar quantification of apo(a) in serum, in multiplex with six other apolipoproteins [3]. This successor RMP is well-suited for the standardization of IVD manufacturer's tests because of its K-IV_2 size-polymorphism and antibody-independence, and does not require dilution of samples with high apo(a) concentrations. The multiplex apo RMP employs a bottom-up proteomics strategy combined with isotope-dilution LC-MS. Specifically, samples are diluted, after which the internal standard is added immediately. Upon reduction and alkylation, proteotypic peptides are formed through proteolysis, and, following clean-up, quantified by LC-MS. The main sources of imprecision we identified arise from pipetting steps, the proteolytic digestion, and the actual

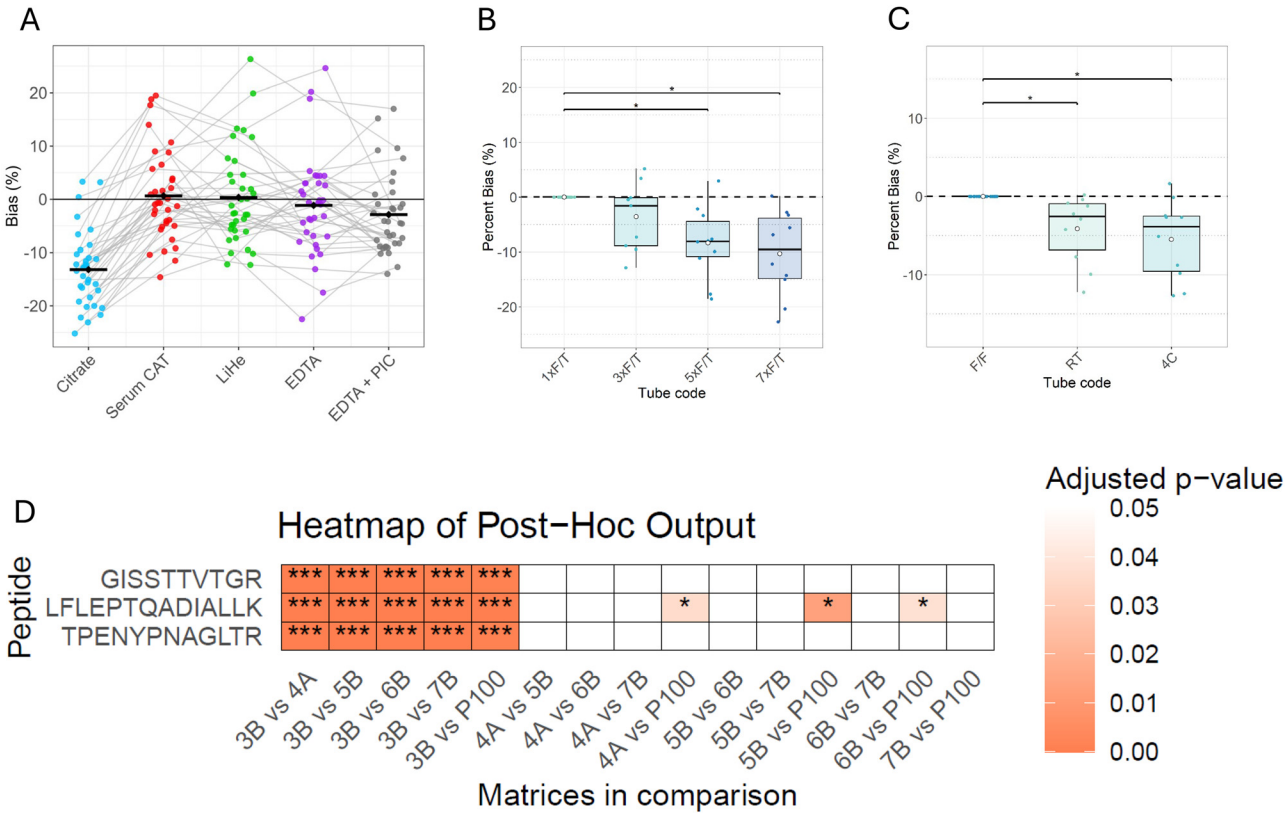


Figure 3: Evaluation of the pre-analytical phase of the MS-based RMP for the quantitation of apo(a). (A) Percent bias observed after quantitation of quantifying peptide LFLEPTQADIALLK in various matrices relative to the gold standard serum gel (5B). (B–C) Stability of LFLEPTQADIALLK, illustrated as percent bias observed following various F/T cycles (B), and under various storage conditions (C). (D) Outcome of the pre-analytical matrix evaluation, illustrated as a heatmap showing the significance of each matrix comparison (*<0.05, **<0.01, ***<0.001). F/T, freeze/thaw.

measurement. Consequently, to further reduce the imprecision of the RMP, its sample preparation has now been semi-automated.

The semi-automated RMP follows a robotized protocol, in which the pipetting steps are always performed in a similar manner, at the same speed, and with standardized waiting steps, reducing both within-run pipetting errors and between-run inter-operator variability and digestion variation. On the other hand, the semi-automated RMP uses a single pipette head for all pipetting steps, while the manual RMP uses multiple pipettes optimized for various volume ranges, which may improve pipetting precision. However, this does not seem to outweigh the precision gain of semi-automation. Using the manual sample preparation procedure, five native human serum samples with concentrations of 46.7, 8.0, 14.9, 238.3, and 343.6 nmol/L were measured and average within-run precision (CV_r) estimates were calculated: 7.3, 5.8, 8.8, 7.7, and 6.6 %, respectively [3]. For the semi-automated sample preparation, repeatability may be estimated as $CV_r=14.522x^{-0.214}$, between-run precision as $CV_{br}=16.987x^{-0.267}$, and within-laboratory precision as $CV_{wl}=23.722x^{-0.252}$. Using the within-run precision formula now defined for the semi-automated RMP, at the above mentioned concentrations, these samples would have CV_r estimates of 6.4, 9.3, 8.1, 4.5, and 4.2 %, respectively. Thus, compared to its manual predecessor, the semi-automated successor RMP shows generally better within-run precision.

Furthermore, for most measurands, the APS are defined as a fixed value, derived from the within-subject variability (CV_i). However, as the CV_i of apo(a) is concentration-dependent, we established a dynamic APS based on the MAU at minimum criteria (MAU_{RMP}) to determine whether the analytical performance of the RMP for the quantitation of apo(a) is acceptable: $MAU_{RMP}=30.5x^{-0.31}$. Similarly, the MU of the RMP is also concentration-dependent, and may be estimated as: $MU_{adj}=\frac{29.044x^{-0.214}}{\sqrt{n}}$. To fulfill the predefined APS, samples should be measured in at least triplicate, but preferably more. Consequently, we intend to perform value assignment of prospective samples in triplicate over two runs (n=6). This should guarantee good analytical performance for the quantitation of apo(a) at minimum criteria.

The semi-automated RMP was calibrated using native human serum samples traceable to former reference material SRM2B. Recoveries indicated accurate value transfer of SRM2B to the native human serum calibrators. The LoB and LoD of the RMP were determined to be 0.73 nmol/L and 1.7 nmol/L, respectively. To maintain apo(a) stability, it is recommended to freeze samples at −80 °C for long-term storage, and to prevent multiple F/T cycles. The semi-automated RMP was analytically validated using various matrices, based on which the use of serum matrices is recommended. Last, a method comparison between the semi-automated MS-based RMP and a ITA was performed. Despite a considerable slope reflecting the calibration bias, the correlation between both methods is very high. As such, the semi-automated RMP appears to be well-suited for the standardization of Lp(a)/apo(a) immunoassays.

Conclusions

Semi-automation had a positive impact on the analytical precision of the RMP for the molar quantitation of apo(a) in serum. The semi-automated RMP fulfills the preselected APS following adjustment for including multiple replicates. Semi-automation also led to a marked gain in efficiency, as evaluated through increased throughput and improved track-and-trace of samples, avoiding time delays during digestion, reduction and alkylation steps, and generally being less labor-intensive. Furthermore, by the introduction of semi-automation, maintaining traceability to former reference material SRM2B and reporting apo(a) in molar units, no absolute changes in apo(a) concentrations are expected as compared to the first-generation ELISA-based apo(a) RMS [19]. As such, the semi-automated RMP is considered suitable for comparison studies, and for the standardization and certification of IVD manufacturer's immunoassays [20, 21].

In this context, an Lp(a)/apo(a) certification program has been developed at the Leiden Apolipoprotein Reference Laboratory (LARL) [22]. Participation of IVD manufacturers in this certification program is encouraged and strongly recommended to reduce inter-method variability and to realize the global standardization of Lp(a)/apo(a) test results, clinical decision limits and on-treatment targets. With the looming of Lp(a)/apo(a)-lowering therapies, standardized, molar quantification of apo(a) is crucial to accurately diagnose and monitor patients at increased risk of cardiovascular disease due to elevated Lp(a) levels.

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Conflict of interest: Prof. dr. C.M. Cobbaert has/had leadership roles: Chair of the IFCC Scientific Division. Past chair of the IFCC WG on APO standardization. Head of the calibration lab running the described apo(a) Reference Measurement Procedure. Dr. L.R. Ruhaak has/had leadership roles: Chair of the IFCC WG APO-MS. Member of the Leiden Apolipoprotein Reference Laboratory, which runs the described apo(a) Reference Measurement Procedure. All other authors state no conflict of interest.
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