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Chemiluminescence immunoassay for sensing lipoprotein-associated phospholipase A2 in cardiovascular risk evaluation

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ABSTRACT

Background: Lipoprotein-associated phospholipase A2 (Lp-PLA_2) is a novel inflammatory biomarker, which is useful as an adjunct identification tool for cardiovascular disease. However, the important limitation of the conventional enzyme-linked immunosorbent assay (PLAC ELISA) for Lp-PLA_2 assay is its relatively low sensitivity and time consuming. A method to measure the Lp-PLA_2 simply, rapidly and sensitively is essential for predicting cardiovascular events in clinic.

Methods: We took advantage of magnetic separation integrated with chemiluminescence to detect Lp-PLA_2. The concentration of Lp-PLA_2 was measured through a one-step process by mixing antibody labelled magnetic beads, antigen and antibody at one time.

Results: Our method realized the sample to answer within 17 min. The detection limit and measurement range were 0.18 ng/ml and 0.18–1350 ng/ml, respectively. The specificity assay showed that no appreciable interference was observed for the substances of bilirubin, triglyceride, hemoglobin, rheumatoid factor and human anti-mouse antibody up to the concentrations of 40 mg/dl, 1000 mg/dl, 2000 mg/dl, 1500 IU/ml and 30 ng/ml, separately. We also tested 122 clinical samples using our method, presenting good overall correlations (R^2 = 0.979) to the PLAC ELISA. It is worth mentioning that, our method was faster, had a wider range of measurement and higher sensitivity compared with the PLAC ELISA.

Conclusions: The Lp-PLA_2 assay is straightforward, sensitive and precise, which is highly suitable to further explore the clinical performance of Lp-PLA_2 in studies of cardiovascular risk management.

1. Introduction

Lipoprotein-associated Phospholipase A_2 (Lp-PLA_2) is a vascular-specific inflammatory enzyme belonging to the A2 Phospholipase superfamily [1]. It is predominantly produced by macrophages, lymphocytes and foam cells in atherosclerotic plaques and expressed in higher concentrations in advanced atherosclerotic lesions than early-stage lesions [2–4]. Lp-PLA_2 can hydrolyze oxidized phosphatidylcholine on low-density lipoprotein (LDL) particles within the arterial intima. This biochemical reaction generates lysophosphatidylcholine and oxidized free fatty acids, and both of them are potent pro-inflammatory products contributing to the formation of atherosclerotic plaques [5–7]. Furthermore, many studies have demonstrated that Lp-PLA_2 has demonstrated modest intra- and inter-individual variation, independent from traditional cardiovascular risk factors and multifarious inflammatory biomarkers, and substantially less than C-reactive protein (CRP). Elevated levels of Lp-PLA_2 in human plasma and serum is a strong risk factor for coronary heart events, a finding that to be used in conjunction with clinical evaluation and patient risk assessment as an aid in predicting risk for coronary heart disease associated with atherosclerosis [8–12].

With the process to ensure Lp-PLA_2 as a biomarker to evaluate cardiovascular risk, the development of detection methods for it is ongoing synchronously. At present, methods for determination of Lp-PLA_2 mainly involve spectrophotometry, latex-enhanced turbidimetry, radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA) [13–18]. Among them, PLAC ELISA is the most common used method and there is one FDA approved product on the market. However, one important limitation for ELISA is its relatively low sensitivity due to the insufficient capture of antigen to the surface-anchored antibody in a heterogeneous system [19]. In clinic, high sensitivity is important, which can satisfy the demand of detecting the low abundance biomarkers in the early stages of diseases or after therapeutic interventions [20]. In addition, multiple steps of incubation and

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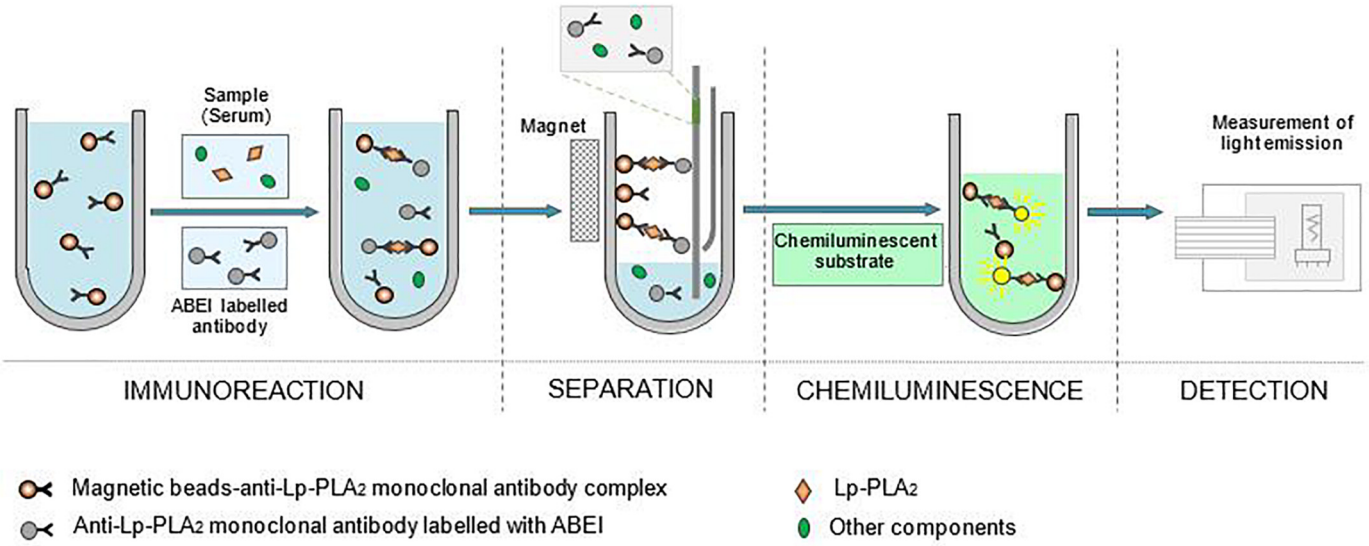

Scheme 1. Schematic representation of Lp-PLA_2 detection through combining magnetic separation and chemiluminescence.

washing which are requiring significant manual processing are unavoidable in a typical ELISA protocol. As a consequence, the entire process usually takes several hours to days to obtain the results [21,22]. Considering the importance of Lp-PLA_2 in predicting cardiovascular events in clinic, a highly-sensitive, straightforward and automated strategy for Lp-PLA_2 detection is thus in urgent needs.

To address this issue, we design a sandwich chemiluminescent immunoassay to measure Lp-PLA_2 through combining magnetic separation and chemiluminescence, which is the first chemiluminescent application for Lp-PLA_2 (Scheme 1). We prepare magnetic beads functionalized with anti-Lp-PLA_2 monoclonal antibody (MBs-Ab_2) and anti-Lp-PLA_2 monoclonal antibody labelled with N-(4-Aminobutyl)-N-ethylisoluminol (ABEI) (Ab_1-ABEI). The functionalized antibodies are stable for minimum four weeks at 2–8 °C. Through one step incubation of Ab_1-ABEI, Lp-PLA_2 in the sample, and MBs-Ab_2, the MBs complex is formed. After magnetic separation and luminous substrate addition, chemiluminescent signals are generated and the intensities of which correlate with the concentration of Lp-PLA_2. The total time to perform this rapid assay is 17 min. We validate this platform and apply it for Lp-PLA_2 quantification in clinical serum samples.

2. Materials and methods

2.1. Materials

Carboxylated magnetic beads were from micromod GmbH ihrem Kundenkreis. ABEI, Proclin 300, MES (2-[N-morpholino] ethane sulfonic acid), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS) were purchased from Sigma Aldrich. BSA, Tris and Phosphate-buffered saline (PBS, pH = 7.2) buffer were from Shanghai Sangon Company. NaN_3, Tween-20 and chemiluminescent substrates (sodium hydroxide and H_2O_2) were bought from Sinopharm Chemical Reagent Co., Ltd. The anti-Lp-PLA_2 antibodies were received from Diazyme. Lp-PLA_2 protein stock and Lp-PLA_2-depleted serum were provided by New Industries Biomedical Engineering Co. Ltd. The bilirubin, hemoglobin and triglycerides were bought from Sigma Aldrich. The rheumatoid factor was purchased from Biorbyt and the human anti-mouse antibody (HAMA) were from Biosources. All chemicals were used as received without further purification. PLAC® Test ELISA Kit was from diaDexus, Inc., whose linear range and detection limit were 151–810 ng/ml and 0.34 ng/ml, respectively. The water we used was deionized water produced by Direct-Q 3UV (Millipore). The washing buffer was made of 0.05 mol/L of Tris solution with 0.05% of Tween-20, and the dilution buffer was composed of BSA (0.5%), NaN_3 (0.1%) or/and Proclin 300 (0.1%).

2.2. Samples

The serum samples from donors were collected from Shenzhen Nanshan People's Hospital. The serum samples were collected into standard sampling tubes or tubes containing separating gel and fasting was not required. Specimens were transported on cold packs (at 2 to 8 °C) and stored up to 60 days frozen at −20 °C or stored at 2–8 °C for up to 1 week after the sample was drawn. Repeated freezing/thawing process should be avoided. All patients provided written informed consent and the study was approved by the institutional review board.

2.3. Instrumentation

The immunoassay procedures were carried out on Maglumi series automatic analyzer (New Industries Biomedical Engineering Co. Ltd). The antibody labelling steps and substances mixing involved the equipment including thermostat (FYL-YS), shaker (ZXWL-100), refrigerated high-speed centrifuge (Thermo) and a model XW-80A vortex mixer (Jingke Industrial).

2.4. Preparation of antibody coated magnetic beads (MBs) and ABEI labelled antibody

0.4 mg EDC (~2 mM) and 0.6 mg of NHS (~5 mM) were added to 1 ml of carboxylated MBs solution (0.1 mg/ml, MES buffer). After incubation for 15 min at room temperature, purified anti-Lp-PLA_2 monoclonal antibody was added by a weight ratio of the resultant solution to the anti-Lp-PLA_2 monoclonal antibody at 1 mg: 12 μg. The mixture was incubated in a constant-temperature shaking bath at 37 °C for 24 h. Then the MBs were collected using magnetic separation and washed with PBS buffer for four times. The prepared anti-Lp-PLA_2 antibody coated MBs with a concentration of 20 mg/ml were preserved at 4 °C for the following application.

The protocols for ABEI labelled anti-Lp-PLA_2 monoclonal antibody also utilized EDC-NHS chemistry. The anti-Lp-PLA_2 monoclonal antibody was dissolved in MES buffer, with the addition of 0.4 mg EDC (~2 mM) and 0.6 mg of NHS (~5 mM). After incubation for 15 min in MES buffer at room temperature, the activated antibody was collected through ultrafiltration and washed with PBS buffer for two times. Next,

the activated antibody (re-dispersed using PBS buffer) was mixed with ABEI. After 2 h at room temperature, the ABEI labelled antibody was gathered using ultrafiltration and washed with PBS buffer for two times. The ABEI labelled anti-Lp-PLA_2 monoclonal antibody were stored at 4 °C for the further application.

Stability of the anti-Lp-PLA_2 antibody coated MBs and ABEI labelled anti-Lp-PLA_2 monoclonal antibody was assessed by analyzing 3 serum with different concentration (100, 250, 500 ng/ml) using the same reagent over a period of 2 weeks stored at 37 °C. The results (Table S1) showed that the average percentage difference of the reagents was maintained within ± 10% for at least 9 days at 37 °C, which meant longer than 6 months at 2–8 °C [23].

2.5. Experimental procedure for the detection of Lp-PLA_2

The Lp-PLA_2 assay was a sandwich chemiluminescent immunoassay. 20 μl Lp-PLA_2 protein solution with different concentrations, 50 μl ABEI labelled anti-Lp-PLA_2 monoclonal antibody solution with the concentration of 0.3 μg/ml and 20 μl of the 1.5 mg/ml suspension of the MBs coated with another anti-Lp-PLA_2 monoclonal antibody were mixed thoroughly and incubated for 15 min at 37 °C, sandwich of immuno-complexes forming. After precipitation in a magnetic field, the supernatant was removed and then a wash cycle was performed. The washing and separation steps were automatically controlled by the cleaning system module, which could be integrated into the automation instrument (Video S1, S2). Subsequently, 200 μl chemiluminescent substrates solution was added and Maglumi series automatic analyzer was applied to measure the relative luminous intensity. The incubation time were optimized, in which immunoreaction times at 10 min, 15 min, 25 min and 35 min were examined, and the results were shown in Fig. S1. When the immunoreaction time was 10 min, the value of chemiluminescence was obviously lower and CV was larger than that whose immunoreaction time was 15 min, 25 min or 35 min. The results received from the system with different incubation time (15 min, 25 min and 35 min) were similar; therefore, 15 min was selected as the incubation time.

To quantify the concentration of Lp-PLA_2 in clinical samples, the standard calibration curve was necessary. The traceable diaDexus calibrator at a concentration of 1500 ng/ml was diluted proportionally in the dilution buffer to obtain 10 different samples with specific concentrations of 0.000, 10.000, 18.700, 34.960, 65.410, 122.380, 229.00, 428.460, 801.676 and 1500.000 ng/ml. All the measurements were repeated 3 times for each sample. The average value of the intensity and the concentrations of the standards were cubic polynomial fitted to generate a ten-point calibration curve.

2.6. Method validation

2.6.1. Study of specimen type

The research on the influence of different specimen type was performed by collecting blood from 30 donors in tubes without additives, serum gel separation tubes, heparin plasma collection tubes and EDTA-2 K plasma collection tubes respectively. All the samples were centrifuged and the supernatant were used to test. The results of the samples in tubes without additives were used as controls to calculate the deviation of other tests.

2.6.2. Determination of inter- and intra-assay precision for the Lp-PLA_2 assay

After constructing the standard calibration curve, the evaluation of the imprecision was the basis for all assay. Inter- and intra-assay precision for the Lp-PLA_2 assay were determined as described in the Clinical Laboratory and Standards Institute (CLSI) EP5-A2. Three human serum pools with low, mid, and high Lp-PLA_2 concentration and two controls containing different concentration of analytes were tested in duplicate in two independent runs per day for 20 testing days. The interval between two runs in one day must not be < 2 h. And three batches of reagents were used for testing, with the first batch of reagents used on day 1–7, the second batch on day 8–14, and the third batch on day 15–20. The mean, standard deviation (SD) and coefficients of variance (CV) were calculated from the measured values. CV ≤10% was acceptable [24,25].

2.6.3. The detection limit and analytical measurement range of Lp-PLA_2 assay

The limit of blank (LoB), limit of detection (LoD) and limit of quantification (LoQ) are important parameters to evaluate the performance of Lp-PLA_2 assay, which were determined according to CLSI EP17-A guideline. To calculate the LoB of the Lp-PLA_2 assay, four batches of the dilution buffer were applied as the zero concentration blank samples. Each sample was repeated 5 times a day for 3 testing days with a minimum of 2 h between each repetition. The results of 60 determinations were arranged from small to large, and the LoB was calculated as the mean of the 57th and 58th values of the results when the type I error was set at 5%.

To determine the LoD of our assay, Lp-PLA_2 protein stock was diluted by Lp-PLA_2-depleted serum to the concentration of 0.50, 1.50, 2.50 and 4.00 ng/ml respectively. The four low-level samples were tested in 3 runs and 5 replicates per run. The LoD was calculated according to the equation (LoB + (1.653× SD of all the LoD samples)). In this equation, 1.653 was the correction factor of a 95% confidence interval for data and SD was the standard deviation of multiple low-level samples [26].

Six samples with low concentration of Lp-PLA_2 (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ng/ml) were used to test LoQ. The samples were tested for 20 days continuously, each test was repeated twice a day and there were a total of 40 tests. According to the formula (CV = SD/Mean × 100%), the CV of results for each sample was calculated. The LoQ was obtained by plotting the CV against the mean concentration and the concentration whose corresponding CV was 20% would be identified as the LoQ.

Furthermore, in order to obtain the measurement range, a sample with the initiate concentration of 1500.0 ng/ml was diluted to the concentration of 0.00, 150.00, 300.00, 450.00, 600.00, 750.00, 900.00, 1050.00, 1200.00, 1350.00 and 1500.00 ng/ml respectively. All the measurements were repeated 3 times for each sample. Then calculated the average of tripartite test results for each sample and used least-squares method to linearly fit the calculated and theoretical concentrations by plotting the expected value on the x-axis versus the mean on the y-axis. Combined with the LoB, we got the analytical range.

2.6.4. Interference of other components in serum for Lp-PLA_2 detection

The common endogenous substances in serum include bilirubin, hemoglobin, triglycerides, rheumatoid factor (RF) and human anti-mouse antibody (HAMA). They might influence the detection of Lp-PLA_2. Following the CLSI EP7-A2, three individual serum samples with “low” (50 ng/ml), “medium” (230 ng/ml), and “high” (500 ng/ml) concentrations of Lp-PLA_2 were spiked with 3 different concentrations of each potential interferent at one time. Three concentrations of interferents were listed in Table S2. Interference rate was calculated according to the equation ((test group results - control group results)/control group results × 100%) and the acceptable standard of the interference rate is < 10%.

2.6.5. Determination of the recovery of the Lp-PLA_2 assay

In the recovery experiments, we prepared serum samples with high (500 ng/ml), medium (150 ng/ml) and low (20 ng/ml) Lp-PLA_2 concentrations respectively and different concentrations (0.00, 20.00 and 200.00 ng/ml) of Lp-PLA_2 standard solution. Afterwards, added 0.1 ml of 0, 200, 2000 ng/ml Lp-PLA_2 standard solution to 0.9 ml of each concentration sample to prepare 1 ml sample to be tested. We repeated each test for two times and calculated the recovery rate by the equation

((mean of measured concentration - value of original concentration before the addition)/analyte addition concentration × 100%).

2.6.6. Patient comparison study

After confirming the accuracy of our platform, the clinical samples were used to compare our method with PLAC ELISA. We measured the concentration of Lp-PLA$_2$ in 122 clinical samples. The final results were calculated based on the standard curve. According to the results, linear regression analysis was applied, in which data was plotted using the measured value of our method on the Y axis and the tested value of PLAC ELISA on the X axis. Furthermore, to evaluate the agreement between these two methods, we performed the Bland-Altman analysis, which was a classical statistical analysis of method comparison studies [27].

2.6.7. Statistical analysis

All statistical analyses were conducted with Microsoft Office Excel 2016 or SPSS 23.0 software. For statistical analyses, Shapiro-Wilk normality test, Wilcoxon signed rank test, polynomial regression analysis, linear regression, correlation analysis, paired *t*-test and Bland-Altman analysis were used as indicated. The P values of statistical tests < 0.05 were considered statistically significant.

3. Results

3.1. The sample type of Lp-PLA$_2$ assay

The concentrations of the samples in tubes without additives were applied as controls to calculate the deviation of data for gel separation serum, heparin plasma and EDTA-2K plasma. The quantitative values of samples collected using heparin or EDTA-2K plasma collection tubes were lower than that using the additive-free blood collection tubes (Fig. 1B, C). Meanwhile, the deviation of data for heparin plasma and EDTA-2K plasma was relatively larger than that for gel separation serum (Fig. 1). To decrease the interference caused by specimen type, we chose the serum from tubes without additives or serum gel separation tubes in the assay.

3.2. Calibration curve establishment and precision analysis

To quantify the concentration of Lp-PLA$_2$ precisely, we established the calibration curve by using ten different concentrations of the standard solution. The chemiluminescent intensities changed gradually with the increasing concentration of Lp-PLA$_2$ (Fig. 2). Using cubic polynomial regression analysis to construct the standard calibration curve ($y = 0.0003 \times ^3 – 1.0045 \times ^2 + 2299.1 \times + 7478.1$), we had a fitted correlation coefficient of 0.9999 for the ten-point curve. It indicated that the luminous intensity of our method could reflect the Lp-PLA$_2$ concentration in the human serum according to the cubic polynomial.

To confirm the precision of our system further, we measured 5 different concentrations of samples with three diverse batches of reagents. The data in Table 1 showed that Coefficient of Variance (CV) values of within-run and between-run were lower than 10%. In particular, the CV values were < 3% except for that of Serum Pool l, indicating satisfying repeatability and precision of our method.

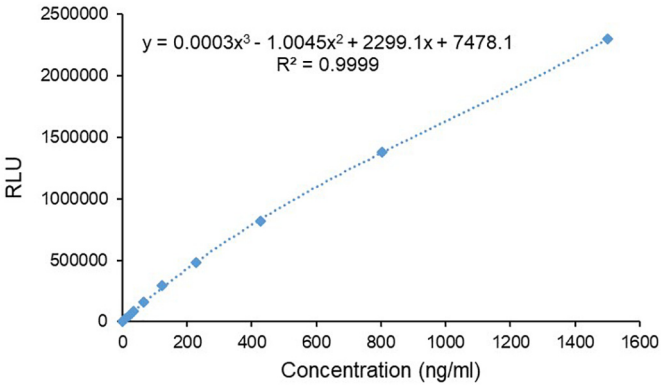

Fig. 2. The standard calibration curve of Lp-PLA$_2$ assay.

Table 1
The results of inter- and intra-assay precision for the Lp-PLA$_2$ assay.

Sample	Mean (ng/ml) (N = 80)	Within-Run		Between-Run		Total	
		SD (ng/ml)	%CV	SD (ng/ml)	%CV	SD (ng/ml)	%CV
Serum pool 1	40.308	1.949	4.84	2.491	6.18	3.163	7.85
Serum pool 2	279.519	5.269	1.89	4.737	1.69	7.085	2.53
Serum pool 3	560.439	6.417	1.15	4.831	0.86	8.033	1.43
Control 1	250.325	5.053	2.02	5.272	2.11	7.302	2.92
Control 2	471.550	7.563	1.60	5.454	1.16	9.324	1.98

3.3. The investigation of sensitivity of Lp-PLA$_2$ assay

According to the experimental results (Table S3, S4 and Fig. S2) and methods described above, the LoB, LoD and LoQ were obtained as 0.180 ng/ml, 0.846 ng/ml and 2.282 ng/ml, respectively. In accordance with the results of analytical measurement range, using linear regression fitting a straight line through the mean and theoretical concentrations to obtain the best-fit value of the slope and intercept (Fig. 3). The curve displayed linearity with $R^2 = 0.9989$ ($P < 0.0001$) displaying that the correlation between the expected value and the measured value was excellent. From the linear regression equation $y = 1.0137 \times -11.443$, we could see that the slope (1.0137) was within 0.97–1.03, demonstrating that our method was suitable for precise quantification of Lp-PLA$_2$ within the measuring range. And the measuring range was identified as 0.18–1350.0 ng/ml defined by the limit of blank and the maximum of the linear curve.

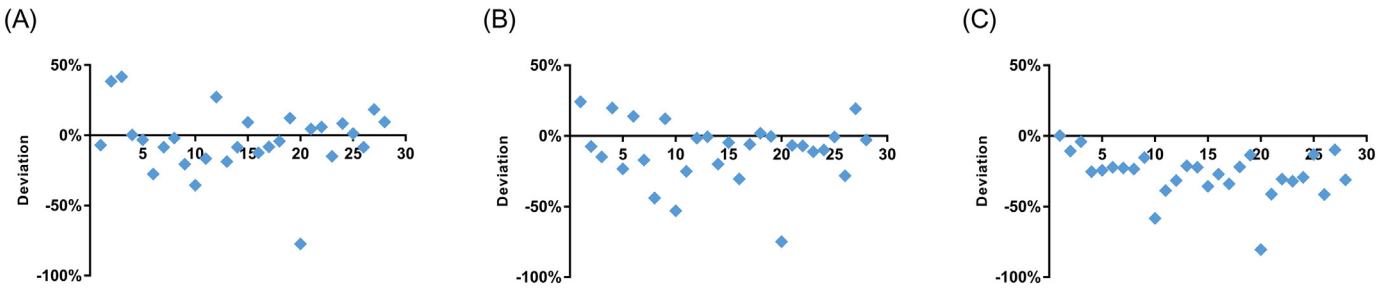

Fig. 1. The deviation of concentration of Lp-PLA$_2$ for gel separation serum (A), heparin plasma (B) and EDTA-2K plasma (C).

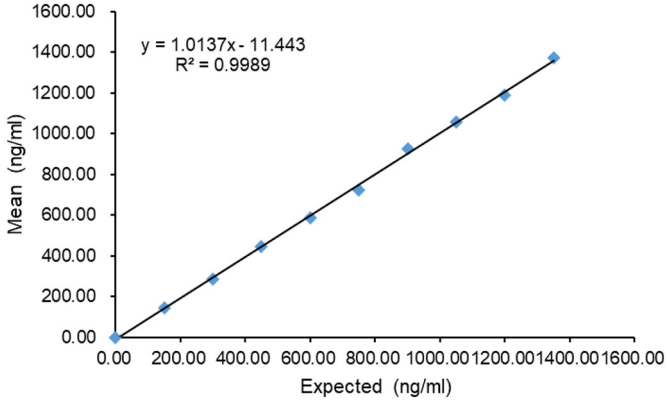

Fig. 3. Linearity of the Lp-PLA$_2$ assay. X-axis is the expected value and y-axis is the measured value.

3.4. The specificity of the system

Apart from sensitivity, the specificity is another significant parameter for evaluating the quality of a detection system. We measured serum samples in the presence of different concentrations of several common substances such as bilirubin, hemoglobin, triglycerides, RF and HAMA. The interference rate of different potential interferents were within ± 10%, meeting the acceptance criteria. No appreciable interference was observed for the substances of bilirubin, triglyceride, hemoglobin, RF and HAMA up to the concentrations of 40 mg/dl, 1000 mg/dl, 2000 mg/dl, 1500 IU/ml and 30 ng/ml, respectively (Fig. 4). Our system presented satisfying specificity for Lp-PLA$_2$ assay, which promoted further exploration in clinical sample detection.

3.5. Recovery of the Lp-PLA$_2$ assay

The recovery rate is an important indicator of the accuracy of detection systems. We tested the recovery of the Lp-PLA$_2$ assay by using different spiked serum samples, which contain "low" (20 ng/ml), "medium" (150 ng/ml) and "high" (500 ng/ml) concentrations of Lp-PLA$_2$. The recovery rate shown in Table 2 was between 94.74% and 103.78%, meeting the requirement that the recovery percentage ranged within 90–110%. The recovery is acceptable for further application in the clinical setting.

3.6. Method comparison

We performed 122 duplicate clinical serum samples by both our method and the PLAC ELISA method (PLAC® Test ELISA Kit) as a reference, which is the only blood test cleared by the FDA to aid in assessing risk for both coronary heart disease (CHD) and ischemic stroke associated with atherosclerosis. In terms of the comparison data, linear regression analysis was applied (Fig. 5A). We obtained the regression equation of $y = 1.0989 \times -2.5863$ and the R^2 of 0.9791 ($P < 0.0001$), suggesting that good correlation between these two methods. In addition, we performed a twofold dilution on high-concentration samples whose concentration was out of detection range of PLAC ELISA and retested the diluted samples by PLAC ELISA and our method. The deviation of PLAC ELISA was bigger than that of our method, which was a serious limitation in clinic (Table S5). Therefore, we could draw a conclusion that our method had a wider analytical range and smaller deviation compared with PLAC ELISA, which was more suitable for clinical application. Furthermore, we used the difference between the two measurements on the Y axis and the average of the two measurements on the X axis to get the Bland-Altman plot (Fig. 5B). We could find that only 2.56% points outside the 95% consistency limit. Furthermore, in the case of large-scale clinical applications in future, it is necessary to use the displacer (whose preparation method was listed in the supplementary material) to liberate Lp-PLA$_2$ from lipoproteins, improving the accuracy of the detection, although our limited results showed no obvious influence with the existence of lipoprotein (Fig. S3). The reasons are that the large-scale clinical samples are more complicated, which may come from the patients with dyslipidemia or inflammation, and the literatures reported Lp-PLA$_2$ enzyme was bound to lipoprotein particles [28].

Table 2
The recovery of the Lp-PLA$_2$ assay for testing Lp-PLA$_2$ in spiked serum.

Sample (ng/ml)	Added amount (ng/ml)	Detection value (ng/ml)			Recovery %
		Rep 1	Rep 2	Mean	
S1 (20)	0	17.999	17.581	17.79	–
	20	36.89	38.69	37.79	100.00%
	200	227.85	222.85	225.35	103.78%
S2 (150)	0	134.607	133.688	134.148	–
	20	153.286	152.906	153.096	94.74%
	200	331.506	323.506	327.506	96.68%
S3 (500)	0	445.636	456.285	450.961	–
	20	470.844	470.744	470.794	99.16%
	200	643.284	657.884	650.584	99.81%

4. Discussion

A lot of guidelines for Cardiovascular disease risk, such as 2010 ACCF/AHA guidelines, 2016 ESC guidelines and 2013 ACCF/AHA guidelines [29–31], support the statement that Lp-PLA$_2$ has recently

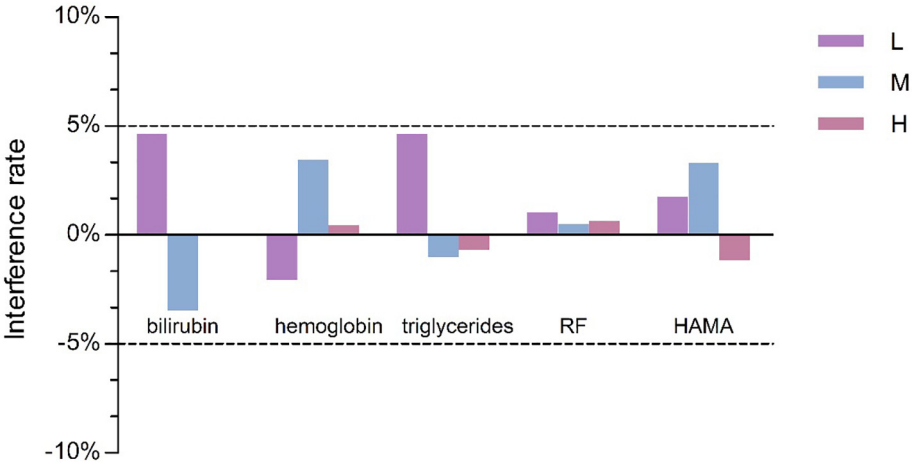

Fig. 4. The specificity of Lp-PLA$_2$ assay. The concentrations of bilirubin, hemoglobin, triglycerides, RF and HAMA were 40 mg/dl, 1000 mg/dl, 2000 mg/dl, 1500 IU/ml and 30 ng/ml, respectively. L means 50 ng/ml, M means 230 ng/ml, and H means 500 ng/ml of Lp-PLA$_2$. The interfering substances show no significant interference (interference rate ≤ ± 5%).

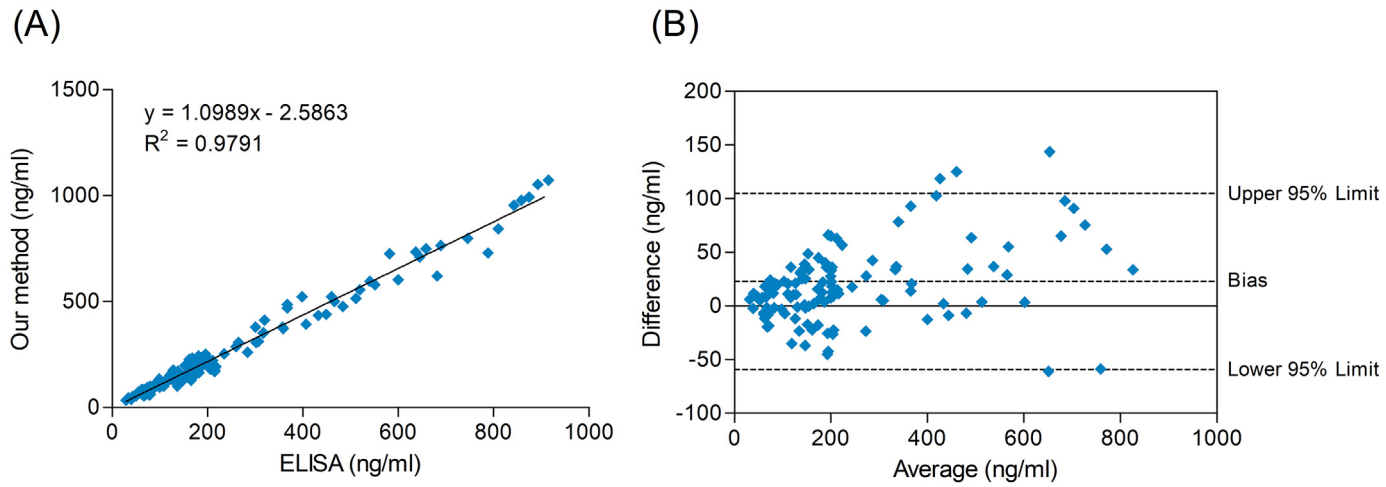

Fig. 5. (A) Linear regression analysis of the detection of Lp-PLA_2 between our method and PLAC ELISA; (B) Bland-Altman analysis for 122 serum samples detected by PLAC ELISA and our method.

emerged as an independent risk factor with high consistency and precision for plaque rupture and atherothrombotic events and it can indicate vascular inflammation specifically. Accordingly, we develop a chemiluminescent immunoassay to measure Lp-PLA_2 through combining magnetic separation and chemiluminescence for the first time. The method adopts a one-step process that is the addition of anti-Lp-PLA_2 antibodies and sample into a reaction cup simultaneously to form a double antibody sandwich mode, which can reduce operation steps and time. And it is applicable to an automatic analyzer to effectively reduce human errors and has higher repeatability compared with the ELISA platform requiring significant manual processing [18].

The Lob of 0.180 ng/ml in our method is well below the range of the PLAC ELISA. Interference studies reveal that there is no significant interference on our assay, even when the highest concentrations of bilirubin, triglyceride, hemoglobin, RF and HAMA are up to 40 mg/dl, 1000 mg/dl, 2000 mg/dl, 1500 IU/ml and 30 ng/ml, respectively. By contrast, immunoturbidimetric assay and spectrophotometry for Lp-PLA_2 assay are impeded by hemoglobin and/or chyle [14,22]. Good overall correlations (R^2 = 0.9791) between our method and PLAC ELISA are confirmed in the method comparison. Linearity is good with R^2 of 0.9989 by linear regression and recoveries falling within ± 10%. Moreover, our method has a wide analytical range from 0.18 to 1350.0 ng/ml which is conducive to risk stratification of cardiovascular events.

Our method for Lp-PLA_2 assay has several advantages over PLAC ELISA. First, the magnetic beads are used as a separation carrier, which can realize the immunoassay, solving the problem of unevenness and a small amount of coated antibody caused by microplate used in the enzyme immunoluminescence. Second, the time of sample to answer is only 17 min in our system, which greatly improves the efficiency at least by several times compared with the PLAC ELISA [18]. Furthermore, PLAC ELISA is restricted by its degree of automation, bringing large human errors and causing significant pre- and post-analytical problems. In addition, our system presents better performance including higher sensitivity and broader analytical range, which is important for detecting the low abundance biomarker in the early stages of diseases or after therapeutic interventions. It is possible to monitor the changes in Lp-PLA_2 levels dynamically and serially, have great potential in evaluating the risk of cardiovascular thromboembolic diseases and taking timely preventive and treatment measures [2].

In future research, the application of our method to evaluate the cardiovascular risk in clinic is essential. Meanwhile, the measurement of Lp-PLA_2 in combination with other biomarkers (such as hs-CRP, low HDL, and high oxidized phospholipids) to better identify high-risk patients is another meaningful part to explore.

5. Conclusions

We have developed a straightforward, automated, rapid, accurate and precise method to detect Lp-PLA_2 by magnetic separation integrated with chemiluminescence. The method adopts a one-step process by mixing MBs, antigen and antibody at one time to enable the sample to answer time within 17 min. It is also possible to utilize an automated instrument to handle and measure the samples which effectively reduce human errors and ensure repeatability. We tested 122 clinical samples, showing good overall correlations between the present method and the PLAC ELISA method. Of note, our method has a wider range of measurement and higher sensitivity compared with the only FDA approved PLAC ELISA, which can satisfy the clinical requirement in detecting the low abundance Lp-PLA_2 in the early stages of disease or after therapeutic interventions. We believe that our platform is highly suitable to further explore the clinical performance of Lp-PLA_2 in studies of cardiovascular risk management.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2018.11.013.

Conflicts of interest

The authors declare no conflicts of interest.

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