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A dual-label time-resolved fluorescence immunoassay for the simultaneous determination of cystatin C and β_2-microglobulin in urine

Y Liu[a], YH Liu[a], WJ Bei[a], K Wang[a], TT Cui[a], HL Li[a], DX Wu[a], SQ Chen[a,b,c], N Tan[a] and JY Chen[a]

[a]Department of Cardiology, Guangdong Cardiovascular Institute, Guangdong Key Laboratory of Coronary Disease, Guangdong General Hospital, Guangzhou, China; [b]National Clinical Research Center for Kidney Disease, State Key Laboratory of Organ Failure Research, Department of Biostatistics, School of Public Health and Tropical Medicine Southern Medical University, Guangzhou, China; [c]Department of Biostatistics, South China College of Cardiovascular Research, Guangdong Society of Interventional Cardiology, Guangzhou, China

ABSTRACT

Background: Contrast media is widely used in clinical diagnostic and interventional procedures, but may cause damage to the kidney, that is, contrast-induced nephropathy. This study was to establish a dual-label time-resolved fluorescence immunoassay (TRFIA) for the simultaneous determination of renal function markers cystatin-C (Cys-C) and β_2-microglobulin (β_2-MG) for the early diagnosis and follow-up surveillance of contrast-induced nephropathy.

Methods: A sandwich immunoassay was used to detect the concentration of Cys-C, and the competitive immunoassay was used to detect the concentration of β2-MG in 50 samples of urine. The performance of this dual-label TRFIA was evaluated and compared with commercial assays.

Results: The sensitivity for Cys-C detection was 1.26 ng/ml, the average recovery was 99.36%; The sensitivity for β2-MG detection was 2.13 ng/ml, the average recovery was 100.18%. Bland–Altman analysis showed that the dual-label TRFIA method and the commercial kits had a good agreement, suggesting they can be used interchangeably in clinical urine analysis.

Conclusion: The present dual-label TRFIA has high sensitivity, specificity and accuracy in clinical sample analysis. This method can be used for the early diagnosis and follow-up surveillance of the contrast-induced nephropathy.

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Introduction

The use of contrast media is becoming an important technique in clinical diagnostic and interventional procedures [1]. However, contrast media may be deleterious: contrast-induced nephropathy is a complex form of acute kidney injury that is defined as an acute reduction in renal function secondary to iodinated contrast media administration [2]. The incidence of contrast-induced nephropathy is reported to be >20% in chronic kidney disease complicated with congestive heart failure, and is associated with increased morbidity, short- and long-term mortality, and healthcare costs [3,4]. The mechanism of contrast-induced nephropathy is multifactorial and includes hemodynamic changes as well as direct renal tubule cell toxicity [5]. The mainstay of preventative strategies is reduction in the amount of contrast used. In addition, early diagnosis is also very important in prevention of renal disease.

Cystatin C (Cys-C) is a 13 kDa non-glycosylated protein belonging to the cysteine protease inhibitor family. It is produced at a constant rate and freely moves from the blood into the glomerulus by filtration [6]. Accordingly, urinary Cys-C is an ideal estimator of the glomerular filtration rate (GFR), and its clinical use has been increasing recently. For example, it is a good predictor of contrast-induced nephropathy after percutaneous coronary interventions in patients with stable angina and diabetic nephropathy [7,8]. Shinde et al. found that Cys-C was a more reliable biomarker than serum creatinine in evaluating the estimated GFR in patients with acute renal failure during the first 24–48 h. The assessment of Cys-C at 24–48 h after contrast media exposure allows an early diagnosis of nephropathy [9].

β_2-microglobulin (β_2-MG) is a low-molecular weight protein readily secreted by lymphocytes and filtered through the glomerulus, and under the normal circumstances is almost completely reabsorbed by proximal tubular cells [10]. The elevation of β_2-MG in urine and serum is mainly due to a decreased GFR and so is regarded as a reliable marker of glomerular filtration and tubular function [11]. Both urinary and serum β_2-microglobulin can be indicative of renal dysplasia [12], while elevated β_2-microglobulin levels in the urine correlates with a decreased number of glomeruli [13].

Many markers and indicators can be used for the early screening and diagnosis of contrast-induced

CONTACT JY Chen chenjiyandr@126.com

nephropathy. A triple-marker approach, using serum creatinine, Cys-C and malondialdehyde had the best performance in indicating delayed graft function [14]. Bartoli et al. substantiated the role of urinary epidermal growth factor, monocyte chemotactic protein-1 and β_2-MG as markers of tubulointerstitial damage in human obstructive nephropathy [15]. Padhy et al. pointed out that the serum neutrophil gelatinase associated lipocalin (NGAL) and Cys-C may act as early markers of contrast-induced nephropathy in patients undergoing percutaneous coronary intervention [16]. Homocysteine, with similar predictive value compared to Cys-C, has been used to predict contrast-induced nephropathy before computed tomography angiography examination [17].

Because no single biomarker achieved adequate sensitivity and specificity for clinical purposes, this study was designed to develop a highly sensitive and specific dual-label time-resolved fluorescence immunoassay (TRFIA) for the simultaneous determination of Cys-C and β_2-MG. The sandwich immunoassay was used to detect the concentration of Cys-C, and the competitive immunoassay was used to detect the concentration of β_2-MG in urine.

Materials and methods

Reagents and chemicals were as follows. Two clones of rabbit monoclonal antibodies against different epitopes of Cys-C, and one clone of mouse monoclonal antibody to β_2-MG were used. Cys-C and β_2-MG standards were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Europium (III) (Norwalk, 1244-302, USA) and samarium (III) (Norwalk, 1244-303, USA) labelling kits were obtained from Perkin Elmer (Waltham, MA, USA). Goat anti-mouse IgG was obtained from Youdi Biotechnology Company (Guangzhou, China). Centrifugal filters with molecular weight cut-off values of 50KDa were purchased from Millipore (Bedford, MA, USA). Sephadex G50 columns were obtained from GE Healthcare (Uppsala, Sweden). Fifty clinical urine samples were obtained from consecutive acute coronary syndrome patients with contrast-induced nephropathy who received percutaneous coronary intervention (32 males, mean/SD age 45.6/12.5, 18 females, aged 54.2/10.3) in the cardiovascular institute of Guangdong Province from June 2015 to May 2016; After collection, the urine was immediately centrifuged and the pH of the supernatant adjusted to 7–9 using 1 mol/L NaOH solution, and then were aliquoted, frozen in liquid nitrogen and stored at −80 °C. The study was approved by the Institutional Review Board of Guangdong Cardiovascular Institute, and all participants gave written informed consent.

Ninety-six well microtiter plates were coated with 200 µl/well of coating buffer (50 mmol/L carbonate buffer, pH 9.6) containing 2.5 µg/ml and 3.0 µg/ml, respectively, of monoclonal antibody for Cys-C and goat-mouse IgG overnight at 4 °C. After coating, plates were washed three times with phosphate buffer containing 0.05% Tween-20 (v/v) followed by addition of blocking buffer 250 µl/well (50 mmol/L Tris-HCl containing 5% BSA, m/v, pH 7.4) for 2hr at 37 °C. The blocking buffer was then removed and the coated plates were vacuum dried and stored at −20 °C.

Antibody labelling was performed using europium (III) Eu^{3+} and samarium (III) Sm^{3+}kits according to the manufacturer's instructions. Briefly, 1 mg of anti-Cys-C antibodies were washed for four times with labelling buffer (50 mmol/L Na_2CO_3, pH 9.0), suspended in 200 µl of labelling buffer and mixed gently with 1000 µg of Eu^{3+} chelates. The mixture was incubated overnight at room temperature, and purified using a Tris-HCl (50 mmol/L, pH 7.8) buffered Sephadex G50 column. The conjugated detection antibody was preserved in Tris-HCl buffer (50 mmol/L, 0.1% BSA, m/v, pH 7.8) at 4 °C. β_2-MG antigen was labelled with Sm^{3+}using the same procedure. Pure reference Cys-C and β_2-MG standards were mixed in equivalent volume in the phosphate saline buffer (50 mmol/L Tris-HCl, pH 7.2, with 1.5% BSA and 0.15% NaN_3) at final concentrations of 0, 1, 10, 100, 500, 1000 ng/ml.

The assay procedure was as follows. In each well, 25 µl of standards, controls or samples were added, followed with 100 µl of Eu^{3+} labelled anti-Cys-C detection antibodies, 50 µl Sm^{3+} labelled β_2-MG antigen and 50 µl anti-β_2-MG antibody. The plate was then incubated for 1hr with continuous horizontal shaking at room temperature. After washing six times, 200 µl/well enhancement solution was added, and the plate was shaken gently for 5 min followed by measurement of fluorescence (Auto DELFIA 1235, Perkin Elmer). Sm^{3+} fluorescence was measured at an excitation wavelength of 340 nm and an emission wavelength of 600 nm. Eu^{3+} fluorescence was measured at an excitation wavelength of 337 nm and an emission wavelength of 615 nm.

The standard curves were constructed using serial dilutions of Cys-C and β_2-MG, both at 0, 1, 10, 100, 500 and 1000 ng/ml. The mean concentration plus 1.645 standard deviations of the blank assayed in duplicate in 20 independent measurements was used as the definition of limit of blank (LOB). The sensitivity was determined by utilising both the measured LOB and test replicates of a sample known to contain a low concentration of Cys-C and β_2-MG. The lower limit of detection was defined as LOB + 1.645($SD_{\text{low concentration sample}}$).

The accuracy of the assay was evaluated by analysing low, median and high concentrations of clinical samples. The intra- and inter-assay variations were obtained from 10 independent experiments. For the specificity tests, different concentrations of urinary albumin, transferrin, α-microglobulin, N-acetyl-β-glucosaminidase and human serum albumin were added to urine, the final concentrations of these interferents were 200, 50, 50, 100 and 500 ng/ml, respectively (these concentrations are

pathologically relevant), and followed by dual-label TRFIA measurement of Cys-C and β_2-MG. Data were obtained from 10 independent experiments. The recovery was assessed by adding different concentrations of Cys-C and β_2-MG standards to the urine samples. Recovery was expressed as the percentage measured of the amount added. The equation was: Recovery (%) = (measured concentration/spiked concentration) × 100.

Performance of the dual-label TRFIA method with commercial Cys-C and β_2-MG quantitative determination kits (both Darui Biotechnology Co. Ltd, Guangzhou, China) were performed using 50 clinical samples. The commercial assays were performed according to the manufacturer's instructions.

Quantitative data are presented as mean ± standard deviation (SD). Comparisons between quantitative data were performed using the paired samples *t*-test. Bland–Altman plots were used to compare the consistency of the dual-label TRFIA method and the commercial kits. SPSS 19.0 was used in the statistical analysis; $p < 0.05$ was considered statistically significant.

Results

Assay validation was as follows. Standard curves plotted for Cys-C and β_2-MG in the TRFIA assay were shown in Figure 1. The linear concentration ranges were 0.42–956.32 ng/ml for Cys-C and 0.86–975.4 ng/ml for β_2-MG. The linear correlation coefficient (R^2) of the Cys-C and β2-MG standard curves was 0.999. Both calibration plots exhibited well-defined linear relationships between the concentration and fluorescent intensity. The sensitivity (as lower limit of detection) of the assay for Cys-C was 1.26 ng/ml, and the sensitivity for β_2-MG was 2.13 ng/ml, which were based on three times the standard deviation of the blank. Accuracy was determined using three clinical samples with low, median and high concentration of Cys-C or β_2-MG, and the results were shown in Table 1. The inter-assay coefficients of variation (CV) for Cys-C were between 3.2 and 5.9%, and the intra-assay values ranged from 3.4 to 6.3%. The inter-assay CVs for β2-MG were between 3.0 and 6.8%, and the intra-assay CV ranged from 3.1 to 8.4%.

Five proteins at various concentrations were investigated as potential interferents. The cross-reactivity rates of urinary albumin, transferrin, α-microglobulin, human serum albumin and N-acetyl-β-glucosaminidase were 0.50, 0.47, 0.35, 0.24 and 0.28%, respectively. The results showed that the dual-labelled TRFIA has high affinity and specificity for Cys-C and β_2-MG. Three clinical urine samples with known concentration of Cys-C and β_2-MG were used to determine recovery. The initial concentration of Cys-C and β_2-MG were 1.45, 352.5, 824.6 ng/ml and 1.25, 322.2, 665.5 ng/ml, respectively. Then, the Cys-C and β_2-MG standards were added, the final concentrations of Cys-C and β_2-MG were 2.0, 500.0, 1000.0 ng/ml for each molecule. The average recoveries for Cys-C were between 97.5 and 100.4%, while β_2-MG values ranged from 99.7 to 102.0% (Table 2). The results showed that this assay was free from interferences in the urine.

Comparison of the TRFIA with commercial assays was determined with 50 urine samples: high agreement

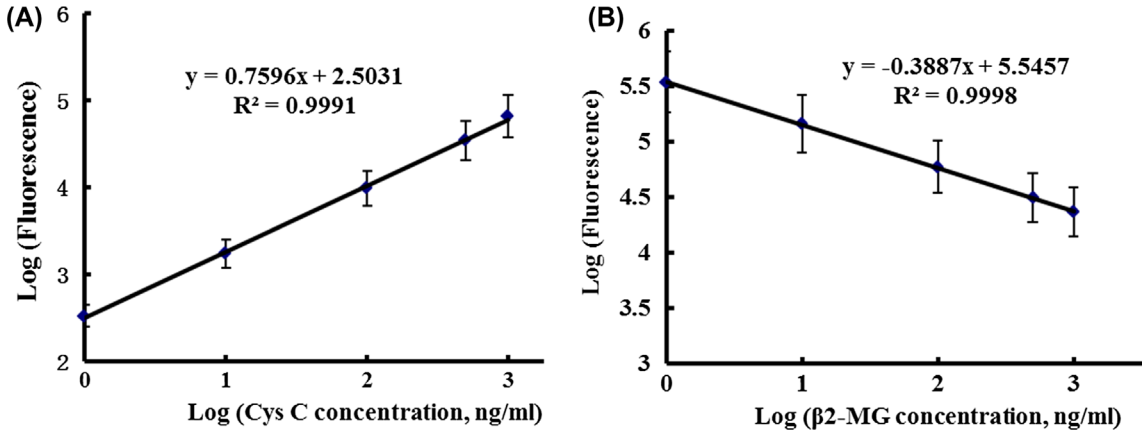

Figure 1. The standard curves of Cys-C (A) and β_2-MG (B).

Table 1. Accuracy experiment of the dual-label time-resolved fluorescence immunoassay process design and results (n = 10).

		Cys C			β_2-MG		
	Concentration	Average (ng/ml)	Standard deviation	CV (%)	Average (ng/ml)	Standard deviation	CV (%)
Inter-assay	Low	3.24	0.19	5.86	3.10	0.21	6.77
	Median	501.53	16.36	3.26	400.25	15.33	3.83
	High	901.25	28.65	3.18	865.24	25.76	2.98
Intra-assay	Low	4.26	0.27	6.34	2.26	0.19	8.41
	Median	498.57	19.64	3.94	456.68	18.87	4.13
	High	925.62	31.44	3.40	852.61	26.85	3.15

Table 2. Recovery experiment of the dual-label time-resolved fluorescence immunoassay process design and results (n = 10).

	Cys C				β_2-MG			
Samples	Fortified (ng/ml)	Determined (ng/ml)	Recovery (%)	Average recovery (%)	Fortified (ng/ml)	Determined (ng/ml)	Recovery (%)	Average recovery (%)
1	2	1.96	98.00	99.36	2	2.04	102.00	100.18
	500	496.86	99.37		500	502.26	100.45	
	1000	1002.56	100.26		1000	1003.25	100.33	
2	2	1.98	99.00		2	2.02	101.00	
	500	497.52	99.50		500	498.67	99.73	
	1000	998.65	99.87		1000	998.57	99.86	
3	2	1.95	97.50		2	1.96	98.00	
	500	502.20	100.44		500	502.22	100.44	
	1000	1003.24	100.32		1000	997.86	99.79	

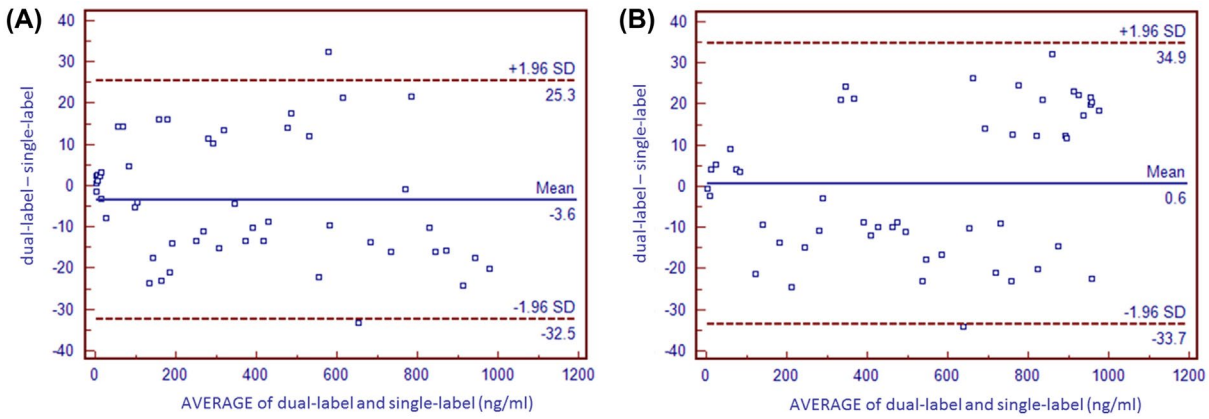

Figure 2. Consistency of Cys-C (A) and β_2-MG (B) results between the dual-labelled TRFIA and the commercial kits.

between the methods were obtained (Figure 2). For Cys-C, 95% consistency limit was 32.5–25.3 ng/ml, and 4% (2/50) of the points were outside this limit. The absolute value of the difference between the dual-label TRFIA method and the commercial kits was 7.2 ng/ml, and the average of the two methods was −3.6 ng/ml (95% confidence interval (95% CI): −7.8, 0.6 ng/ml). While for β_2-MG, 95% consistency limit was −33.7–34.9 ng/ml, and 2% (1/50) of the points were outside this limit. The absolute value of the difference between the dual-label TRFIA method and the commercial kits was 1.2 ng/ml, and the average of the two methods was 0.6 ng/ml (95% CI: −4.4, 5.6 ng/ml). The results indicated that the dual-label TRFIA method and the commercial kits have good agreement and can be used interchangeably in clinical urine samples.

Discussion

The most commonly used detection method of Cys-C is the particle-enhanced nephelometric immunoassay. Nephelometric immunoassay, chemiluminescence and ELISA methods have been used to detect β_2-MG [18]. However, new detection methods have emerged. Mi et al. reported a simple and sensitive photoelectrochemical immunosensor based on TiO_2 nanotube arrays for the sensitive detection of CysC [19]. Li et al. developed the time-resolved fluorescence immunoassay to detect β_2-MG [20]. TRFIA has been widely used as an 'ideal' immunoassay method in human diagnostics, such as the detection of viruses [21]. Through the use of lanthanide chelates, the non-specific background is differentiated from the time-resolved fluorescence of the specific signal due to different emission wavelengths and kinetics, enabling development of a dual-label TRFIA using Sm^{3+} and Eu^{3+} chelate, permitting simultaneous detection of two molecules [22,23].

In this study, a highly sensitive dual-label TRFIA, using Eu^{3+} and Sm^{3+} chelates, was developed for the simultaneous determination of Cys-C and β_2-MG. The rapid determination of Cys-C and β_2-MG within 1–2 h would help earlier diagnosis decisions with sensitivities of 1.26 ng/ml for Cys-C and 2.13 ng/ml for β_2-MG. The assay has well-defined linear relationships between the analyte concentrations and fluorescent intensities with broad detection ranges. Moreover, there is no cross-reaction with common potential interferents. Comparison with commercial kits, the dual-label TRFIA method has a good consistency with the commercial kits and can be used interchangeably in clinical urine samples.

In conclusion, the present dual-label TRFIA method was a simple and sensitive fluoroimmunoassay for the determination of Cys-C and β_2-MG, and this method has a good consistency with the commercial kits. Compared with the single marker detection, this simultaneous method has advantages for the early diagnosis and follow-up surveillance of the CIN. This work represents

an advance in biomedical science because it provides an accurate, simple and rapid method to diagnose and monitor contrast-induced nephropathy.

Summary table

What is known about this subject:
- The current clinical diagnosis (using serum creatinine level and creatinine clearance rate) of contrast-induced nephropathy has limitations.
- Current and general diagnosis biomarkers of this condition, especially in urine, are inaccurate.

What this paper adds:
- Urine Cys-C and β_2-MG can be detected with a time-resolved fluorescence immunoassay

Disclosure statement

The authors report no conflicts of interest.

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