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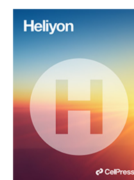
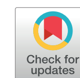


Research article

Assessment of ELISA-based method for the routine examination of serum indoxyl sulfate in patients with chronic kidney disease



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Summary

The IS ELISA kit from Leadgene exhibits a comparable capability, high agreement and correlation to UPLC-MS/MS in the quantification of total serum IS levels and would be suitable for dynamic monitoring of serum IS levels in patients with CKD.

A R T I C L E I N F O

Keywords:
Indoxyl sulfate
Chronic kidney disease
ELISA
UPLC-MS/MS

A B S T R A C T

Introduction: Indoxyl sulfate (IS), a protein-bound uremic toxin, is associated with kidney function and chronic kidney disease (CKD)-related complications. Currently, serum IS levels are primarily quantified using mass spectrometry-based methods, which are not feasible for routine clinical examinations.
Methods: The efficiencies of three commercial ELISA kits in determination of serum IS were validated by comparing with ultra-performance liquid chromatography (UPLC)-MS/MS-based method using Bland-Altman analysis. The associations between kidney parameters and serum IS levels determined by ELISA kit from Leadgene and UPLC-MS/MS were evaluated by Spearman correlation coefficient in a CKD validation cohort.
Results: ELISA kit from Leadgene showed clinical agreement with UPLC-MS/MS in the determination of serum IS levels ($p = 0.084$). In patients with CKD, Spearman's correlation analysis revealed a perfect correlation between the IS levels determined using the Leadgene ELISA kit and UPLC-MS/MS ($r = 0.964$, $p < 0.0001$). IS levels determined using the Leadgene ELISA kit were associated with the estimated glomerular filtration rate ($r = -0.772$, $p < 0.0001$) and serum creatinine concentration ($r = 0.824$, $p < 0.0001$) in patients with CKD, and on dialysis ($r = 0.557$, $p = 0.006$).
Conclusions: The Leadgene ELISA kit exhibits comparable efficacy to UPLC-MS/MS in quantifying serum IS levels, supporting that ELISA would be a personalized method for monitoring the dynamic changes in serum IS levels in dialysis patients to prevent the progression of CKD.

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1. Introduction

Indoxyl sulfate (IS), a metabolite of tryptophan obtained from the diet, is a protein-bound uremic toxin. IS is generated in the liver and enters the circulation after the hydroxylation and sulfation of indole produced by intestinal bacteria. Normally, serum IS is absorbed by renal tubular cells through organic anion transporters 1 and 3 and further excreted in urine (Wu et al., 2017). However, serum IS accumulates when kidney injury occurs and leads to renal failure. The increasing in serum IS levels subsequently aggravates the progression of kidney diseases and worsens therapeutic outcomes (Cheng et al., 2020). In addition to affecting renal function, IS increases smooth muscle cell proliferation, promotes aortic calcification, and increases the risk of cardiovascular disease (CVD) in patients with chronic kidney disease (CKD) (Adijiang et al., 2008; Lekawanvijit et al., 2010; Yamamoto et al., 2006). Thus, the serum IS level is considered as a biomarker not only for renal function deterioration but also for the prevalence of cardiovascular events in patients with CKD (Hung et al., 2017; Lin et al., 2012). Continuous detection of serum IS levels might help evaluate disease progression in patients with CKD. Unfortunately, the serum IS level is not routinely examined in clinical practice.

Currently, both high-performance liquid chromatography (HPLC)- and mass spectrometry (MS)/MS-based methodologies are applied to determine serum IS levels with high accuracy and repeatability (Al Za'abi et al., 2013; Lin et al., 2019; Zhang et al., 2017). However, the requirement for specialized equipment, the need of specialized technicians, complicated sample preparation methods for blood fluid, and higher cost might restrict the application of HPLC- and MS/MS-based methodologies in routine clinical examination of IS levels. Recently, an enzymatic method for the quantification of total serum IS levels was developed and showed a strong correlation with the HPLC-based method (Fushimi et al., 2019). However, the effects of other coexisting substances in enzymatic reactions on IS quantification remain elusive. On the other hand, the enzyme-linked immunosorbent assay (ELISA) is an analytical biochemistry assay which is commonly used in routine clinical examination due to its cost-effectiveness and high specificity, sensitivity, and efficiency. IS ELISA kits are commercially available, but the performances of these kits in the determination of serum IS levels remain unknown. Thus, the aim of this study was to identify commercial IS ELISA kits which could be applied to routine examination of serum IS levels. In the present study, a Bland-Altman analysis was applied to evaluate the agreement between ELISA- and ultra-performance liquid chromatography (UPLC)-MS/MS-based methods. A correlation analysis was also performed to assess the association between renal function parameters and serum IS levels determined using commercial ELISA kit and UPLC-MS/MS in patients with CKD.

2. Materials and methods

2.1. Chemical and reagents

Acetonitrile (liquid chromatography-MS-grade) was purchased from JT Baker (Center Valley, PA, USA), and formic acid was purchased from Fluka (St. Louis, MO, USA). The indoxyl sulfate potassium was obtained from Sigma-Aldrich (St. Louis, MO, USA) and 3-indoxyl sulfate-d4 potassium salt (IS-d4) was purchased from Toronto Research Chemicals (North York, ON, Canada).

Table 1. The linearity, sensitivity, and repeatability of three commercial ELISA kits.

	Range of linearity (μmol/L)	Linearity (R^2)	Sensitivity (LOD) (ng/ml)[1]	Repeatability (%CV)[2]
BlueGene	0–21.2	0.987	0.1 (~0.4 nmol/L)	10.8
Leadgene	0.2–8.0	0.999	50 (~200 nmol/L)	3.3
Mybiosource	12.4–398.0	0.997	1000 (~4000 nmol/L)	16.1

[1] Obtained from manufacturer instruction of each ELISA kits.
[2] Intra-assay %CV was calculated based on triplicate measurements of clinical sample.

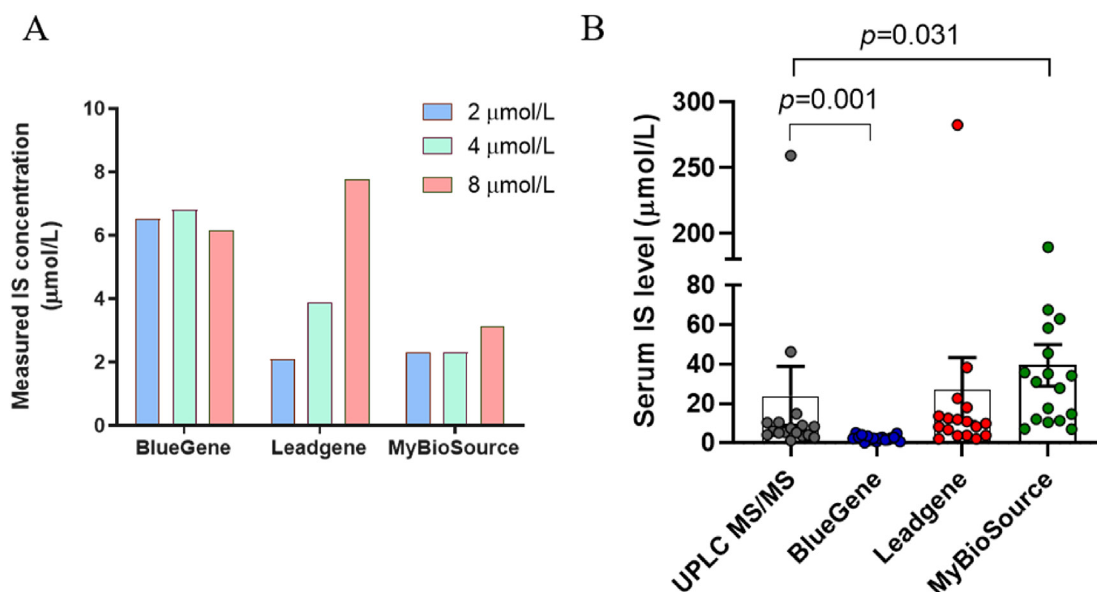

Figure 1. Quantification of serum IS levels using UPLC-MS/MS- and ELISA-based methodology. (A) The indoxyl sulfate potassium was prepared to concentrations of 2, 4, and 8 μmol/L and determined by commercial ELISA kits according to the manufacturer's instructions. (B) The serum IS levels in 17 clinical samples in group 1 were determined using UPLC-MS/MS and commercial ELISA kits. The data were presented as means ± SEM, and the significance of differences between the IS levels determined by UPLC-MS/MS and commercial IS ELISA kits was evaluated by Wilcoxon signed-rank test.

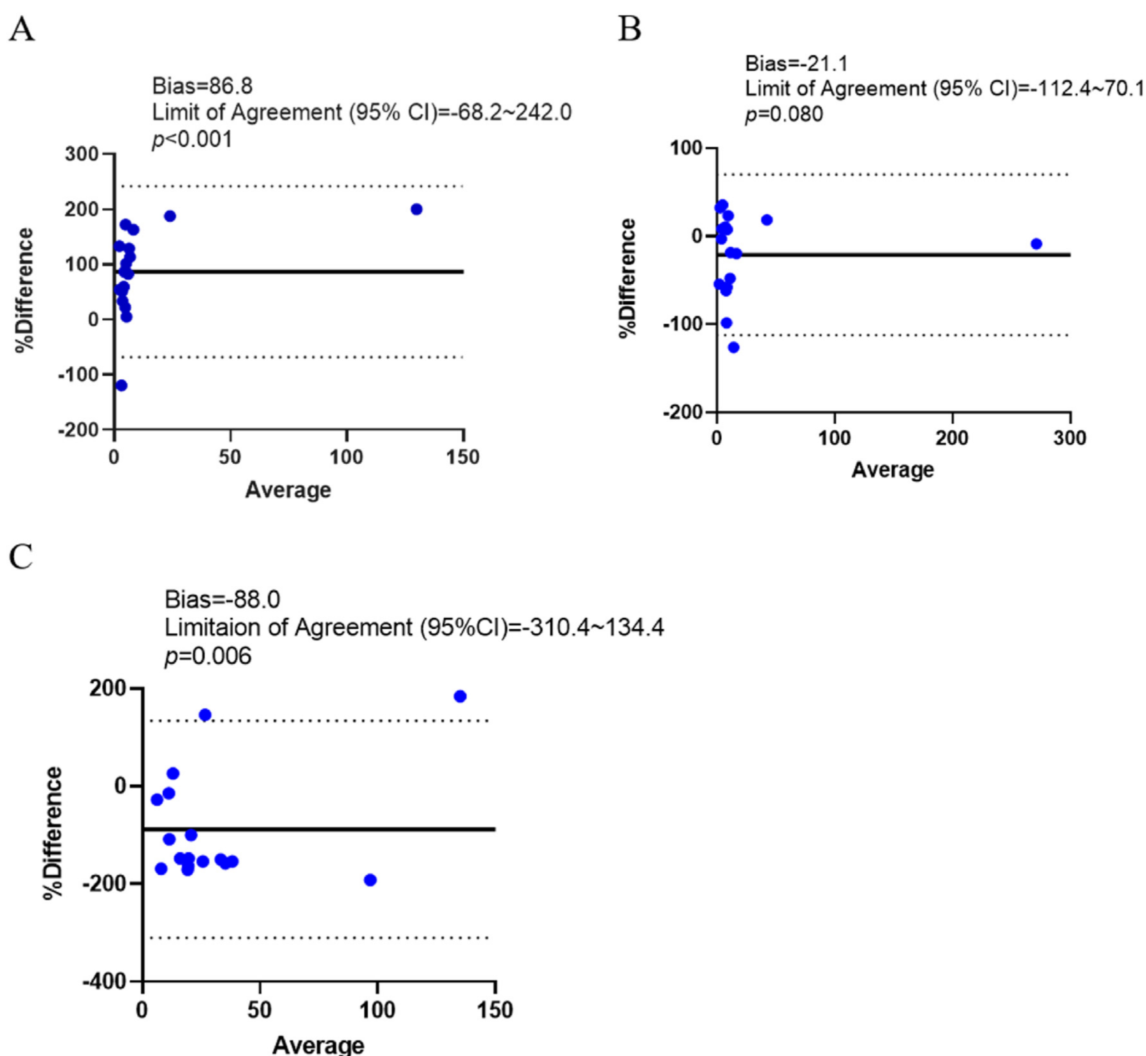

Figure 2. Bland-Altman analysis of serum IS levels determined using UPLC-MS/MS- and ELISA-based methods. Bland-Altman plots of serum IS levels determined by UPLC-MS/MS versus those by Bluegene (A), Leadgene (B), and Mybio-source (C) ELISA kits. The data were presented as the means ± 1.96*SD and agreements between UPLC-MS/MS- and ELISA-base methods were evaluated by one-sample t test.

Table 2. Correlation analysis of serum IS levels determined by UPLC-MS/MS and commercial ELISA kits.

ELISA kit (n = 17)	Spearman correlation	*p* value
BlueGene	-0.441	0.076
Leadgene	0.706	0.002
MyBioSource	-0.358	0.158

2.2. Clinical samples

Two groups of patients were enrolled in the present study. The first group consisted of seventeen clinical samples without CKD evaluation was enrolled during routine physical examination at Tainan Municipal Hospital, Tainan, Taiwan to evaluate the performance of three commercial ELISA kits in determination of serum IS levels. An independent validation group consisted of thirty-four CKD patients, including 4 of stage I, 6 of stage II, 1 of stage III, and 23 of stage V, was enrolled at Cardinal-Tien Hospital, New Taipei City, Taiwan to validate the associations between renal function parameters and serum IS levels determined by Leadgene ELISA kit and UPLC MS/MS. Renal function parameters, including estimated glomerular filtration rate (eGFR) and serum creatinine level, were obtained during routine laboratory testing. Serum samples were collected and stored at -80 °C until conducting IS quantification. Patient demographics of two groups, and total enrollment were shown in Supplementary Table 1. The study was conducted according to the guidelines of the Declaration of Helsinki, approved by the Institutional Review Board of Tainan Municipal Hospital, Show Chwan Health Care system (Ethical approval number: SCHMH_IRB No: 1060603) and Cardinal-Tien Hospital (Ethical approval number: CTH-107-3-5-027 and CTH-108-2-5-002), and written informed consent was provided by all participants.

2.3. Quantification of total serum IS using ultra-performance liquid chromatography (UPLC)-MS/MS

The total serum IS levels were determined using a Xevo TQ-S triple-quadrupole mass spectrometer (Waters, Milford, MA, USA) coupled with a Waters Acquity UPLC system and a stepwave ion transfer optics unit. The experiment was performed in Chang Gung Memorial Hospital (Department of Laboratory Medicine, Linkou Medical Center, Tai-wan) using the standard protocol described in a previous study (Lin et al., 2019). Briefly, a standard curve of IS with concentrations of 0–5 mg/L (0–19.9 μmol/L) and IS-d4 (internal standard) stock solutions of 10 mg/mL (39.2 μmol/L) was first prepared in double-distilled water and acetonitrile, respectively. The serum sample was mixed with acetonitrile containing 0.2 mg/L (0.8 μmol/L) IS-d4 at a ratio of 1:10. The

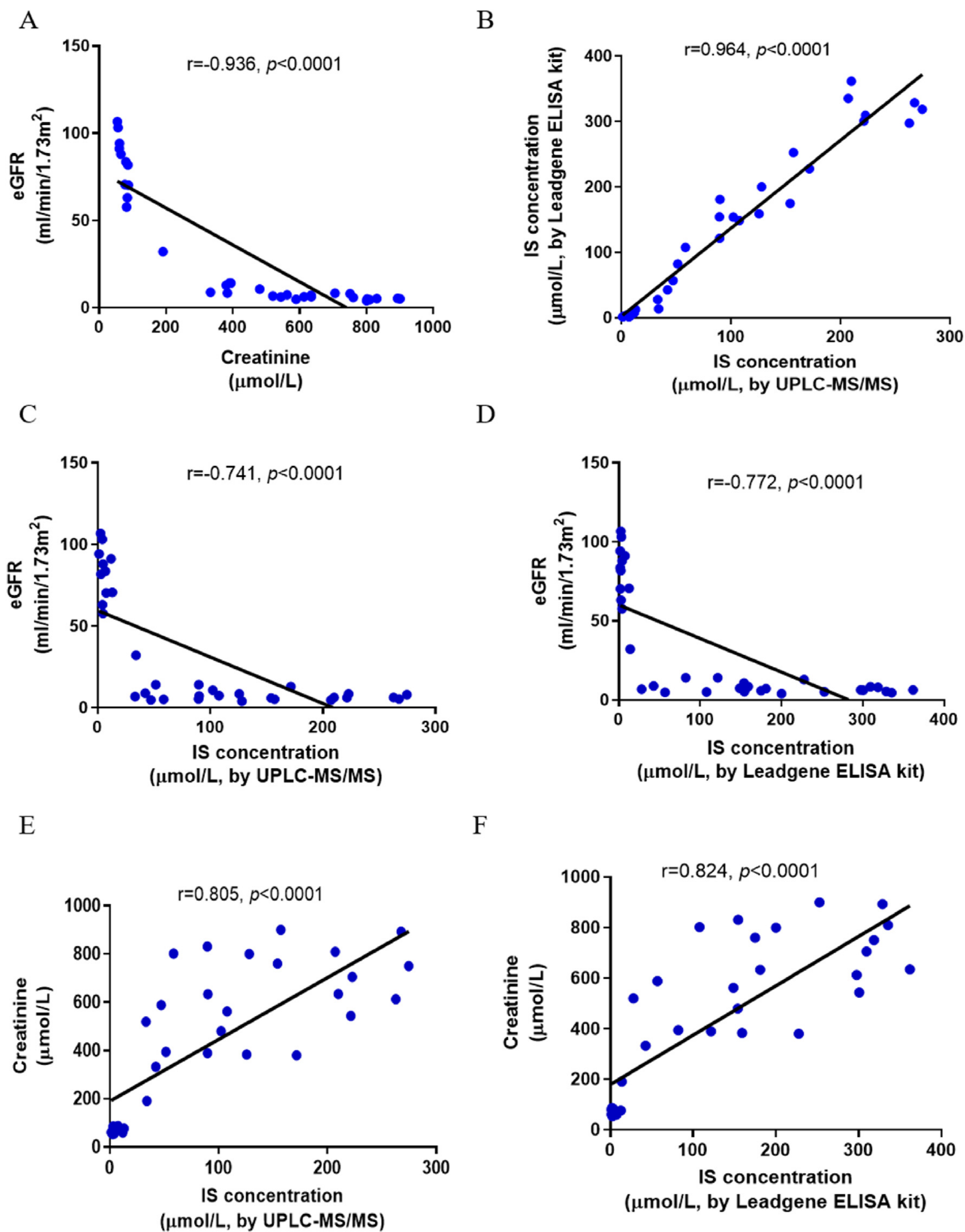

Figure 3. Correlation analysis of serum IS levels and renal function parameters in patients with CKD. Correlations between the eGFR and serum creatinine level (A), IS levels determined by UPLC-MS/MS and Leadgene ELISA kit (B), eGFR and UPLC-MS/MS-determined IS levels (C), eGFR and Leadgene ELISA-determined IS levels (D), serum creatinine level and UPLC-MS/MS-determined IS levels (E), and serum creatinine level and Leadgene ELI-SA-determined IS levels (F) were analyzed by Spearman's correlation coefficients.

supernatant was collected after centrifugation, diluted 10-fold with double-distilled water, and analyzed by a UPLC-MS/MS system. UPLC was performed using an Acquity UPLC BEHC 18 column (2.1 × 100 mm, 1.7-mm particle size, Waters) with a linear gradient of mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The analytes were subjected to negative electrospray ionization, and the IS levels were determined in multiple reaction monitoring mode based on the IS standard curve.

2.4. ELISA quantification of total serum IS

The ELISA kits from BlueGene Biotech (Abdelrahman et al., 2019; Ali et al., 2019; Lakshmanan et al., 2021) and Mybio-source (Al Za'abi et al., 2021; Barisione et al., 2016; Choroszy et al., 2022) had been cited in scientific research, whereas the ELISA kit from Leadgene was certified by in-vitro diagnostic medical devices (IVD) certification. Therefore, the kits from three manufacturers are selected for further

Table 3. Correlation analysis of renal function parameters and serum IS concentration in patients with advanced renal disease.

Criteria	Methodology	eGFR		Creatinine	
		Spearman correlation	*p* value	Spearman correlation	*p* value
Total enrollment[1] (n = 51)	UPLC-MS/MS	-0.837	<0.0001	0.867	<0.0001
	Leadgene	-0.869	<0.0001	0.877	<0.0001
eGFR[2] (n = 37)	UPLC-MS/MS	-0.755	<0.0001	0.825	<0.0001
	Leadgene	-0.784	<0.0001	0.840	<0.0001
Creatinine[3] (n = 35)	UPLC-MS/MS	-0.712	<0.0001	0.795	<0.0001
	Leadgene	-0.747	<0.0001	0.813	<0.0001
CKD stage V(n = 23)	UPLC-MS/MS	-0.221	0.310	0.451	0.031
	Leadgene	-0.347	0.105	0.557	0.006

[1] Patients of Group 1 and Group 2 were included.
[2] Patients with eGFR <60 ml/min/1.73m2 were selected for analysis.
[3] Patients with serum creatinine level >106 mol/L were selected for analysis.

evaluation. The standard curves of commercial ELISA kits purchased from BlueGene Biotech (Cat. No. E01I0039, Shanghai, China), Leadgene (Cat. No. 00201, Taiwan), and Mybio-source (Cat. No. MBS019983, San Diego, CA, USA) were prepared according to the manufacturer's instructions (Supplementary Figure S1). The total serum IS levels and the concentrations of the commercial IS standard were determined and expressed as μmol/L. The repeatability of commercial ELISA kit was indicated by intra-assay coefficient of variation (intra assay %CV) calculated based on triplicate measurements. The range of linearity, linearity, sensitivity, and repeatability of commercial ELISA kits was shown in Table 1.

2.5. Statistical analysis

The significance of differences between analytical data determined using UPLC-MS/MS- and ELISA-based methods was evaluated using the Wilcoxon signed-rank test. The agreements of analytical data obtained from UPLC-MS/MS- and ELISA-based assays were validated by Bland-Altman plot combined with one-sample t test. The associations between analytical data and renal function parameters were addressed using Spearman correlation coefficient. All statistical analyses were conducted by SPSS software (version 19.0; SPSS, Inc., Chicago, IL).

3. Results

3.1. ELISA kit from Leagene exhibits a comparable efficacy to UPLC-MS/MS in the quantification of total serum IS levels

To test the specificity of commercial IS ELISA kits, the indoxyl sulfate potassium was first prepared at indicated concentration and determined by IS ELISA kits according to the manufacturer's instructions. The results showed that the level of IS determined by ELISA kit from Leadgene, but not those from Bluegene and MyBioSource, was consistent with the given concentration of indoxyl sulfate potassium (Figure 1A). Next, the performances of three IS ELISA kits in determination of serum IS levels were evaluated by comparing with UPLC-MS/MS based methodology. The average (±SEM) of ELISA-determined serum IS concentration in group 1 was 2.4 ± 0.3 μmol/L for the Bluegene ELISA kit, 27.0 ± 16.1 μmol/L for the Leadgene ELISA kit, and 39.3 ± 10.5 μmol/L for the MyBioSourse ELISA kit (Figure 1B). The intra-assay %CV was 10.8 for the Bluegene ELISA kit, 3.3 for the Leadgene ELISA kit, and 16.1 for the MyBioSourse ELISA kit (Table 1). Of the three tested ELISA kits, the IS levels determined by ELISA from Leadgene were comparable to that by UPLC-MS/MS (27.0 ± 16.1 vs. 23.7 ± 14.9 μmol/L, p = 0.135, Figure 1B). The result suggested a comparable efficacy between Leadgene ELISA kit and UPLC-MS/MS in the quantification of total serum IS.

3.2. Leadgene ELISA kit exhibits clinical agreement with the UPLC-MS/MS-based method in determination of total serum IS levels

Next, the agreement of ELISA- and UPLC-MS/MS-based methods in the determination of total serum IS levels was evaluated by Bland-Altman analysis coupled with one-sample t test. The Bland-Altman bias with 95% confidence interval was 86.8% (-68.2%–242.0%) for the Bluegene ELISA kit (Figure 2A), -21.1% (-112.4%–70.1%) for the Leadgene ELISA kit (Figure 2B), and -88.0% (-310.4%–134.4%) for the MyBiosource ELISA kit (Figure 2C). The IS levels determined using the ELISA kit from Leadgene fell within the nonsignificant range (Figure 2B, p = 0.080). Furthermore, the associations between ELISA- and UPLC-MS/MS-determined IS levels were further assessed by Spearman's correlation analysis. As shown in Table 2, the UPLC-MS/MS-determined serum IS levels correlated positively with those determined by ELISA kit from Leadgene (r = 0.706, p = 0.002) but not from BlueGene (r = -0.441, p = 0.076) and MyBioSource (r = -0.358, p = 0.158). Taken together, the result showed that Leadgene ELISA kit exhibits clinical agreement and correlates with UPLC-MS/MS-based methodology in the quantification of total serum IS levels. However, the performance of ELISA kits from Mybiosource and BlueGene did not meet the minimum requirement for quantification of serum IS and therefore were excluded for further evaluation.

3.3. Serum IS level is correlated with renal parameters in CKD

The eGFR and serum creatinine levels are two of the major indicators of renal function of CKD. Generally, the eGFR exhibited an inverse correlation with the serum creatinine level (r = -0.936, p < 0.0001, Figure 3A) (Jin et al., 2008). Next, the serum IS levels of thirty-four CKD patients were determined by either UPLC-MS/MS and ELISA kit from Leadgene. The result showed that serum IS levels determined by Leadgene ELISA kit were highly correlated with that determined by UPLC-MS/MS (r = 0.964, p < 0.0001, Figure 3B). The percentage difference of serum IS levels determined by two methodologies was -6.7% ± 46.6% and fell within the nonsignificant range (p = 0.407, Supplementary Figure S2). The eGFR correlated negatively with serum IS levels determined using UPLC-MS/MS (r = -0.741, p < 0.0001, Figure 3C) and the Leadgene IS ELISA kit (r = -0.772, p < 0.0001, Figure 3D). Both UPLC-MS/MS- (r = 0.805, p < 0.0001, Figure 3E) and Leadgene IS ELISA-determined IS levels (r = 0.824, p < 0.0001, Figure 3F) exhibited positive correlations with serum creatinine level. Furthermore, the renal function parameters showed strong correlations with IS levels determined by UPLC MS/MS and ELISA kit from Leadgene in subjects with an abnormal eGFR and serum creatinine (Table 3).

The associations between serum IS levels and renal parameters of patients with end stage renal disease were also examined and shown in Table 3 and Supplementary Figure S3. In patients with stage V of CKD, total serum IS determined by UPLC-MS/MS (r = 0.451, p = 0.031) and ELISA kit from Leadgene (r = 0.557, p = 0.006) correlated positively with serum creatinine, but not with eGFR. Taken together, the results showed that Leadgene IS ELISA kit would be suitable for routine IS examination in patients with CKD.

4. Discussion

In the present study, three IS ELISA kits are tested for their efficacy in the determination of serum IS concentration to meet the clinical demand for a routine examination of serum IS levels, particularly in patients with CKD. The analytical data are evaluated using Bland-Altman and Spearman correlation analyses to respectively assess the agreement and correlation between ELISA- and UPLC-MS/MS-based methodologies. Of three tested IS ELISA kits, kit from Leadgene, but not Mybiosource and

BlueGene, exhibits a comparable performance to UPLC-MS/MS-based method. The factors that lead to the differential performance of IS ELISA kits between brands remain unclear, partially because IS is a small molecule (~213 daltons) with a limited epitope for generating specific antibody. In addition, IS is a metabolite of tryptophan and primarily binds to plasma proteins. Thus, the low molecular weight and complexity of IS and plasma protein complexes might influence the specificity of antibodies against IS, which is one of the major factors affecting the performance of an ELISA kit (Schrijver and Kramps, 1998). The antibodies cross reactions occurred during analysis might also be a possible reason explaining the poor accuracy of Mybiosource and BlueGene IS ELISA.

IS is a uremic toxin produced from the metabolization of tryptophan to indole by intestinal bacteria. In the circulation, more than 90% of IS is associated with plasma proteins, which are cleared through tubular secretion in the native kidney (Sirich et al., 2013). However, dialytic clearance of serum IS levels in patients with CKD is limited because only free unbound IS diffuses across the dialysis membrane (Lesaffer et al., 2000). Indeed, a high level of IS is detected in hemodialysis patients, and the accumulation of IS in patients with CKD might deteriorate renal function, leading to the progression of end-stage renal disease and CVD (Leong and Sirich, 2016). Specifically, accumulating of IS has been reported to associate with overall and cardiovascular mortality in CKD patients (Barreto et al., 2009). Thus, serum IS level may be s an indicator of cardiovascular disease or other complications for patients with end-stage kidney disease. Future studies are still needed to address the correlation between serum IS and progression of renal pathology of CKD.

Currently, reduction of intestinal absorption of IS is the only way to slow the accumulation of IS in circulation. A low-protein diet with reduced animal protein provides a lower amount of substrate for protein degradation (Marzocco et al., 2013) and alters the microbial population (Di Iorio et al., 2019; Sirich et al., 2014), thus decreasing the production of IS. On the other hand, the carbonaceous oral adsorbent AST-120 reduces IS levels by absorbing indole in the intestine (Takara et al., 1985). AST-120 has been reported to exert beneficial effects on both patients with CKD and CVD (Schulman et al., 2016; Toyoda et al., 2020) but controversially fails to lower the serum IS level (Fujii et al., 2009; Yamaguchi et al., 2017). Monitoring the serum IS levels remain an important need since both dietary modification and AST-120 control the intestinal absorption of IS rather than clearance of serum IS, which accumulates during the disease progression. The routine evaluation of IS levels might help to assess the effectiveness of treatment, such as dialysis or combined approach to decrease IS levels (Rocchetti et al., 2020), and predict disease progression in patients with CKD.

By using ELISA-based method, the IS concentration of a total 24 serum samples with triplicate analysis could be determined in one single plate. Moreover, the entire procedure of ELISA could be completed within one day. We therefore conclude that ELISA-based methodology might be an effective and reliable method, and could be applied to routine clinical IS examination.

Declarations

Author contribution statement

Conceived and designed the experiments, H.Y.S. and K.C.Y.; Performed the experiments, S.D.D., J.Y.P. and C.H.W.; Analyzed and interpreted the data, S.D.D., J.Y.P., C.H.W., H.Y.S. and K.C.Y.; Contributed reagents, materials, analysis tools or data, C.Y.A.L., C.J.L., Y.C.H. and L.Y.S.; Wrote the paper, H.Y.S., S.D.D., J.Y.P., 6 C.H.W., C.Y.A.L., C.J.L., Y.C.H., L.Y.S. and K.C.Y.

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Data availability statement

The authors do not have permission to share data.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e12220.

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