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Assessment of the Trueness and Inter-Laboratory Precision of Routine Uric Acid Assays Using 4 Frozen Pooled Serum Samples Measured by the Japan Society of Clinical Chemistry's HPLC Method

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Background: Reference procedures are required for evaluating the accuracy of routine analytical systems for uric acid (UA). External quality assessment (EQA) for UA has only been conducted with quality controls in China, and the results have not been published. This study was designed to investigate both the trueness and inter-laboratory precision of UA measurements among routine analytical systems using a candidate reference method.

Methods: We performed the HPLC method recommended by the Japan Society of Clinical Chemistry (JSCC). Next, we evaluated its analytical performance and validated its trueness. The performance of 4 routine analytical systems (5 instruments per system, n = 20) for UA was assessed by using 4 frozen pooled serum samples measured by the HPLC method according to biologically relevant quality goals.

Results: Within-run, between-run, inter-day, and total CV of the method were less than 0.3%, 0.4%, 1.8%, and 2.6%, respectively. The UA measurements were consistent with the target values of standard reference material (SRM) 909b, the sixth ring trial for Reference Laboratories (RELA-2008) specimen, and national primary reference materials. The 4 frozen pooled serum samples were homogeneous, stable, and commutable. All routine systems achieved the desirable performance goal (total error < 11.9%).

Conclusions: We successfully reproduced the JSCC's HPLC method, which was simple, specific, precise, and accurate. We recommend this method as a reference method for UA measurement in human serum. Four routine analytical systems for UA measurement had acceptable traceability, and their UA results showed good concordance.

Key Words: Uric acid, HPLC, Standardization

INTRODUCTION

Uric acid (UA) is the major end product of purine metabolism. Serum levels of UA are elevated in gout, lead poisoning [1], Down syndrome [2], and hypothyroidism [3]. Moreover, many epidemiological studies have suggested that hyperuricemia, which may be an independent risk factor for metabolic syndrome [4], is associated with kidney disease, cardiovascular disease [5], cerebrovascular disease [6], obesity, diabetes, hypertriglyceridemia, and hypertension. On the other hand, hypouricemia can be seen in Wilson's disease [7], xanthinuria, and the syndrome of inappropriate antidiuretic hormone secretion (SIADH) [8]. Therefore, the accurate measurement of UA in human serum plays an important role in the diagnosis, treatment, and prognosis of diseases associated with disorders of purine metabolism.

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Table 1. Reference methods established by different laboratories for uric acid

Organization or author	Reference method	Reference material(s) for calibration	Reference material(s) for trueness
Duncan et al. [9] (1982)	Spectrophotometry	NIST SRM 913a	None*
DGKC [10] (1985)	ID-GC/MS	NIST SRM 913a	NIST SRM 909b, DGKC-reference sera
NIST [11] (1990)	ID-GC/MS	NIST SRM 913a	NIST SRM 909b
Ghent university [12] (1993)	ID-GC/MS	NIST SRM 913a	NIST SRM 909b, DGKC-reference sera
JSCC [13] (1993)	HPLC-UV	NIST SRM 913a	None*
National Institute of Metrology, China [14] (2007)	ID-LC/MS	GBW09202	NIST SRM 909b
National Center for Clinical Laboratories, China [15] (2009)	ID-LC/MS/MS	NIST SRM 913a	NIST SRM 909b

*None means that the trueness of the methods had not been evaluated.
Abbreviations: DGKC, German Cognitive linguistics Association; NIST, National Institute of Standards and Technology; JSCC, Japan Society of Clinical Chemistry; ID-GC/MS, isotope dilution gas chromatography-mass spectrometry; ID-LC/MS, isotope dilution liquid chromatography-mass spectrometry; ID-LC/MS/MS, isotope dilution liquid chromatography-tandem mass spectrometry; SRM, standard reference material.

Numerous methods have been reported for the measurement of UA in human serum. The uricase method is widely used in auto-analyzers; the direct method quantifies the decrease in ultraviolet (UV) absorbance at 293 nm (the absorbance maximum of UA), and the indirect method quantifies hydrogen peroxide formed by uricase activity. However, enzymatic methods can be affected by the presence of interfering compounds, and many clinical laboratories use calibrators that are not matched with appropriate reagents and analyzers. Therefore, reference procedures are necessary in order to evaluate the accuracy of routine analytical systems for UA. External quality assessment (EQA) for UA has only been conducted by the use of quality controls in China, and the EQA results have not been published so far. Therefore, this study was designed to investigate both the trueness and inter-laboratory precision of UA results among routine analytical systems through a candidate reference method.

Different reference methods have been established in order to provide a reliable standard and to improve the accuracy of UA measurement in serum (Table 1) [9-15]. In the present work, we searched for a simple, rapid, and economical reference method that also had good analytical performance. Duncan et al. [9] optimized and evaluated a manual UV uricase method for measuring UA. This method exhibited satisfactory analytical recoveries (98-105%) and precision (day-to-day precision, 1.5% [590 μmol/L] to 2.7% [180 μmol/L]) and was resistant to endogenous interference, including ascorbate and glutathione levels. As such, this method was once recommended as a reference method for UA. However, the disadvantages of this manual ultraviolet uricase method were the interference from xanthine and poor precision at low UA concentrations [16]. All isotope dilution mass spectrometry (ID-MS) methods for measuring UA in serum have very high accuracy and precision. Nevertheless, HPLC methods are superior to ID-MS methods with regard to their speed, simplicity,

economy, and compact size. These features are particularly important in developing countries and regions where ID-MS methods are difficult to perform.

Several HPLC methods for measuring UA have been established as candidate reference methods; however, most employed complex operational procedures [17]. In 1993, the Japan Society of Clinical Chemistry (JSCC) recommended a reversed-phase HPLC with UV detection as a national standard for UA detection [13]. Although this method is not on the Joint Committee for Traceability in Laboratory Medicine's (JCTLM) list, the HPLC assay is simple, rapid, economical, and precise. To date, the analytical performance and trueness of the JSCC's HPLC method have not been evaluated (Table 1). Thus, we performed the JSCC's HPLC method using the same or similar chemicals and apparatus in China to observe whether it is reproducible and re-valuated its analytical performance and trueness using several approaches. Next, we applied this HPLC method to investigate the both trueness and inter-laboratory precision of UA results among 4 routine analytical systems with 4 frozen pooled serum that were standardized by this method.

METHODS

1. Chemical and apparatus preparation
The HPLC apparatus (Shimadzu Co. Ltd., Tokyo, Japan) consisted of a model LC-20AT pump, an SIL-20A autosampler, a CTO-10AS column oven, and a model SPD-20A spectrophotometer with a UV variable wavelength detector connected to a model LC-solution chromatoprocessor. Glass autosampler vials (1.5-mL capacity) were purchased from Agilent (Agilent Technologies Inc., Santa Clara, CA, USA). Samples were centrifuged in a Beckman coulter Allegra X-22 refrigerated benchtop centrifuge (Beckman Coulter Inc., Brea, CA, USA). Solutions were

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prepared with Milli-Q (18.2 MΩ cm) water and filtered through 0.2-μm filters from Sartorius AG (Goettingen, Germany).

Standard reference material (SRM) 913a (certified chemical purity±uncertainty, 99.6%±0.1%) and SRM 909b were obtained from National Institute of Standards and Technology (NIST). National primary reference materials (GBW09174, GBW09175, and GBW09176) were donated by the National Center for Clinical Laboratories (NCCL) (Beijing, China). Perchloric acid (purity 70%), methanol (purity >99.9%), phosphoric acid (purity >85%), and lithium carbonate (purity >99%) were provided by Sigma (St. Louis, MO, USA). Sodium phosphate dibasic dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$) (purity >99%) was purchased from Sinopharm Chemical Reagent Company (Beijing, China). Uricase (50 U/mg) was supplied by Roche (Mannheim, Germany).

2. Calibrator preparation for the HPLC method

SRM 913a used for HPLC calibration was dissolved in 0.01 mol/L lithium carbonate so that the concentration of UA in the stock solution was approximately 1 g/L (5,950 μmol/L). Five standard solutions were prepared from the stock solution by dilution with Milli-Q water and were used to construct a calibration curve using their peak areas.

3. Sample treatment

Each serum sample or standard solution (0.2 mL of each) was mixed with 2 mL of 0.3 mol/L perchloric acid [18]. The mixed solutions were cooled in an ice-bath for 30 min, and then vortex-mixed for 10 sec and centrifuged at approximately 4,270 g at 4°C for 10 min. The supernatants were collected and centrifuged for a second time. Then, 0.3 mL of the supernatants was diluted with an equal volume of 0.2 mol/L $Na_2HPO_4 \cdot 12H_2O$ in glass vials. During the deproteinizing procedure, a precipitated product can be seen in the supernatant after the first centrifugation. Therefore, the JSCC method suggested two centrifugation steps [13].

4. HPLC assay

The HPLC method was performed using a Shimadzu LC-20A series apparatus (Shimadzu Co. Ltd.). The spectrophotometer was set at 284 nm. All separations were performed at 20°C on a Shimadzu Inertsil ODS-SP column (150 mm×4.6 mm, particle diameter 5 μm). The mobile phase was 0.2 mL/L of methanol in 74 mmol/L of phosphate-buffered saline (pH 2.2, 25.0°C). The flow rate was 1.0 mL/min. The injection volume was 50 μL.

5. Method validation

For method validation, we assessed specificity, linearity, precision, recovery, accuracy, and system suitability. The UA chromatogram from human serum samples treated with uricase was observed to evaluate the specificity [19]. The linear range was evaluated by generating a standard curve based on the UA concentrations of a series of standard solutions. The precision was investigated according to NCCLS EP5-A2 [20] by analyzing RANDOX quality controls (Randox Laboratories Ltd., Crumlin Co., Antrim, UK) at normal and pathological levels. Each level was run in duplicate with 2 runs per day for 20 days, and the interval between each successive run was at least 2 hr.

Serum pools were prepared by dividing a homogeneous pool of human serum into 5 portions. One was retained as a reference pool. The other 4 serum pools were adjusted to the desired concentration with the SRM 913a aqueous standard solution. Each of the 5 pools was analyzed 3 times, and the means of the UA results were calculated to assess the recovery.

The trueness of the HPLC method was controlled and validated by analyzing SRM 909b, the RELA-2008 specimen, and national primary reference materials (GBW09174, GBW09175, and GBW09176). The values of national primary reference materials were determined by isotope dilution liquid chromatography-tandem mass spectrometry (ID-LC/MS/MS), which was established by the NCCL of China.

System suitability tests were used to verify whether the system's resolution and reproducibility were adequate. The parameters used in this test were retention time, theoretical plate, capacity factor, and tailing factor.

6. Preparation, biological characterization, and UA measurement of four frozen pooled serum samples

Four pooled human serum samples with different UA concentrations were prepared and assessed by the HPLC method in order to evaluate the various routine analytical systems. Four UA concentrations in fresh fasting sera without hemolysis, lipemia, or choloplania were collected based on the suggested distribution of data for comparison of the methods recommended in NCCLS EP9-A2 [21]. All sera were negative for hepatitis B surface antigen (HBsAg), HIV, and hepatitis C virus (HCV). Four pooled human serum were mixed and centrifuged. Then, the supernatants were filtered with 0.2-μm filters and divided into 300-μL aliquots per tube, which were sealed and stored at -70°C.

The homogeneity and stability of the 4 frozen pooled serum samples were evaluated with a Beckman Coulter Synchron DXC800 homogeneous system (Beckman Coulter Inc.) accord-

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ing to ISO Guide 35 [22]. Ten vials of each concentration of the 4 frozen pooled serum samples were analyzed 3 times to determine their homogeneity. In order to assess the stability, randomly chosen samples were stored for different time periods at room temperature, 4℃, -20℃, and -70℃. The homogeneity and stability of the 4 frozen pooled serum samples were analyzed using 1-way ANOVA and linear regression analysis. Then, between-bottle uncertainty (u_{bb}) and long-term storage uncertainty (u_{lts}) were calculated. Commutability of the 4 frozen pooled serum samples was assessed according to CLSI EP14-A2 [23]. Thirty-nine single donor samples spanning the relevant UA concentration range (85-856 µmol/L) were selected. The 4 pooled samples and the 39 donor samples were analyzed by pairs of methods (HPLC and 5 routine analytical systems: Roche Modular P800 (Roche Diagnostics GmbH, Branchburg, NJ, USA), Beckman DXC800 (Beckman Coulter Inc.), Vitros 250 (Ortho-Clinical Diagnostics Inc., Rochester, NY, USA), Hitachi 7600/Wako (Kabu-shiki-gaisha Hitachi Seisakusho, Tokyo, Japan), and Dade RXL-MAX (Dade Behring Inc., Newark, DE, USA). Each routine analytical system was performed with only one instrument. We used regression analysis to determine whether the 4 prepared frozen pooled serum samples fell within the 95% predicted interval defined by the 39 donor samples.

The 4 frozen pooled sera were measured by the HPLC method. Each pool was tested twice daily for 5 successive days, and target values of the 4 frozen pooled sera are reported as mean±expanded uncertainty (U). According to ISO Guide 35, the combined uncertainty (u_c) of the 4 frozen pooled serum samples consisted of the uncertainty in the certified value of SRM 913a (u_{cal}), the uncertainty due to characterization (u_{char}), u_{bb}, u_{lts}, and bias compared with the national primary reference materials of the HPLC. The u_{char} is mainly derived from SD of the measured values. The coverage factor (k)=2. Therefore, U=k× u_c=2×$[u_{cal}^2+SD^2+u_{bb}^2+u_{lts}^2+bias^2]^{1/2}$.

7. Performance assessment of 4 routine analytical systems

The precision, bias, and total error (TE) of 4 routine analytical systems, including Roche (instruments: Modular P800=4, cobas 8000=1), Beckman (instruments: DXC800=5), Vitros (in-

Table 2. Analytical goals for uric acid measurement derived from data on biological variation

Quality level	Imprecision (%)	Bias (%)	Total error (%)
Optimal	2.2	2.4	5.9
Desirable	4.3	4.8	11.9
Minimal	6.5	7.2	17.8

struments: 5.1 FS=2, Vitros 5600=1, Vitros 350=1, Vitros 950=1), and Hitachi/Wako (instruments: Hitachi 7600=4, Hitachi 7180=1) homogeneous systems, were investigated using the 4 frozen pooled serum samples. Each routine analytical system consisted of 5 instruments. Each machine was matched with its respective calibrators and reagents. The 4 frozen pooled serum samples were analyzed in duplicate after confirmation that each instrument was calibrated and quality-controlled. The means of the results (2 replicates×5 instruments=10 results) from each routine analytical system were used to calculate the precision and bias compared with the target values for the 4 routine analytical systems.

8. Statistical methods

All results were entered into SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA). The performance of each routine analytical system was assessed by quality goals based on biological variability suggested by Fraser [24]. The minimum, desirable, and optimum analytical goals for precision, bias, and TE for UA derived from biological variation data are shown in Table 2 [25].

RESULTS

1. Specificity of the HPLC method

The UA chromatogram from the human serum sample disappeared after treatment with uricase (Fig. 1).

2. Linearity of the HPLC method

The linearity of the HPLC method ranged from 2.08 µmol/L to 1,785 µmol/L with aqueous standard solutions (r^2=0.999992).
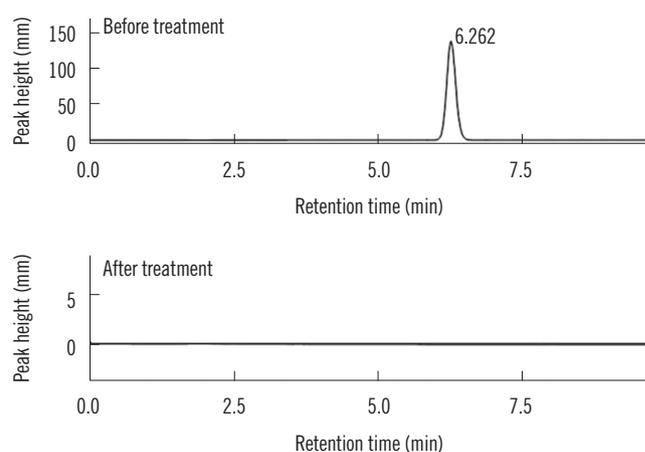

Fig. 1. Uric acid chromatograms of human serum samples before and after treatment with uricase.

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Table 3. Precision of the HPLC method for uric acid measurement

RANDOX quality controls	Mean concentration (μmol/L)	Within-run CV (%)	Between-run CV (%)	Inter-day CV (%)	Total CV (%)
Normal level	331	0.10	0.24	1.80	1.82
Pathological level	521	0.22	0.35	1.79	2.60

3. Precision of the HPLC method

The within-run, between-run, inter-day, and total CV of the HPLC method were less than 0.3%, 0.4%, 1.8%, and 2.6%, respectively, both at normal and pathological levels when calculated from the peak area (Table 3). The within-run precision in both previously reported JSCC methods [13] (0.7-0.9%) and the present HPLC method (0.1-0.22%) were less than 1% when evaluated by peak area. However, it was 1.5-1.7% in the previous method and 1.25-1.27% in the present HPLC method when evaluated by peak height.

4. Recovery of the HPLC method

The 5 serum pools had approximate UA concentrations of 120 μmol/L, 246 μmol/L, 380 μmol/L, 466 μmol/L, and 547 μmol/L. The average relative recovery rate was 99.6-100.6%. In the previously reported JSCC method, the analytical recoveries were measured by adding $[2-^{14}C]$ UA to 10 mL of a serum pool and to a standard solution; the recoveries were 98.5% and 99.9%, respectively [13].

5. Trueness of the HPLC

The concentrations (mean±SD) of UA in SRM 909b were 270± 1.54 μmol/L (n=15) for level 1 and 716±3.36 μmol/L (n=15) for level 2. The results were within the concentration range certified by NIST for UA in SRM 909b level 1 (277±12 μmol/L) and level 2 (733±23 μmol/L) [26]. The proportional biases of UA in RELA (2008) specimen level 1 and level 2 were 0.35% and -0.69%, when compared with the mean results obtained from participating laboratories. The UA levels measured by the HPLC method had biases of 0.29%, -0.74%, and 0.06% respectively, compared with the target values of national primary reference materials (273.4 μmol/L, 335.7 μmol/L, and 494.9 μmol/L) [27].

6. System suitability test

The CV of system-suitability test parameters was satisfactory (Table 4).

7. Biological characteristics of the 4 frozen pooled serum samples

No stabilizing agent or preservative was added to the 4 frozen

Table 4. System suitability test parameters

Parameters	UA (150 μmol/L, n=5)		UA (250 μmol/L, n=5)	
	Mean±SD	CV (%)	Mean±SD	CV (%)
Retention time (min)	6.251±0.0013	0.021	6.253±0.0009	0.014
Theoretical plates	3,680.553±21.65	0.588	3,678.211±20.43	0.555
Capacity factor	3.392±0.009	0.265	4.015±0.010	0.249
Tailing factor	0.99±0.001	0.101	1.02±0.003	0.294

Abbreviation: UA, uric acid.

pooled serum samples in this study, so the matrix effect was negligible. The four frozen pooled serum samples were homogeneous ($P>0.05$) and stable for at least 115 days when stored at -20℃ and 15 months when stored at -70℃ ($P>0.05$ for both). The 4 frozen pooled serum samples had good commutability between HPLC and 5 the routine analytical systems. Specifically, the UA results of the 4 frozen pooled serum samples all fell within the 95% predicted interval defined by the 39 donor samples. The mean±U of the 4 frozen pooled serum samples of UA were 282±5 μmol/L, 383±7 μmol/L, 431±10 μmol/L, and 520±12 μmol/L.

8. Precision, bias, and TE of 4 routine analytical systems

The 4 routine analytical systems (5 instruments per system, n= 20) all achieved the desired performance goal with measurement of the 4 frozen pooled serum samples (CV <4.3%, bias <4.8%, and TE <11.9%) (Table 5 and Fig. 2).

DISCUSSION

In this study, we successfully replicated the JSCC's HPLC method for UA measurement in human serum, evaluated its analytical performance, and validated its trueness for the first time in China. Both the JSCC's previously reported HPLC method and the present HPLC method had high levels of precision and recovery. Although the accuracy of the JSCC's HPLC method was only confirmed through evaluating the recovery, we verified its trueness by analysis of SRM 909b, the RELA (2008) specimen, and national primary reference materials. The CV calculated by the peak height was approximately 2 times higher than that calculated by the peak area. This could be because changes in pH, concentrations of the mobile phase and methanol, and retention

Table 5. Performance evaluation of 4 homogeneous routine analytical systems for uric acid measurement with 4 frozen pooled serum samples

	Target value (µmol/L)	Roche (n=5)	Beckman (n=5)	Vitros (n=5)	Hitachi (n=5)
Mean	282	272.45	284.67	282.32	277.97
SD		5.68	5.51	4.05	4.54
CV (%)		2.09	1.94	1.43	1.63
Relative bias (%)		-3.29	1.04	0.21	-1.33
TE (%)		6.74	4.24	2.57	4.02
Mean	383	375.14	381.87	379.87	377.95
SD		8.98	6.02	4.63	2.28
CV (%)		2.39	1.58	1.22	0.60
Relative bias (%)		-2.10	-0.34	-0.86	-1.37
TE (%)		6.04	2.95	2.87	2.36
Mean	431	423.16	425.87	424.71	425.20
SD		6.32	7.09	6.42	2.07
CV (%)		1.49	1.66	1.51	0.49
Relative bias (%)		-1.76	-1.13	-1.40	-1.28
TE (%)		4.22	3.87	3.89	2.09
Mean	520	513.03	507.00	510.78	512.66
SD		11.07	9.28	10.09	3.66
CV (%)		2.16	1.83	1.98	0.71
Relative bias (%)		-1.34	-2.50	-1.77	-1.41
TE (%)		4.90	5.52	5.04	2.58

Desired performance goals (CV <4.3%, bias <4.8%, and TE <11.9%).
Abbreviation: TE, total error.

time affected the results calculated by peak height but not those calculated by peak area.

Initially, we tried to use the same sample treatment procedure and chromatographic conditions with the same (or similar) chemicals and apparatuses to assess the performance of the JSCC's HPLC method. We determined that the method was simple, rapid, and specific and had high recovery, precision, and accuracy, so we did not make any modifications. We identified the following differences between the previously reported HPLC method and that in the present study: 1) The HPLC apparatus was different. The previous study used a Toyosoda HPLC apparatus (Toyosoda Co. Ltd., Tokyo, Japan) and a Toyosoda ODS-120A reversed-phase column (25 cm×4.6 mm, particle diameter 5 µm). Our study used a Shimadzu HPLC apparatus (Shimadzu Co. Ltd.) and a Shimadzu Inertsil ODS-SP column (150 mm×4.6 mm; particle diameter, 5 µm). 2) The purity of perchloric acid was 60% in the previous study and 70% in the present study.
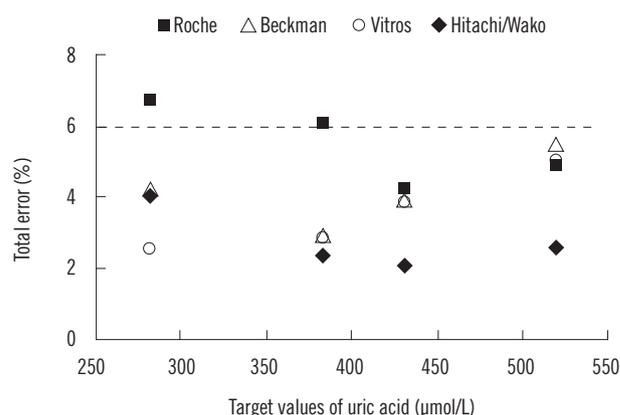
Fig. 2. Total error of 4 routine analytical systems for uric acid versus target values of the 4 frozen pooled serum samples measured by the HPLC method. The dotted line represents the optimum (5.9%) performance goal based on biologic variability.

In addition, no peaks aside from the UA peak was observed in the standard (SRM913a) or serum samples, and no peak was observed in the blank samples (Milli-Q water and mobile phase). Thus, the matrix effects were negligible. On the basis of these findings, we concluded that the JSCC's HPLC method is reproducible. Therefore, we recommend it as a candidate reference method for UA measurement in human serum in developing countries and poor regions.

We used this method to evaluate the analytical performance of 4 routine systems for UA measurement of 4 frozen pooled serum samples. Four routine analytical systems for UA measurement all achieved the desired performance goals (CV <4.3%, bias <4.8%, and TE <11.9%) [25]. The results were similar to those of an earlier study [28], where UA instruments displayed good performance; all participating laboratories met the minimum bias performance goal (bias <7.2%), and greater than 87.5% of the participating laboratories met the desired bias goal (bias <4.8%) based on biological variability versus a target value (UA 320 µmol/L) assigned by ID-MS and uricase methods. The routine analytical systems for UA measurement displayed good analytical performance and consistency, likely because nearly all of the instruments used the same enzymatic methodology. The lower threshold of the reference interval for UA was less than 282 µmol/L, and hypouricemia is a clinical concern. One limitation of this study was that frozen pooled sera with low UA concentrations were not prepared, and the number of participating laboratories was small. Further investigation is needed to confirm our results.

In summary, this study was the first preliminary evaluation of routine analytical systems for UA measurement by the JSCC's

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HPLC method in China.

Authors' Disclosures of Potential Conflicts of Interest

Acknowledgments

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