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Evaluation of a kinetic uricase method for serum uric acid assay by predicting background absorbance of uricase reaction solution with an integrated method*

LIAO Fei†[1,2], ZHAO Yun-sheng[1,2], ZHAO Li-na[1,2], TAO Jia[1], ZHU Xiao-yun[2], LIU Lan[3]

([1]*Laboratory of Bioinformatics and Molecular Engineering, Chongqing University of Medical Sciences, Chongqing 400016, China*)

([2]*Department of Biochemistry and Molecular Biology, Chongqing University of Medical Sciences, Chongqing 400016, China*)

([3]*Affiliated Children's Hospital, Chongqing University of Medical Sciences, Chongqing 400016, China*)

†E-mail: liaofeish@vip.sina.com; liaofeish@yahoo.com

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Abstract: A patented kinetic uricase method was evaluated for serum uric acid assay. Initial absorbance of the reaction mixture before uricase action (A_0) was obtained by correcting the absorbance at 293 nm measured before the addition of uricase solution, and background absorbance (A_b) was predicted by an integrated method. Uric acid concentration in reaction solution was calculated from ΔA, the difference between A_0 and A_b, using the absorptivity preset for uric acid. This kinetic uricase method exhibited CV<4.3% and recovery of 100%. Lipids, bilirubin, hemoglobin, ascorbic acid, reduced glutathione and xanthine <0.32 mmol/L in serum had no significant effects. ΔA linearly responded to 1.2 to 37.5 μmol/L uric acid in reaction solution containing 15 μl serum. The slope of linear response was consistent with the absorptivity preset for uric acid while the intercept was consistent with that for serum alone. Uric acid concentrations in clinic sera by different uricase methods positively correlated to each other. By Bland-Altman analysis, this kinetic uricase method accorded with that by quantifying the total change of UV absorbance on the completion of uricase reaction. These results demonstrated that this kinetic uricase method is reliable for serum uric acid assay with enhanced resistance to both xanthine and other common errors, wider range of linear response and much lower cost.

Key words: Background absorbance, Bland-Altman analysis, Kinetic uricase method, Reaction curve fitting, Serum uric acid assay
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INTRODUCTION

Uricase method is widely used to monitor serum uric acid for laboratory diagnosis of gout, kidney function and hyperuricemia-associated diseases. It is easy to quantify uric acid by its distinctive absorbance at 293 nm or to quantify hydrogen peroxide, one of the uricase products, by peroxidase-coupled analysis. There is a direct uricase method for quantifying the decrease of UV absorbance at 293 nm of uric acid upon uricase action and an indirect uricase method for quantifying the amount of hydrogen peroxide formed by uricase action. Indirect uricase method is sensitive to bilirubin, ascorbate, glutathione, lipids, hemoglobin, and peroxide in serum besides xanthine (Aoki *et al*., 1992; Bartl *et al*., 1979; Kroll and Elin, 1994; Sanders *et al*., 1980; Spain and Wu, 1986). The direct equilibrium method by quantifying the difference between initial UV absorbance of the reaction solution before uricase action (A_0) and background absorbance after completion of uricase reaction (A_b) is resistant to most of these interferences except xanthine, but has lower efficiency and precision (Duncan *et al*., 1982). The kinetic uricase method by fitting the classical Michaelis-Menten rate equation to the uricase reaction curve of UV absorbance is limited by background absorbance and unsatisfactory lower limit (Hamilton and Pardue, 1982).

By predicting A_b of the uricase reaction solution with an integrated method (Liao *et al*., 2001; 2003a; 2003b; 2005a), a new kinetic uricase method was

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proposed for uric acid assay with A_0 estimated through extrapolation according to the initial rate and preset lag time of steady-state reaction (Liao, 2005; Liao *et al*., 2005b). This kinetic uricase method showed much lower cost and resistance to most common errors. However, it was unclear whether this kinetic uricase method accorded with other established uricase methods for uric acid assay in clinic sera. Hyperuricemic serum is rich in xanthine (Hande *et al*., 1979), but this direct kinetic uricase method is sensitive to xanthine when uric acid concentration in reaction solution is comparable to uricase Michelis-Menten constant (K_m) (Liao, 2005; Liao *et al*., 2005b). Moreover, the detection limit is much higher than the lower limit of linear response, and the use of lower uricase activity to reduce the detection limit results in lower efficiency (Liao *et al*., 2005b). Herein, by correcting the absorbance measured before the addition of uricase solution to determine the desired A_0 and using uricase of higher K_m, this kinetic uricase method was evaluated for uric acid assay in clinic sera.

MATERIALS AND METHODS

Chemicals and materials

Bacillus fastidious uricase (EC 1.7.3.3) with K_m of 65 μmol/L was Fluka 94310 (Zhao *et al*., 2005), uric acid was from ICN. Other chemicals were domestic analytical reagents.

Specimens

Clinic sera were collected in the clinic laboratory of the First Affiliated Hospital of Chongqing University of Medical Sciences, and stored below –10 °C, thawed and centrifuged to remove denatured proteins before analysis. By this kinetic uricase method, uric acid from 0.68 to 0.99 mmol/L in six clinic sera showed a decrease of (5.4±3.0)% after 6-day storage at 4 °C (P<0.05 by paired t-test). Therefore, 189 clinic sera with the widest distribution of uric acid concentrations (screened by the indirect equilibrium method) in the pool of over 600 sera were analyzed by the following three methods within 12 h to examine their agreements with each other.

Indirect equilibrium method

The two-reagent kit containing ascorbate oxidase for serum uric acid assay by peroxidase-coupled assay of hydrogen peroxide was from Biosino Biotechnology and Science INC (Changping, Beijing 102200, China). It was used on HITACHI 7170 Autoanalyzer with 8 μl serum in 398 μl reaction solution for analysis. For the comparison of recoveries and the ranges of linear response with all methods, the reaction solution was amplified to 1.2 ml with the same sample ratio to measure absorbance using common spectrophotometer.

Direct equilibrium method

URCA uric acid Flex® cartridge from Dade Behring (Newark, DE 19714, USA) was used on Dade Behring Dimension® RXL Clinical Chemistry System to measure the total change of absorbance at 293 nm upon uricase action (Duncan *et al*., 1982). Operations followed the stated protocol with 17 μl serum in 406 μl reaction solution for analysis.

Kinetic uricase method by predicting background absorbance

1. Monitoring uricase reaction curve and estimating A_0

Absorptivity of uric acid at 293 nm was fixed at 11.5 (L·cm)/mmol throughout all analysis. The buffer and assay of uricase activity were the same as before (Liao *et al*., 2005a; Zhao *et al*., 2005). *Bacillus fastidious* uricase at final 0.03 U/ml had no detectable absorbance at 293 nm. Final reaction mixture contained 1.18 ml buffer, 15 μl clinic serum, and 5 μl uricase solution to give final 0.020 U/ml uricase unless stated otherwise. Reaction solution without uricase was mixed in quartz cell before the absorbance at 293 nm was taken, and the subtraction of 0.3% from this absorbance to correct the effect of volume change upon the addition of uricase solution yielded the desired A_0. After the addition of uricase solution, the reaction was initiated by mixing solutions in quartz cell. The reaction curve was monitored 30 s after the initiation of reaction at 10 to 30 s interval within 5 min at (25±0.5) °C unless stated otherwise. The highest absorbance was limited to 1.270. For direct assay of A_b, the absorbance after 40 min reaction at uricase activity of no less than 0.020 U/ml was measured again and directly taken as A_b.

2. Estimation of A_b by the integrated method

K_m for *Bacillus fastidious* uricase was fixed at 65 μmol/L unless stated otherwise (Zhao *et al*., 2005). By taking that more than 45 s after the initiation of

reaction as the initial datum for analysis, background absorbance (A_b) was estimated by fitting integrated Michaelis-Menten rate equation with the predictor variable of reaction time to uricase reaction curve that contained more than five data with absorbance drop above 0.001 (Liao, 2005; Liao *et al*., 2005b). If there were less than five absorbance data with change above 0.001 at uricase >0.02 U/ml for reaction curve fitting, the absorbance after 5 min reaction minus 0.001 was directly taken as A_b. Uric acid concentration was calculated from ΔA, the difference between A_0 and A_b, using absorptivity preset for uric acid at 293 nm.

3. Programming

The program was written in Visual Basic 6.0 with plotting of data to examine outliers. Both K_m and the range of data for fitting with the integrated rate equation were adjustable. Results were output to a specified text file for further analysis.

Statistical analysis

The results were mean±*SD*. The lower limit was thrice the standard error of estimate, the upper limit was that with *CV*<5% and deviation from the linear plot below twice the standard error of estimate, respectively. The agreements between any two methods were examined according to Bland-Altman using SAS 8.0 (Bland and Altman, 1995).

RESULTS

Estimation of A_b by the integrated method

In the pool of over 600 clinic sera, there were only two sera needing 1:1 dilution to give A_0<1.270. After the desired dilution, A_0 varied from 0.300 to 1.130 while A_b varied from 0.220 to 1.030. The percentage of residual substrate to initial substrate under analysis was the primary factor to determine the reliability of A_b estimated by the integrated method while the lag time to monitor reaction curve showed no effects. With initial uric acid <30 μmol/L, residual substrate <1/5 of the initial one usually gave A_b with deviation of <2% from that by direct assay after 40 min reaction. A_b was resistant to deviation of 15 μmol/L in preset K_m if residual uric acid was below one-fifth of initial uric acid that was <15 μmol/L, and the lower concentration of initial uric acid for analysis gave stronger resistance. The estimation of A_b was sensitive to outliers in the reaction curve, and lower content of initial uric acid for analysis led to higher sensitivity to outliers.

For all tested clinic sera to give A_0 below 1.270 after necessary dilution, uric acid in reaction solution at the time 45 s after the addition of uricase was <25 μmol/L, and residual uric acid after 5 min reaction at 0.020 U/ml uricase was low enough for reliable estimation of A_b. With uric acid from 0.23 mmol/L to 0.97 mmol/L in clinic sera and reaction curve monitored within 5 min, the increase of uricase up to 0.030 U/ml resulted in no differences in A_b from those at 0.020 U/ml uricase (n=3, P>0.1 by paired t-test), respectively, but reaction duration for reliable estimation of A_b with these sera was reduced to within 3.5 min at 0.030 U/ml uricase. The shift of reaction temperature to 21 °C at 0.020 U/ml uricase to monitor reaction within 5 min still led to no changes of A_b in the presence of 15 μmol/L uric acid in reaction solution.

Comparisons of this kinetic uricase method to other uricase methods

1. Reproducibility

For serum uric acid from 0.35 to 1.0 mmol/L, the within-run variation and between-run *CV* were <4.3% (n=5), and comparable to automated analysis with other uricase methods.

2. Recoveries and the effects of common serum interferences

By this kinetic uricase method, the recoveries for 0.83 mmol/L serum uric acid were consistent to 100% (P>0.3 by t-test). There were no interferences from common serum substances including lipids, bilirubin, glutathione, ascorbate besides xanthine <0.32 mmol/L (Table 1). Except for slight negative error by lipids and xanthine, the direct equilibrium method showed similar pattern of resistance to these interferences, and the recoveries were consistent to 100% (P>0.3 by t-test). However, there were usually negative deviations with the indirect equilibrium method except that lipids led to slight positive deviation, and the recovery for 0.83 mmol/L serum uric acid was ((95.3±2.7)%, n=4), smaller than 100% (P<0.05 by t-test).

3. Ranges of linear response

By this kinetic uricase method with data monitored within 5 min at 0.020 U/ml uricase, ΔA linearly responded to the concentration of extra uric acid (C_u, μmol/L) added into reaction solution containing 15 μl serum (ΔA=0.029+0.0116×C_u, r>0.9996, s<0.005).

Table 1 Effects of common substances on serum uric acid assay by different uricase methods

Candidate interferences	C_{de} (mmol/L)	C_{dk} (mmol/L)	C_{ie} (mmol/L)
Serum A+water[a]	0.978±0.012	0.976±0.011	0.934±0.005
Serum A+6.0 mmol/L glutathione[a]	0.974±0.011	0.966±0.010	0.897±0.006*
Serum A+3.0 mmol/L ascorbate[a]	0.985±0.018	0.980±0.012	0.827±0.013*
Serum A+45 g/L beef serum albumin[a]	0.982±0.015	0.980±0.016	0.930±0.008
Serum A+0.32 mmol/L xanthine[a]	0.896±0.020*	0.974±0.016	0.916±0.008**
Serum A+0.85 mmol/L EDTA[a]	0.980±0.012	0.978±0.009	0.940±0.010
Serum B+water[b]	0.490±0.006	0.490±0.007	0.486±0.004
Serum C (Hb 1.35 g/L)+water[b]	0.074±0.002	0.064±0.004	0.066±0.002
Serum B+Serum C[c]	0.563±0.009	0.555±0.008	0.510±0.006*
Serum D (TB 0.17 mmol/L)+water[b]	0.080±0.002	0.068±0.009	0.064±0.002
Serum B+Serum D[c]	0.571±0.010	0.558±0.012	0.538±0.007**
Serum E (TG 12.0 mmol/L)+water[b]	0.097±0.005	0.085±0.008	0.080±0.002
Serum B+Serum E[c]	0.561±0.010**	0.570±0.018	0.576±0.004**

C_{de}: Serum uric acid concentration by the direct equilibrium method; C_{ie}: Serum uric acid concentration by the indirect equilibrium method; C_{dk}: Serum uric acid concentration by this kinetic uricase method. C_{de}, C_{dk} and C_{ie} (in mmol/L) were from assays in triplicate. *Indicated $P<0.01$ and **indicated $P<0.05$ vs that without indicated interference, respectively. Serum A and Serum B were artificial hyperuricemic sera made by adding different amounts of solid uric acid into 20 ml serum from healthy individual. Serum C, Serum D, Serum E were clinic sera rich in endogenous interferences, of total bilirubin (TB), total triglycerides (TG), hemoglobin (Hb), respectively. [a]Serum A was mixed with water alone or aqueous solution of specified candidate interference at 10:1 ratio to give indicated final concentrations in serum; [b]Serum B or serum rich in endogenous interference (Serum C, Serum D or Serum E, respectively) was mixed with water at 1:1 ratio to determine the amount of endogenous uric acid; [c]Serum B was mixed with serum rich in candidate endogenous interference (Serum C, Serum D or Serum E, respectively) at 1:1 ratio to test the effect of interference

The slope was consistent with the absorptivity preset for uric acid. ΔA for 15 μl serum alone was 0.030±0.001 (n=3). The correction by 0.3% of the mixture absorbance before the addition of uricase solution to determine A_0 yielded the smallest difference between the intercept and ΔA for serum alone. After the contribution from serum alone was corrected, uric acid at 0.6 μmol/L in reaction solution yielded ΔA of 0.007±0.001 (n=3). This signal of ΔA was below the lower limit of linear response. The upper limit for uric acid in reaction solution was >37.5 μmol/L, which suggested that with sample ratio of 1.25%, serum uric acid from 0.10 to 3.0 mmol/L was suitable for analysis without dilution given measurable A_0. When 0.030 U/ml uricase was used, the upper limit was increased to >50 μmol/L in reaction solution while the lower limit was the same.

With the indirect equilibrium method, there was negative deviation at uric acid concentration >25 μmol/L in reaction solution, and the range of linear response was for 0.06 to 1.2 mmol/L serum uric acid. The direct equilibrium method gave linear response to uric acid in reaction solution from 2.3 to 50 μmol/L ($\Delta A_{293}=0.033+0.0116\times C_u$, $r>0.9996$, $s<0.009$), enabling serum uric acid from 0.06 to 1.2 mmol/L to be suitable for analysis without dilution. However, it exhibited the highest standard error of estimate for linear response.

Agreements among different uricase methods

Taking 189 clinic sera into account, there were close positive correlations among serum uric acid concentrations by the direct equilibrium method (C_{de}), by the indirect equilibrium method (C_{ie}) and by this kinetic uricase method (C_{dk}). Deviations between any two methods were <0.25 mmol/L. The slopes for these correlation equations were comparable to their differences in recoveries for 0.83 mmol/L uric acid. However, Bland-Altman analysis demonstrated that there was close correlations of the differences between C_{ie} and C_{de} to their averages (Fig.1a) while there was no significant correlation of the differences between C_{dk} and C_{de} to their averages (Fig.1b).

DISCUSSION

With uricase of higher K_m, there was stronger resistance of this kinetic uricase method to the deviation in K_m preset for fitting to the reaction curve and the variation of residual substrate for analysis. Xanthine showed comparable inhibition potency on uricase from either *Candida utilis* or *Bacillus fastidious* (Liao *et al*., 2005a; Zhao *et al*., 2005). By this kinetic uricase method using *Bacillus fastidious* uricase of higher K_m, xanthine in reaction solution at 4.0 μmol/L showed no effects while there was negative deviation

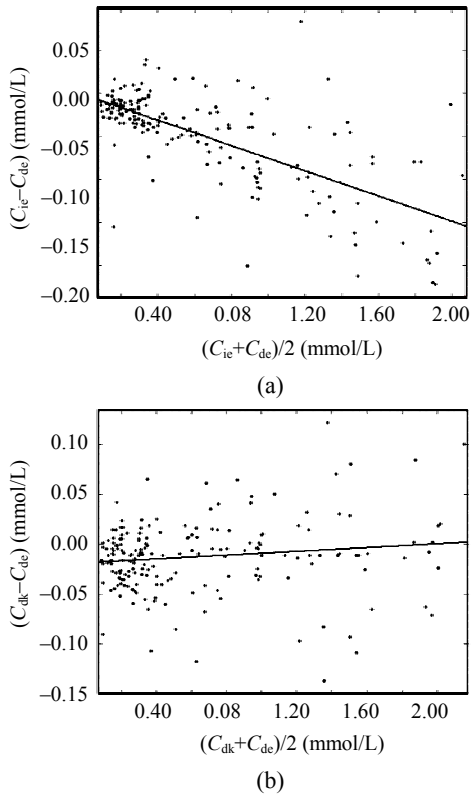

Fig.1 Bland-Altman analysis of the agreement between different uricase methods for the assay of uric acid in 189 clinic sera. (a) The indirect equilibrium method (C_{ie}) vs the direct equilibrium method (C_{de}). Bland-Altman analysis gave $C_{ie}-C_{de}=-0.002-0.073\times((C_{ie}-C_{de})/2)$ ($r>0.698$, $s=0.039$) while the correlation equation between them was $C_{ie}=-0.001+0.927\times C_{de}$ ($r>0.997$, $s=0.037$); (b) This direct kinetic uricase method (C_{dk}) vs the direct equilibrium method (C_{de}). Bland-Altman analysis gave $C_{dk}-C_{de}=-0.019+0.010\times((C_{de}+C_{dk})/2)$ ($r<0.137$, $s=0.037$) while the correlation equation between them was $C_{dk}=-0.018+1.007\times C_{de}$ ($r>0.998$, $s=0.037$)

when *Candida utilis* uricase with K_m of 12.5 μmol/L was used (Liao *et al*., 2005b). Moreover, the detection limit for uric acid was below the lower limit of linear response and was independent of uricase activity when A_0 was determined by correcting the absorbance measured before the addition of small amount of uricase solution. The standard error of estimate for this kinetic uricase method was below one-third of that by fitting classical Michaelis-Menten rate equation to uricase reaction curve (Hamilton and Pardue, 1982), and it was comparable to the lowest one of the common uricase method (Sanders *et al*., 1980). The upper limit for uric acid in reaction solution at 0.030 U/ml uricase was comparable to the highest one of other practical uricase methods (Duncan *et al*., 1982; Sanders *et al*., 1980). The upper limit was 30 times of the lower limit at 0.02 U/ml uricase, 40 times of the lower limit at 0.03 U/ml uricase, respectively, suggesting much wider range of linear response than the indirect equilibrium method. Therefore, the use of uricase of higher K_m and alternative way to determine A_0 improved this kinetic uricase method.

Each reducing agent in serum is quite low, but the total equivalents of reducing agents in serum are not negligible. There was negative deviation with the indirect uricase method due to reducing agents like glutathione and ascorbate. The recovery for higher concentration of serum uric acid by the indirect equilibrium method was lower than those for direct uricase methods. Moreover, hyperuricemic serum is usually rich in xanthine (Hande *et al*., 1979). The equilibrium method is preferred to uricase of lower K_m, which showed higher sensitivity to xanthine (Liao *et al*., 2005a; Zhao *et al*., 2005). Thus the indirect equilibrium method may be prone to negative deviation by the actions of common serum interferences. The direct equilibrium method showed stronger resistance to common errors than other available uricase methods, and was once used as a candidate reference method for uric acid assay (Duncan *et al*., 1982). Bland-Altman analysis suggested that this kinetic uricase method agreed with the direct equilibrium method aside from its even stronger resistance to serum xanthine while the indirect equilibrium method disagreed with the direct equilibrium method (Bland and Altman, 1995). Therefore, this kinetic uricase method may exhibit higher resistance to common errors than other uricase methods.

This kinetic uricase method can reliably predict A_b with clinic sera, and is resistant to the variation of uricase activity or reaction temperature. The standard error of estimate by this kinetic uricase method is smaller than that by the direct equilibrium method (Duncan *et al*., 1982) or by fitting classical Michaelis-Menten rate equation to uricase reaction curve (Hamilton and Pardue, 1982). The estimation of both A_0 and A_b for the same reaction solution led to close positive covariance between A_0 and A_b, which may give lower random error in ΔA (Liao *et al*., 2005b). This property and the resistance to common errors may account for its satisfactory reproducibility. Within 5 min reaction, uricase activity above 0.5 U/ml was required for the upper limit of 50 μmol/L

uric acid in reaction solution by the direct equilibrium method (Duncan *et al*., 1982). This kinetic uricase method needed uricase <0.030 U/ml within 3.5 min reaction to give comparable upper limit. There is no need for other auxiliary enzyme or toxic chemical. Therefore, this kinetic uricase method showed much lower cost for even higher efficiency.

Autoanalyzer like Dimension® series from Dade Behring was supplied with UV light at 293 nm, which may enable automated analysis with this kinetic uricase method. This kinetic uricase method preferred for analyzing data with substrate below one-fifth of uricase K_m. In the pool of over 600 clinic sera screened by the indirect equilibrium method, the lowest uric acid concentration was ~0.07 mmol/L while the highest uric acid concentration was ~2.16 mmol/L. This dynamic range suggested that sample ratio of 2% was desirable and that uric acid in reaction solution should be <50 μmol/L for most cases. At the initial datum for analysis by this kinetic uricase method, 40 s after the addition of uricase, uric acid concentration should be lower that the initial one. Thus uricase with K_m slightly above 0.20 mmol/L and resistance to xanthine as stronger as possible was desired. With such an uricase, this kinetic uricase method may be reliable for serum uric acid assay in routine practice with enhanced resistance to both xanthine and other common errors, wider range of linear response, improved reproducibility and much lower cost for even higher efficiency. Such uricases were already available, and they conferred on the resistance of this kinetic uricase method to xanthine as high as 30 μmol/L in reaction solution (Zhao *et al*., 2006). Therefore, this kinetic uricase method may be developed into a practical method for routine assay of serum uric acid after optimization and standardization.

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