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DETERMINATION OF SERUM CREATININE IN SELECTED GROUPS OF PATIENTS BY MEANS OF HPLC WITH UV DETECTION — COMPARISON WITH JAFFÉ AND ENZYMATIC ASSAYS

Xenie ŠTRAMOVÁ and Roman KANĎÁR[1]
Department of Biological and Biochemical Sciences,
The University of Pardubice, CZ–532 10 Pardubice

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A reversed-phase high-performance liquid chromatography method for the determination of creatinine in human serum samples has been developed. Results from measurements of by a high-performance liquid chromatography on 232 serum samples were compared with enzymatic and Jaffé methods. The serum samples were deproteinized with ethanol. For the separation, a column MAG 1, 250 mm x 4.6 mm, Labiospher PSI 100 C18, 5 μm, was used. The mixture of ethanol and 25 mmol l^{-1} sodium hydrogenphosphate (3:97 v/v), pH 6.5 was used as a mobile phase. The analytical performance of this method is satisfactory: the intra-assay and inter-assay coefficients of variation were below 6 %. Quantitative recoveries of spiked serum samples were between 101.8 and 106.0 %. The limit of quantification was 10.0 μmol l^{-1}. The results obtained by chromatographic method correlated well with Jaffé assays. Enzymatic method gave at average significantly higher values depending on a group of patients. The presented high-performance

[1] To whom correspondence should be addressed.

liquid chromatographic assay is useful for the analysis of samples where the classical Jaffé or enzymatic methods do not give reliable results and can be a candidate for reference method.

Introduction

Creatinine (synonyms: 2-amino-1-methyl-2-imidazolin-4-one; 2-imino-1-methyl-imidazolidin-4-one; 2-imino-N-methylhydantoin) is a break-down product of the creatine catabolism (Fig. 1). In humans it occurs in muscles and blood. About 2 % of the body's creatine is converted to creatinine every day. Since the muscle mass is relatively constant, the creatinine level in the blood remains practically unchanged, and creatinine is excreted in urine at a constant rate of about 25 mg per kg of body weight per day. Creatinine is thus a good indicator of kidney function. In renal diseases, the creatinine concentration in the blood is increased due to reduced clearance by the kidneys [1-5].

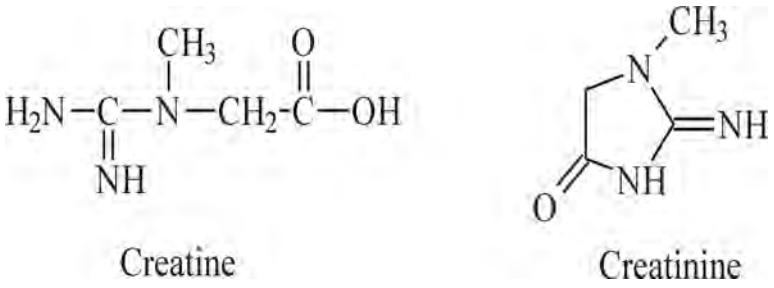

Fig. 1 Chemical structures of creatine and creatinine

The measurement of serum creatinine is based on the Jaffé alkaline picrate reaction. This photometrical method is simple and rapid; however, it is not sufficiently specific because a number of other substances react with picrate too [6-8]. More specific are enzymatic methods, but there have been reported various substances that interfere too [9]. Hence many high-performance liquid chromatographic (HPLC) [10-12], gas chromatographic (GC) [13] and/or high-performance capillary electrophoretic (HPCE) [14,15] methods for the determination of creatinine in biological fluids have been developed.

In this paper, we present a simple, rapid, accurate and eco-friendly reversed-phase HPLC method for the determination of serum creatinine.

Materials and Methods

Reagents and Chemicals

Creatinine, uric acid, hydrochloric acid, metaphosphoric acid, *ortho*-phosphoric acid, perchloric acid, trichloroacetic acid, oxalic acid, 5-sulfosalicylic acid, sodium dihydrogenphosphate, sodium hydrogenphosphate, sodium acetate, ammonium acetate, sodium hydroxide were obtained from Sigma Chemical Company (St. Louis, MO, USA). HPLC-gradient grade methanol, ethanol and acetonitrile were from Merck KgaA (Darmstadt, Germany). Lyophilized serum creatinine controls chemTRAK® (Lot TLM10081, TLM 10082) were from MAS® Controls (Passau, Germany). All the other chemicals were of analytical grade.

Instrumentation

Chromatographic analysis was performed with a liquid chromatograph (Shimadzu, Kyoto, Japan), LC-10ADvp solvent delivery system, SIL-10ADvp cooled autosampler, CTO-10ASvp column oven, SPD-10Avp variable wavelength spectrophotometric detector and SCL-10Avp system controller. The data were collected digitally with Clarity chromatography software (DataApex, Prague, the Czech Republic).

Subject and Samples

A total of 232 patients (116 women in the age of 21-96 years, mean age 69 years and 116 men in the age of 19-91 years, mean age 65 years) were included in the present study. Serum samples were obtained from the Department of Clinical Biochemistry and Diagnosis, Regional Hospital in Pardubice within routine investigation. The Hospital Committee on Human research (Regional Hospital of Pardubice) approved this research study according to the Helsinki Declaration. The patients were divided into four groups: patients with non-insulin dependent diabetes mellitus (n = 70, 35 women in the age of 39-90 years, mean age 68 years and 35 men in the age of 38-90 years, mean age 65 years), patients with hyperbilirubinemia (n = 26, 13 women in the age of 29-96 years, mean age 73 years and 13 men in the age of 19-91 years, mean age 56 years), patients with cardiovascular diseases (n = 64, 32 women in the age of 36-96 years, mean age 78.5 years and 32 men in the age of 36-85 years, mean age 67 years) and a control group of patients (n = 72, 36 women in the age of 21-88 years, mean age 66.5 years and 36 men in the age of 21-84 years, mean age 64 years).

Blood Samples Collection

Blood was collected into tubes containing gel (Vacuette No. 455071, Greiner Labortechnik Co., Kremsmünster, Austria). Serum was separated from blood clot by centrifugation (1700×g, 15 min, room temperature). Serum samples were stored at the temperature –80 °C.

Sample Preparation

Cold ethanol (400 μl) was carefully added to serum (100 μl). After incubation (4 °C, 10 min) and centrifugation (22 000×g, 15 min, 4 °C), supernatants (100 μl) were diluted with deionized water (400 μl), filtered through a nylon filter (pore size 0.20 μm, 4 mm diameter, Supelco, Bellefonte, PA, USA) and transferred into 1.0 ml vials.

A stock solution of creatinine (about 2000 μmol l^{-1}) was prepared in deionized water. The stock solutions were further diluted with deionized water to give a series of working standards. To 100 μl of the standard, cold ethanol was carefully added (400 μl). This way prepared standards were subjected to the same procedure as described for serum supernatants.

For the recovery experiment, 1 volume of creatinine standard of different concentrations was added to 19 volumes of serum. Next steps were the same as for serum sample preparation.

Chromatography Method

Chromatography of creatinine was accomplished using an isocratic elution on a MAG 1, 250 mm × 4.6 mm, Labiospher PSI 100 C18, 5 μm analytical column fitted a MAP, 20 mm × 4.6 mm, Labiospher PSI 100 C18, 5 μm guard column (Labio a.s., Prague, the Czech Republic) and a PEEK pre-column filter, pore size 0.5 μm (Supelco, Bellefonte, PA, USA) at 25 °C. The mobile phase consisted of 3.0 % ethanol in 25 mmol l^{-1} sodium hydrogenphosphate (v/v), pH 6.50 ± 0.05. The flow rate was kept constant at 0.5 ml min^{-1}. The optimum response of creatinine was observed when wavelength was set at 234 nm (absorbance maximum of creatinine). The amount of creatinine was quantified from the corresponding peak area using Clarity chromatography software (DataApex, Prague, the Czech Republic). The concentration of creatinine in the samples was determined from the calibration curve.

Enzymatic Determination of Creatinine

Creatinine in the serum samples was measured with the set CREATININE ENZYMATIC LIQUID (Erba Lachema, Brno, the Czech Republic). In the first reaction, creatinase and sarcosine oxidase are used in the enzymatic hydrolysis of endogenous creatine to produce hydrogen peroxide that is eliminated by catalase. Then creatininase and 4-aminoantipyrine are added, and only the creatine generated from creatinine by creatininase is hydrolyzed sequentially by creatinase and sarcosine oxidase to produce hydrogen peroxide. This newly formed hydrogen peroxide is measured in a coupled reaction catalyzed by peroxidase, with N-ethyl-*N*-sulphopropyl-*m*-toluidine as a chromogen. The absorbance of the produced complex is read at 546 nm against blank (diode-array spectrophotometer Agilent 8453, Santa Clara, CA, USA).

Determination of Creatinine Using a Photometrical Jaffé method

Serum creatinine was determined by standard procedures using an automatic biochemistry analyzer Dimension® RxL Max® (Siemens Healthcare Diagnostic Ltd., Deerfield, IL, USA).

Statistical Analysis

The data are presented as mean values ± S.D. Differences between the women and men were analyzed with the use of the Mann-Whitney Rank Sum Test and analysis of correlation was carried out using Spearman Rank Order Correlation (software QCexpert, Trilobyte, Pardubice, the Czech Republic). A $p < 0.05$ value was considered statistically significant.

Results and Discussion

The Effectiveness of Various Protein Precipitants, Creatinine Stability

Commonly used protein precipitants are acids [10,16]. Organic solvents have been used instead acids [15,17]. We have tested number of protein precipitants with regard to stability and recovery. Cold protein precipitant (10% metaphosphoric acid, 1.0 mol l^{-1} perchloric acid, 10 % trichloroacetic acid, 10 % 5-sulfosalicylic acid, acetonitrile, methanol and ethanol) was carefully added (400 µl) to serum or mixed standards (100 µl). After incubation (4 °C, 10 min) and centrifugation (22 000×g, 15 min, 4 °C), supernatants (100 µl) were diluted with deionized water

(400 μl), and filtered through a nylon filter (pore size 0.20 μm, 4 mm diameter) and transferred into 1.0 ml vials. Only ethanol as a protein precipitant led to satisfactory recoveries and stability. All others of tested protein precipitants interfered with HPLC analysis.

High-Performance Liquid Chromatographic Assay of Creatinine

Creatinine was separated on a reverse-phase column using an isocratic system of ethanol and sodium hydrogenphosphate. The mobile phase was optimized in order to obtain the best separation of the analytes in the shortest time. Standard solutions of creatinine as well as pooled serum were used for study of the mobile phase composition. Several mobile phases (namely different buffers containing ethanol) were tested. We prefer the use of an eco-friendly mobile phase, because the organic solvents such as acetonitrile and methanol are considered as significant pollutants. Optimization of the separation was obtained after studying the effect of sodium hydrogenphosphate concentration (from 5.0 to 100.0 mmol l^{-1}) and ethanol concentration. The retention behavior was studied in dependence of pH value of the mobile phase in the range 3.0-6.8. The optimum pH 6.5 was chosen for the best separation and detection of creatinine. The column temperature was changed from 20 to 45 °C. The mobile phase conditions leading to the best separation were: 3 % ethanol in 25 mmol l^{-1} sodium hydrogenphosphate, pH 6.50 ± 0.05. The optimal temperature interval was from 20 to 25 °C. The criteria were the resolution, stability of the absorbance, and the analysis speed. We can establish that the presented method is highly robust. Three different columns, two of them of C18 type and one of them of cation-exchange type, were tested in the research: a Discovery (Supelco, Bellefonte, PA, USA), a MAG 1, Labiospher and a Supelcosil LC-SCX. The MAG 1, Labiospher column was selected for further experiments. Two lengths of selected column (150 and 250 mm) were tested, and that of 250 mm length yielded the best resolution with acceptable retention times for creatinine in human serum. The 250 mm column provided a proper separation between tested analytes and interferences. An HPLC chromatogram of creatinine in human serum is shown in Fig. 2.

Precision of creatinine analysis for serum samples is shown in Table I. To determine the within-day precision, the serum samples were analyzed ten times in the same day under the same conditions. Similarly, results on the between-day precision were obtained on the same serum samples, which were analyzed in ten different days. The coefficients of variation were below 6 %. The spike recoveries ranged between 101.8 % and106.0 % (Fig. 3). The calibration curve (11-point for a determination of analytical parameters and 7-point for routine analysis) was linear in the whole range tested (10.0-1000.0 μmol l^{-1}). The regression lines obtained from the combination of 5 standard curves were $y = 5.7541x + 6.4914$

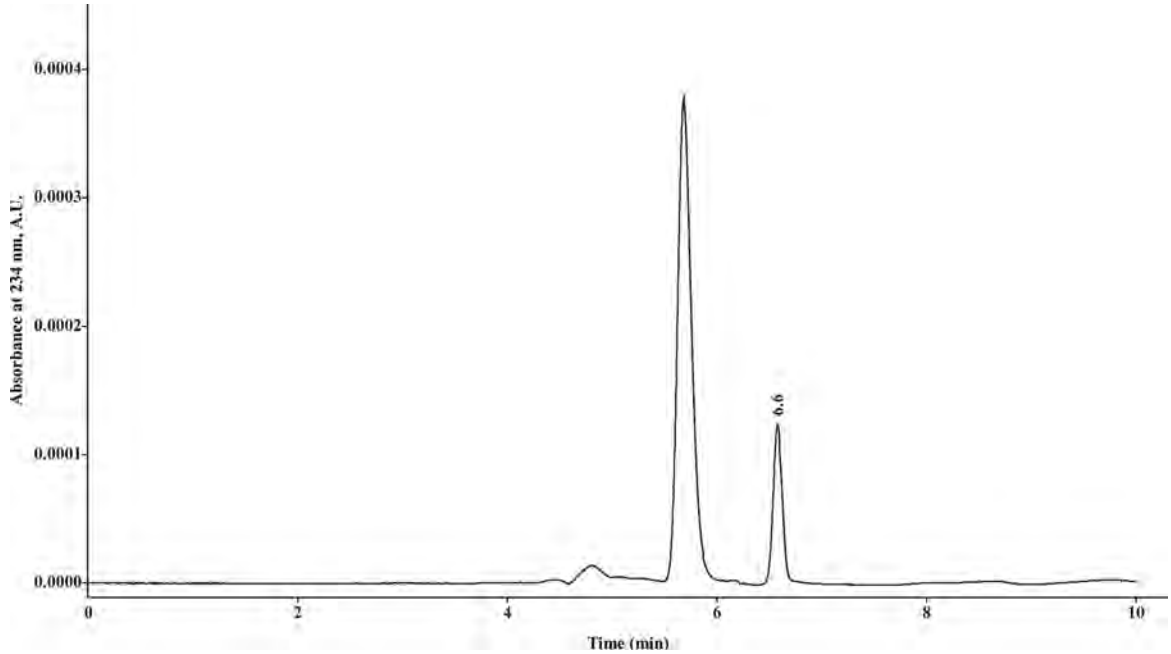

Fig. 2 HPLC chromatogram of creatinine (142.8 μmol l^{-1}) in human serum. The peak at 6.6 min corresponds to creatinine and that before corresponds to uric acid (retention time about 5.9 min). HPLC conditions: an isocratic elution (mobile phase: 3 % ethanol in 25 mmol l^{-1} sodium hydrogenphosphate, pH 6.50), the stationary phase was an analytical column MAG 1, 250 mm × 4.6 mm, Labiospher PSI 100 C18, 5 μm fitted a MAP, 20 mm × 4.6 mm, Labiospher PSI 100 C18, 5 μm guard column and a PEEK pre-column filter, pore size 0.5 μm, the flow rate was kept constant at 0.5 ml min^{-1}, separation proceeded at 25 °C and creatinine was monitored at 234 nm

Table I Precision of creatinine in human serum

n	Mean ± *S.D.*, μmol l^{-1}	*CV*, %
A) Precision (within-day)		
10	105.4 ± 1.4	1.3
10	6.2.6 ± 1.1	1.8
B) Precision (between-day)		
10	63.0 ± 3.5	5.6
10	458.9 ± 21.7	4.7

μmol l^{-1}. The mean slope, intercept and correlation coefficient (*R*) for the calibration curves were 5.7541 (95% confidence interval, 5.4812 to 6.0145), –1.1 μmol l^{-1} (–3.8 to 1.8 μmol l^{-1}), and 0.9998. The lowest concentration that could be quantified with acceptable accuracy and precision was 10.0 μmol l^{-1} (4.0 pmol/inject). Furthermore, limit of detection for creatinine, defined as signal-to-noise (*S/N*) ratio of 3:1, was 3.0 μmol l^{-1} (1.2 pmol/inject) (detector sensitivity: 0.001 absorbance units full scale, AUFS).

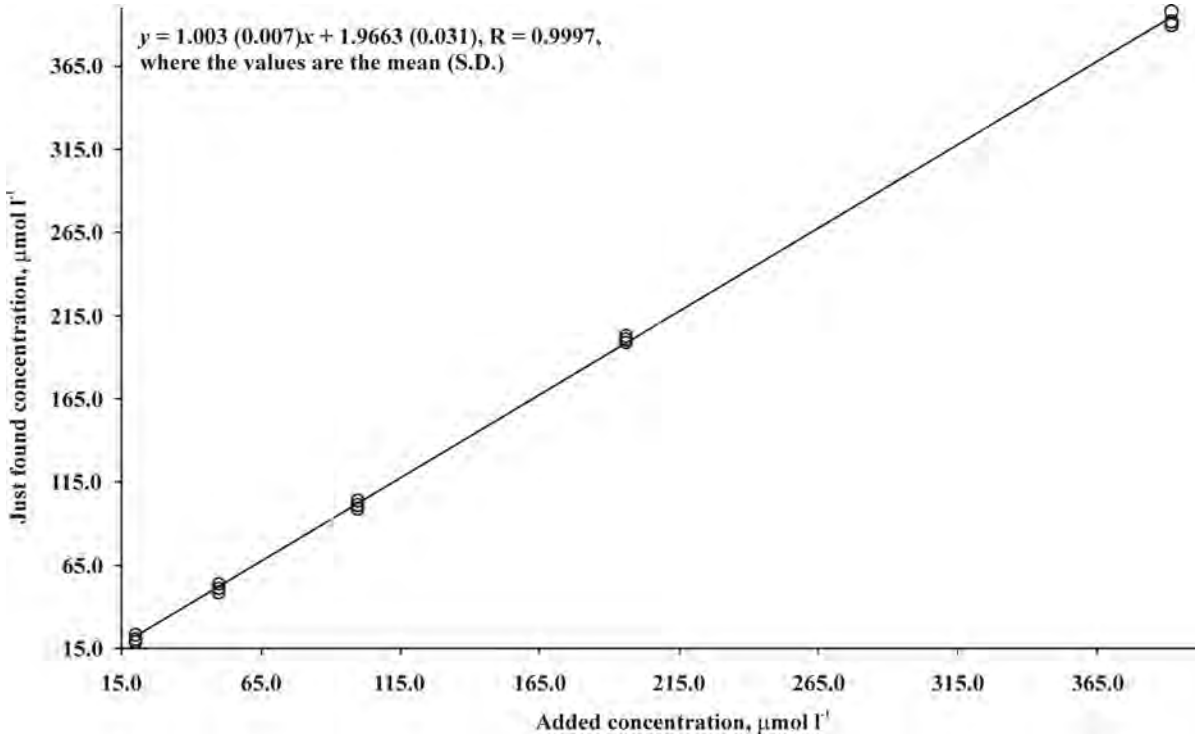

Fig. 3 Recovery experiment. Values of triplicate assays are recorded. Slope corresponds to the mean recovery 102.9 % (*S.D.* = 2.0 %)

Determination of Creatinine in Human Serum

Levels of serum creatinine in selected patients, a comparison of serum creatinine levels between patients and a control group as well correlations between age and serum creatinine are shown in Tables II and III. Reference ranges of serum creatinine in adults are approximately 45-110 μmol l^{-1}. Women usually have lower levels than men, because they usually have less muscle mass. Our results confirmed this fact however we found no significant differences in patients with hyperbilirubinemia and cardiovascular disease. We assume that the rate of disease is more significant than gender. We observed significant correlation between creatinine concentration and age in all patients and patients with cardiovascular disease as well as in women of all patients, patients with non-insulin dependent diabetes mellitus and a control group (Table III).

Comparison with Enzymatic and Jaffé Methods

The values obtained using photometrical Jaffé method are comparable to those obtained by the present HPLC (Table IV and Fig. 4). There is less than 10 % difference in creatinine levels measured by photometrical Jaffé method and HPLC

Table II Group chacteristic

	n	Age, years Mean±*S.D.*	Creatinine, μmol l^{-1} Mean±*S.D.*	P* Patients vs. Control group	P* F vs. M
Patients with NIDDM (all)	70	65.0 ± 1.5	90.6 ± 6.2	0.509	
Patients with NIDDM (F)	35	68.0 ± 5.6	80.2 ± 6.1	40724	
Patients with NIDDM (M)	35	65.0 ± 2.8	110.7 ± 11.2	0.840	*0.003*
Patients with hyperbilirubinemia (all)	26	60.0 ± 6.9	92.9 ± 12.3	0.328	
Patients with hyperbilirubinemia (F)	13	73.0 ± 13.5	92.9 ± 32.7	0.659	
Patients with hyperbilirubinemia (M)	13	56.0 ± 12.2	92.5 ± 15.9	*0.037*	0.430
Patients with CVD (all)	64	72.5 ± 3.1	100.6 ± 8.7	0.308	
Patients with CVD (F)	32	78.5 ± 5.4	99.3 ± 11.2	0.167	
Patients with CVD (M)	32	67.0 ± 5.4	114.1 ± 11.7	0.974	0.486
Control group (all)	72	64.5 ± 3.1	96.9 ± 8.4	-	
Control group (F)	36	66.5 ± 6.6	82.1 ± 8.4	-	
Control group (M)	36	64.0 ± 4.8	110.2 ± 13.2	-	*0.025*

NIDDM – non-insulin dependent diabetes mellitus, CVD – cardiovascular diseases, F – female, M – male, * – Mann–Whitney rank sum test

Table III Correlations between age and serum creatinine levels

	Correlation Spearman's rho*	Regression analysis	
		R	*F*-test (*P*)
All patients	*0.3827*	0.1366	*0.0204*
All patients (F)	*0.4370*	0.1795	*0.0348*
All patients (M)	0.3438	0.0840	0.3187
Patients with NIDDM (all)	0.2981	0.2263	0.0655
Patients with NIDDM (F)	*0.5601*	0.3593	*0.0434*

Table III – Continued

	Correlation Spearman's rho*	Regression analysis	
		R	*F*-test (*P*)
Patients with NIDDM (M)	0.1350	0.1059	0.5449
Patients with hyperbilirubinemia (all)	0.3546	0.3400	0.0888
Patients with hyperbilirubinemia (F)	0.3462	0.3516	0.2388
Patients with hyperbilirubinemia (M)	–0.1687	0.1143	0.7236
Patients with CVD (all)	*0.4670*	0.3010	*0.0175*
Patients with CVD (F)	0.5037	0.3441	0.0538
Patients with CVD (M)	0.5061	0.3188	0.0860
Control group (all)	0.5073	0.1884	0.1131
Control group (F)	*0.6710*	0.6313	*< 0.001*
Control group (M)	0.3012	0.0842	0.6255

* – Spearman's rank correlation coefficient

one. The major advantages of HPLC method against Jaffé method lie in elimination of interfering substances and simultaneous determination other clinically important analytes such as uric acid, creatine, and creatine phosphate [10-12, 14, 16,17]. On the other hand, the values obtained using enzymatic method gave at average higher values (Table V and Fig. 5). A big problem of the set CREATININE ENZYMATIC LIQUID is low absorbance (< 0.1) at concentrations in reference interval. The working range of 4.6-5700 μmol l^{-1} is for photometrical method inadequately wide, therefore, a concentration reading in the range of reference values (45-110 μmol l^{-1}) is incorrect.

The presented method is cheap (about 2000$/1000 analyses; i.e. HPLC instrument, analytical column, mobile phase, filters, and chemicals for sample preparation costs) and sample preparation includes only a protein precipitation with ethanol, a dilution with deionized water and a filtration. Within 2 years we have carried out more than 1000 analyses and have found out that lifetime of the analytical column is more than 1000 injects. Therefore we can establish that the method is inexpensive and suitable for clinical tests.

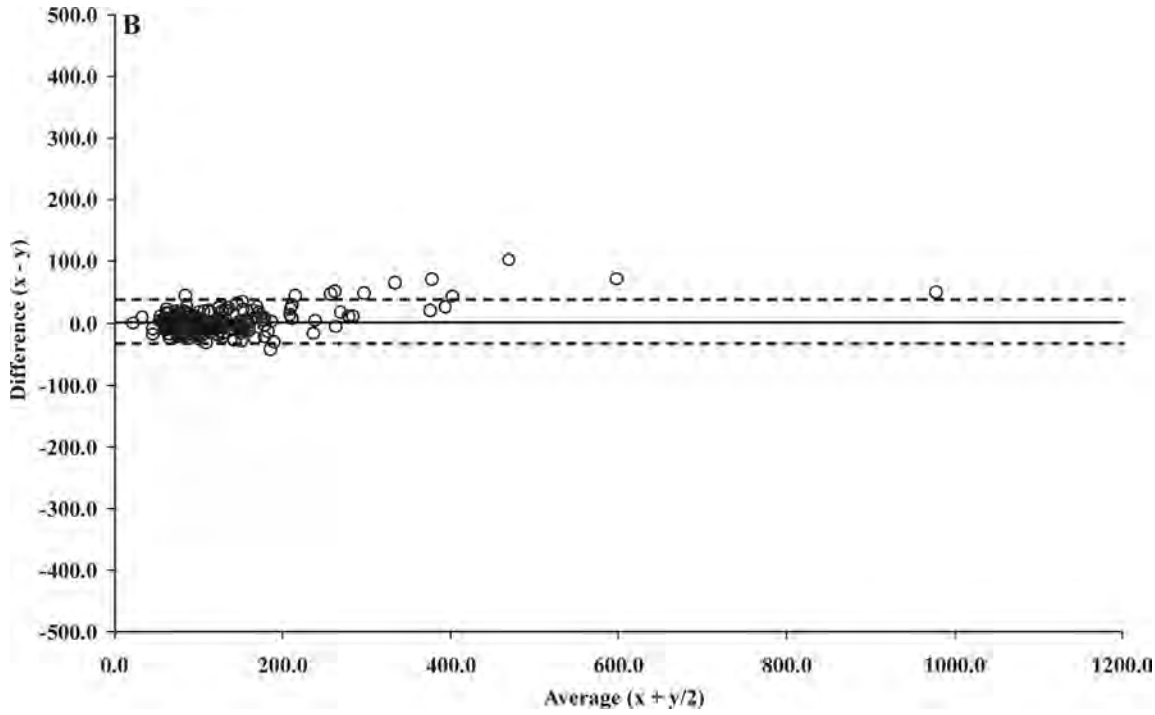

Fig. 4 Bland-Altman difference plot. Average difference ±1.96 standard deviation of the difference. x = HPLC method; y = photometrical Jaffé method; n = 232

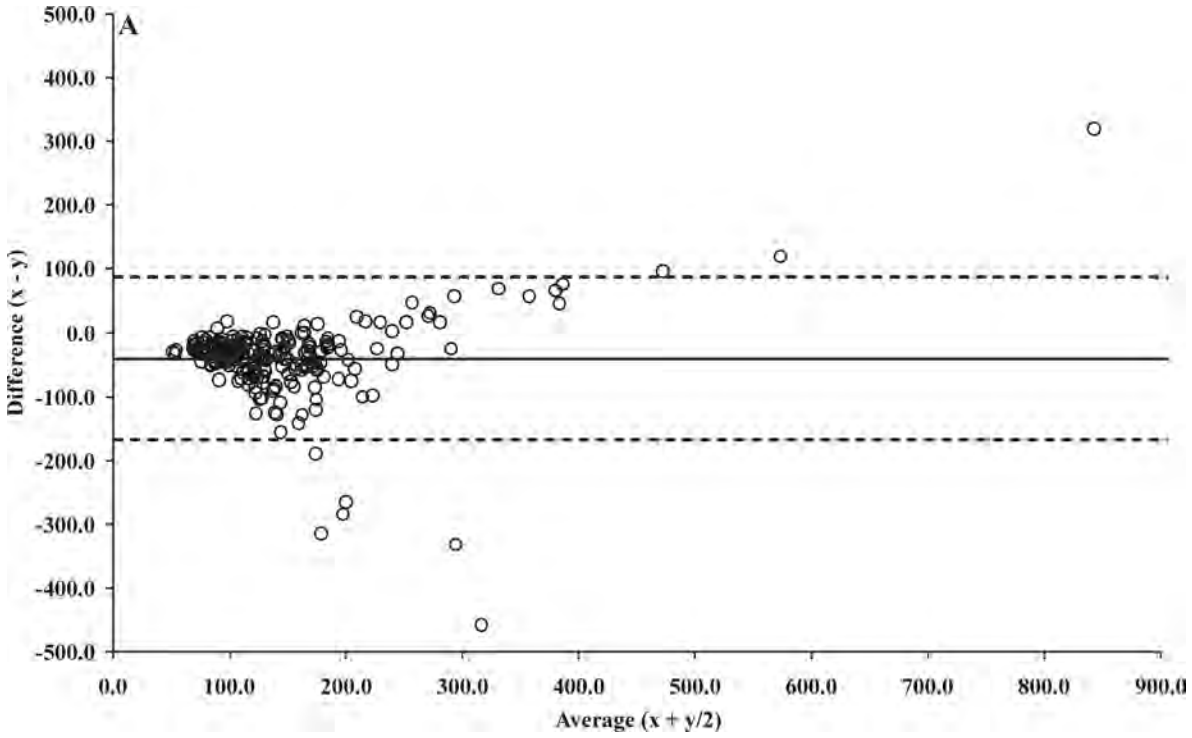

Fig. 5 Bland-Altman difference plot. Average difference ±1.96 standard deviation of the difference. x = HPLC method; y = enzymatic method; n = 232

Table IV Comparison of HPLC method with Jaffé method

	Correlation Spearman's rho*	Regression analysis	
		R	*F*-test (*P*)
All patients	0.952	0.957	$y = 0.775x + 25.2$
All patients (F)	0.931	0.964	$y = 0.739x + 25.7$
All patients (M)	0.954	0.958	$y = 0.821x + 23.1$
Patients with NIDDM (all)	0.949	0.960	$y = 0.846x + 21.3$
Patients with NIDDM (F)	0.904	0.963	$y = 0.782x + 23.0$
Patients with NIDDM (M)	0.963	0.972	$y = 0.882x + 21.1$
Patients with hyperbilirubinemia (all)	0.912	0.925	$y = 0.873x + 15.5$
Patients with hyperbilirubinemia (F)	0.962	0.942	$y = 0.887x + 11.0$
Patients with hyperbilirubinemia (M)	0.832	0.724	$y = 0.885x + 17.2$
Patients with CVD (all)	0.926	0.946	$y = 0.811x + 21.5$
Patients with CVD (F)	0.943	0.943	$y = 0.830x + 16.0$
Patients with CVD (M)	0.925	0.954	$y = 0.792x + 27.5$
Control group (all)	0.974	0.990	$y = 0.927x + 0.458$
Control group (F)	0.867	0.945	$y = 0.905x + 1.03$
Control group (M)	0.964	0.995	$y = 0.926x + 2.47$

Table V Comparison of HPLC method with enzymatic method

	Correlation Spearman's rho*	Regression analysis	
		R	*F*-test (*P*)
All patients	0.789	0.825	$y = 0.575x + 93.7$
All patients (F)	0.784	0.848	$y = 0.551x + 90.7$
All patients (M)	0.753	0.810	$y = 0.604x + 95.6$
Patients with NIDDM (all)	0.766	0.466	$y = 0.711x + 75.7$
Patients with NIDDM (F)	0.773	0.562	$y = 0.648x + 72.5$
Patients with NIDDM (M)	0.766	0.378	$y = 0.704x + 82.1$
Patients with hyperbilirubinemia (all)	0.852	0.829	$y = 0.834x + 49.7$
Patients with hyperbilirubinemia (F)	0.835	0.830	$y = 0.837x + 47.6$

Table V – Continued

	Correlation Spearman's rho*	Regression analysis	
		R	*F*-test (*P*)
Patients with hyperbilirubinemia (M)	0.783	0.704	$y = 0.911x + 44.5$
Patients with CVD (all)	0.485	0.304	$y = 0.515x + 120.9$
Patients with CVD (F)	0.534	0.625	$y = 0.560x + 98.8$
Patients with CVD (M)	0.333	0.200	$y = 0.458x + 146.3$
Control group (all)	0.739	0.778	$y = 0.629x + 74.0$
Control group (F)	0.517	0.187	$y = 0.479x + 91.5$
Control group (M)	0.849	0.960	$y = 0.648x + 69.9$

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References

[1] Wyss M., Kaddurah-Daouk R.: Physiol. Rev. **80**, 1107 (2000).
[2] Wannamethee S.G., Shaper A.G., Perry I.J.: Stroke **28**, 557 (1997).
[3] Jacobi D., Lavigne C., Halimi J.M., Fierrard H., Andres C., Couet C., Maillot F.: Clin. Prac. **80**, 102 (2008).
[4] Buysschaert M., Joudi I., Wallemacq P., Hermans M.P.: Diabetes Metab. **29**, 377 (2003).
[5] Lee B.W., Ihm S.H., Choi M.G., Yoo H.J.: Diabetes Res. Clin. Prac. **78**, 428 (2007).
[6] Ambrose R.T., Ketchum D.F., Smith J.W.: Clin. Chem. **29**, 256 (1983).
[7] Delanghe J.R., Louagie H.K., De Buyzere M.L., Leroux-Roels G.G.: Clin. Chim. Acta **224**, 33 (1994).
[8] Weber J.A., van Zanten A.P.: Clin. Chem. **37**, 695 (1991).
[9] Junge W., Wilke B., Halabi A., Klein G.: Clin. Chim. Acta **344**, 137 (2004).
[10] Jen F.J., Hsiao S.L., Liu K.H.: Talanta **16**, 711 (2002).
[11] Hewavitharana A.K., Bruce H.L.: J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. **784**, 275 (2003).

[12] George S.K., Dipu M.T., Mehra U.R., Singh P., Verma A.K., Ramqaokar J.S.: J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. **832**, 134 (2006).
[13] Tsikas D., Wolf A., Mitschke A., Gutzki F.M., Will W., Bader M.: J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. **878**, 2582 (2010).
[14] Zinellu A., Sotgia S., Zinellu E., Chessa R., Deiana L., Carru C.: J. Sep. Sci. **29**, 704 (2006).
[15] Costa A.C.O., Costa J.L., Tonin F.G., Tavares M.F.M., Micke G.A.: J. Chromatogr. A **1171**, 140 (2007).
[16] Zuo Y., Wang C., Zhou J., Sachdeva A., Ruelos V.C.: Anal. Sci. **24**, 1589 (2008).
[17] Zuo Y., Yang Y., Zhu Z., He W., Aydin Z.: Talanta **83**, 1707 (2010).