SCIENTIFIC PAPERS
OF THE UNIVERSITY OF PARDUBICE
Series A
Faculty of Chemical Technology
19 (2013)

AN ASSAY OF CREATINE IN HUMAN SERUM USING AN HPLC WITH UV DETECTION

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Received August, 30, 2013

An ion-exchange high-performance liquid chromatography with ultraviolet detection for the determination of creatine in human serum has been developed. The human serum was deproteinized with perchloric acid. For the separation, a cation-exchange column LC-SCX, 250 mm × 4.6 mm, 5 μm, was used. The mixture of ethanol and 10 mmol l^{-1} ammonium acetate (15:85, v/v), pH 4.00 ± 0.05 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 10 %. Quantitative recoveries of spiked serum samples were between 97.1 % and 103.5 %. The calibration curve was linear in the whole range tested. The limit of quantification was 2 μmol l^{-1}. The preliminary reference ranges of serum creatine in a group of blood donors are 17-89 μmol l^{-1} for women and 21-97 μmol l^{-1} for men.

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Introduction

Creatine (synonyms: 2-[carbamimidoyl(methyl)amino]acetic acid; *N*-carbamimidoyl-*N*-methylglycine; "-methylguanidoacetic acid; *N*-amidinosarcosine) has an important role in energy metabolism of skeletal muscle and brain (Fig. 1). It is synthesized mainly in the liver, pancreas and kidney, is transported to tissues by the creatine transporter and is non-enzymatically converted to creatinine. In humans, more than 95 % of creatine is stored in the skeletal muscle. The physiologically normal concentration range for serum creatine is below 100 μmol l^{-1}, but during kidney dysfunction or muscle disorder its concentration may raise up to a value higher than 1 mmo l^{-1}. The determination of the creatine levels, therefore, plays an important role in clinical studies [1-5]. The measurements of serum creatine are based on enzymatic conversions of creatine and the monitoring of ultraviolet (UV) absorption of co-factors. These photometrical methods are simple and rapid, but there have been reported various substances that interfere [6, 7]. Hence many high-performance liquid chromatographic (HPLC) [8,9], gas chromatographic (GC) [10,11] and/or high-performance capillary electrophoretic (HPCE) [12,13] methods for the determination of creatine in biological fluids have been developed. High polarity and non-volatility of creatine, as well as background interference of biological matrix is a big problem at determination of creatine in human serum. Therefore, several LC-MS/MS methods [14,15] have been developed for analysis of creatine in biological samples. The LC-MS/MS technology is expensive; therefore, it is not yet as widely used as is HPLC with UV detection; thus it is not suitable for the routine monitoring of creatine levels in patients. Many types of chromatographic columns are available for the determination of creatine in biological samples, reversed-phase columns are still standard choice. In case of use of reversed-phase columns, the addition of ion-pairing agents to mobile phase is commonly used in order to improve chromatographic separation of creatine. Cation-exchange columns have a lower performance than reversed-phase columns, but are more selective.

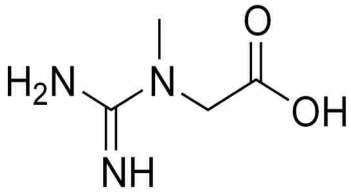

Fig. 1 Structure of creatine

The aim of this work is to develop a simple, rapid, accurate and eco-friendly cation-exchange HPLC method for measuring creatine levels in human serum. Reference values for a normal population have been established.

Material and Methods

Reagents and Chemicals

Creatine, metaphosphoric acid, perchloric acid, trichloroacetic acid, 5-sulfosalicylic acid, sodium dihydrogenphosphate, sodium hydrogenphosphate, potassium dihydrogenphosphate, potassium hydrogenphosphate, sodium acetate, ammonium acetate were obtained from Sigma Chemical Company (St. Louis, MO, USA). HPLC-gradient grade methanol, ethanol and acetonitrile were from Merck (Darmstadt, Germany). Lyophilized serum creatine controls chemTRAK® were from MAS® Controls (Passau, Germany). All other chemicals were of analytical grade.

Instrumentation

Chromatographic analyses were performed with a liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an LC-20AD solvent delivery system, an SIL-20ACHT cooled autosampler, a CTO-20AC column oven, an SPD-20A variable wavelength spectrophotometric detector and a CBM-20A system controller. The data were collected digitally with Clarity chromatography software (DataApex, Prague, the Czech Republic).

Subject and Samples

Samples of serum were obtained from a group of blood donors (n = 100, 55 women in the age 23-49 years, mean age 34 years, and 45 men in the age 19-56 years, mean age 37 years). None of the studied subjects exhibited renal, hepatic, gastrointestinal, pulmonary, cardiovascular or oncological diseases. All study participants gave written informed consent to participate in this study, which was approved according to the Helsinki Declaration by the Hospital Committee on Human Research (Regional Hospital of Pardubice).

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 of –80 °C.

Sample Preparation

The volume of 200 μl serum was pipetted into a well-capped 1.5ml polypropylene tube. The volume of 400 μl cold perchloric acid (1 mol l^{-1}) was added, the solution was vortexed for 60 s, and centrifuged (22 000 g, 10 min, 4 °C). The volume of 100 μl supernatant was 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 1.0ml vial.

A stock solution of creatine (about 10 mmol l^{-1}) was prepared in deionized water. The stock solution was further diluted with deionized water to give a series of working standards. To 200 μl of the standard, cold perchloric acid (1 mol l^{-1}) 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 creatine standard of different concentrations was added to 19 volumes of serum. Next steps were the same as for serum sample preparation.

Chromatography Method

The chromatographic analysis of creatine was accomplished using an isocratic elution on an LC-SCX cation-exchange column, 250 mm × 4.6 mm, 5 μm, fitted a PEEK pre-column filter, pore size 0.5 μm (all from Supelco, Bellefonte, PA, USA) at 25/C. The mobile phase (pH = 4.00 ± 0.05) was a mixture of ethanol and 10 mmol l_{-1} ammonium acetate (15:85, v/v). The flow rate was kept constant at 0.5 ml min^{-1}. The optimum response of creatine was observed when the wavelength was set at 210 nm (absorbance maximum of creatine). The amount of creatine was quantified from corresponding peak area using Clarity chromatography software. The concentration of creatine in the samples was determined from the calibration curve.

Statistical Analysis

The data are presented as median ± IQR (interquartile range; the differences between the upper and lower quartiles). Differences between women and men were analyzed using the Mann-Whitney Rank Sum Test. The correlation analysis was carried out using Spearman Rank Order Correlation, and the regression analysis was carried out using the least squares method (software SigmaStat for Windows, Version 3.5, systat Software Inc., Point Richmond, CA, USA). A $P < 0.05$ was considered statistically significant.

Results and Discussion

The Effectiveness of Various Protein Precipitants

Commonly used protein precipitants are acids [16,17], organic solvents have been used instead acids [3,14]. A number of protein precipitants with regard to stability, chromatographic separation and recovery of added creatine were tested. Cold protein precipitant (1mmol l^{-1} perchloric acid, 10 % metaphosphoric acid, 10 % trichloroacetic acid, 10 % 5-sulfosalicylic acid, acetonitrile, methanol and ethanol) was carefully added (400 µl) to serum or standards (200 µl). Organic acids (trichloroacetic acid and 5-sulfosalicylic acid) interfered with chromatographic analysis. Organic solvents and metaphosphoric acid are not enough effective as precipitating agents. Only 1mol l^{-1} perchloric acid led to satisfactory deproteinatization and recoveries of added creatine.

High-Performance Liquid Chromatographic Assay of Creatine

Creatine was separated on a cation-exchange column using an isocratic system of ethanol and ammonium acetate. The mobile phase was optimized in order to obtain the best separation of the analytes in the shortest time. Standard solutions of creatine 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 ammonium acetate concentration (from 5.0 to 100 mmol l^{-1}) and ethanol concentration. The retention behavior was studied in dependence of pH value of the mobile phase in the range 3.0-7.0. The optimum pH 4.00 was chosen for the best separation and detection of creatine. The column temperature was changed from 20 to 45 °C. The mobile phase conditions leading to the best separation were 15 % ethanol in 10mmol l^{-1} ammonium acetate, pH 4.00. 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 presented method is robust. Except the cation-exchange column, two columns, both of C18 type, were tested in the research: a Discovery (Supelco, Bellefonte, PA, USA) and a MAG 1, Labiospher (Labio, Prague, the Czech Republic). Creatine contains ionic components; therefore, it is too polar to be retained in a reversed-phase mode. In such situations, ion-pairing reagent selectively increased the retention of charged analytes. Routinely, alkyl sulfonate with 5-12 carbon atoms is used. The higher number of carbon atoms in the alkyl sulfonate, the stronger the retention effect on

the creatine. 1-Octanesulfonic acid as an ion-pairing reagent was tested. In comparison with a cation-exchange chromatography, ion-pairing chromatography uses expensive ion-pairing agents; moreover it is less selective and robust. The reversed-phase column must have been equilibrated at least 24 h to achieve baseline stability. HPLC chromatograms of creatine in human serum are shown in Figs 2 and 3.

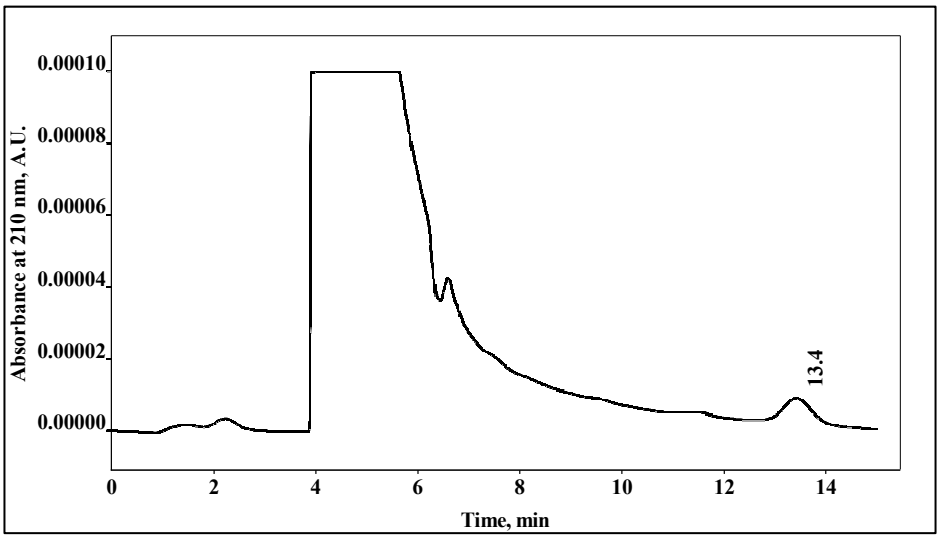

Fig. 2 HPLC chromatogram of creatine (40.8 μmol l^{-1}) in human serum. The peak at 13.4 min corresponds to creatine. HPLC conditions: an isocratic elution (mobile phase: 15 % ethanol in 10 mmol l^{-1} ammonium acetate, pH 4.00), the stationary phase was an analytical column LC-SCX, 250 mm × 4.6 mm, 5 μm, fitted 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 creatine was monitored at 210 nm

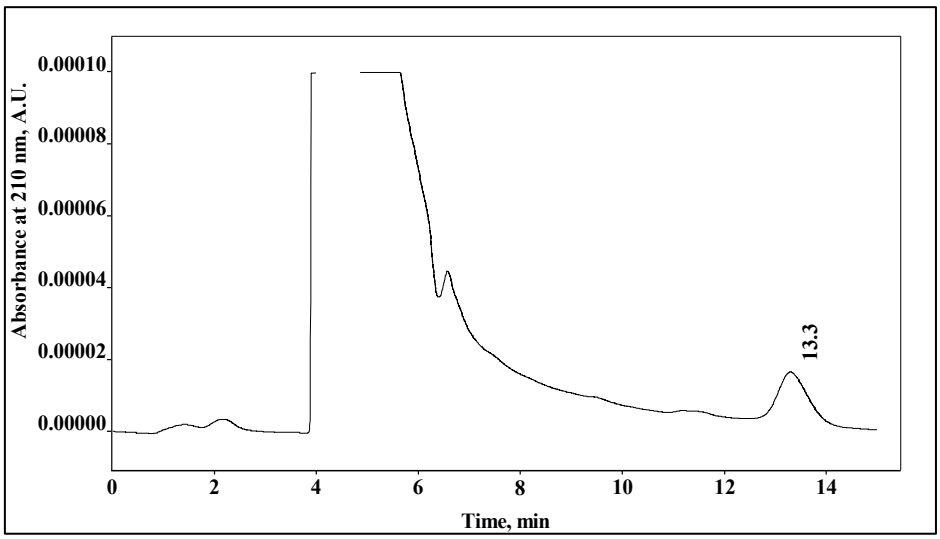

Fig. 3 HPLC chromatogram of creatine (88.9 μmol l^{-1}) in the spiked human serum with creatine standard (50 μmol l^{-1} addition); for HPLC conditions, see Fig. 2

Table I Precision of creatine in human serum

n	Mean ± *S.D.*, μmol l^{-1}	CV, %
A) Precision (within-day)		
10	20.5 ± 1.0	4.9
10	84.6 ± 2.9	3.4
B) Precision (between-day)		
10	83.3 ± 5.2	6.2

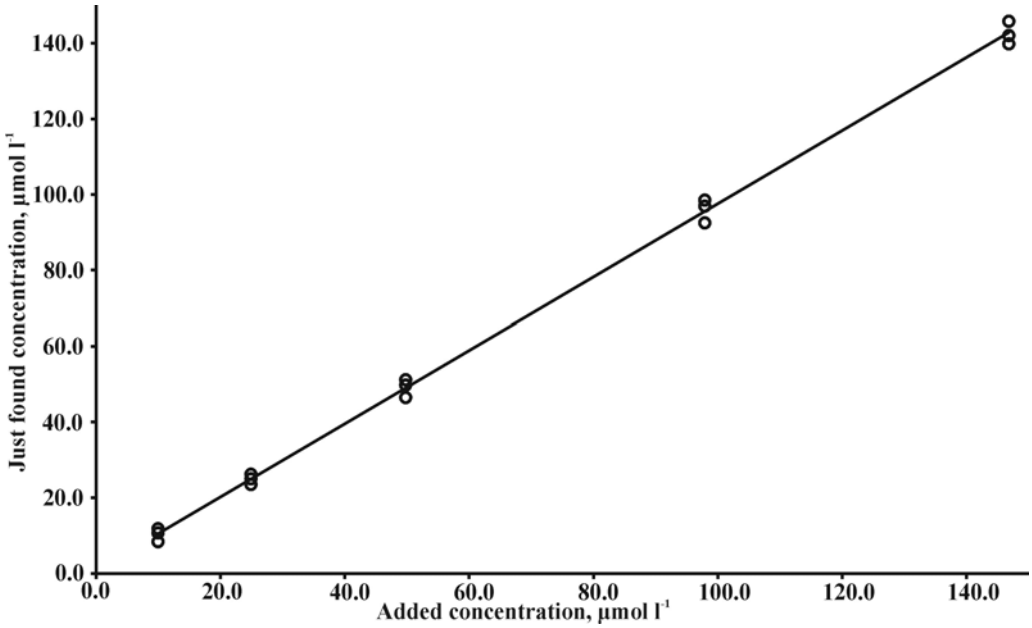

Fig. 4 Recovery experiment. Values of triplicate assays are recorded. Slopes correspond to the mean recovery 99.5 % (*S.D.* = 2.5 %). $y = 0.9670x + 0.8745$, $R = 0.9991$

Precision of creatine 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 10 %. The spike recoveries ranged between 97.1 % and 103.5 % (Fig. 4). The calibration curve (11-point for a determination of analytical parameters and 7-point for routine analyses, Fig. 5) was linear in the whole range tested (2.0-200.0 μmol l^{-1}). The regression lines obtained from the combination of 5 standard curves were $y = 4.4629x - 3.0129$ μmol l^{-1}. The mean slope, intercept and correlation coefficient (R) for the

calibration curves were 4.4629 (95 % confidence interval, 4.1125 to 4.8479), –3.0129 μmol l^{-1} (–4.1254 to –1.8795 μmol l^{-1}) and 0.9998. Accuracy of calibration curves was verified by means of a commercial control serum. The lowest concentration that could be quantified with acceptable accuracy and precision was 2.0 μmol l^{-1}. Since the injection volume was 10 μl, the mass quantification limit was 1.3 pmol. Furthermore, limit of detection for creatine, defined as a signal-to-noise (*S/N*) ratio of 3:1, was 0.6 μmol l^{-1} (0.4 pmol).

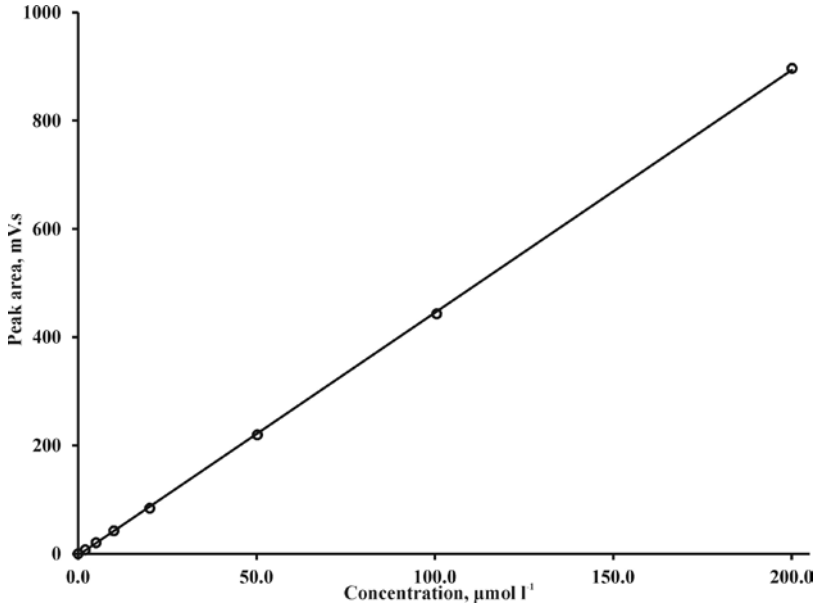

Fig 5 Typical calibration curve for HPLC quantification of creatine. $y = 4.4819x - 3.2509$, $R = 0.9999$

Table II Group characteristic

	n	Age, years Median ± *IQR*[a]	Creatine, μmol l^{-1} Median ± *IQR*	*P*[b] F vs. M
Blood donors (all)	100	35.0 ± 4.5	52.6 ± 28.2	
Blood donors (F)	55	34.0 ± 5.0	51.7 ± 25.8	
Blood donors (M)	45	37.0 ± 6.5	54.1 ± 30.3	0.091

F – female, M – male, [a]*IQR*, interquartile range is the difference between the upper quartile and the lower quartile
[b]Mann-Whitney Rank Sum Test

Determination of Creatine in Human Serum

Levels of serum creatine in blood donors and a comparison of serum concentrations between women and men are in Table II. Reference ranges of serum creatine in adults are approximately 10-100 μmol l^{-1}. Our preliminary reference ranges of serum creatine in a group of blood donors are 17-97 μmol l^{-1}. Women usually have lower levels than men, because they usually have less muscle mass. Our results confirmed this fact (Table II). We observed no significant correlation between creatine concentrations and age (R = 0.0214, P = 0.895).

Conclusion

A reliable, selective, and sensitive cation-exchange HPLC method with spectrophotometric detection for the determination of creatine in human serum was developed. Sample preparation includes only a protein precipitation with perchloric acid, a dilution with deionized water, and a filtration. Therefore we can establish that the method is inexpensive and suitable for clinical tests.

Acknowledgement

The Ministry of Education, Youth and Sports of the Czech Republic (Grant SGFChT07/2013) supported this work. The authors thank staffs of the Department of Transfusion, Regional Hospital in Pardubice for the serum samples.

References

[1] See H.H., Schmidt-Marzinkowski J., Pormsila W., Morand R., Krähenbühl S., Hauser P.C.: Anal. Chim. Acta **727**, 78 (2012).
[2] Jiang Y., Sun C., Ding X., Yuan D., Chen K., Gao B., Chen Y., Sun A.: J. Pharm. Biomed. Anal. **66**, 258 (2012).
[3] Boenzi S., Rizzo C., Di Ciommo V.M., Martinelli D., Goffredo B.M., Marca G., Dionisi-Vici C.: J. Pharm. Biomed. Anal. **56**, 792 (2011).
[4] Wyss M., Kaddurah-Daouk R.: Phys. Rev. **80**, 1107 (2000).
[5] Nasrallah F., Feki M., Briand G., Kaabachi N.: Clin. Biochem. **43**, 1356 (2010).
[6] Beyer C., Hoenderdos A., Mairuhu W.M., Statius L.W.: Clin. Chim. Acta **136**, 263 (1984).
[7] Sundaram P.V., Igloi M.P.: Clin. Chim. Acta **94**, 295 (1979).
[8] Karatzaferi C., De Haan A., Offringa C., Sargeant A.J.: J. Chromatogr. B

730, 183 (1999).
[9] Shingfield K.J., Offer N.W.: J. Chromatogr. B **723**, 81 (1999).
[10] Almeida L.S., Verhoeven N.M., Roos B., Valongo C., Cardoso M.L., Vilarinho L., Salomons G.S., Jakobs C.: Mol. Genet. Metab. **82**, 214 (2004).
[11] Struys E.A., Jansen E.E., Ten Brink H.J., Verhoeven N.M., Van der Knaap M.S., Jakobs C.: J. Pharm. Biomed. Anal. **18**, 659 (1998).
[12] Rodríguez J., Berzas J.J., Castañeda G., Mora N., Rodríguez M.J.: Anal. Chim. Acta **521**, 53 (2004).
[13] Zinellu A., Sotgia S., Zinellu E., Chessa R., Deiana L., Carru C.: J. Sep. Sci. **29**, 704 (2006).
[14] Cognat S., Cheillan D., Piraud M., Roos B., Jakobs C., Vianey-Saban C.: Clin. Chem. **50**, 1459 (2004).
[15] Carducci C., Santagata S., Leuzzi V., Carducci C., Artiola C., Giovanniello T., Battini R., Antonozzi I.: Clin. Chim. Acta **364**, 180 (2006).
[16] Kågedal B., Olsson B.: J. Chromatogr. **527**, 21 (1990).
[17] Harmoinen A., Sillanaukee P., Jokela H.: Clin. Chem. **37**, 563 (1991).