DIAGNOSTICS OF CEREBRAL CREATINE DEFICIENCY SYNDROMES USING TANDEM MASS SPECTROMETRY IN URINE AND DRY URINE SPOT

DIAGNOSTIKA SYNDRÓMOV DEFICITU KREATÍNU V MOZGU POMOCOU TANDEMOVEJ HMOTNOSTNEJ SPEKTROMETRIE V MOČI A SUCHEJ KVAPKE MOČU

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ABSTRACT

Background: Creatine deficiency syndromes (CDS) are a group of inborn errors of metabolism and include disorders of creatine synthesis and transport. A common feature of these syndromes is a lack of creatine in the brain with neurological manifestation of diseases. Specific CDS biomarkers are creatine and guanidinoacetic acid (GAA), and their determination in body fluids is essential for proper diagnosis and treatment. The disadvantage is creatine instability in liquid urine sample.
Aim: This work is aimed at investigating the possibility of using a dry urine spot to determine the concentration of creatine, GAA and creatinine in urine sample, and testing the stability of these analytes in a liquid matrix and a dry urine spot at different storage temperatures.
Methods: Isotope dilution tandem mass spectrometry was used for the determinatin of the analytes.
Results: GAA and creatinine are stable analytes in the liquid matrix as well as in dry urine spot. Creatine was stable at room temperature up to 3 hours in liquid matrix, in dry urine spot up to 24 hours. Better stability was at 4 °C, and all analytes show a good stability when stored at -20 °C. Intra- and interday precision was up to 4.4 %, and accuracy up to 13.5 % for all analytes using a dry urine spot. LOQ for creatine, GAA and creatinine were at 0.018 mmol/l, 0.016 mmol/l and 0.40 mmol/l, respectively.
Conclusion: The results demonstrate the advantage of using a dry urine spot for determination of CDS biomarkers, especially for creatine due to better stability.

Key words: Dry urine spot. Creatine deficiency syndromes. Tandem mass spectrometry.

ABSTRAKT

Východiská: Syndrómy spojené s deficitom kreatínu (CDS) patria medzi dedičné metabolické poruchy a zahŕňajú poruchy syntézy a transportu kreatínu. Spoločným znakom týchto syndrómov je deficit kreatínu v mozgu, ktorý vedie k neurologickej manifestácii týchto ochorení u postihnutých jedincov. Špecifickými biomarkermi CDS sú kreatín a kyselina guanidinooctová (GAA). Ich stanovenie v telesných tekutinách je nevyhnutné pre správnu diagnostiku a liečbu. Nevýhodou je nestabilita kreatínu v tekutých vzorkách moču.
Cieľ: Táto práca je zameraná na preskúmanie možnosti použitia suchej kvapky moču na stanovenie koncentrácie kreatínu, GAA a kreatinínu vo vzorkách moču a overenie stability uvedených analytov v tekutej matrici moču, ako aj suchej kvapke moču pri rôznych skladovacích teplotách.
Metódy: Na stanovenie analytov bola použitá metóda tandemovej hmotnostnej spektrometrie s využitím izotopovo značených vnútorných štandardov.
Výsledky: GAA a kreatinín sú stabilné analyty v tekutej matrici, ako aj v suchej kvapke moču. Kreatín bol stabilný v tekutej matrici pri izbovej teplote do 3 hodín, v suchej kvapke moču do 24 hodín. O niečo lepšiu stabilitu vykazoval pri 4 °C. Všetky tri analyty boli stabilné pri skladovaní pri -20 °C. Opakovateľnosť a medziľahlá presnosť merania boli do 4,4 %, správnosť do 13,5 % pre všetky analyty s použitím suchej kvapky moču. LOQ pre kreatín, GAA a kreatinín boli 0,018 mmol/l, 0,016 mmol/l a 0,40 mmol/l, v uvedenom poradí.
Záver: Výsledky dokazujú výhodu použitia suchej kvapky moču na stanovenie biomarkerov CDS, najmä z hľadiska stability kreatínu.

Kľúčové slová: Suchá kvapka moču. Syndrómy deficitu kreatínu. Tademová hmotnostná spektrometria.

INTRODUCTION

Creatine deficiency syndromes (CDS) are a group of inborn errors of metabolism and include disorders of creatine synthesis and transport. A common feature of these syndromes is a lack of creatine in the brain with neurological manifestation of diseases. The CDS group includes two autosomal recessive disorders of creatine synthesis – arginine glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT) enzyme deficiency, as well as an X-linked creatine transporter deficiency disorder (CRTR). Creatine deficit in the brain is detectable by magnetic resonance spectroscopy. The main symptoms of CDS include intellectual disability, seizures, epilepsy, autistic behaviour, and speech delay [1-5]. The determination of creatine and guanidinoacetic acid (GAA) as specific biomarkers of inborn errors of creatine metabolism and transport is significant from the point of view of establishing the correct diagnosis and treatment [5-7]. A characteristic diagnostic biomarker of AGAT deficiency is a reduced concentration of

GAA in plasma, urine, and cerebrospinal fluid, while GAA concentration is high in GAMT deficiency. For CRTR, the main diagnostic marker is an increased urinary creatine/creatinine ratio. Plasma creatine is low in AGAT and GAMT deficiency, normal for CRTR [2, 3, 7].

Several analytical methods have been developed to determine GAA and creatine in biological samples, such as gas chromatography with mass spectrometric detection [9-12], liquid chromatography with tandem mass spectrometry (LC-MS/MS) [8, 12-16] or MS/MS with direct sample injection [17]. Tandem mass spectrometry enables rapid determination of creatine and GAA in urine, plasma and cerebrospinal fluid. When determining the content of creatine in urine, the disadvantage is its instability and increasing concentration over time probably due to bacterial contamination. If the creatine determination in urine sample is not carried out immediately after collection, it is necessary to freeze the sample immediately and reduce the number of thaws to a minimum [10, 18].

This work is aimed at investigating the possibility of using a dry urine spot (DUS) to determine the concentration of creatine, guanidinoacetic acid and creatinine in urine sample using tandem mass spectrometry and testing the stability of these analytes in a liquid matrix and a dry urine spot at different storage temperatures. Results obtained for liqud and dry urine samples were compared using quality control samples with declared content of examined analytes and urine samples from patients with confirmed GAMT deficiency.

MATERIALS AND METHODS

Chemicals

Creatine, creatinine and GAA were purchased from Sigma-Aldrich (Switzerland and USA), internal standards d_5-creatine 99.7% (methyl-d_3; glycine-2,2-d_2), d_2-GAA 97.5% (guanidinoacetic-2,2-d_2 acid) from CDN Isotopoes (Quebec, Canada), and d_3-creatinine 98% (N-methyl-d_3) from CIL (Andover, MA, USA). LC-MS grade water and methanol were purchased from Fisher Scientific (Loughborough, UK), and formic acid 98% from Mikrochem (Pezinok, Slovakia). Lyophilized human urine - Control Special Assay in Urine, used as a quality control, was purchased from MCA Laboratory of the Queen Beatrix Hospital (BN Winterswijk, The Netherlands).

Standard solutions preparation

Creatine, GAA and creatinine stock solutions with concentration of 7.63 mmol/l, 8.54 mmol/l and 9.28 mmol/l, respectively were prepared in deionized water and stored at -20 °C as well as internal standards d_5-creatine, d_2-GAA and d_3-creatinine stock solutions with concentation of 6.49 mmol/l, 8.19 mmol/l and 8.61 mmol/l, respectively. For processing of liquid urine matrix one internal standards (IS) working solution with concentration of d_5-creatine, d_2-GAA and d_3-creatinine at 0.13 mmol/l, 0.02 mmol/l and 0.22 mmol/l was prepared by dilution of IS stock solutions in mixture of 80:20 (vol. %) methanol:water and stored at -20 °C. Extraction solution for DUS with concentration of d_5-creatine, d_2-GAA and d_3-creatinine at 2.6 μmol/l, 4 μmol/l and 43 μmol/l was prepared by dilution of IS stock solutions in mixture of 80:20 (vol. %) methanol:water containing 0.1 % formic acid. To investigate the linearity of matrix calibration curves, the set of calibration solutions containing all three standards of analytes was prepared by diluting an appropriate amount of analyte standards in deionized water for the most concentrated calibration solution, from which the other calibration solutions were subsequently prepared by gradual dilution. The concentrations of creatine, GAA and creatinine in calibration solutions were in the range of 0.077 – 39.35 mmol/l, 0.039 – 19.87 mmol/l and 0.872 – 446.4 mmol/l, respectively. The set of spiked urine samples was prepared by spiking of 900 μl urine with 100 μl of calibration solutions.

Sample preparation

Anonymized spot urine samples from voluntary donors and two patients with GAMT defficiency were collected in urine containers and stored at -20 °C until analysis. The study was approved by the Ethics Committee of the Faculty of Science of the Comenius University under the number ECH19024.

For working with liquid urine matrix, the sample preparation procedure for MS/MS analysis was as follows. 50 μl of thawed urine aliquots were placed into microvials and filled up to 1 ml with deionized water. 20 μl of diluted urine samples were placed into 96-well plate (PP-microplate 96 well, flat bottom, Greiner Bio One, Germany), and 20 μl of IS work solution were added. An additional 60 μl mixture of 80:20 (vol. %) methanol:water contai-

ning 0.1 % formic acid were added, plate was shaken shortly, sealed and then placed in the autosampler for MS/MS analysis.

Dry urine spot samples were prepared by dropping 20 µl of thawed urine sample onto standardized filter paper (Whatman 903, Cardiff, UK). After drying (2 hours, room temperature), or at specified time intervals, a small disk (3 mm diameter, corresponds to 1 µl of urine) was punching out and dropping into microtiter plate. Extraction was performed shaking with extraction solution enriched with IS (20 min, 650 rpm, room temperature). The extracts were subsequently transferred to a new microtiter plate and placed in an autosampler for analysis.

To test the stability of analytes in a liquid urine sample and in a DUS, urine samples from two voluntary donors were analyzed in two parallels at different time intervals at three storage temperatures (room temperature, -4 °C and -20 °C).

Table 1 Concentrations of creatine, GAA and creatinine in urine control samples

Level	creatine (mmol/l)	GAA (mmol/l)	creatinine (mmol/l)
Level 1	0.484	0.731	7.17
Level 2	5.575	0.112	3.93
Level 3	2.788	0.057	1.97

Lyophilized human urine control sample at two concentration levels Level 1 and Level 2 was processed according to the instructions, and the third concentration level – Level 3 was obtained by diluting Level 2 with deionized water twice. Control samples were stored at -20 °C.

HPLC-MS/MS experimental conditions

A triple quadrupole mass spectrometer Finnigan TSQ Quantiva (Thermo Fisher Scientific Inc.) equipped with a heated electrospray ionization (HESI) source was used. Sample injection was performed using Dionex Ultimate 3000 RS Autosampler and an HPLC pump Dionex Ultimate 3000 RS (Thermo Fisher Scientific Inc.), connected with the heated HESI by a PEEK capillary (1 m × 0.127 mm i. d.). Data were acquired and processed using Thermo Xcalibur software (Thermo Fisher Scientific Inc.).

The heated electrospray source was operated in positive ion mode at the source parameters: 3500 V spray voltage, 325 °C capillary temperature, 144 °C vaporizer temperature, and using nitrogen as sheath gas, auxilliary gas and ion sweep gas at pressure 20, 7 and 1 instrument units, respectively. The pressure of collision gas argon was 0.6 mTorr.

Analytes were delivered to MS/MS with a mixture of methanol and water (80:20) acidified with 0.1% formic acid at an isocratic flow rate of 120 µl/min from 0 to 1.1 min. The tray temperature was set at 5 °C. The injected volume was 10 µl.

MS/MS analysis was performed in selective reaction monitoring mode (SRM). Optimized SRM transition parameters for the $[M+1]^+$ ions of analytes and internal standards are listed in Table 2.

Equations used for calculation

The following equations were used to calculate the concentrations of individual analytes in urine samples using one-point calibration:

$$c_a = \frac{A_a}{A_{IS}} \times c_{IS} \times F$$

$$F = \frac{f_{IS}}{f_a}$$

$$f = \frac{A}{c}$$

Notes: c – concentration, A – peak area, f – response factor, F – relative response factor; a – analyte, IS – internal standard. Analytes response factor f_a was calculated using a certified reference material of lyophilized urine at three different concentration levels

Intraday precision s and interday precision s_r were calculated according following equations:

Table 2 SRM parameters for analytes and their corresponding internal standards

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)	Tube lens (V)
creatine	132.15	90.05	12	62
d_5-creatine	137.15	95.10	13	62
GAA	118.15	101.05	10	57
d_2-GAA	120.15	103.05	10	57
creatinine	114.15	44.15	19	57
d_3-creatinine	117.15	47.20	19	57

$$s = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{(n-1)}}$$

Notes: x_i – measured value, $\bar{x}$ – mean of measured values, n – number of measurements

$$s_r = \sqrt{\frac{\sum_{d=1}^{D}\sum_{r=1}^{n}(x_{dr} - \bar{x}_d)^2}{D(n-1)}}$$

Notes: D – number of days, x_{dr} – measured value on day d, $\bar{x}_d$ – mean of measured values on day d

Limits of detection (LOD) and quantification (LOQ) were calculated using linear regression parameters (from the first five points of the calibration curve) according equations:

$$LOD = 3 \times \frac{s}{b}$$
$$LOQ = 10 \times \frac{s}{b}$$

Notes: s - standard error of the regression, b – slope

RESULTS AND DISCUSSION

The results show (Fig. 1) that GAA and creatinine are stable in both liquid matrix and DUS at room temperature (RT) and 4 °C. Creatine in the liquid matrix is stable at RT for only about 3 hours, then its concentration increases and after 24 hours it is about 3 times higher. In DUS, creatine is stable up to 24 hours at RT and more than 3-fold increase in concentration was noted after a week; when stored at 4 °C there was about 1.5-fold increase after a week. All three analytes show a decrease in concentration when stored at -20 °C mainly in the liquid matrix due to precipitation, so sonication of the sample is recommended to redissolve these precipitates [19].

A certified reference material of lyophilized urine at three different concentration levels (Level 1 – 3) was used to evaluate intraday precision, interday precision, and accuracy for DUS with 6 replicates, for interday precision on 3 different days (Tab. 3). Achieved detection and quantification limits are also listed in Table 3.

The maximum value authorized by EMA guideline [20] for bias and for coefficient of variability (*CV*) is 15 %, and at the level of the quantification limit should be within 20 %. Bias and *CV* values are acceptable according to the EMA validation requirements for all analytes, bias does not exceed the value of 13.5 % (for GAA at concetration level 0.731 mmol/l), intraday *CV* ranges from 0.7 – 3.8 % and 1.4 – 4.4 % for interday *CV*.

Matrix calibration curves were linear in the range of 0.005 – 3.94 mmol/l for creatine, 0.021 – 2 mmol/l for GAA, and 0.87 – 45.5 mmol/l for creatinine, with coefficient of determination R^2 of 0.9996, 0.9997, and 0.9999 for creatine, GAA, and creatinine, respectively. A Mandel's fitting test was also performed to confirm linearity, when the calculated value F_{calc} must be lower than critical value F_{crit}. The values for creatine were F_{calc} = 7.05 and F_{crit} = 12.25, for GAA F_{calc} = 0.77 and F_{crit} = 12.25, and for creatinine F_{calc} = 6.12 and F_{crit} = 13.75.

The results of analysis of urine samples from two patients with genetically confirmed GAMT deficiency show good agreement using a liquid matrix and a DUS (Tab. 4). Elevated GAA/creatinine ratio in the urine is a biomarker of this disorder, but the several-fold increased urinary creatine/creatinine ratio compared to the reference value is due to creatine supplementation in these patients (Tab. 5).

Table 3 Validation parameters of MS/MS method for determination of creatine, guanidinoacetic acid (GAA) and creatinine in dry urine spot

Analyte	Bias (%)			Intraday CV (%)			Interday CV (%)			LOD (mmol/l)	LOQ (mmol/l)
	L1	L2	L3	L1	L2	L3	L1	L2	L3		
creatine	10.3	0.3	-2.7	0.8	2.9	1.8	1.4	3.4	2.5	0.005	0.018
GAA	13.5	-5.8	-9.3	0.64	3.8	2.7	2.0	4.4	3.4	0.005	0.016
creatinine	6.1	0.4	-2.8	0.7	1.8	2.1	2.0	2.9	3.0	0.12	0.40

Legend: *CV* – coefficient of variability, L1 – Level 1, L2 – Level 2, L3 – Level 3, *LOD* – limit of detection, *LOQ* – limit of quantification.

Table 4 Concentrations of creatine, GAA and creatinine in urine of two patients with GAMT deficiency determined by MS/MS in DUS and liquid matrix

Patient No.	creatine			GAA			creatinine		
	DUS (mmol/l)	Liquid (mmol/l)	Bias (%)	DUS (mmol/l)	Liquid (mmol/l)	Bias (%)	DUS (mmol/l)	Liquid (mmol/l)	Bias (%)
1	198.43	221.57	-10.4	4.32	4.38	-1.4	18.21	17.30	5.3
2	223.77	223.74	0	4.85	5.2	-6.7	18.28	16.88	8.3

Table 5 Reference values of creatine/creatinine and GAA/creatinine ratio in urine and determined values in two GAMT deficiency patients by the MS/MS method in DUS

Patient No.	creatine (mmol/mol creatinine)	GAA (mmol/mol creatinine)
1	10897	237.2
2	12241	265.3
Upper limit [8]	1550	150.0

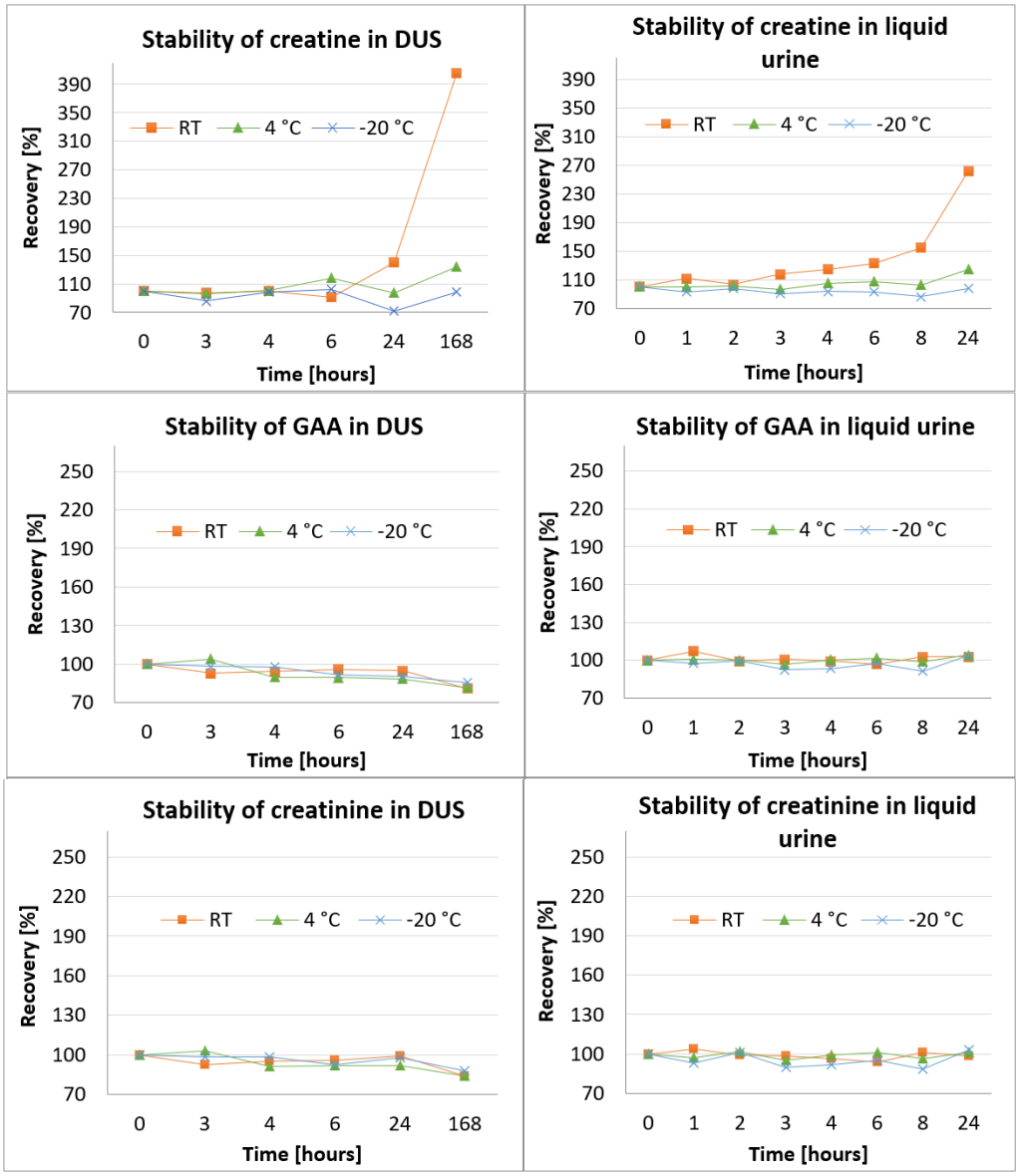

Figure 1 Stability of creatine, GAA and creatinine in dry urine spot and liquid urine

CONCLUSION

The presented results point to the possibility of using dry urine spot to determine biomarkers of creatine metabolism and transport disorders, especially from the point of view of creatine stability. A more significant creatine increase was observed in DUS at room temperature after 24 hours, while in the liquid matrix a similar increase was observed after only three hours. GAA and creatinine show good stability in both matrices. Accuracy and precision parameters of MS/MS determination of creatine, GAA and creatinine in DUS meet the recommendation of EMA guideline on bioanalytical method validation and do not exceed 15 %. The comparison of the values obtained by MS/MS analysis of DUS and liquid urine of two patient with GAMT deficiency is also satisfactory, the differences do not exceed 10.4 % and the results confirm the diagnosis.

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