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GC–MS determination of creatinine in human biological fluids as pentafluorobenzyl derivative in clinical studies and biomonitoring: Inter-laboratory comparison in urine with Jaffé, HPLC and enzymatic assays☆

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ABSTRACT

In consideration of its relatively constant urinary excretion rate, creatinine in urine is a useful biochemical parameter to correct the urinary excretion rate of endogenous and exogenous biomolecules. Assays based on the reaction of creatinine and picric acid first reported by Jaffé in 1886 still belong to the most frequently used laboratory approaches for creatinine measurement in urine. Further analytical methods for creatinine include HPLC–UV, GC–MS, and LC–MS and LC–MS/MS approaches. In the present article we report on the development, validation and biomedical application of a new GC–MS method for the reliable quantitative determination of creatinine in human urine, plasma and serum. This method is based on the derivatization of creatinine (d_0-Crea) and the internal standard [*methyl*-trideutero]creatinine (d_3-Crea) with pentafluorobenzyl (PFB) bromide in the biological sample directly or after dilution with phosphate buffered saline, extraction of the reaction products with toluene and quantification in 1-μl aliquots of the toluene extract by selected-ion monitoring of *m/z* 112 for d_0-Crea-PFB and *m/z* 115 for d_3-Crea-PFB in the electron-capture negative-ion chemical ionization mode. The limit of detection of the method is 100 amol of creatinine. In an inter-laboratory study on urine samples from 100 healthy subjects, the GC–MS method was used to test the reliability of currently used Jaffé, enzymatic and HPLC assays in clinical and occupational studies. The results of the inter-laboratory study indicate that all three tested methods allow for satisfactory quantification of creatinine in human urine. The GC–MS method is suitable for use as a reference method for urinary creatinine in humans. In serum, creatine was found to contribute to creatinine up to 20% when measured by the present GC–MS method. The application of the GC–MS method can be extended to other biological samples such as saliva.

1. Introduction

Creatinine (2-amino-1,5-dihydro-1-methyl-4H-imidazol-4-one, MW 113.12; see Scheme 1) is the end-product of the creatine catabolism. In humans, creatinine and creatine occur in muscle tissues and blood. Creatinine is excreted in the urine with a fairly constant rate of about 25 mg per kg of body weight per day. Because of this particular physiological property, creatinine in urine is used since several decades for the correction of excretion rates or urinary concentrations of numerous endogenous and exogenous substances. This correction is indispensable in clinical and occupational studies in which urine specimens from spontaneous micturition are mostly available [1,2]. The urinary creatinine concentration is usually expressed in the dimensions of mM or g/l, the conversion factor being 1 mM = 0.113 g/l. In addition, creatinine in serum or plasma (below about 100 μM in healthy subjects) is of particular interest, as its circulating concentration provides important information about the filter function of the kidneys. In accordance with recommendations by the World Health Organisation [2], the American Conference of Governmental Industrial Hygienists [3] as well as by the Human Biomonitoring Commission of the German Federal Environmental Agency [4], the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) has recently adopted a creatinine range of 0.3 g/l–3 g/l (approximately 2.5–25 mM) in urine as a criterion for specimen acceptance in human biomonitoring studies [5]. The mean concentration of creatinine in urine samples of

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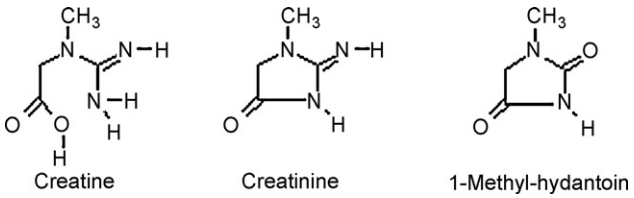


Scheme 1. Chemical structures of creatine, creatinine and 1-methyl-hydantoin.

healthy adults is approximately 1.3–1.5 g/l (12–13 mM) [6,7]. Apart from the use of urinary creatinine as an exclusion criterion for diluted or concentrated samples, creatinine-based biological limit values have been established for some biomarkers. These compounds include 2-thio-4-thiazolidine carboxylic acid (carbon disulfide exposure), several chlorocatechols (chlorobenzene exposure), *N*-methyl acetamide (*N*,*N*-dimethylacetamide exposure), methoxyacetic acid (ethylene glycol monomethyl ether and ether acetate exposure), mandelic acid and phenylglyoxylic acid (styrene and ethylbenzene exposure), dimethylbenzoic acids (trimethylbenzene exposure), fluoride, mercury and aluminium [5].

The spectrophotometric method based on the famous Jaffé reaction [8] belongs to the oldest and most commonly used assays for creatinine. However, the great interest in creatinine has resulted in many different analytical methods. They include batch and automated spectrophotometric and enzymatic assays as well as instrumental methods including HPLC, GC–MS, LC–MS and LC–MS/MS [9–25].

With regard to the analytical quality of the creatinine determination, the results of the German External Quality Assurance Scheme (G-EQUAS; www.g-equas.de), as organised on behalf of the German Society for Occupational Medicine and Environmental Medicine (DGAUM) (c/o Institute and Out-Patient Clinic for Occupational, Social and Environmental Medicine of the University of Erlangen-Nuremberg), over the last decade have shown highly varying overall success rates of 60–90% among the participating laboratories. In view of the aforementioned importance of creatinine as an inclusion criterion and due to its use as a correction factor for biological reference and limit values, the round robin results reveal considerable and unsatisfying variations between laboratories and methods.

To investigate the performance of and to identify possible systematic differences between analytical procedures for creatinine determination, a new GC–MS method for the quantitative determination of urinary creatinine was developed and validated as a reference technique. An inter-laboratory comparison with three participants was carried out including the newly developed GC–MS method (referred to as *GC–MS*), a photometric assay according to the Jaffé method (referred to as *JAFFÉ*), an enzymatic method based on a multi-step reaction resulting in the oxidation of 4-aminophenazone with hydrogen peroxide (referred to as *ENZYM*), and an HPLC–UV method (referred to as *HPLC*)

2. Experimental

2.1. Chemicals

2,3,4,5,6-Pentafluorobenzyl bromide (PFB-Br), creatine, 1-methyl-hydantoin, unlabelled creatinine (d_0-Crea) and the trideutero-creatinine, i.e., [*methyl*-$^2H_3$]creatinine (d_3-Crea), with a declared isotopic purity of > 99 atom% ^2H, were obtained from Aldrich (Steinheim, Germany). Stock solutions of d_0-Crea and d_3-Crea (20 and 10 mM) were prepared in 10 mM HCl and were stored in a refrigerator at 8 °C. Toluene and acetone were purchased from Baker (Deventer, The Netherlands).

2.2. Biological samples

Urine samples (n = 100) with broadly varying creatinine concentration were taken from a previously reported human study [26] and used for inter-laboratory comparison. The urine specimens were centrifuged to separate particles and the clear supernatants were divided into 1-ml aliquots. Each of the three participating laboratories received one aliquot. Urine samples were shipped at frozen state on dry ice. The samples were kept frozen at −18 °C to −27 °C until analysis.

Urine, plasma and serum samples used in method development and validation were obtained from healthy volunteers being members of the Institute of Clinical Pharmacology. Volunteers had given their informed consent. Samples were kept frozen at −18 °C until analysis. More information is provided in Section 3.

2.3. Sample derivatization procedure

In quantitative analyses the following derivatization procedure was used. Urine samples (10 μl) were diluted with 90 μl of 67 mM phosphate buffered saline (PBS), pH 7.4. To 100-μl aliquots of diluted urine samples placed in 1.8-ml glass vials, 10-μl aliquots of a 10-mM solution of d_3-Crea in 10 mM HCl were added, followed by addition of acetone (400 μl) and PFB-Br (10 μl). Glass vials were tightly closed and heated for 60 min at 50 °C. After cooling to room temperature acetone was evaporated under a nitrogen stream and reaction products were extracted from the remaining aqueous phase by vortex-mixing with toluene (1000 μl) using a Heidolph vortex mixer model Reax 2000 (Schwabach, Germany) at the highest vortex speed (i.e., stage 9). Samples were then centrifuged (5 min, 800 × *g*, 4 °C) and aliquots (700 μl) of the organic phase were transferred into 1.8-ml autosampler glass vials for GC–MS analysis.

2.4. Validation of the method

The GC–MS method was validated for creatinine in human urine and plasma in relevant concentrations ranges. Validation experiments included standardization of the internal standard, determination of accuracy and precision, as well as studies on potential interference by the creatinine-relatives creatine and 1-methyl-hydantoin. For the sake of clarity, validation experiments are described in Section 3.

2.5. Creatinine methods used in comparison studies

The GC–MS method was compared with three different analytical approaches for urinary creatinine which were performed as described below.

A spectrophotometric Jaffé assay (PHOTO) for the direct measurement of creatinine in urine was applied as described elsewhere [26]. Briefly, urine (100 μl) was added to saturated picric acid (2 ml) and 10 wt% aqueous NaOH (150 μl). After a 10-min incubation period at room temperature, the samples were diluted with ultra-pure water (7.75 ml) and left for another 5 min at room temperature. Aliquots (1 ml) of each sample were then transferred to a polypropylene cuvette and the absorption at λ = 546 nm was measured (Spectrophotometer UV-1602, Shimadzu, Duisburg, Germany). The creatinine concentration was calibrated by comparison with aqueous creatinine standards in the concentration range between 0.5–3.0 g/l (4.4–26.5 mM). The accuracy of this creatinine procedure was demonstrated by successful participation and certification in round robins of the German External Quality Assurance Scheme (G-EQUAS; c/o Institute and Out-Patient Clinic for Occupational, Social and Environmental Medicine of the University of Erlangen-Nuremberg).

The LOD value of this Jaffé method according to DIN 32 645 (1994) was 0.9 mM (0.1 g/l), and the inter-assay imprecision (RSD, %; $n = 10$) was 5% at 4.9 mM (0.55 g/l) and 6% at 9.6 mM (1.09 g/l).

The second analytical method (HPLC) compared was an HPLC–UV method after minor modifications of previously described methods [12,22]. Briefly, 10-μl aliquots of 1:5 (v/v)-diluted urine samples were analyzed by an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a degasser, an isocratic pump, a column oven (kept at 25 °C) and a variable UV–vis wavelength detector. The mobile phase contained 50 mM ammonium sulphate in water-methanol (95:5, v/v, pH 5.5) and was pumped at a flow rate of 1 ml/min. Separation was performed on a Synergi Hydro-RP80A column (250 × 4.6 mm, 4 μm particles) from Macherey-Nagel (Düren, Germany) and analytes were detected at 236 nm. In this HPLC system, creatinine eluted at about 3.4 min. The LOD value {according to DIN 32 645 (1994)} of the HPLC method was 0.18 mM (0.02 g/l) and the inter-assay imprecision was 3% at 6.8 mM (0.77 g/l, $n = 10$).

The third method (ENZYM) for the quantification of creatinine in urine was an automated photometric assay based on the enzyme-catalyzed formation of a quinonimine dye from 4-aminophenazone, 2,4,6-triiodine-3-hydroxybenzoic acid and hydrogen peroxide, the latter being liberated from the conversion of creatinine to sarcosin and subsequent oxidation of sarcosin. This *CREA plus* assay is commercially available from Roche diagnostics (Mannheim, Germany) and involves the use of an automated Roche/Hitachi Cobas® 900 series analyser. The LOD value of this method (according to the manufacturer) is 0.03 mM (0.003 g/l) and the inter-assay imprecision is 2.1% at 2.16 mM (0.244 g/l, $n = 10$). The accuracy of this procedure was tested and confirmed in round robins of the DGAUM (c/o Institute and Out-Patient Clinic for Occupational, Social and Environmental Medicine of the University of Erlangen-Nuremberg).

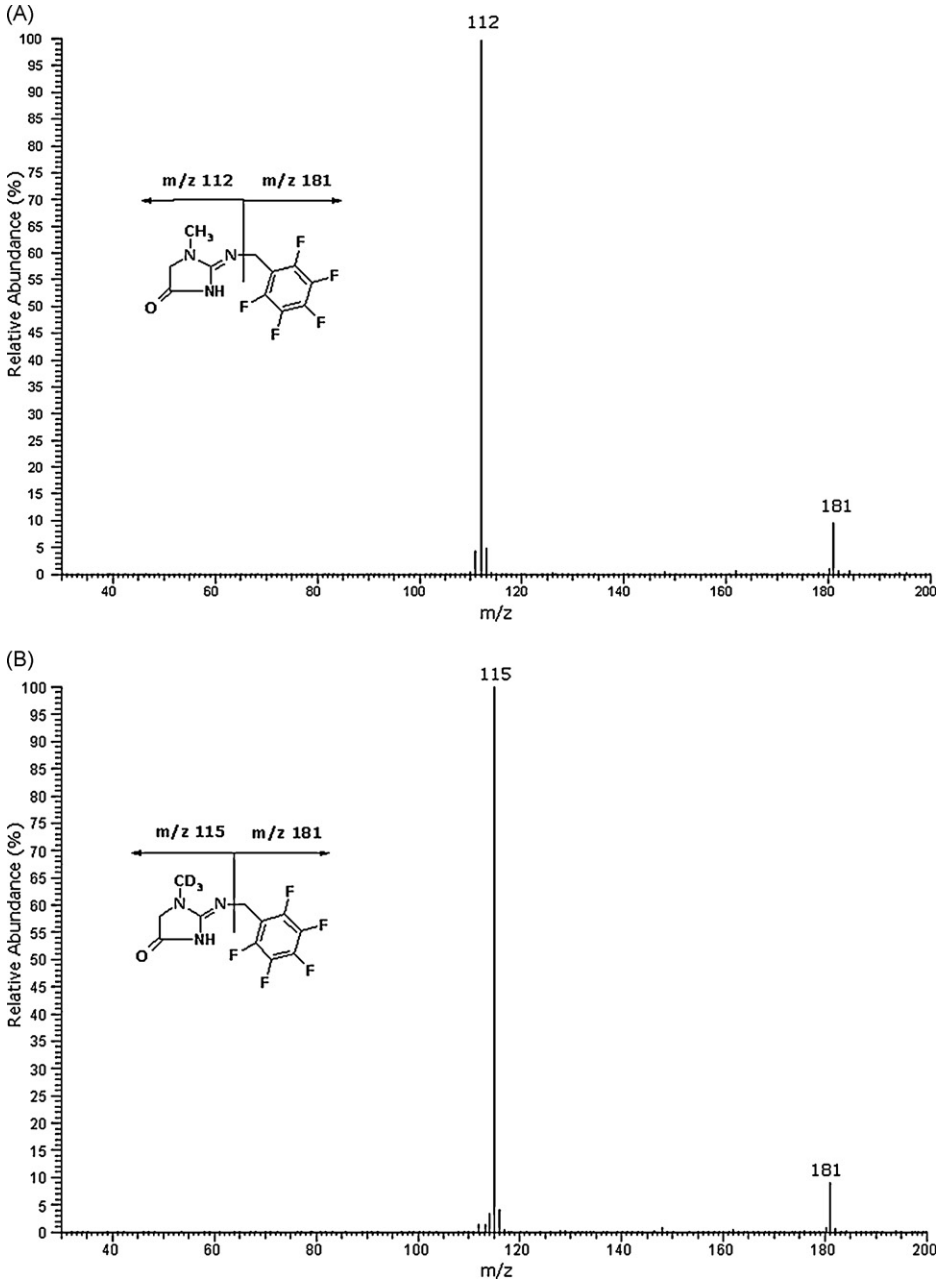


Fig. 1. ECNICI mass spectra of the PFB derivatives of synthetic unlabelled creatinine (A) and trideutero-creatinine (B).

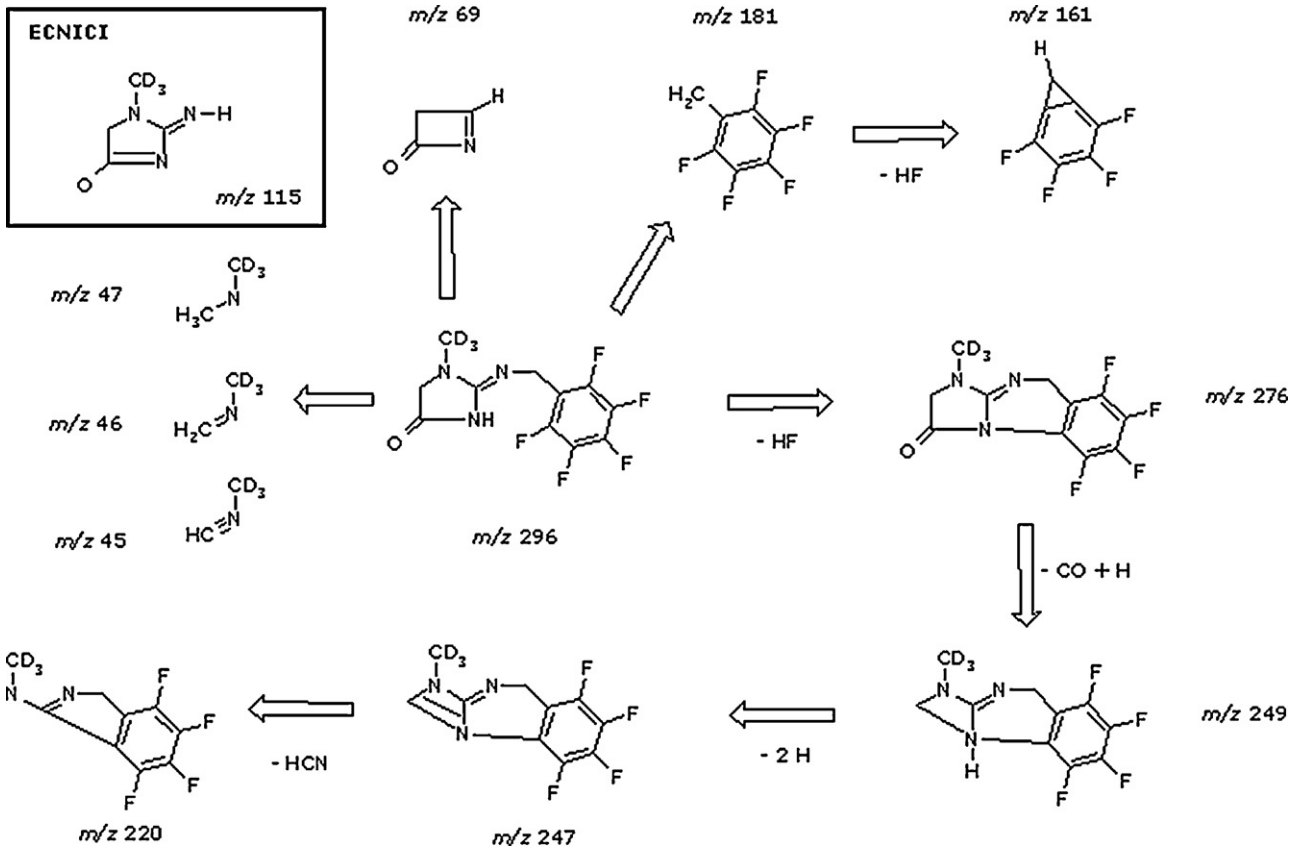
Table 1
Major ions present in the GC–MS electron ionization mass spectra of the pentafluorobenzyl derivatives of d_0-creatinine and d_3-creatinine in the range m/z 20–400[a].

d_0-Crea-PFB, m/z (%)	Ion assignment	d_3-Crea-PFB, m/z (%)	Ion assignment
42 (90)	$[CH_3NCH]^+$	45 (35)	$[CD_3NCH]^+$
43 (28)	$[CH_3NCH_2]^+$	46 (20)	$[CD_3NCH_2]^+$
44 (100)	$[CH_3NCH_3]^+$	47 (80)	$[CD_3NCH_3]^+$
69 (20)	$[COCH_2CHN]^+$	69 (15)	$[COCH_2CHN]^+$
161 (15)	$[C_6F_4CH]^+$	161 (10)	$[C_6F_4CH]^+$
181 (35)	$[C_6F_5CH_2]^+$	181 (30)	$[C_6F_5CH_2]^+$
217 (25)	$[CH_3NCNCH_2C_6F_4]^+$	220 (32)	$[CD_3NCNCH_2C_6F_4]^+$
244 (35)	$[M–F–CO–2H]^+$	247 (50)	$[M_{(d3)}–F–CO–2H]^+$
246 (12)	$[M–F–CO]^+$	249 (20)	$[M_{(d3)}–F–CO]^+$
273 (75)	$[M–HF]^+$	276 (100)	$[M_{(d3)}–HF]^+$
274 (30)	$[M+1–HF]^+$	277 (45)	$[M_{(d3)}+1–HF]^+$
293 (40)	$[M]^+$	296 (75)	$[M_{(d3)}]^+$
294 (7)	$[M+1]^+$	297 (10)	$[M_{(d3)}+1]^+$

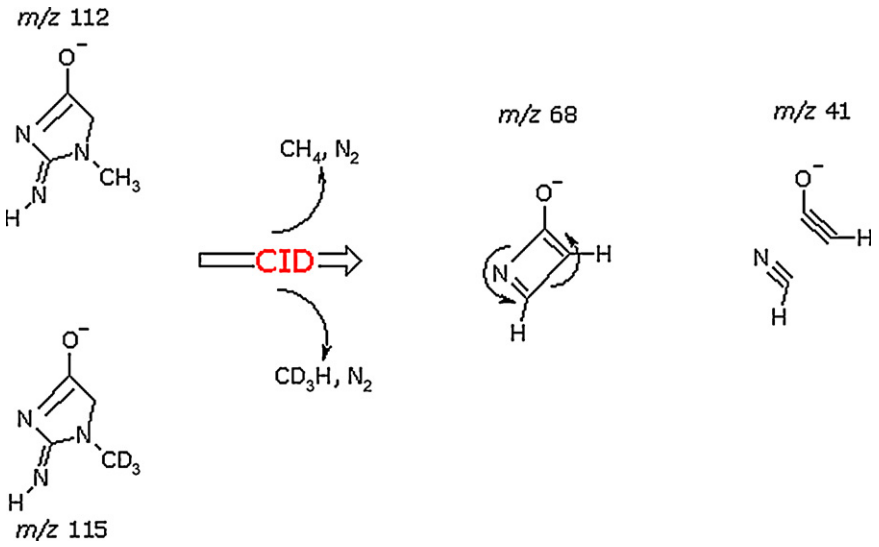
[a] M and $M_{(d3)}$ are the molecular masses of d_0-creatinine and d_3-creatinine, respectively.

2.6. GC–MS conditions

GC–MS analyses were performed on a ThermoElectron DSQ quadrupole mass spectrometer connected directly to a ThermoElectron Focus gas chromatograph and to an autosampler AS 3000 (ThermoElectron, Dreieich, Germany). Two fused-silica capillary columns from Macherey-Nagel (Düren, Germany) were used in this study. Optima® 17 (30 m × 0.25 mm i.d., 0.25-μm film thickness) columns were used in method validation and in the inter-laboratory comparison study. An Optima® δ6 (30 m × 0.25 mm i.d., 0.25-μm film thickness) column was compared with an Optima® 17 column by analyzing 26 urine samples. Routinely, aliquots (0.5 μl) of the toluene extracts were injected in the splitless mode. The following oven temperature program was used with helium (constant flow rate of 1 ml/min) as the carrier gas: 0.5 min at 120 °C, then increased to 220 °C at a rate of 30 °C/min, and to 330 °C at a rate of 70 °C/min; the final temperature was held for 1 min. Interface, injector and ion source were kept at 260 °C, 200 °C and 250 °C, respectively. Electron energy and electron current were set to 50 eV and 120 μA, respectively, for electron-capture negative-ion chemical ionization (ECNICI) with methane (2.4 ml/min) as the reagent gas. Quantification by GC–MS in the ECNICI mode was performed by selected-ion monitoring (SIM) of the ions at m/z 112.1 for d_0-Crea-PFB and m/z 115.1 for d_3-Crea-PFB using a dwell-time of 100 ms for each ion and an electron multiplier voltage of 1.6 kV. The concentration of unlabelled creatinine ([d_0-Crea]) in a sample was calculated by the formula: [d_0-Crea] = [d_3-Crea] × PAR, whereas [d_3-Crea] is the known concentration of the internal standard in the sample and PAR is the value of the peak area ratio of the peak with m/z 112.1 for d_0-Crea-PFB and m/z 115.1 for d_3-Crea-PFB. Electron ionization (EI)



Scheme 2. Proposed structures for the ions of the PFB derivative of trideutero-creatinine found in the EI and ECNICI (inset) mass spectra. For simplicity the charge of the ions is not indicated.



Scheme 3. Proposed structures for the product ions at *m*/*z* 68 and *m*/*z* 41 by collision-induced dissociation (CID) of the precursor ions at *m*/*z* 112 and *m*/*z* 115 of the PFB derivative of unlabelled and deuterium-labelled creatinine found in the ECNICI mode.

was performed using the conditions applied in ECNICI except for the electron energy (70 eV) and the ion source temperature (225 °C)

GC–MS and GC–tandem MS analyses were also performed on a triple-stage quadrupole mass spectrometer ThermoQuest TSQ 7000 (Finnigan MAT, San Jose, CA) directly interfaced with a Trace 2000 series gas chromatograph equipped with an autosampler AS 2000 (CE Instruments, Austin, TX). Interface, injector and ion source were kept at 280 °C, 280 °C and 180 °C, respectively. Electron energy and electron current was set to 200 eV and 300 μA, respectively. Methane (530 Pa) and argon (0.13 Pa collision pressure) were used as reagent and collision gases, respectively. Collision energy was set to 25 eV. In quantitative analyses the dwell time was 100 ms for each ion both in GC–MS and in GC–tandem MS. The other GC conditions were as described above for the DSQ GC–MS instrument.

3. Results and discussion

3.1. Derivatization and GC–MS analysis of creatinine

3.1.1. GC–MS and GC–tandem MS characterization of reaction products

Derivatization of 10-mM solutions of d_0-Crea and d_3-Crea in 67 mM PBS, pH 7.4, with PFB-Br in acetone at 50 °C resulted each in a single GC peak of which the mass spectrum contained conjugated ions. This finding suggests that creatinine reacts with PFB-Br to produce a single reaction product. The characteristic ions of the PFB derivatives of d_0-Crea (d_0-Crea-PFB) and d_3-Crea (d_3-Crea-PFB) in the EI mass spectra are summarized in Table 1. The ECNICI mass spectra of the PFB derivatives of d_0-Crea and d_3-Crea are shown in Fig. 1. After EI intense ions at *m*/*z* 293 and *m*/*z* 296 were obtained which are most likely the molecular cations of the singly pentafluorobenzylated d_0-Crea and d_3-Crea, respectively. Furthermore, the EI mass spectra of these derivatives suggest that PFB-Br alkylates the 2-amino but not the ring-imino group of creatinine (Scheme 2). Unlike EI, ECNICI of d_0-Crea-PFB and d_3-Crea-PFB produced only two anions, i.e., *m*/*z* 181 which is the pentafluorobenzyl anion $[C_6F_5CH_2]^-$, and *m*/*z* 112 and *m*/*z* 115, respectively, due to the $[M–PFB]^-$. It is likely that ECNICI is accompanied by rearrangement to finally produce a conjugation-stabilized hydroxylate ion (shown as an inset in Scheme 2). In the present study quantitative analyses were performed exclusively in the ECNICI mode by SIM of *m*/*z* 112.1 for unlabelled synthetic and endogenous creatinine and of *m*/*z* 115.1 for the internal standard d_3-Crea.

Under GC–tandem MS conditions in the ECNICI mode, collision-induced dissociation (CID) of *m*/*z* 112.1 and *m*/*z* 115.1 resulted in virtually identical product ion mass spectra, i.e., in product ions with identical *m*/*z* values, suggesting that they do not carry any deuterium atom in their molecules. At a collision energy of 25 eV, the most intense products ions were *m*/*z* 68 (intensity 35%) and *m*/*z* 41 (intensity 100%). The mechanisms by which these ions are formed have not been investigated. We assume (see Scheme 3) that the anion *m*/*z* 68 corresponds to the cation *m*/*z* 69 which was obtained by EI of the d_0-Crea-PFB and d_3-Crea-PFB derivatives (see Scheme 2). The product ion at *m*/*z* 41 could be $HC{\equiv}C–O^-$, the anion of ethinol, which could be formed from the anion *m*/*z* 68 by neutral loss of methane (CH_4, 16 Da from d_0-Crea-PFB; CD_3H, 19 Da from d_3-Crea-PFB) and molecular nitrogen (N_2, 28 Da) after rearrangement.

3.1.2. Optimization of the derivatization procedure for urinary creatinine

The derivatization procedure applied to creatinine in the present study for GC–MS analysis is based on the derivatization procedure originally reported by one of our groups for nitrite and nitrate in various biological systems including human urine, plasma and serum [27]. Maximum derivatization yield of creatinine in 100-μl

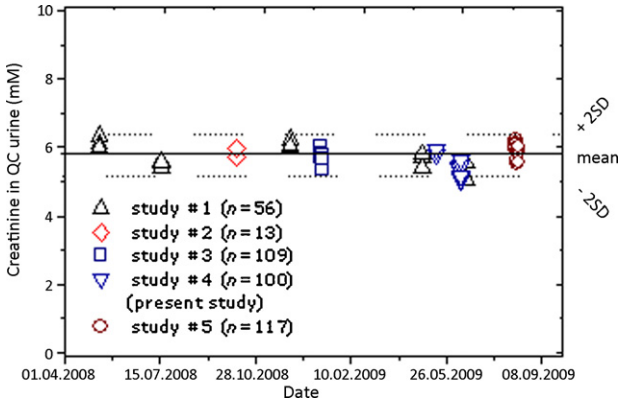


Fig. 2. Time course of the creatinine concentration measured by the present GC–MS method in unspiked urine samples used as quality control (QC) samples in the frame of clinical studies performed within the period May 2008 to July 2009. The concentration of the internal standard d_3-creatinine in all urine samples was 10 mM.

Table 2
Intra-assay accuracy (recovery) and imprecision (RSD) of the GC–MS method for creatinine in urine [a] samples of two healthy subjects with different basal concentrations.

Creatinine added (mM)	Creatinine measured (mM) (mean ± SD, $n = 3$)	Recovery[b] (%)	Imprecision (%)
Subject #1			
0	1.94 ± 0.02	N.A.	0.9
1.2	3.32 ± 0.17	115	5.0
2.4	4.27 ± 0.19	97.1	4.4
4.8	6.50 ± 0.24	95.0	3.7
7.2	9.11 ± 0.24	99.6	2.6
9.4	11.5 ± 0.26	101.7	2.3
11.8	13.5 ± 0.06	98.0	0.4
Subject #2			
0	9.93 ± 0.29	N.A.	2.9
1.2	11.0 ± 0.17	89.2	1.5
2.4	12.9 ± 0.15	124	1.2
4.8	14.8 ± 0.30	101.5	2.0
7.2	16.9 ± 0.15	96.8	0.9
9.4	19.5 ± 0.25	101.7	1.3
11.8	20.7 ± 0.44	91.3	2.1

N.A., not applicable.
[a] Ten-fold dilution (v/v), 20 mM d_3-Crea.
[b] Recovery (%) is calculated as: [(creatinine measured − creatinine basal):creatinine added] × 100.

aliquots of biological samples was achieved by using 10 μl of the derivatization reagent PFB-Br and 400 μl of the solvent acetone, i.e., 4 times the volume of the creatinine-containing sample. Under these conditions a homogenous solution is obtained except for proteins which precipitate if present in the matrix. Derivatization was performed without removal of precipitated proteins.

By using a 1:10 (v/v)-diluted human urine sample (pH 5.6) spiked with 10 mM of d_3-Crea we optimized reaction temperature and time for the derivatization of urinary creatinine with PFB-Br. Like nitrite and nitrate, the reaction temperature of 50 °C was found to be optimum for creatinine. As the optimum derivatization time for nitrite and nitrate with PFB-Br had been determined in a previous study [27] to be 5 and 60 min, respectively, the derivatization time was varied in this range (5, 10, 30, 45, 60 min). The concentration of endogenous creatinine in the urine samples examined was independent of the incubation time (i.e., 8.6 ± 0.1 mM). However, the largest peak areas were obtained after 45 and 60 min of incubation. Therefore, a derivatization time of 60 min was considered optimum.

Routinely, GC–MS analysis of nitrite and nitrate as PFB derivatives is carried out on 100-μl aliquots of a biological sample [27]. Because of the considerably higher concentration of creatinine in human urine as compared to nitrate, we wanted to use 10-fold diluted urine samples (v/v) prior to derivatization of 100-μl aliquots of the diluted urine. The applicability of the method was tested for 10-fold, 2-fold and undiluted urine. We obtained very similar creatinine concentrations for diluted and undiluted urine samples, i.e. 8.46 ± 0.18 mM, 8.40 ± 0.05 mM and 8.56 ± 0.06 mM, respectively. For routine quantitative analyses we decided to use 100-μl aliquots of 10-fold diluted urine samples (see below).

As urine samples may have very different pH values which usually range between 5 and 7, the yield of the PFB derivatives of d_3-Crea and d_0-Crea was investigated using 67 mM PBS of varying pH value (range 3–9). The yield was found to depend upon the sample pH value, with maximum yield being obtained for pH values in the range 6–9. In case of dilution we routinely used 67 mM PBS, pH 7.4, with physiological saline or distilled water also yielding very comparable results (data not shown).

3.2. Validation of the GC–MS method for creatinine in urine and plasma

3.2.1. Characterization of the internal standard [methyl-$^2H_3$]creatinine

A 10-mM solution of d_3-Crea in PBS was derivatized and analyzed by SIM of m/z 112.1 and m/z 115.1. The value of

Table 3
Intra-assay accuracy (recovery) and imprecision (RSD) of the GC–MS method for creatinine in plasma[a] samples of a healthy subject across two concentration ranges.

Creatinine added (μM)	Creatinine measured (μM)(mean ± SD, $n = 3$)	Recovery[b] (%)	Imprecision (%)
Range 0–120 μM			
0	58.4 ± 2.2	N.A.	3.8
12	69.3 ± 1.3	90.8	1.9
24	85.3 ± 1.8	112	2.1
48	107 ± 0.1	101	0.1
72	125 ± 1.7	92.5	1.4
96	144 ± 1.9	89.2	1.3
120	177 ± 2.3	98.8	1.3
Range 0–1200 μM			
0	60.1 ± 2.9	N.A.	4.8
120	173 ± 9.2	94.1	5.3
240	295 ± 11.1	97.9	3.8
480	518 ± 15.7	95.4	3.0
720	777 ± 24.2	99.6	3.1
960	1015 ± 35.8	99.5	3.5
1200	1231 ± 48.3	97.6	3.9

N.A., not applicable.
[a] 100-μl aliquots, 200 μM d_3-Crea.
[b] Recovery (%) is calculated as: [(creatinine measured − creatinine basal):creatinine added] × 100.

Table 4
Intra- and inter-assay imprecision (RSD) of the GC–MS method for creatinine in human urine[a].

	Monday	Tuesday	Wednesday	Thursday	Friday	Inter-assay, Mean ± SD (mM)	RSD (%)
	Creatinine (mM)						
	9.63	9.82	10.2	9.29	9.34	9.66 ± 0.37	3.8
	9.35	9.36	9.93	9.46	9.10	9.44 ± 0.30	3.2
	9.60	9.80	9.65	9.57	9.73	9.61 ± 0.13	1.4
Intra-assay							
Mean ± SD (mM)	9.53 ± 0.15	9.66 ± 0.26	9.93 ± 0.28	9.44 ± 0.14	9.29 ± 0.17	9.57 ± 0.24	2.5
RSD (%)	1.6	2.7	2.8	1.5	1.8		

[a] Ten-fold dilution (v/v), 20 mM d_3-Crea

the peak area ratio (PAR) of *m*/*z* 112.1 to *m*/*z* 115.1 was determined to be 0.0044 ± 0.0001 for d_3-Crea, suggesting that [*methyl*-$^2H_3$]creatinine has an isotopic purity of about 99.6% at ^2H. This finding confirms the declared isotopic purity of >99 atom% ^2H. Using d_3-Crea at 10 mM, the PAR was linearly dependent upon the concentration of d_0-Crea (0–40 mM) in various matrices including PBS and PBS-diluted urine samples (data not shown), indicating the utility of d_3-Crea as an internal standard in relevant concentration ranges for creatinine.

3.2.2. Accuracy and precision of the method

The results from the validation of the GC–MS method are summarized in Tables 2–4. The concentration of d_3-Crea was chosen as 20 mM in urine and 200 μM in plasma. Urine samples we diluted 10-fold with PBS, whereas plasma samples were used without dilution. Methods accuracy and precision were evaluated for relevant concentration ranges.

Intra-assay validation was performed using two urine samples (i.e., urine #1 and urine #2) from two healthy subjects with different creatinine concentration, i.e., 1.94 and 9.93 mM, as measured by the present method. Thus, urine samples were spiked with unlabelled creatinine at added concentrations up to about 21 mM. Table 2 indicates that creatinine can be accurately and precisely measured in human urine by this GC–MS method. Linear regression analysis between measured (*y*) and added (*x*) creatinine concentration yielded the regression equations $y = 1.95 + 0.97x$ ($r = 0.99994$) for urine #1 and $y = 10.2 + 0.95x$ ($r = 0.99495$) for urine #2, revealing high linearity and accuracy in the investigated concentration range. The relative lower limit of quantitation (rLLOQ) [28] of the method for creatinine in human urine was estimated to be 12.1% from the data of Table 2 for subject #2, i.e., (1.2 mM/9.93 mM) × 100. The rLLOQ value describes that fraction of the basal concentration of an endogenous analyte that can be still determined with analytically acceptable accuracy and precision upon addition to the biological sample [28].

Intra-assay validation was performed using plasma from a healthy subject within a narrow (up to 120 μM creatinine added) and a wide (up to 1200 μM creatinine added) concentration range. Table 3 indicates that creatinine can be accurately and precisely measured in human plasma in the ranges investigated. Linear regression analysis between measured (*y*) and added (*x*) creatinine concentration yielded the regression equations $y = 59.3 + 0.94x$ ($r = 0.99633$) for the narrow and $y = 57.2 + 0.99x$ ($r = 0.99971$) for the wide concentration range. These data reveal that the GC–MS method possesses high linearity and accuracy in human plasma of normal and elevated creatinine concentrations. In eight healthy adult subjects we measured by this GC–MS method creatinine plasma concentrations of 86.5 ± 19.4 μM (range 57–115 μM).

Using the urine sample #2 (see above) the inter-assay precision of the method was investigated within a working week. In parallel we also investigated the inter-assay precision of the method for a plasma sample of a healthy subject (see above). The data of Tables 4 and 5 indicate that the present GC–MS method possesses analytically satisfactory precision for urine and plasma creatinine.

To determine the LOD value of the GC–MS method we diluted with toluene the toluene phase of a derivatized urine sample spiked with 10 mM d_3-Crea. Assuming complete derivatization of d_3-Crea and quantitative extraction of the PFB derivative, 500 amol injected 5 times in the GC–MS apparatus using the Optima δ6 column and analyzed in the SIM mode were detected at a signal-to-noise (S/N) ratio of 14:1 with an imprecision (RSD) of 18% for the peak with *m*/*z* 115, suggesting an LOD value of about 100 amol (S/N 3:1) of creatinine.

3.2.3. Quality control for urinary creatinine in clinical studies

For use as quality control (QC) in the frame of clinical studies we collected urine for 24 h and stored on ice until fractionation into 1-ml aliquots which were then stored at −18 °C. Creatinine in urine samples from clinical studies were analyzed in parallel with at least two QC samples. From May 2008 to July 2009 creatinine was analyzed in 395 urine samples in total from 4 clinical studies and from the inter-laboratory study in the present work. In QC and clinical study samples d_3-Crea was used at a final concentration of 10 mM, and all samples were diluted 10-fold with PBS. Fig. 2 shows the results of the analysis of the QC samples in the respective period. The creatinine concentration in the entire series of the QC samples was 5.74 ± 0.29 mM which indicates an inter-assay imprecision (RSD) of 5.1%.

Table 5
Intra- and inter-assay imprecision (RSD) of the GC–MS method for creatinine in human plasma[a].

	Monday	Tuesday	Wednesday	Thursday	Friday	Inter-assay, Mean ± SD (μM)	RSD (%)
	Creatinine (μM)						
	52.9	58.7	51.9	56.1	57.6	55.4 ± 2.9	5.2
	57.7	58.3	56.8	55.5	60.7	57.8 ± 1.9	3.3
	58.9	56.9	52.1	55.4	61.8	57.0 ± 3.6	6.3
Intra-assay							
Mean ± SD (μM)	56.5 ± 3.2	58.0 ± 0.9	53.6 ± 2.8	55.7 ± 0.38	60.0 ± 2.2	56.7 ± 2.5	4.4
RSD (%)	5.7	1.6	5.2	0.7	3.7		

[a] 100 μl plasma, 200 μM d_3-Crea

3.3. Methods comparison

3.3.1. Comparison of two GC columns

By using the current GC–MS method, most creatinine analyses were performed on Optima 17 GC columns (phenylmethylpolysiloxane, 50% phenyl) on which the PFB derivatives of creatinine showed considerable tailing (see below). Upon the recent commercial availability of the Optima δ GC columns, which possess autoselectivity according to the manufacturer (www.mn-net.com/tabid/5788/default.aspx), we compared the Optima 17 with the Optima δ6 of the same length, inner diameter and film thickness. Twenty-six urine samples of the inter-laboratory study (see Section 3.5) were analyzed consecutively in duplicate under practically identical GC–MS conditions. GC–MS chromatograms from the analysis of the same urine sample by Optima 17 and Optima δ6 GC columns are shown in Fig. 3. The PFB derivative of creatinine emerged from the Optima δ6 column as a symmetric peak and earlier than from the Optima 17 column. In addition, using the same electron multiplier voltage the area of the creatinine peak was considerably higher using the Optima δ6 column as compared with the Optima 17 column. Fig. 4 shows a very close correlation ($r = 0.99974$) between these columns, with the Optima δ6 column, however, yielding creatinine concentrations being consistently lower (by about 14%) compared to those obtained by the

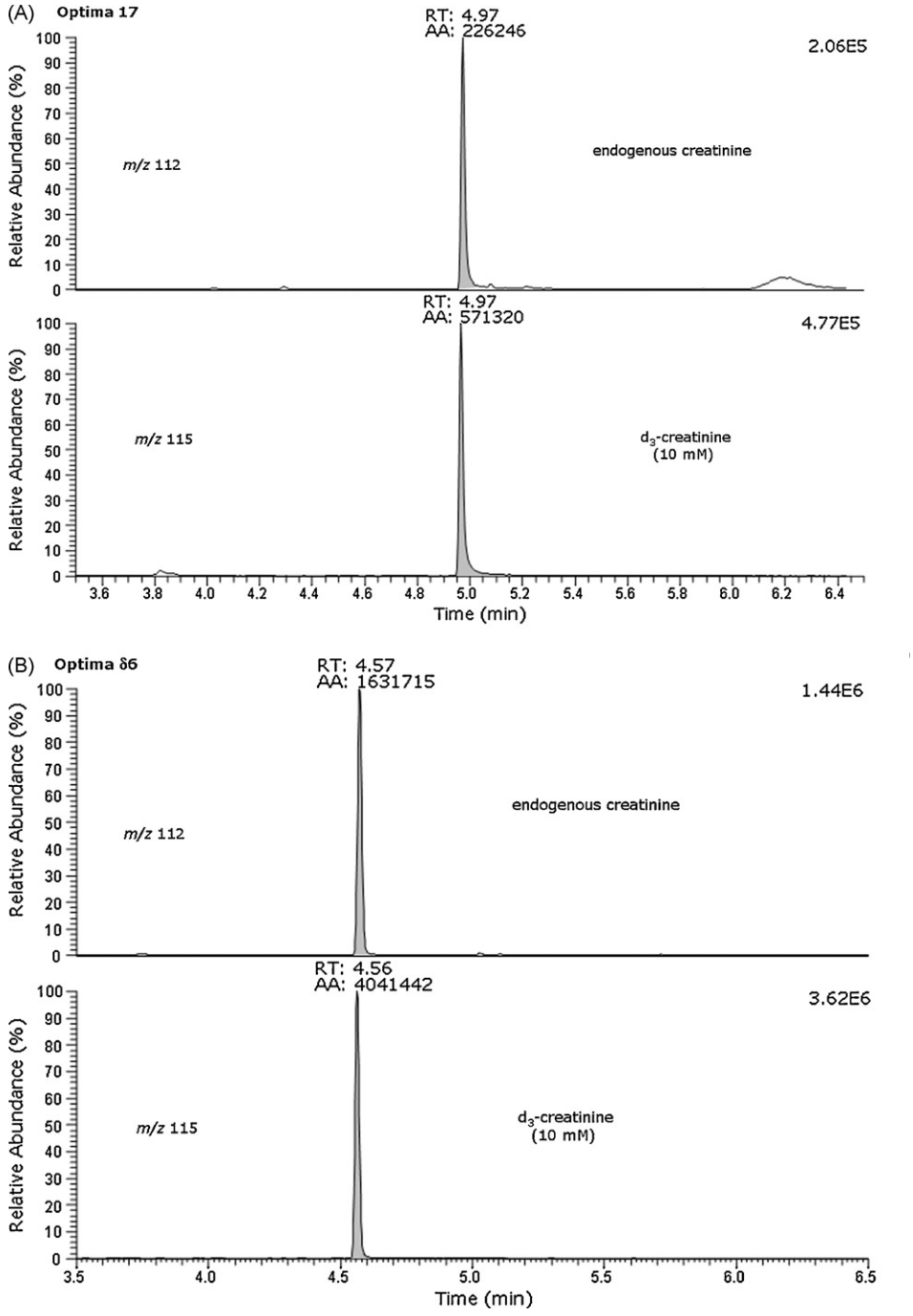


Fig. 3. Partial GC–MS chromatograms from the quantitative determination of creatinine in urine of a healthy subject as PFB derivative in the ECNICI mode using two fused-silica capillary columns of different polarity, i.e., Optima 17 (A) and Optima δ6 (B). Selected-ion monitoring of *m*/*z* 112.1 for endogenous creatinine (upper tracing) and *m*/*z* 115.1 for the internal standard d_3-creatinine (lower tracing) which was added to the urine sample at a final concentration of 10 mM.

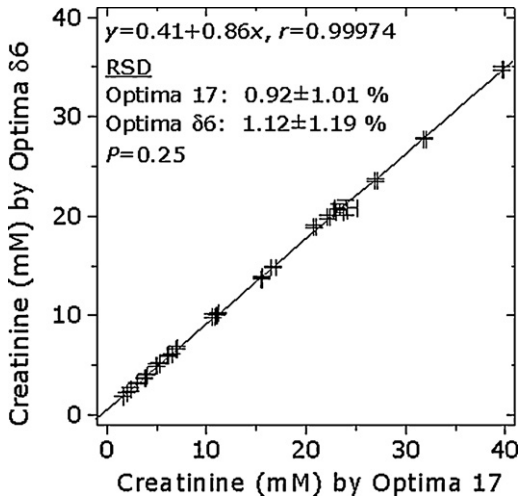


Fig. 4. Correlation between the creatinine concentrations measured in 26 urine samples by GC–MS using the Optima 17 and the Optima δ6 GC columns.

Optima 17 column. We have no explanation for this unexpected finding. It is possible that Optima δ6 and Optima 17 columns require different GC conditions for optimum analysis. It should be noted that Optimum 17 columns were used in method validation. Due to the symmetric peak of the PFB derivative of creatinine on the Optima δ6 we have assumed that this column would be associated with a considerably higher precision as compared to the Optima 17. However, the precision (RSD, %) by which the 26 urine samples were analyzed was not statistically significantly higher with the Optima δ6 column as compared with the Optima 17 column (0.92% versus 1.12%, $P = 0.25$; see Fig. 4).

3.3.2. Comparison of GC–MS with GC–tandem MS

Urine and plasma samples ($n = 176$ in total) which were first analyzed by GC–MS in the present study were subsequently analyzed by GC–tandem MS in the selected-reaction monitoring (SRM) mode, i.e., by monitoring the same product ion at m/z 41 which was formed from the precursor ions at m/z 112 for endogenous and externally added creatinine and at m/z 115 for the internal standard. With exception of very few samples there was a very good agreement between the GC–MS and GC–tandem MS results. In pair analysis revealed that, in average, the GC–MS provided data which were only by 2.8% lower than those obtained by GC–tandem MS. This finding suggests that the GC–MS method is free of interferences and allows accurate quantitative determination of creatinine in human urine and plasma samples (see however below).

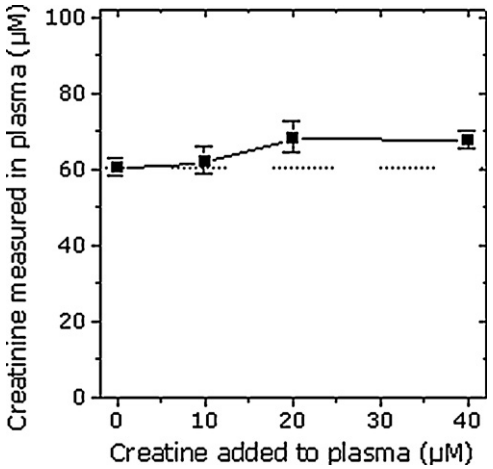


Fig. 5. Contribution of externally added creatine to plasma creatinine analyzed as PFB derivative by the present GC–MS method.

3.4. Interference studies

Creatine, the precursor of creatinine, occurs physiologically in serum and urine. Creatine and creatinine concentrations are sim-

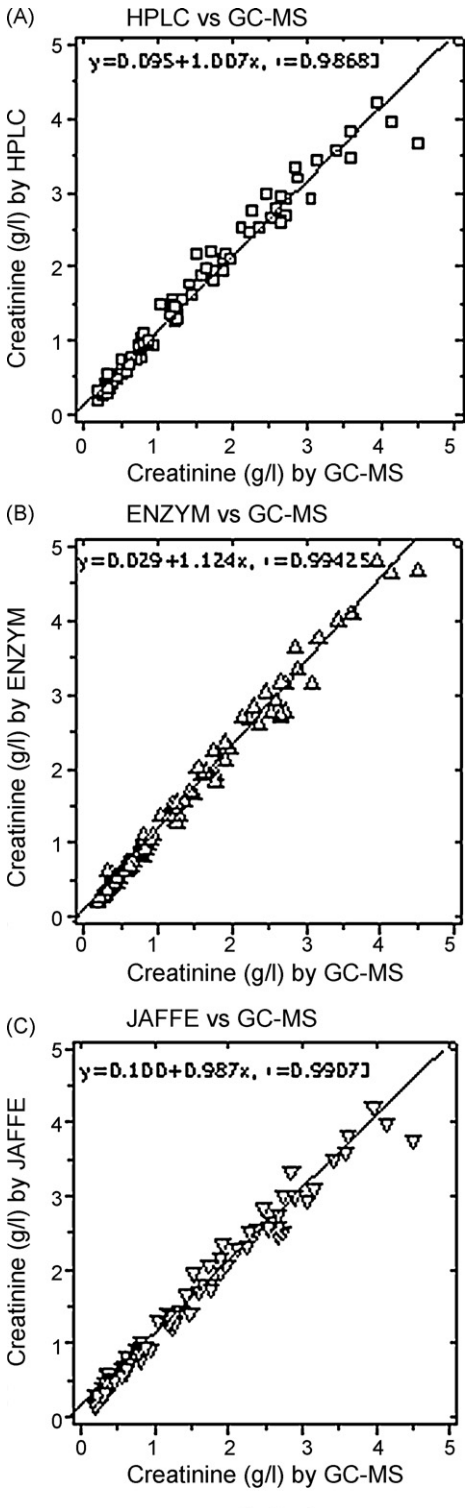


Fig. 6. Inter-laboratory study – linear regression analysis. Linear regression analysis between the urinary creatinine concentrations measured in the inter-laboratory study by the tested assays and those measured by the present GC–MS method serving as the reference method. See also Fig. 7.

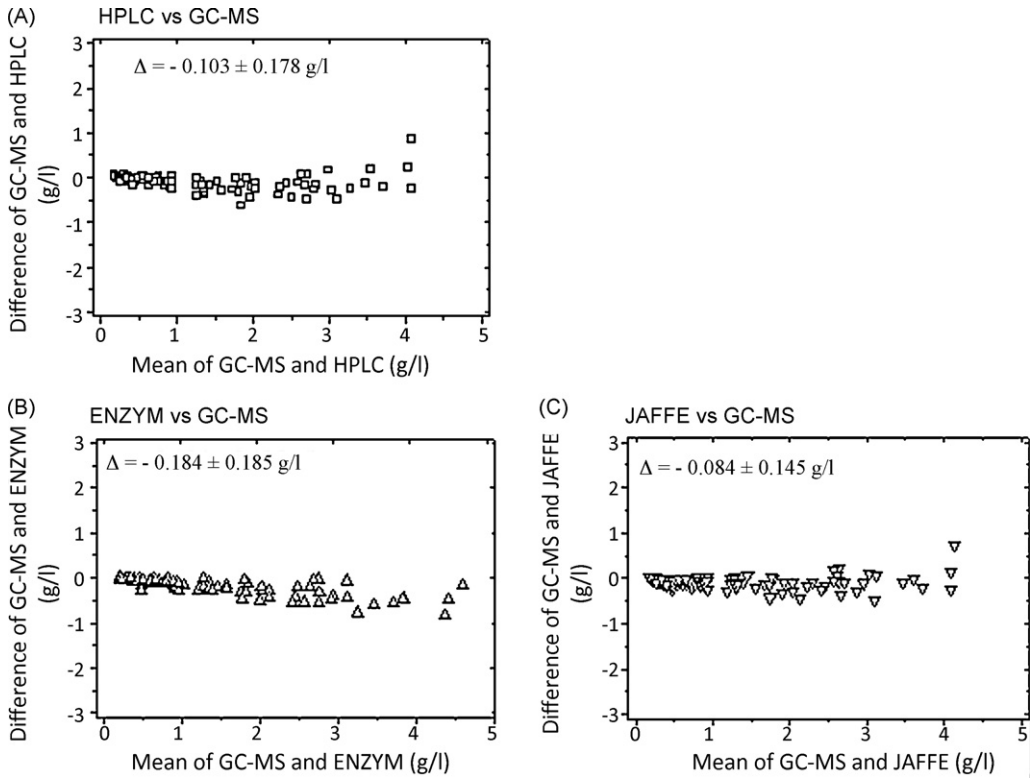


Fig. 7. Inter-laboratory study – Bland–Altman approach. Bland–Altman plots for urinary creatinine concentrations measured in the inter-laboratory study by the tested assays and those measured by the present GC–MS method serving as the reference method. Δ is the difference between the compared methods. See also Fig. 6.

ilar in serum, whereas the excretion rate of creatine in urine is about twenty times lower than that of creatinine, for instance about 0.7 mmol/24 h [29] versus 13 mmol/24 h. In aqueous solution creatinine and creatine are in a pH-dependent equilibrium. At a pH value of 6.2 the creatinine-to-creatine molar ratio has been determined to be 1.11:1 [30]. We investigated by the present GC–MS method a potential contribution of creatine to creatinine. A possible interference by the structurally related 1-methylhydantoin was also evaluated. However, 1-methyl-hydantoin was found not to interfere with the creatinine measurement (data not shown).

PFB-Br derivatization of creatine (0–200 μM) and d_3-Crea (200 μM) in 67 mM PBS, pH 7.4, and GC–MS analysis confirmed the formation of the PFB derivative of creatinine from creatine. Linear regression analysis of measured creatinine (y) versus added creatine (x) resulted in the regression equation $y = -0.14 + 0.19x$ ($r = 0.99835$), suggesting a relatively constant contribution of about 19% of creatine to creatinine under the chosen conditions. GC–MS analyses of mixtures of 10 mM creatine, 10 mM of d_0-Crea and 10 mM d_3-Crea in 67 mM PBS of varying pH values in the range 4–9 resulted in a d_0-Crea concentration of 13.5 ± 0.5 mM (RSD, 3.7%), suggesting a mean contribution by 35% of creatine to creatinine. The relatively constant contribution of creatine to creatinine in the pH range 4–9 is in agreement with the creatine/creatinine equilibrium reported by Edgar and Shiver [30].

Samples from pooled plasma were spiked with creatine at concentrations of 0, 10, 20 and 40 μM, and analyzed for creatinine by the present GC–MS method in triplicate. Fig. 5 shows that synthetic creatine contributes to creatinine by 1.6 μM (1.16%), 7.6 μM (38%) and 7.3 μM (18%), respectively, when measured as PFB derivative by this GC–MS method. The discrepancy between plasma and aqueous buffered solutions suggests that creatine-contribution to creatinine is likely to be the result of two or more different but interdependent factors, including the pH-dependent creatine/creatinine equilibrium [30] and the likely reaction of creatine with PFB-Br. Presumably, creatine can react with PFB-Br through its carboxylic as well as through its amino/imino groups (see Scheme 1). Reaction of creatine with PFB-Br through its amino/imino groups is likely to decrease the extent of creatine-contribution to creatinine, because such a PFB derivative would be not able to further react intramolecularly to form the Crea-PFB derivative. On the other hand, however, reaction of the carboxylic group of creatine with PFB-Br would form an activated PFB ester which could be attacked by the free amino group to finally form the Crea-PFB derivative. It cannot be excluded that these reactions take place differently in plasma than in aqueous buffers such as in PBS.

Table 6
Summary of the results from the inter-laboratory comparison study on creatinine in human urine using the GC–MS method as the reference method.

(A) Linear regression analysis					
HPLC	$y = 0.095 + 1.007x$,	$r = 0.98683$			
ENZYM	$y = 0.029 + 1.124x$,	$r = 0.99425$			
JAFFE	$y = 0.100 + 0.987x$,	$r = 0.99073$			
(B) Bland–Altman method					
	Mean	Difference	Linear regression of (y) versus (x)		
HPLC	1.301 ± 1.075	−0.103 ± 0.178	$y = -0.076 - 0.021x$	$r = -0.125$	$P = 0.216$
ENZYM	1.344 ± 1.135	−0.184 ± 0.184	$y = -0.019 - 0.122x$	$r = -0.753$	$P < 0.001$
JAFFE	1.293 ± 1.063	−0.084 ± 0.145	$y = -0.089 + 0.043x$	$r = +0.031$	$P = 0.758$

3.5. Inter-laboratory comparison

Three creatinine assays were compared with the present GC–MS method for creatinine in urine samples from 100 healthy subjects and evaluated by using linear regression analysis as well as the Bland–Altman method [31]. The results of the inter-laboratory study are shown in Figs. 6 and 7 and are summarized in Table 6. These results indicate close correlations between the tested creatinine assays and the GC–MS method reported here, with correlation coefficients ranging between $r = 0.987$ and $r = 0.994$ and curve slopes ranging between 0.987 and 1.124. The deviations are higher at creatinine concentrations above 3.0 g/l. Using the Bland–Altman approach (Fig. 7B; Table 6) a low but systematic difference in the results of about +12% was observed for the automated enzymatic assay in comparison with the GC–MS method. However, these differences were small and within the analytical imprecision range of the methods applied. This result is indicative of the consistent and successful certification of the Jaffé method and the enzymatic assay in round robins of the German External Quality Assurance Scheme. The inter-laboratory study suggests that each of the creatinine assays tested is suitable for the reliable quantification of creatinine in human urine across the relevant concentration range.

4. Conclusions

Knowledge of the creatinine concentration in urine is of eminent importance in clinical and occupational or environmental medical studies where urine is mostly collected after spontaneous micturition. In this article we report on a new GC–MS method which is based on the derivatization of the imino group of endogenous creatinine and the commercially available deuterium-labelled internal standard with PFB-Br, either in the native sample or in diluted urine. The procedure also applies to human plasma or serum and saliva. This method should be applicable to any biological matrix, provided derivatization with PFB-Br is performed at pH values around 7. The GC–MS method is accurate and precise and is a candidate for a reference method for creatinine in human urine.

The inter-laboratory comparison of creatinine assays, including the most frequently used photometric Jaffé method, with the GC–MS method revealed that all tested creatinine assays are virtually equally suited for creatinine measurement in human urine specimens and are recommendable for biomonitoring purposes as well as for use in clinical studies. The Jaffé method for creatinine [8] has been reported more than 120 years ago. There exist numerous modifications of the original method which belong for several decades to the most frequently used creatinine assays. In consideration of the importance of urinary creatinine concentration in occupational and environmental medicine as an exclusion criterion for urine specimens, and in clinical studies, adoption and validation of Jaffé assays and performance of quality control are advisable to any laboratory interested in creatinine measurement. This can be done for example by using certified reference material or by participating in round robins.

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