ENZYMATIC SYNTHESIS OF [METHYL-^{2}H$_3$] CREATININE

C. Arcelloni, R. Paroni, P.A. Bonini,

F. Magni and M. Galli Kienle[#]

Scientific Institute H. San Raffaele, Via Olgettina 60, 20132 Milan, Italy. # Department of Medical Chemistry and Biochemistry, University of Milan, Milan, Italy.

SUMMARY

2-Amino-1,5-dihydro-1-[methyl-^{2}H$_3$] 4H-imidazol-4-one ([methyl-^{2}H$_3$] creatinine) was obtained by acidification and heating (120 °C, 120 min) of the [methyl -^{2}H$_3$] N-amidinosarcosine (creatine) synthesised by methylation of guanidoacetate (GA) with S-adenosyl-[methyl-^{2}H$_3$] L-methionine ([methyl-^{2}H$_3$] AdoMet) in the presence of rat liver guanidoacetate methyltransferase (GAA-MT). Isotopic enrichment was 94.7 %.

INTRODUCTION

"Definitive Methods" are those by which the analyte is measured with the highest accuracy and precision and, in this respect, Isotope Dilution - Mass Spectrometry (ID-MS) allows one

to obtain definitive concentration values for various   analytes.
The particularly high accuracy of methods based on this technique
depends upon the use, as internal standard, of the same analyte
to be quantifiably labelled with stable isotope(s). The addition
of the standard occurs before any manipulation of the sample and
therefore the ratio between the labelled and unlabelled compound
remains constant during the entire procedure. Different ID-GC-MS
methods have been already developed for the determination of
creatinine (1,2), a very critical analyte for the clinical che-
mist, because routine methods are affected by various interferen-
ces (3). In addition, several "Reference Methods" based on High
Performance Liquid Chromatography (HPLC) were set up for this
compound, which are more accurate and precise than the routine
methods (4-8). Since the association of liquid chromatography
with mass spectrometry (LC-MS) combines the simplicity of LC
with the accuracy and specificity of the mass spectrometric de-
tection, we became interested in studying the conditions of crea-
tinine analysis by this technique.

    To reach this goal, the first step is the synthesis of a
suitable isotopically labelled internal standard. This paper de-
scribes the enzymatic synthesis of [methyl-^{2}H$_3$] creatine, its
transformation into creatinine, the HPLC purification and the LC-
MS analysis of the deuterated compound.

EXPERIMENTAL

Reagents and apparatus

    Commercial [^{2}H$_3$] iodomethane (99.5-atom percent enrichment)
was purchased from MDS Isotopes, Germany. The resins Sephadex
G-75, Hydroxyapatite and AG 50W-4X (100-200 mesh, hydrogen form)
were from BioRad (Richmond, CA, USA). Standard Reference Material
(SRM 914a) creatinine was from the National Institute of Stan-
dards and Technology (NIST, Gaithersburg, MD 20899), while crea-
tine and all other reagents for enzymatic synthesis were from

Sigma (St.Louis, MO). Solvents "HPLC-grade" were from Merck (Darmstadt, Germany). Authentic S-adenosyl-L-homocysteine (SAH), S-adenosyl-L-methionine sulfate and S-methylthioadenosine (MTA) were kindly supplied by Bioresearch S.p.A. (Liscate, Italy). Sprague-Dawley rats used to obtain the liver enzyme were from Charles River (Calco, Italy). Centricon 10 (10000 MW cut-off) ultrafiltration devices (Amicon, Denver, MA, USA) were used for protein fractions concentration. Proteins were evaluated with the method of Bradford (9).

The HPLC analyses were performed with a low pressure apparatus mod 1050 (Hewlett Packard, Palo Alto, CA) connected to a diode-array multi channel detector Jasco mod 320 and to a normal phase column Supelcosil LC-Si (250X2.1 mm, 5μ) (Supelco Inc., Bellefonte, CA). The eluent was an ethanol:methanol (70:30, v:v) mixture at a flow-rate of 0.4 ml/min.

For the analysis of deuterated AdoMet an ion exchange column Whatman Partisil SCX (250X4 mm, 10 μ) was used under previously described conditions (10).

Mass spectrometry was performed with a quadrupolar mass spectrometer Hewlett Packard HP 5988A, equipped with a particle beam interface PB 59880A and a low pressure HPLC pump HP 1050. Analyses were carried out in Positive Ion Chemical Ionization (PICI) mode with methane as chemical reactant gas, at an electron energy of 100 eV and with a source temperature of 200 °C. The particle beam desolvation chamber temperature was 55 °C, the helium pressure was 35 Psi and the source pressure 1 torr.

NMR analysis was carried out on a Bruker AM-500 spectrometer. The spectrum was registred in D_2O at 303 K.

Synthesis of S-adenosyl-[methyl-²H₃] L-methionine ([Methyl-²H₃] AdoMet)

[Methyl-²H₃] AdoMet sulfate was synthesised by methylation

of SAH with [^{2}H$_3$] iodomethane according to the method of Matos

et al. (11). The labelled AdoMet was freeze-dried and was then

stored at -40 °C until used. The sulfate showed a single peak

when analyzed by ion exchange HPLC under previously reported con-

ditions (10). The almost complete absence of the signals of the

methyl group of AdoMet (2.80 and 2.83 ppm; H$_2$O signal at 4.55

ppm) in the NMR spectrum of the deuterated analogue, confirmed

the high isotopic enrichment.

Purification of the enzyme guanidinoacetate methyltransferase
(E.C. 2.1.1.3) from rat liver

The enzyme involved in the transfer of the methyl-[^{2}H$_3$]

rat liver as previously described (12, 13) with some modifica-

tions.

Rat livers were rapidly collected after the sacrifice, rin-

sed in a buffer solution (sodium acetate 75 mM, pH 5.0), weighed

and homogenized (2.5:1, v:w) in a Waring blender with the same

buffer. After centrifugation at 10000 g for 30 min the superna-

tant was separated and precipitated with ammonium sulphate (19.5

g/100 ml). After centrifugation (10000 g for 15 min) the precipi-

tate was removed and discarded, while the supernatant was again

purified by ammonium sulphate precipitation (10.5 g/100 ml). The

precipitate was then redissolved in a small volume of 10 mM po-

tassium phosphate buffer (pH 7.4) containing 1mM dithiothreitol

and 1 mM EDTA and dialyzed against the same buffer for 2 h. The

enzyme was then adsorbed on a column of Sephadex G-75 (1.2X50

cm) equilibrated with 5 mM potassium phosphate (pH 7.4), 20 mM

KCl, 1 mM dithiothreitol, and 1 mM EDTA at a flow-rate of 0.6

ml/min and connected to a UV detector set at 280 nm. The column

was previously calibrated with a mixture of bovine serum albumin

(MW 67000), ovalbumin (MW 43000), chymotripsinogen A (MW 25000)

and ribonuclease A (MW 13700) (Fig 1). Fractions containing the

enzyme (MW 31000) were collected, concentrated by ultrafiltra-
tion and frozen at −80 °C. Under these conditions the prepara-
tion was stable for several months. Protein evaluation was car-
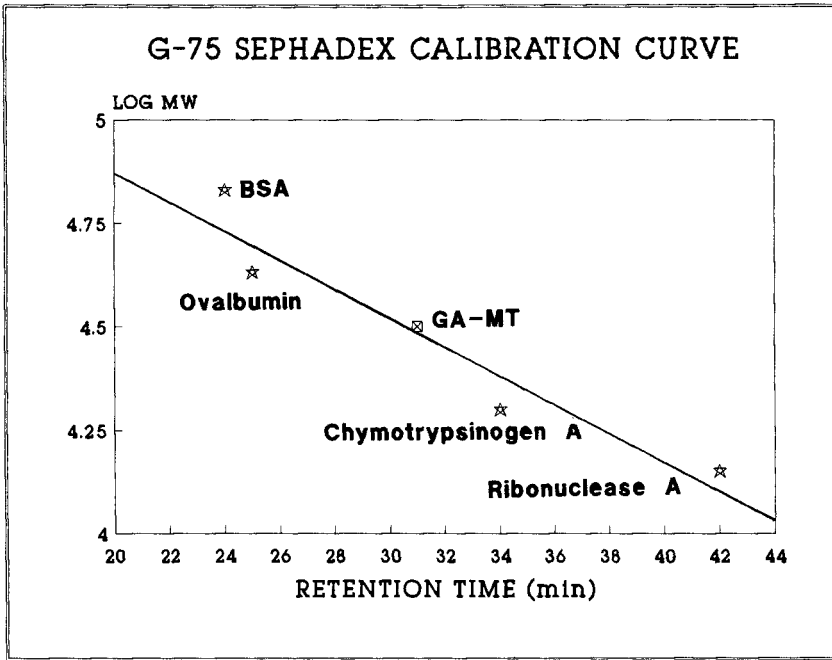ried out after each step of purification.

G-75 SEPHADEX CALIBRATION CURVE

LOG MW

☆ BSA

☆
Ovalbumin

⊠ GA-MT

☆
Chymotrypsinogen A

Ribonuclease A   ☆

RETENTION TIME (min)

FIGURE 1: Calibration curve for Sephadex G-75 (1.2 x 50 cm) size
exclusion chromatography column. Standard protein mixture (1 ml):
BSA (7mg/ml), ovalbumin (7mg/ml), chymotripsinogen A (3mg/ml),
ribonuclease A (10mg/ml). Mobile phase 5 mM potassium phosphate
(pH 7.4), 20 mM KCl, 1 mM dithiotreitol, 1 mM EDTA. Flow rate 0.6
ml/min, detector UV 280 nm.

RESULTS

Synthesis of [methyl-²H₃] creatinine

[Methyl-²H₃] Creatine was enzymatically synthesised from

[methyl-^{2}H$_3$] AdoMet and guanidinoacetate. By acidification and heating creatine was then cyclized to creatinine (Fig. 2).
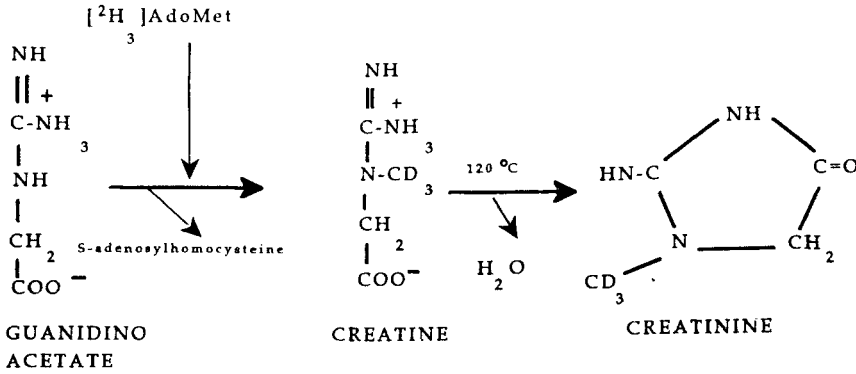

FIGURE 2: Enzymatic synthesis of creatinine

In order to obtain the maximum yield of creatinine forma-tion, we studied the reaction kinetics at various protein/sub-strate ratios (Fig. 3). Among those tested, the optimal condi-tions were: 180 min incubation at 37°C with 5 mg of proteins in a final volume of 0.25 ml. Incubations were carried out in the pre-sence of 2.5 mM final concentration of deuterated AdoMet, 3 mM guanidoacetate, 8 mM GSH and 2 mM potassium phosphate, pH 7.4. The reaction was stopped by adding 0.2 ml of 10% TCA. After cen-trifugation, the supernatant was neutralized with 0.25 ml of Tris buffer 1 M and purified on an ion exchange column AG 50W-X4, H$^+$ form, 100-200 mesh (2.5X1cm). The resin was previously equili-brated with 3 M ammonia followed by neutralization with water. The sample was adsorbed onto the column and eluted with water (4 X 1ml). Fractions 2 and 3, containing [methyl-^{2}H$_3$] creatine, we-re pooled, acidified (1 M HCl, 80 μl), heated at 120°C for 120 min and quantified by the Jaffe' method (14). The deuterated standard was dried under vacuum for storage, and redissolved in methanol before use.
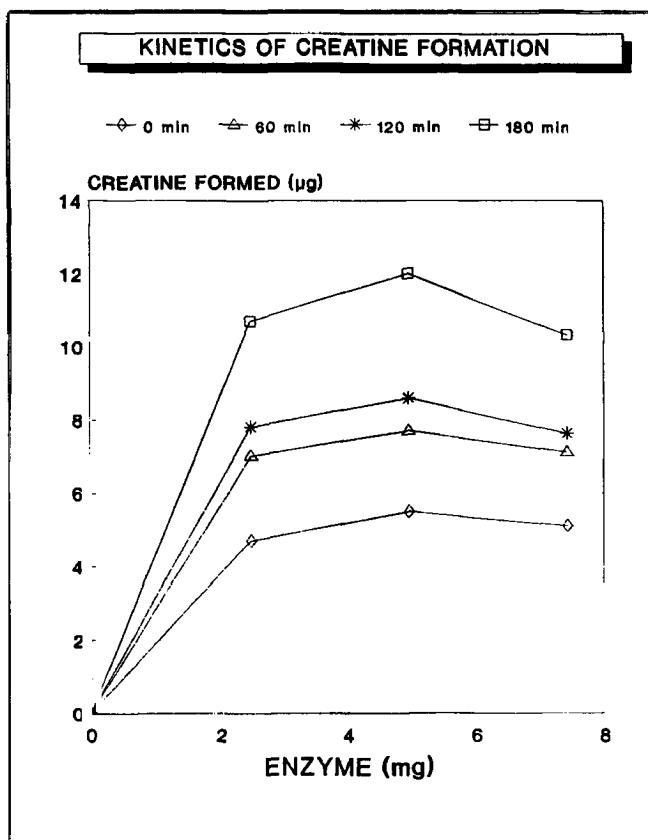
FIGURE 3: Kinetics of creatine formation carried out for 0, 60, 120, 180 min with different concentrations of GAA-MT. Incubation conditions: deuterated AdoMet 2.5 mM, guanidinoacetate 3.0 mM, GSH 8.0 mM, in a final volume of 0.25 ml of potassium phosphate (10 mM, pH 7.4). Temperature 37°C.

Purification, identification and quantification of [methyl-^{2}H$_3$] creatinine

Labelled creatinine recovered as described above from a preparative incubation (final volume 75 ml) was further purified by HPLC with a semi-preparative silica column under the conditions already described. Chromatographic analysis and absorbance

spectrum of the NBS standard creatinine and of the synthesised deuterated compound are reported in Fig. 4, and show a very similar behaviour of the latter with the authentic non-labelled standard. Under the described HPLC conditions creatinine showed a retention time (R_t) of 4.4 min, while creatine was not detected within 20 min.

The identity of [methyl-^{2}H$_3$] creatinine was checked by particle beam CI-LC-MS (Fig. 5A). Selected Ion Monitoring (SIM) analysis at m/z 114 and 117 gave an enrichment of the 94.7±0.3 %
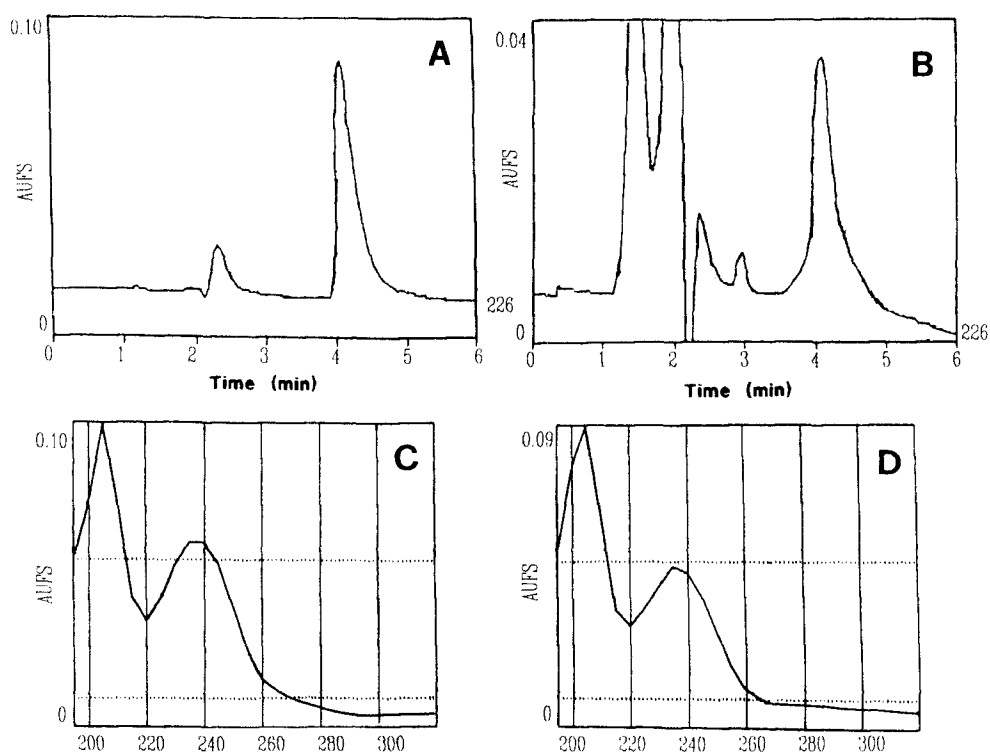

FIGURE 4: Panel **A**: HPLC analysis of NBS creatinine standard (20 μl= 400 ng). Column SupelcoSil LC-Si (250 x 2.1 mm), mobile phase ethanol:methanol (70:30, v:v), flow rate 0.4 ml/min. Detector diode-array multi channel Jasco mod. 320. Panel **B**: HPLC analysis of [methyl-^{2}H$_3$] creatinine. Analytical conditions as in Panel A. Panel **C** and **D**: absorbance spectra (200-300 nm) of creatinine and deuterated creatinine, respectively.

(mean±SE, n=3), expressed as atom percent excess (APE) (15) (Fig. 5B).

After a rough quantification by HPLC, the exact quantification of the deuterated standard synthesised was assessed by means of a calibration curve constructed adding increasing amounts of
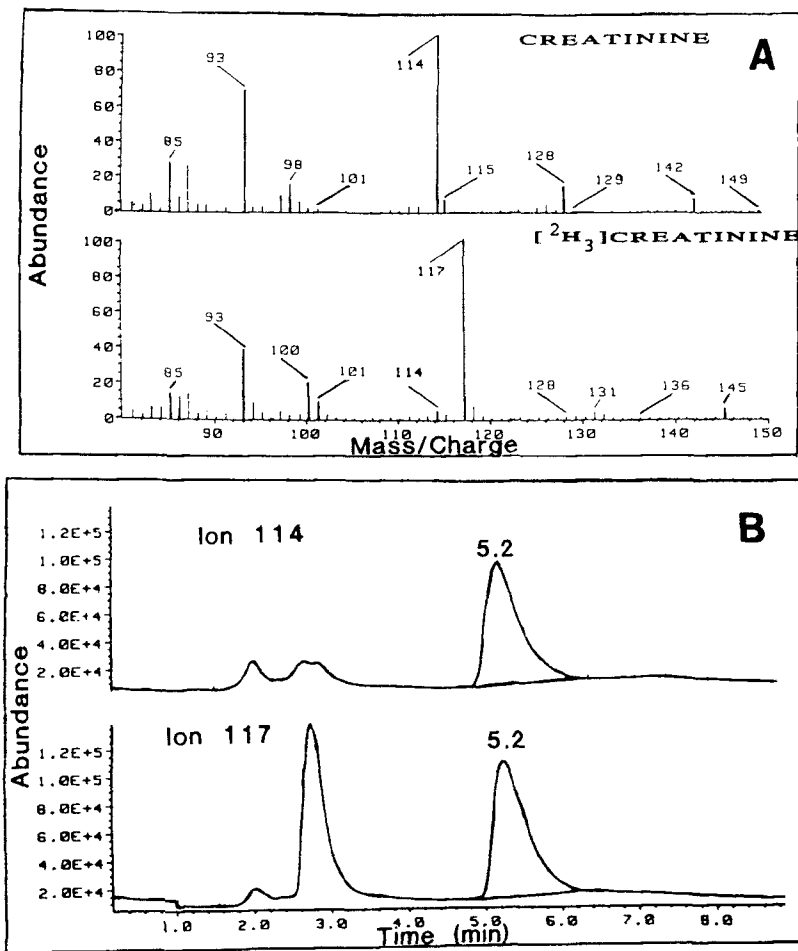

FIGURE 5: Panel **A**: Mass spectra of standard creatinine (upper part) and deuterated creatinine (lower part) obtained in Positive Ion Chemical Ionization with PB-LC-MS. Ion source temperature 200 °C; electron energy 100 eV; PB desolvation temperature 43 °C; He pressure 35 Psi; source pressure 1 torr. Panel **B**: Selected Ion Monitoring traces of creatinine and [methyl-²H₃] creatinine; analytical conditions as in panel A.

non-labelled creatinine to a costant amount of the unknown deute-
rated solution, in the range 0.8-1.2 of $[H_3]/[^2H_3]$ ratio. The
curve (Fig. 6) was found to be linear in the tested range.

The reaction yield calculated under the described condi-
tions was 14.2 %, relative to the deuterated AdoMet when the en-
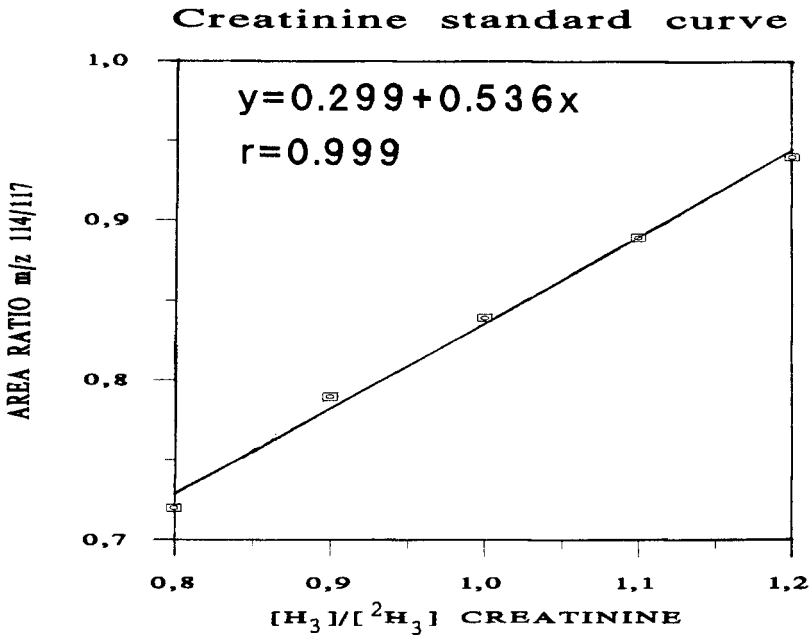zyme fraction deriving from the Sephadex purification was used.

Creatinine standard curve

$$y = 0.299 + 0.536x$$
$$r = 0.999$$

AREA RATIO m/z 114/117

$[H_3]/[^2H_3]$ CREATININE

FIGURE 6: Creatinine standard curve obtained adding increasing a-
mount of non-labelled creatinine to a constant amount of unknown
deuterated solution. $[H_3]/[^2H_3]$ creatinine range: 0.8-1.2.

DISCUSSION

Creatinine is one of the analytes that causes major problems
in clinical laboratories. Results obtained quantifying creatinine
in a sample by different methods markedly differ from each other
and, therefore, there is the need to obtain absolute methods
to certify the concentrations of standards (16). ID-MS is the

most suitable technique to reach the goal of a "definitive" con-
centration value but precision and linearity of this determina-
tion are higher when the molecular weight of the internal stan-
dard differs from that of the unlabelled analyte by more than one
mass unit.

Many labelled analogues of creatinine were synthesised for
this pourpose. Bjorkhem et al. (2) described the synthesis of
$[^{15}N_2]$ creatinine that they utilized for the quantification of
creatinine after conversion into the di-trifluoroacetate of the
(2-hydroxy, 2-methyl)ethyl derivative. Sieckmann (1) used instead
$[^{13}C,^{15}N_2]$ creatinine as internal standard in GC-MS analysis
after formation of creatinine trimethylsilyl derivative. $[^{13}C_2]$
Creatinine was also used as internal standard by Welch et al.
(17) who performed GC-MS analysis after its conversion into the
ethyl ester of N-(4,6-dimethyl-2-pyrimidinyl)-N-methylglycine.
According to these authors attempts to use $[methyl-^2H_3]$ creati-
nine as internal standard gave inconsistent results possibly due
to an isotope effect of deuterium occurring during the derivati-
zation step.

We are interested in the developement of a method to be pro-
posed as Reference Method for creatinine analysis. The LC-MS ap-
paratus, giving wider range of applications than a GC-MS instru-
ment, may be more widely used in the clinical laboratories and
allows the direct analysis of the deproteinized serum, avoiding
the derivatization step. Under the above described conditions,
deuterated creatinine was found to be a suitable internal stan-
dard; imprecision described by other authors with ID-GC-MS (17),
and attributed to the isotope effect of deuterium was not obser-
ved. Moreover the direct analysis of creatinine by LC-MS was not
influenced by the presence of residual creatine which, besides
having a different molecular weight (MW 131), is not detectable
under the LC conditions employed.

In respect to previously described chemical syntheses the enzymatic preparation of [methyl-^{2}H$_3$] creatinine gave a lower reaction yield, but is much less expensive because deuteration is carried out with [^{2}H$_3$] iodomethane in the high yield transformation of SAH to AdoMet. Moreover, 60-70% of unreacted [methyl-^{2}H$_3$] AdoMet can be easily recovered from the ion exchange resin used to separate creatine after the enzyme incubation.

When the enzyme fraction deriving from the Sephadex column is further purified on a hydroxyapatite column, a ten times enhancement of the specific activity is obtained. Therefore in principle a higher yield might be obtained with the more purified enzyme. Nevertheless, the relevant loss of proteins occurring during hydroxyapatite purification (12, 13) is not compensated for by the advantage of an increased activity.

It is worth noting that conditions described here allow a rather rapid preparation of the samples to be injected into the MS instrument and the analysis can be carried out in 6-7 min. Moreover the well known problems of LC-MS carried out with salt solutions as the eluting mixture are avoided by the use of a direct phase analysis utilising methanol:ethanol as the eluent.

In conclusion accuracy, precision and repeatibility of the method will be easily determined and will likely reach the standard requested for a definitive method.

REFERENCES

1) Siekmann, L. - J Clin. Chem. Clin. Biochem. <u>23</u>: 1137-1144 (1985).

2) Bjorkhem, I., Blomstrand, R., Ohman, G. - Clin. Chem. <u>23</u>: 2114-2121 (1977).

3) Henkel, E., Kogenge, E., Meier, H. - Z. Anal. Chem. <u>301</u>: 175-177 (1980).

4) Zhiri, A., Hanot, O., Wellman-Bednowska, M., Siest, G.- Clin. Chem. <u>31</u>: 109-112 (1985).

5) Achari, R., Mayersahn, M., Conrad, K.A. - J. Chromatogr. Sci. <u>21</u>: 278-281 (1983).

6) Patel, C.P., George, R.C. - J. Chromatogr. <u>145</u>: 41-49 (1978).

7) Paroni, R., Arcelloni, C., Fermo, I., Bonini, P.A. - Clin. Chem. <u>36</u>: 830-840 (1990).

8) Rosano, T.G., Ambrose, R.T., Wu, A.H.B., Swift, T.A., Yadegari, P. - Clin. Chem. <u>36</u>: 1951-1965 (1990).

9) Bradford, M.M. - Anal. Biochem. <u>72</u>: 248-250 (1976).

10) Giulidori, P., Galli Kienle, M., Catto, E., Stramentinoli, G. - J Biol. Chem. <u>259</u>: 4205-4211 (1984).

11) Matos, J.R., Raushel, M.F., Wong, C. - Biotechnology and Applied Biochemistry <u>9</u>: 39-52 (1987).

12) Cantoni, G.L., Vignos Jr, P.J. - J. Biol. Chem. <u>209</u>: 647-659 (1954).

13) Im, Y.S., Chiang, P.K., Cantoni, G.- J. Biol. Chem. <u>254</u>: 11047-11050 (1979).

14) Jaffe',M. - Z. Physiol. Chem. <u>10</u>: 391-400 (1886).

15) Campbell, I.M. - Bioorg. Chem. <u>3</u>: 386-397 (1974).

16) Bjorkhem, I., Blomstrand, R., Eriksson, S., Falk, O., Kallner, A., Svensson, L., Ohman, G.- Scand. J. Clin. Lab. Invest. <u>40</u>: 529-534 (1980).

17) Welch, M.J., Cohen, A., Hertz, H.S., Ng, K.J., Shaffer, R., Van Der Lijn, P., White, E. Anal. Chem. <u>58</u>: 1681-1685 (1986).