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Note

Specific high-performance liquid chromatographic method for the determination of creatinine in rat plasma

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The determination of creatinine, the final excretion product of creatine, in various body fluids is an important diagnostic parameter in human diseases, and is often used in biochemical, pharmacological and toxicological research, also in laboratory animals.

Creatinine has been reviewed by Narayanan and Appleton [1] and recently by Spencer [2]. Various colorimetric [3–5] and enzymatic methods [2] have been introduced, but the majority of laboratories still base their determination of creatinine on the alkaline creatinine–picrate reaction, first reported by Jaffe [6], a hundred years ago. Several endogenous [7–9] and exogenous compounds [10,11] are known to interfere with the colorimetric determination of creatinine. Various techniques for the elimination of non-creatinine chromogens have been proposed, including dialysis, adsorption, treatment with strong cation-exchange resins, chemical modification and extraction [1,2]. However, interferences, often producing elevated values, still exist, even in reaction rate-based colorimetric methods. These interferences, probably additive in nature, are highly significant when low "true" concentrations have to be determined. For example, this is the case in rat plasma where the normal concentration of creatinine is less than half the value in human plasma.

During the past decade, several high-performance liquid chromatographic (HPLC) methods have been developed for the determination of creatinine. Cation-exchange and reversed-phase techniques have often been used, but other types of column have also been described [2]. None of these methods has been evaluated with respect to rat plasma.

The present paper describes a method that has been developed and evaluated

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for the specific determination of creatinine in rat plasma. The inter-animal variation, precision, accuracy, linearity and various chromatographic parameters have been investigated, and the effects have been tested of endogenous compounds known to interfere in the Jaffe procedure. The method has been applied to the determination of the half-life ($t_{1/2}$) in rat plasma of the specific activity of intravenously injected [^{14}C]creatinine.

EXPERIMENTAL

Chemicals

Creatinine was obtained from Sigma (St. Louis, MO, U.S.A.). Methanol (HPLC grade) was obtained from Rathburn (Walkerburn, U.K.). The ammonium acetate (p.a.) was obtained from Merck (Darmstadt, F.R.G.) and creatininase (EC 3.5.2.10) from Sigma. Carbonyl[^{14}C]creatinine hydrochloride (specific activity 12.0 mCi/mmol, 444 MBq/mmol, Amersham International, U.K.) was used for recovery corrections. The liquid scintillation cocktail used was Opti-Fluor™ (United Technologies, Packard). Ultrapure reagent-grade water was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

Instruments

HPLC analyses were performed on a Hewlett-Packard 1084-B liquid chromatograph equipped with a 250 mm × 4 mm I.D., 7 μm, RP-18 Hibar LiChroCART® column (Merck) protected by a 20 mm × 4 mm I.D. RP-18 guard column. Liquid scintillation counting was performed on a Packard 460 CD Tri-Carb liquid scintillation system equipped with dpm and luminescence options.

Chromatographic conditions

The sample injection volume was 75 μl. The mobile phase consisted of an ammonium acetate buffer (0.01 *M*, pH 6.50–6.60) at a flow-rate of 1.0 ml/min. The temperature of the column was 10–15 °C, and the wavelength setting was 236 nm.

For the determination of capacity factors and selectivities, tritiated water was injected and the time of elution of unretained compounds was established by collection of 5-s fractions and liquid scintillation counting. Five samples were injected for this purpose.

Animals

Wistar rats [Mol:Wist (SPF) Møllegaard Breeding Centre, Ejby, Denmark] had free access to acidified water (citric acid, pH 3.5) and a pelleted diet (Chow 101, Institute of Toxicology, National Food Agency). The room temperature was 23 ± 1 °C and the relative humidity 60 ± 5%, with six to eight changes of air per hour and artificial light from 9 p.m. to 9 a.m.

Blood samples

Blood was obtained from the periorbital plexus of rats in carbon dioxide narcosis. Lyophilized heparin (Løvens Kemiske Fabrik, Copenhagen, Denmark) was used for anticoagulation.

Procedures for preparation of samples

Routine analysis. A 50-μl volume of [^{14}C]creatinine (ca. 2000 dpm) was added to 200 μl of rat plasma. The radioactivity was determined by liquid scintillation counting on 25 μl of this mixture. The remaining plasma was deproteinized by addition of 1000 μl of methanol followed by refrigeration and centrifugation. The supernatant was evaporated to dryness under a stream of nitrogen and the residue was redissolved in 225 μl of water and filtered through Millipore HV 0.45-μm disposable membrane filters. The recovery of radioactivity was determined after counting on 25 μl of this solution. Liquid scintillation counting was performed after addition of 4 ml of Opti-Fluor and followed by quench correction and conversion of the values into dpm. The remaining sample was used for the HPLC analysis. A standard curve in the concentration range 0–400 μ*M* was established using four series of aqueous creatinine samples

Standard addition. Accuracy and linearity were investigated using 24 random samples of rat plasma, each spiked with creatinine corresponding to increases in concentration of 0, 5, 10, 15, 20, 25, 30, 35 and 40 μ*M* in the following way: 50 μl of the solution of [^{14}C]creatinine (ca. 2000 dpm) were added to 200 μl of the respective aqueous standards and evaporated to dryness. The residue was redissolved in 200 μl of rat plasma and 50 μl of water and analysed according to the procedure of routine analysis.

Plasma half-life of the specific activity of [^{14}C] creatinine. Rats in cardon dioxide narcosis were injected intravenously with an aqueous 0.9% (w/v) saline solution of [^{14}C]creatinine corresponding to 0.5 μCi. Blood samples were taken after 20, 40, 60 and 90 min. The plasma was deproteinized, evaporated and redissolved as described for the routine analysis. Then 4 ml of Opti-Fluor were added to 3×15 μl of this solution for liquid scintillation counting, and 150 μl were used for HPLC analysis.

Calculations

Capacity factors and selectivities were calculated for creatinine (peak 2, Fig. 1A) and the two neighbouring compounds (peaks 1 and 3, Fig. 1A) in the chromatogram.

Peak heights (in cm) of creatinine were corrected for recovery, after processing of the samples of plasma, for the addition of [^{14}C]creatinine and dilution.

Linear regression was performed on the four aqueous standard curves and each of the 24 plasma standard addition series, the latter without values corresponding to zero addition.

The intercept of each standard addition curve with the *y*-axis and the "measured" zero addition height were expressed as concentrations, C_c (calculated) and C_m (measured), respectively. Further, the increase in concentration corresponding to each standard addition was calculated, and the means were analysed by linear regression. All calculations were also performed with respect to integrated peak areas.

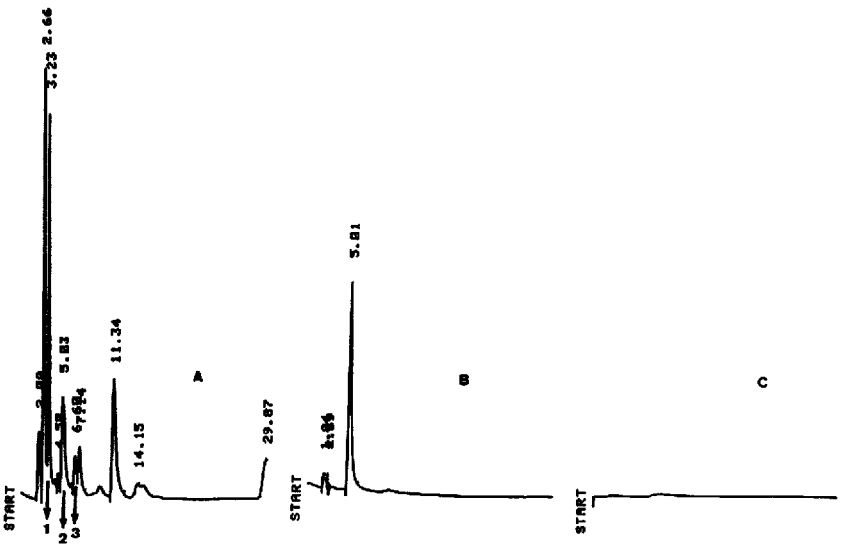

Fig. 1. Chromatograms of samples of rat plasma. Retention times are in minutes. (A) Typical chromatogram of rat plasma processed as described in the text. Peak 2, with a time of retention of 5.03 min, is creatinine, peaks 1 and 3 coelute with uric acid and tyrosine, respectively. Absorbance, 0.0128 a.u.f.s. (B) and (C) are chromatograms of isolated peak 2 obtained after collection of fractions and blank treatment or creatininase treatment, respectively. The concentration of injected material in (B) was ca. 8 μM. The treatment with creatininase was performed in a 0.3 M potassium phosphate buffer (pH 6.5) at 37°C with 5 units of enzyme activity for 1 h. Blank treatment was performed analogously but without enzyme. The reaction was stopped by addition of methanol. Absorbance, 0.0032 a.u.f.s.

RESULTS AND DISCUSSION

The recovery following deproteinization, centrifugation, decanting, drying and redissolution was 79.6±8.1% (n=216). The creatinine not recovered was present in the sediment, as confirmed by two additional washings with 225 μl of water–methanol (1:4). However, the washing procedure and the further dilution of samples for HPLC analysis were judged to be too time-consuming and inconvenient. Therefore the addition of [^{14}C]creatinine was necessary.

Stopped-flow scanning from 190 to 540 nm of aqueous standards and plasma creatinine showed maximum absorption at 236 nm under the chromatographic conditions applied. This wavelength was found to be suitable for analysis with respect to baseline stability, although stronger absorption was found below 200 nm.

The separation was optimized by varying the pH value and ionic strength of the mobile phase (details are not given here). The separation was further optimized by adjusting the temperature of the column to 10–15°C by means of a cooling jacket.

By comparing the time of retention and the stopped-flow scanning properties of samples of plasma and aqueous standards, the compounds with times of reten-

TABLE I

CHROMATOGRAPHIC PARAMETERS OF COMPOUNDS 1, 2 AND 3 IN RAT PLASMA ($n=70$)

Parameter	Value (mean ± S.D.)	Relative standard deviation (%)
k_1	0.384 ± 0.015	3.91
k_2	0.549 ± 0.021	3.83
k_3	1.007 ± 0.113	11.22
$\alpha_{2,1}$	1.429 ± 0.037	2.59
$\alpha_{3,2}$	1.833 ± 0.198	10.80
Creatinine retention time (s)	302.1 ± 4.1	1.36

tion of 3.23 (peak 1) and 6.60 min (peak 2) in Fig. 1A were identified as uric acid and tyrosine, respectively.

Table I shows the chromatographic parameters and a highly reproducible retention time of creatinine, indicating a remarkable stability of the column (ca. 1000 injections during a period of six months).

The peak heights were found to be better than the integrated peak areas with respect to reproducibility and agreement between respective concentrations.

The aqueous standard curve was linear in the range of concentration between zero and at least 400 μM. The linear regression equation was $y=0.255x-0.164$ ($r=0.9999$, $n=4$). By linear regression analysis of the means of peak heights of aqueous standards (0–40 μM) (a) and of the increases in concentration by standard additions (b), the following equations were obtained: $y_a=0.254x-0.014$ ($r_a=0.9998$, $n_a=4$) and $y_b=0.249x+0.130$ ($r_b=0.9994$, $n_b=24$). The limit of detection of the concentration of injected material was less than 1 μM for injection volumes of 75 μl with a signal-to-noise ratio higher than 3.5.

The within-day and the day-to-day precision values (both shown in Table II) are excellent. The low inter-animal variation indicates a well defined reference material for experimental toxicological studies. The level corresponds to a reference value less than half the one traditionally reported for rat plasma/serum [12], but is in perfect agreement with the results of Meyer et al. [13], who have used a different HPLC technique.

The "identities" of C_m and C_c values (Table III), also illustrated by the linear regression equations, are in accordance with the accuracy and linearity of the

TABLE II

PRECISION DATA FOR THE DETERMINATION OF CREATININE IN RAT PLASMA

	Concentration (mean ± S.D.) (μM)	n	Relative standard deviation (%)
Inter-animal variation	25.8 ± 2.3	93	8.91
Within-day precision	25.4 ± 0.7	10	2.76
Day-to-day precision	24.8 ± 1.2	10	4.84

TABLE III

MEANS AND STANDARD DEVIATIONS OF MEASURED AND CALCULATED CONCENTRATIONS OF CREATININE IN RAT PLASMA ($n=24$)

Measured concentration (C_m) (μM)	Calculated concentration (C_c) (μM)	C_c/C_m (%)
27.62 ± 2.93	28.47 ± 2.83	102.40 ± 6.92

analysis and do not indicate any interferences from endogenous compounds. The possible interferences were excluded by comparing chromatograms of untreated and enzyme-treated isolated creatinine peaks (Fig. 1B and C). Other hypothetical interferences from endogenous compounds could be excluded by addition of creatinine standards to creatininase-treated plasma, after which chromatography showed a normal aqueous standard curve (results not shown).

When compounds known to interfere with the Jaffe procedure were added to plasma in concentrations up to 100 mM, there was no interference with the determination of creatinine, and the interference of all inert volatile substances can per se be excluded. The Jaffe-interfering endogenous compounds tested included: acetone, glucose, fructose, ascorbic acid, β-hydroxybutyric acid, pyruvic acid, oxalic acid, hippuric acid, urea, uric acid, creatine, bilirubin, hemolytic and lipaemic material. Fig. 2 illustrates the application of the method to the determination of $t_{1/2}$ in rat plasma of the specific activity of intravenously injected [^{14}C]creatinine, which may be an important parameter for the elucidation of the mechanisms

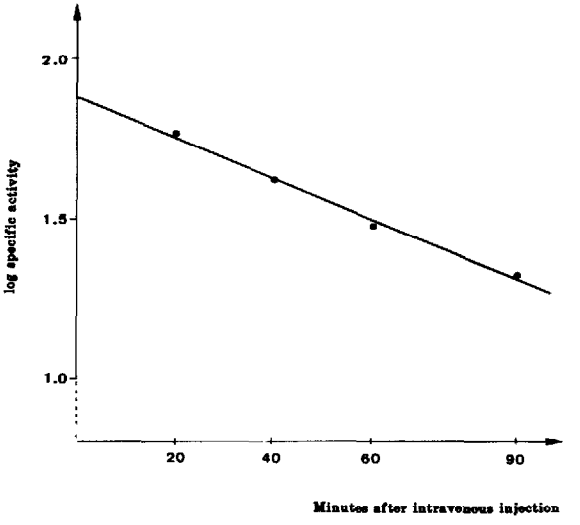

Fig. 2. Linear relation between log specific activity (pCi/nmol) of intravenously injected [^{14}C]creatinine and the time after injection of 0.5 μCi [^{14}C]creatinine per animal. $y = -6.38 \cdot 10^{-3}x + 1.884$, $r = 0.9960$, corresponding to $t_{1/2} = 47.2$ min.

underlying the action of chemical compounds affecting the metabolism of creatinine [14]. This is impossible by existing colorimetric methods.

CONCLUSIONS

The method described is specific, sensitive and reproducible with respect to the determination of creatinine in rat plasma. This has not been evidenced by any other method. It is a reliable, time-consuming method well suited for investigational purposes, but not for clinical, chemical routine work. It may be used as a method of reference for determination of "true" creatinine in human body fluids.

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REFERENCES

1 S. Narayanan and H.D. Appleton, Clin. Chem., 26 (1980) 1119.
2 K. Spencer, Ann. Clin. Biochem., 23 (1986) 1.
3 J.F. Van Pilsum, R.P. Martin, E. Kito and J. Hess, J. Biol. Chem., 222 (1956) 225.
4 M.X. Sullivan and F. Irreverre, J. Biol. Chem., 233 (1958) 530.
5 A.C. Parekh and C. Sims, Clin. Chem., 23 (1977) 2066.
6 M. Jaffe, Hoppe-Seyler's Z. Physiol. Chem., 10 (1886) 391.
7 J. Bierens de Haan, Anal. Lett., 5 (1972) 815.
8 N.A. Daugherty, K.B. Hammond and I.M. Osberg, Clin. Chem., 24 (1978) 392.
9 I.M. Osberg and K.B. Hammond, Clin. Chem., 24 (1978) 1196.
10 R.K. Reichards, T.D. Bjornsson and L.D. Waterbury, Clin. Pharmacol. Ther., 23 (1978) 430.
11 N.A. Roach, M.H. Kroll and R.J. Elin, Clin. Chim. Acta, 151 (1985) 301.
12 S.T. Wolford, R.A. Schroer, F.X. Gohs, P.P. Gallo, M. Brodeck, H.B. Falk and R. Ruhren, J. Toxicol. Environ. Health, 18 (1986) 161.
13 M.H. Meyer, R.A. Meyer, R.W. Gray and R.L. Irwin, Anal. Biochem., 144 (1985) 285.
14 F. Tarding and H.R. Lam, in preparation.