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Note

Determination of creatine in body fluids and muscle

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The non-enzymatic methods generally employed for determining creatine in biological samples lack specificity, because of interference from several compounds normally present. The widely used Folin method [1] for determination of creatinine and creatine in serum and urine is based on the Jaffé reaction after conversion of creatine into creatinine [2, 3]. Unfortunately, though, it is subject to gross errors when applied to urine which contains a large amount of creatine [4, 5]. Adult human males usually excrete very little creatine in the urine. However, during fasting and in certain muscle diseases blood creatine rises above the levels that can be reabsorbed by the kidneys, and creatinuria results [6]. During studies of muscle diseases, evaluation of the methods available for measuring creatinine and creatine in biological samples led us to the conclusion that they can be measured respectively the Jaffé picrate [2] and the α-naphthol—diacetyl reaction [7] with satisfactory results if preliminary ion-exchange chromatography is carried out. In this laboratory we have recently developed a satisfactory method for determination of creatinine in plasma and urine [8].

This paper now describes a method for creatine determination in body fluids. It includes prior removal of interfering substances from biological samples, using a strong cation-echange resin, followed by separation of creatinine from creatine by a weak cation-exchanger. The ease and speed of analysis make this method very attractive for routine clinical determination of creatine and creatinine in muscle pathologies.

MATERIALS

Chemicals

Creatine monohydrate, creatinine and α-naphthol were purchased from

Merck (Darmstadt, F.R.G.). Guanidinoacetic acid was obtained from Fluka (Buchs, Switzerland) arginine from Mann Research Labs. (New York, NY, U.S.A.) and picric acid aqueous solution (1.2%) from BDH Chemicals (Poole, U.K.).

All chemicals used in the procedure and those screened in the interference study were reagent grade. Creatine standard solutions in the range 4.5—450 μmol/l, were prepared with distilled water from a stock solution of 0.1 g/l.

Ion-exchange resins Duolite C 225 (52—100 mesh, H^+) and Bio-Rex 70 (100—200 mesh, H^+) were obtained from BDH and from Bio-Rad Labs. (Richmond, CA, U.S.A.), respectively.

Reagents for creatine determination by the α-naphthol—diacetyl reaction

An alkali stock solution is prepared by dissolving 60 g of sodium hydroxide and 160 g of anhydrous sodium carbonate in 1 l of water and stored in a warm place.

A 1.6% (w/v) solution of α-naphthol in the alkali stock solution is prepared immediately before use and filtered. It should not be darker than a pale straw-yellow and can be used for at least 2 h after preparation.

A 1% (v/v) stock solution of diacetyl in water is kept at 4°C and diluted 1:20 with water immediately before use.

PROCEDURE

Isolation and separation of creatine and creatinine from biological materials.

Urine. A 1 ml volume of urine, adjusted to pH 5—6 with hydrochloric acid, was applied to a column (15 × 0.5 cm I.D.) packed with Duolite C-225 (52—100 mesh, H^+). The column was washed with 15 ml of water and eluted with 15 ml of 2 *M* ammonium hydroxide. The first 3 ml were discarded, and 6 ml of the next 12 ml were passed through a column (10 × 1 cm I.D.) packed with Bio-Rex 70 (100—200 mesh, H^+); 30 ml of 0.1 *M* acetic acid were used for elution. The first 5 ml were discarded; the next 10 ml, which contained creatine, and the last 15 ml, containing creatinine, were collected in 1-ml fractions. Each fraction was used for assay of creatine and creatinine by the α-naphthol—diacetyl reaction and the Jaffé picrate reaction, respectively. Creatine and creatinine, used as standards, 0.885 mmol/l, each dissolved in 2 *M* ammonium hydroxide, were applied to the Bio-Rex 70 column.

Plasma and muscle. Plasma (1 ml) was deproteinized with 0.6 *M* perchloric acid (1:5, v/v) and rat muscle homogenate (0.2 ml) in 1.15% potassium chloride (1:8, w/v) with 0.6 *M* perchloric acid (1:1, v/v). After standing 10 min in ice, samples were centrifuged at 3000 *g* for 10 min. Portions of the supernatants were then applied to the first chromatographic column and processed as described for urine.

Recovery

The recovery of creatine added to control human plasma, to pooled patient's plasma, or urine (diluted twenty times) was determined by diluting portions of each of these specimens (1) with an equal volume of a standard solution of creatine (45 mmol/l) and (2) with an equal volume of water. Creatine was then

isolated from the samples by the two ion-exchange procedures, and measured by the α-naphthol—diacetyl reaction, as follows. A portion of each eluate (7 ml) was added to 2 ml of α-naphthol solution and 1 ml of diacetyl solution; the procedure was calibrated against similarly treated creatine standards with absorbance measured at 525 nm against a column blank. Recoveries were calculated from the results.

Determination of other interfering components in eluates

The check of purity of eluates from the ion-exchange columns, control plasma, urine (diluted twenty times) and pooled control rat muscles were processed as above. Final eluates were desalted by passing through ion-exchange resin Duolite C-225 (200—400 mesh) as previously described [8]. After washing with distilled water (50 ml), the absorbed creatine was eluted with 50 ml of 2 *M* ammonia. Eluates were dried under vacuum and spotted on precoated silica gel 60 F 254 thin-layer aluminium sheets (20 × 20 cm, Merck). The developing solvent was a mixture amyl alcohol—pyridine—water (21:42:37). The detection reagent was prepared as follows: (A) 1% α-naphthol in 8% sodium hydroxide (dissolved in 80% ethyl alcohol), (B) 0.1% diacetyl dissolved in 90% ethyl alcohol. Immediately before use 1 volume of A was mixed with 1 volume of B. Plates were placed for 3 min at 100° C.

RESULTS AND DISCUSSION

As shown in Fig. 1, creatine adsorbed on the Bio-Rex 70 column from plasma, urine, muscle or aqueous solutions of creatine was eluted (99 ± 1%) with 6 ml of 0.1 *M* acetic acid. The following 8 ml of eluate (from fractions 19 to 26) containing creatinine can be analysed by the Jaffé reaction, with minor modifications [9].

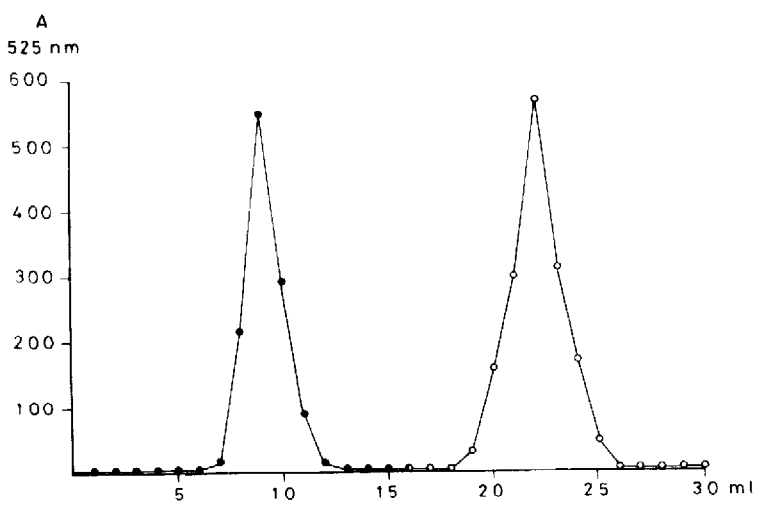

Fig. 1. Elution pattern of creatine (•) and creatinine (○) (aqueous solutions, 100 μg each) absorbed on the Bio-Rex 70 column; analyses are as described under Materials and Procedure. Fractions of 1 ml.

Recovery studies

Recoveries of creatine added at two different amounts (2.5 mg/l and 5.0 mg/l) to seven different control plasma, pooled patient's plasma and urine (diluted twenty times) and pooled control rat muscle, isolated by the two-step cation-exchange procedure and assayed by the α-naphthol—diacetyl reaction, were, respectively, 95 ± 2%, 98 ± 2% and 94 ± 3%. These results indicate that the isolation procedure was quantitative.

Precision and accuracy

Reproducibility of the method was checked by assaying pooled plasma and urine samples twenty times. Mean values of 7.20 mg/l and 23.52 mg/l for pooled plasma were found by this method with standard deviations (S.D.) of ± 0.05 mg/l and ± 0.05 mg/l and coefficients of variation (C.V.) of ± 0.69% and ± 0.21%. For urine, mean values were 46.10 mg/l and 411.0 mg/l with S.D. ± 0.09 mg and ± 2.0 mg/l and C.V. ± 0.19% and ± 0.50%, respectively.

These pooled samples, stored frozen at −20° C, were determined in duplicate over a one-month period, with the following results: 7.25 mg/l and 23.00 mg/l for mean values of pooled plasma; S.D. = ± 0.04 mg/l and ± 0.05 mg/l; C.V. = ± 0.55% and ± 0.22%. For urine pools, the mean values were 45.10 mg/l and 415.12 mg/l; S.D. = 0.05 mg/l and ± 1.92 mg/l; C.V. = ± 0.11% and ± 0.46%.

Reproducibility and accuracy were checked employing pooled rat skeletal muscles. Mean values of 22.29 μmol/g were found; S.D. = ± 0.30 μmol/g; and C.V. = ± 1.34%. Duplicate determinations of these pooled muscles, kept frozen at −20° C as described for plasma and urine, led to the following results: 23.09 μmol/g and 20.53 μmol/g for mean values of pooled muscles; S.D. = ± 0.29 μmol/g and ± 0.25 μmol/g; C.V. = ± 1.26% and ± 1.22%.

Calibration

Standard curves for the procedure, prepared from the aqueous standards, are linear up to 50 mg/l (plasma) and 800 mg/l (urine) and pass through the origin. Calibration was done daily to ensure maximum precision.

Application

The method has been applied for routine clinical analysis of urine of patients with Duchenne muscular dystrophy and plasma of uraemic patients. Creatine concentrations in urine and plasma of these patients are reported in Table I, together with normal values. In order to clarify the relationship between creatine and creatinine in healthy and diseased subjects Table I also reports results of a previous study on creatinine determination [8] and creatine levels in pooled rat skeletal muscle.

Interferences

Not many compounds are likely to interfere in this procedure, because of the double passage through two different cation-exchange resins. Anions, proteins and neutral molecules wash through the cation-exchange resin Duolite C-225. Thin-layer chromatography of desalted eluates from control human plasma and urine, indicates that they contain only creatine. The endogenous and exogenous materials we tested (Table II) did not interfere in creatine determination.

TABLE I

CREATININE AND CREATINE IN PLASMA AND URINE OF NORMAL HUMAN CONTROLS AND PATIENTS

Clinical diagnosis	Creatinine		Creatine	
	Plasma (mmol/l ± S.E.)	Urine (mmol/l ± S.E.)	Plasma (mmol/l ± S.E.)	Urine (mmol/l ± S.E.)
Duchenne muscular dystrophy (n = 50)	n.d *	3 30 ± 0.2	n.d.	3 56 ± 0.2
Uraemia (n = 50)	0.74 ± 0.02	n.d.	0.10 ± 0.03	n.d.
Normal controls (n = 50)	0.09 ± 0.001	6 50 ± 0.5	0.04 ± 0.001	0.24 ± 0.006
	Creatinine (μmol/g)			
Pooled control rat skeletal muscle	22.29 ± 1.2			

*n.d. = not detectable

TABLE II

COMPOUNDS TESTED FOR INTERFERENCE WITH CREATINE ASSAY BY THE α-NAPHTHOL—DIACETYL REACTION

Compound	Test concentration (mg/l)
Creatinine	50
Arginine	60
Guanidine	60
Methionine	50
Pyruvic acid	400
Urea	600
Glutathione	80
2,3-Diphosphoglyceric acid	6000
Guanidinacetic acid	50
Uric acid	70

Creatine is the end-product of the metabolism of glycine and arginine. It is eliminated via the kidneys by glomerular filtration, but is normally reabsorbed in renal tubules, resulting in very little creatine in the urine. Quantitative determination of creatine in plasma and urine is important in the diagnosis of muscular diseases. High serum creatine values have been reported in patients with amyotrophic lateral sclerosis, progressive dystrophia musculorum and dermatomyositis [10]. Urine creatine levels are reported by the same authors to be high in glomerulonephritis. Studies are in progress on the correlation between creatine and creatinine in urine and muscle in muscle-wasting diseases.

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