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A MICROCOLORIMETRIC DETERMINATION OF CREATININE IN SERUM OR PLASMA BY THE JAFFÉ'S REACTION

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A micro procedure requiring 200 μl of sera for the analysis of creatinine concentration in a large humber of samples collected in a mass campaign during the screening of the people for Balkan endemic nephropathy has been developed.

The reliability of the micro procedure was proved by examination of the precision 1.79 mg/100 ml (SD = 0.12), the exactitude was from 0.5 to 6.0 mg/100 ml.

The experiments on rat's plasma showed a very high correlation between the results of the macro and micro procedure (r = 0.92).

An average creatinine concentration found in 82 healthy men was 1.24 mg/100 ml (SD = 0.18), in 15 healthy women 1.08 mg/100 ml (SD = 0.20) and in 51 healthy children 0.79 mg/100 ml (SD = 0.27) of serum.

Creatinine concentration did not considerably change when serum samples were stored at 4 °C for 48 hours or at —18 °C for one year.

Creatininemia is considered as one of the four criteria for diagnosis of Balkan endemic nephropathy (1). Today there are several methods for the quantitative determination of creatinine (anhydride methylguanidinoacetic acid) in sera and other biological materials (2—8). All of them require a relatively high quantity of serum or plasma and are easily applied in clinical practice. However, according to our experience in mass campaigns, when the screening for Balkan endemic nephropathy is carried out, it is impractical or even impossible to obtain the amount of blood needed for creatinine macro determination from all people.

Consequently, the purpose of this study was to select and adapt a procedure for the quantitative determination of serum creatinine in a smaller volume (200 μl) of serum or plasma. The decision was made to introduce and modify the macro method as described by *Owen* and co-workers (9) which was already described by *Jaffé* as early as 1886 (10) and introduced by *Folin* in 1914 (2) and *Folin* and *Wu* in 1919 (11) for the determination in urine and blood and by *Van Pilsum* end co-workers (8) for the determination in biological fluids. The method is unspecific, because picric acid forms a coloured complex with various chromogen compounds (ascorbic acid, pyruvate, aceton, levulose etc.) jointly called pseudocreatinine. The creatinine concentration in biological material is therefore seemingly higher. By this method the total *Jaffé's* chromogen is measured and is called creatinine. The error arising out of this increases creatinine value by about 15 per cent (7). When deproteinization with the trichloroacetic acid (TCA) at pH lower than 2 is performed, the creatinine recovery is expected to be nearly total (2).

MATERIAL AND METHODS

The experiments were carried out on human and rat's sera and plasma.

Bloood samples

Human blood samples were obtained by venepuncture from 148 apparently healthy subjects (men, women and children). Serum was separated after a mild centrifugation (1500 rpm) of the clotted blood sample on the day of withdrawal. For the examination of the effect of storage on the creatinine concentration, samples of sera were stored at 4° C up to 48 hours and at —18° C for approximately one year. All other creatinine analyses were performed within one hour after the sampling.

From anesthetized albino rats, blood was taken by heart puncture in heparinized tubes and plasma was separated after a mild centrifugation.

Chemicals and apparatus

A stock solution of 3N trichloroacetic acid (TCA) (Kemika, Zagreb, analytical grade), a saturated picric acid solution (Merck, analytical grade) and a 4.1 N sodium hydroxide solution (Kemika, Zagreb, analytical grade) were prepared for all the experiments. A standard solution of creatinine (1 mg/ml) for all measurements was prepared in 0.1 N hydrochloric acid (both Kemika, Zagreb).

The measurements were performed with a spectrophotometer UNICAM SP 600, Series 2 with an adaptor for microcuvettes (2.5 × 10 × 25 mm).

Micro procedure

Aliquots of 200 μl of serum or plasma were pipetted by an automatic Jencons pipette with a disposable plastic tip into a tube to which 200 μl of distilled water and 200μl of 3N TCA were added. Subsequently, the precipitated protein was spun at 4500 rpm for 20 minutes. For the anasysis 300 μl of the supernatant was taken to which 100 μl of a saturated picric acid solution was added. At zero time 100 μl of 4.1 N sodium hydroxide was added, thus the final pH reaction mixture becoming 12.4. After 15 minutes of incubation at room temperature the absorbance was measured at 546 nm against a blank sample. The blank sample (200 μl of distilled water and 100 μl of TCA) was previously treated in the same way as the supernatant of the test. All the measurements were carried out in duplicates.

For the determination of the creatinine recovery the procedure did not differ.

Creatinine concentration in a given sample was calculated on the basis of a coefficient of the regression line slope.

Macro procedure

The entire procedure did not differ essentially from that described by *Owen* and co-workers in 1954. For each measurement 2 ml of serum or plasma was required. After the addition of 2 ml of distilled water and 2 ml of 3N TCA, the sample was incubated at 90—95° C for 15 minutes and then cooled at room temperature and filtered through the filter paper (black filter paper — Selecta). An aliquot of 3 ml of the filtrate was mixed with 1 ml of saturated picric acid and 1 ml of 4.1 N sodium hydroxide. The absorbance was measured at the same wavelength in 10 $\times$10 $\times$ 45 mm cuvette on the same spectrophotometer.

RESULTS AND DISCUSSION

Known concentrations of creatinine in the standard solution were determined by both micro and macro procedure. The dependence between the absorbance and the creatinine concentration was found to be remarkably high for the concentrations ranging from 0.5 to 10 mg/100 ml.

From the equation of a line

$$A \text{ (absorbance)} = a \cdot c + b$$

where a represents a slope of the line and b an intercept the creatinine concentration c may be calculated in mg/100 ml.

In our experimental conditions, when b is equal to 0, it comes out that creatinine concentration (in mg/100 ml) is equivalent to the absorbance divided by 0.023.

The precision of the micro and macro procedure was determined on the basis of 36 analyses of the same sample of pooled rats' plasma. The mean value of the creatinine concentration for the micro procedure was 1.79 mg/100 ml (SD = ± 0.12) the coefficient of variation being 0.1 per cent. For the macro procedure this value was 1.70 mg/100 ml (SD = ± 0.15) the coefficient of variation being 0.09 per cent.

To study the recovery of creatinine when determined by the micro procedure known amounts of creatinine were added to the sample of rat's plasma. The results are shown in Table 1.

Table 1

Recovery of creatinine added to the rat's plasma samples as determined by the micro procedure

Creatinine (mg/100 ml) Added	Found Individual values	$\bar{X}$	Percent of recovery
0	0.98 0.92 1.10	1.00	—
1	1.92 1.89 2.16	1.99	100
2	3.29 3.00 2.95	3.08	103
3	4.00 3.85 3.95	3.90	98

The concentration of the endogenous creatinine in this particular sample of rat's plasma was exceptionally low when compared with normal values obtained in 20 rats (Table 2). This, however, does not influence the results of creatinine recovery, which was shown to be nearly 100 per cent.

In another set of experiments, known concentrations of creatinine were added to pooled rat's plasma and concentrations were measured by the micro method. When theoretically calculated values were plotted against those obtained in the experiment, a straight line was obtained (Figure 1).

The correlation between the creatinine concentrations determined by micro and macro procedures was examined in 20 samples of pooled rat's plasma to which different concentrations of creatinine were added.

The results are presented in Figure 2. There is a positive correlation between these two procedures which is highly statistically significant ($r = 0.99$).

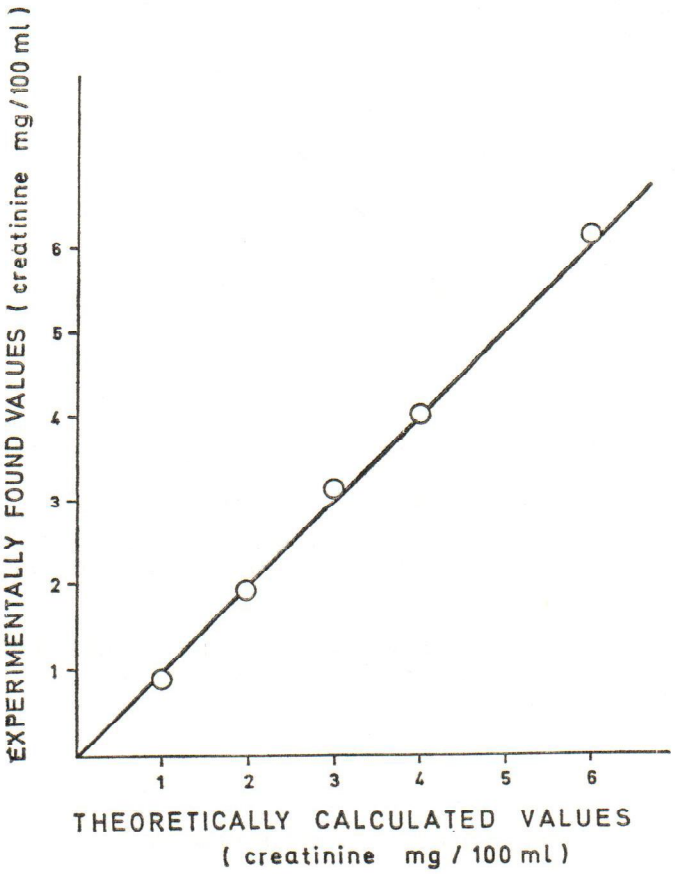

Fig. 1. *Recovery of creatinine determined by the micro procedure in rat's plasma. Each point represents the mean value of three measurements*

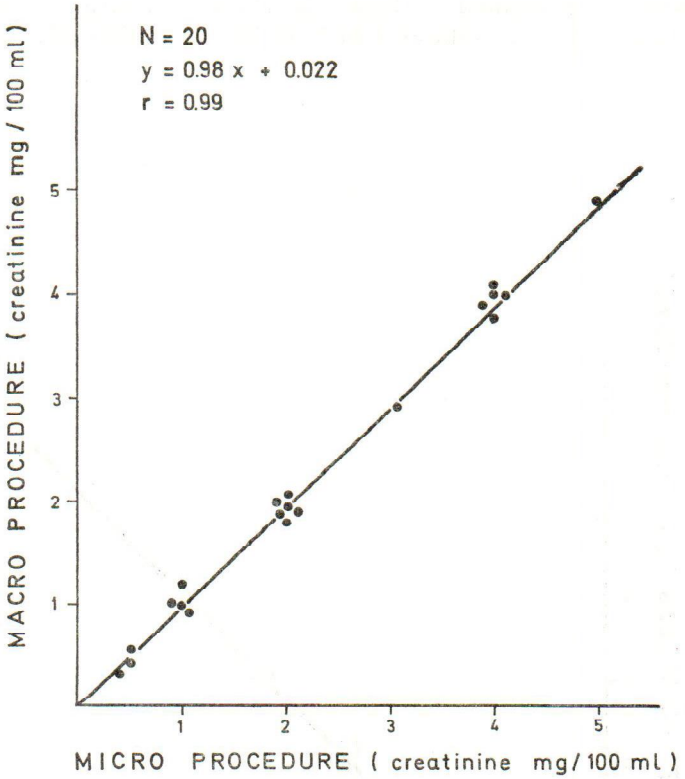

Fig. 2. *Correlation between micro and macro procedure for the creatinine determination in pooled rats' plasma to which known concentrations of of creatinine were added*

The correlation between the two procedures was also examined in the plasma of 20 albino rats. The results are shown in Table 2.

Table 2

Creatinine concentration in the plasma of 20 male albino rats determined by both, micro and macro procedure

Procedure	Creatinine concentration (mg/100 ml)			
	N	$\overline{X}$	SD	Range
Macro	20	1.57*	0.18	1.22 — 1.92
Micro	20	1.58*	0.15	1.36 — 1.84

* $t = 0.171, p > 0.8$

The correlation coefficient for the two procedures was also very high ($r = 0.92$).

In order to determine the range of concentrations of creatinine in human sera by the micro method, blood samples from a group of 148 clinically healthy men, women and children were analyzed. The results are presented in Table 3.

Table 3

Creatinine concentration in sera of clinically healthy human subjects determined by the micro procedure

Subjects	N	Age (years)		Creatinine mg in 100 ml of serum		
		$\bar{X}$	Range	$\bar{X}$	SD	Range
Men	82	37	21—61	1.24	0.18	0.95 — 1.73
Women	15	44	23—55	1.08	0.20	0.82 — 1.60
Children	51	10	4—14	0.79	0.27	0.43 — 1.43

These results are in agreement with those obtained by many other authors (12—14). From these and from the previously described experiment it may be concluded that the micro proccedure, which requires only 200 μl of serum or plasma, satisfies most of the criteria for a large number of creatinine determinations in people living in the endemic area. However, in practice it is very difficult to perform the analysis on the day of collecting blood samples. Therefore, the effect of storage of serum samples on creatinine concentration was tested. The results are presented in Table 4.

Table 4

The effect of storage of human serum samples on creatinine concentration determined by the micro procedure

Storge			N	Creatinine concentration (mg/100 ml)		
Duration	Temparature			$\bar{X}$	SD	$SE_{\bar{x}}$
1 hr	Room		5	1.16	0.21	0.09
24 hrs	4 °C		5	1.15	0.13	0.06
48 hrs	4 °C		5	1.13	0.11	0.05
1 year	—18 °C	1977	30	1.17	0.21	0.39
		1978	30	1.12	0.22	0.41

It appears, therefore, that the storage of the serum at 4° C for two-days or rat — 18° C for a year does not considerably affect the creatinine concentration.

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Sažetak

MIKROKOLORIMETRIJSKA METODA ZA ODREĐIVANJE KREATININA U SERUMU ILI PLAZMI JAFFEOVOM REAKCIJOM

U svrhu pojednostavljenja postupka i smanjivanja količine krvi potrebne za analizu prilagodili smo i pojednostavnili uobičajeni makropostupak za određivanje kreatinina u serumu ljudi. Za analizu uvedenom mikrometodom potrebno je 200 μl seruma ili plazme, količina što se može dobiti ubodom u jagodicu prsta. Pojednostavljenje postupka ubrzalo je analizu čime je olakšano određivanje kreatinina u svih ljudi u toku akcija perlustracije stanovništva što se provode u svrhu utvrđivanja endemske nefropatije.

Pouzdanost mikrometode provjerena je usporednim određivanjem alikvotnih uzoraka makropostupkom i mikropostupkom. Korelacija je bila vrlo visoka (r = 0,92). Prosječna vrijednost kreatinina izmjerena u 82 zdrava muškarca iznosila je 1,24 mg/100 ml (SD = 0,18), u 15 zdravih žena vrijednost kreatinina bila je 1,08 mg/100 ml (SD = 0,20) a u 51 zdravog djeteta 0,79 mg/100 ml (SD = 0,27). Pohranjivanje uzoraka seruma na 4° C tijekom 48 sati odnosno na —18° C kroz godinu dana, nije značajnije utjecalo na izmjenu koncentracije kreatinina.

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