Journal of Chromatography, 614 (1993) 221–226
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6745

High-performance liquid chromatographic determination of creatinine in serum, and a correlation of the results with those of the Jaffé and enzymic methods

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(First received May 7th, 1992; revised manuscript received January 11th, 1993)

ABSTRACT

A high-performance liquid chromatographic method for the determination of creatinine in control sera is reported, based on its separation from deproteinated serum components on the ion-exchange material HEMA Bio 1000 SB and ultraviolet detection at 230 nm. Groups of eleven to fourteen samples of human serum and several control materials were simultaneously analysed by the Jaffé, enzymic ultraviolet and enzymic peroxidase aminophenazone methods. Another group (52–115 sera) was analysed for correlations with spectrophotometric methods. The precision of the chromatographic method ranges between 2.0 and 1.0% (relative standard deviation) for serum creatinine concentrations of 115.1 to 471 μmol/l, respectively. A very good accuracy was found in analyses of reference materials Kontrollogen-L and -LP. Some results of analyses of the other control sera were higher and the other lower than those obtained by the Jaffé and enzymic methods, because both interferences and enzyme inhibitors were encountered. Correlations between the chromatographic and spectrophotometric methods were good.

INTRODUCTION

The determination of creatinine is one of the most important clinical analyses in human medicine. The creatinine concentration in serum and urine is one of the parameters used in the evaluation of renal functions. The methods for determination of creatinine have been reviewed [1,2]. One of the most common procedures is based on removal of proteins from the serum followed by Jaffé reaction [3], *i.e.* the formation of an adduct of creatinine with picric acid in alkaline solution, whose absorbance is measured at 500 nm.

The original Jaffé method has been modified many times since its introduction for several reasons: the clinical importance of creatinine, the simplicity of its determination and the need to improve the rather poor selectivity of the procedure [1,4–10]. The interferences are now well known, and a number of rapidly and slowly reac-

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0378-4347/93/$06.00

ting pseudocreatinine chromogens have been systematically described [11,12]. Other chemical methods have been developed for determination of creatinine, but none has been able to replace the Jaffé reaction (see Table I) [11].

Only recently have enzymic methods begun to compete with the Jaffé procedure; their principles have been known since the end of the 1930s [13] but their commercial availability is recent. There are two different groups of enzymic methods. One is based on creatinine hydrolysis by creatininase, and the creatine formed is either directly determined or is further hydrolysed to yield sarcosine, which is oxidized to formaldehyde, glycine and hydrogen peroxide; the latter is then determined by the Trinder reaction [peroxidase aminophenazone (PAP) method] [14]. The other group of methods employs creatinine iminohydrolase, which hydrolyses creatinine with liberation of ammonia, which is either directly determined or subjected to the glutamate dehydrogenase reaction, and the loss in NADH is monitored (UV method) [15].

The enzymic methods are substantially more selective than the chemical procedures, but there are still interferences, different ones for the two groups. An extensive review of the chemical and enzymic methods has been given by Sonntag [16]. Enzymic reactions have recently been used in the construction of enzyme electrodes [17–22].

TABLE I

METHODS FOR DETERMINATION OF CREATININE (MODIFIED ACCORDING TO WISSER [11])

Chemical methods
Jaffé reaction
Direct (end-point or kinetic)
Indirect (Fuller's earth (Lloyd reagent))
Modification of creatinine into methylguanidine and Sakaguchi reaction
Reaction with 1,4-naphthoquinone-2-sulphonate
Enzymic methods
Hydrolysis to creatine
Determination of creatine
Hydrolysis of creatine to sarcosine, oxidation of sarcosine and determination of hydrogen peroxide (PAP method)
Hydrolysis to methylhydantoin and ammonia
Determination of ammonia
Glutamate dehydrogenase reaction (UV method)
Physico-chemical methods
Chromatographic methods (HPLC, HPTLC, GC–MS)
Isotope dilution MS
Electrophoretic methods

The physicochemical methods for determination of creatinine are more complicated and instrumentally more demanding. So far, isotope dilution mass spectrometry (MS) [23,24] and high-performance chromatographic methods, especially high-performance liquid chromatography (HPLC) [25–43], high-performance thin-layer chromatography (HPTLC) [44], and micellar electrokinetic chromatography [45], have been used to determine creatinine selectively in body fluids. These are the only methods estimating true creatinine, and thus they have been selected as reference methods (*e.g.* refs. 23, 39 and 40).

This paper describes a simple, rapid and reliable HPLC determination of creatinine in serum using a stationary phase of hydroxyethylmethacrylate-based polymer modified with sulphobutyl groups, HEMA 1000 SB. This material behaves as a cation exchanger, but also exhibits hydrophobic properties that permit a fine tuning of the separation conditions. Another advantage is that HEMA SB permits separations over a broad pH range. The HEMA materials [46] thus permit a great variety of applications. The results obtained in this HPLC determination were correlated with those of the common kinetic Jaffé procedure and the enzyme PAP [14] and UV [15] determinations.

EXPERIMENTAL

Chemicals and biological materials

Three types of diagnostic kit were used: (1) Test-Combination Creatinine PAP (Boehringer Mannheim, Mannheim, Germany); (2) Creatinine-Jaffé ohne Enteiweissung (Centronic, Notzing, Germany); (3) Creatinine-Duo UV GLDH-UV Test (Biomed, Munich, Germany).

For calibration purposes, the creatinine substance (No. 914 reference material from NBS, Washington, DC, USA), commercially available water solutions of creatinine (*e.g.* Calibrator,

Centronic), and serum-based calibrators (*e.g.* Calibrator for automatic systems, Boehringer 172219) were used. The other calibrators were part of the diagnostic kits. As control materials, various control sera were evaluated: Monitrol II E (Baxter, Switzerland), Kontrollogen-L, Kontrollogen-LP and Precinorm U (all from Boehringer), Duotrol 5036/1 (Biomed), Humatrol N (Human, Taunusstein, Germany) and Exatest I and Exatest Standard K (Imuna, Šarišské Michalany, Czech Republic). The test human serum samples were chosen from clinical departments.

All the other chemicals were reagent grade, from Lachema (Brno, Czech Republic). Doubly distilled water was used.

Apparatus and chromatographic conditions

The liquid chromatograph consisted of an LC-XPD pump, an LC-UV variable-wavelength detector (both from Pye Unicam, Cambridge, UK), a Rheodyne 7125 sampling valve (Cotati, CA, USA) with a 5-μl loop and an LKB 2220 recording integrator (Bromma, Sweden). A HEMA Bio 1000 SB stainless-steel column (80 mm × 8 mm I.D., 10 μm particle size) with an ion-exchange capacity of 1.6–2.2 mmol/g (Tessek, Prague, Czech Republic) was used. The mobile phase was 0.016 *M* aqueous ammonium carbonate solution (pH 9.1), and the flow-rate 0.7 ml/min. Creatinine was detected photometrically at 234 nm. All the chromatographic experiments were performed at laboratory temperature (20 ± 2°C).

For the spectrophotometric measurements in the Jaffé and enzymic methods, the automatic analysers Technicon RA 1000 (Bayer, Technicon, Tarrytown, USA) and Cobas Mira Plus (Roche, Switzerland) were used.

Procedure

Lyophilized standard serum samples were dissolved in appropriate volumes of redistilled water in an ultrasonic bath, 2-ml aliquots of the solution were mixed with 1 ml of 10% trichloroacetic acid (TCA), and the protein precipitate was centrifuged off at 1500 *g* for 10 min. A 5-μl volume of the transparent supernatant was injected onto the column. The standard solutions of creatinine were prepared by dissolving 11.31, 16.965 and 56.55 mg of the substance in 1 l of redistilled water (concentrations of 100, 150 and 500 μmol/l, respectively). All serum samples and standard solutions were stored in a refrigerator at 4°C.

RESULTS AND DISCUSSION

Creatinine can be separated from the serum components on ion exchangers [25–28], isocratically or with pH programming [27]. Silica-gel columns have also been employed (*e.g.* ref. 29). Reversed-phase separations with C_{18} stationary phases are the most common (*e.g.* refs. 30–37). They employ mobile phases of aqueous buffers, usually phosphate or acetate mixed with acetonitrile or methanol.

However, these procedures sometimes have a disadvantage in the short retention time of creatinine, which leads to interferences from some serum components. The retention time can be increased by adding an ion-pairing agent [37], but the limit of detection of the UV absorbance measurement is then much higher. At pH 8, creatinine exhibits absorption maxima at *ca.* 210 nm (ε = 6310 l mol^{-1} cm^{-1}) and 230 nm (ε = 7943 l mol^{-1} cm^{-1}) and thus is usually detected at wavelengths of *ca.* 230 nm. To improve the detection limit in ion-pair chromatography, creatinine can be derivatized post-column with ninhydrin, with measurement of fluorescence of the derivative [37].

Modified poly(hydroxyethylmethacrylate), HEMA Bio 1000 SB, used in the present study showed improved pH stability: there was a baseline separation of creatinine from serum and urine components with the mobile phase of aqueous ammonium carbonate solution (Fig. 1). Creatinine was the last-eluted compound and there were no interferences from other serum components. The time of analysis was 5 min. Owing to the low absorptivity of the mobile phase, UV detection at 234 nm is very sensitive: the limit of detection was 0.6 μmol/l (or 1.32 ng in 20 μl injected) at a signal-to-noise ratio of 3 and 2 μmol/l (or 4.4 ng) at signal-to-noise ratio of 10.

The aqueous standard solutions of creatinine,

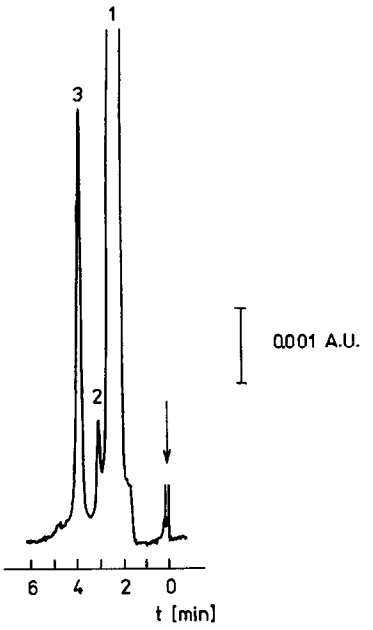

Fig. 1. Chromatogram of human serum. Conditions as in Experimental. Peaks: 1, 2 = serum components; 3 = creatinine.

all the control sera and the real serum samples were analysed under the above conditions. The chromatograms were evaluated from the peak height using absolute calibration. The precision was evaluated in series of 11 to 19 determinations

TABLE II

PRECISION OF CREATININE DETERMINATION IN SERUM

Method	n	x (μmol/l)	S.D. (μmol/l)	R.S.D. (%)
HPLC	19	115.1	2.3	2.0
	11	189.9	2.8	1.5
	15	471.0	4.7	1.0
Jaffé	34	69.9	2.5	3.6
	20	162.9	1.6	0.9
	20	437.1	4.8	1.1
Enzymic UV	28	76.5	5.3	7.0
	20	180.8	4.4	2.4
	20	548.9	6.7	1.2
Enzymic PAP	30	74.3	1.5	2.0
	30	241.2	3.9	1.6
	30	407.8	2.0	0.5

TABLE III

DAY-TO-DAY REPRODUCIBILITY OF CREATININE DETERMINATION IN SERUM

Method	n	x (μmol/l)	S.D. (μmol/l)	R.S.D. (%)
HPLC	15	111.5	2.8	2.5
	15	189.9	2.8	1.5
	15	270.0	4.0	1.5
Jaffé	13	121.1	3.8	4.0
	13	293.1	8.8	3.0
	13	476.0	16.3	3.4
Enzymic UV	15	122.2	3.9	3.2
	15	340.7	11.0	3.2
	15	556.9	22.4	4.0
Enzymic PAP	15	91.8	2.2	2.4
	15	275.0	10.5	3.8
	15	453.2	19.4	4.2

with HPLC and 20 to 34 determinations with the other methods (Table II). The precision of our HPLC method is equal to or slightly better than that of the Jaffé and both the automated enzymic methods. The same holds for the day-to-day reproducibility (Table III). Thirteen to fifteen subsequent determinations were carried out at three different levels of creatinine over ten days. It could be concluded that the day-to-day reproducibility of the HPLC method is comparable with those of the other methods.

The accuracy of the methods was estimated from a comparison of the results with the values given for the commercial control materials (Table IV). Two materials were reference HPLC standards (Kontrollogen-L and -LP). The agreement between our results and declared values was very good. There is a rather great discrepancy between the values from the HPLC and the spectrophotometric methods. In comparison with the enzymic methods, the results are both positive and negative; various types of interferences and enzyme inhibitors can mutually interact. In correlation analysis, groups with different numbers of sera were evaluated. It limits the interpretation of data obtained (Table V). The best correlations were found between the Jaffé and the two enzymic methods. The HPLC results correlated

TABLE IV

DETERMINATION OF CREATININE IN CONTROL SERA

Values in parentheses are mean values of our measurements; $n = 10$.

Serum	Creatinine concentration (μmol/l)				
	HPLC	HPLC[a]	Jaffé	Enzymic UV	Enzymic PAP
Monitrol II E	(480.5)	–	535	–	512 (418.2)
Duotrol	(152.0)	–	159.3[b] (118.0)	140 (172.4)	– –
Precinorm U	(168.4)	171[c]	155[d] (136.8)	– (175.2)	153 (169.1)
Boehringer	(133.6)	–	164 (99.9)	– (161.1)	133 –
Kontrollogen-L	(116.5)	116[e]	148.8 133[b] 129[d]	–	115
Kontrollogen-LP	(294.6)	295[e]	324.6 301[b] 305	–	295
Humatrol N/013	(93.3)	–	100 (126) 92.1[f]	–	77.2[g]
Exatest I	(115.1)	–	120[f]		
Exatest K	(249.9)		(275)		

[a] Declared values.
[b] Jaffé without deproteination.
[c] Isotope dilution MS.
[d] Jaffé (Merck) with deproteination; other given values: 186 μmol/l (Jaffé without deproteination), 185 μmol (Jaffé with deproteination by TCA).
[e] Both HPLC and GC–MS.
[f] Jaffé end-point.
[g] Enzyme iminohydrolase.

TABLE V

CORRELATION OF THE METHODS

Methods	Equation	n	r
Jaffé *vs.* HPLC	$y = 0.982x - 9.14$	12	0.9862
Enzymic PAP *vs.* HPLC	$y = 1.017x + 13.90$	9	0.9757
Enzymic UV *vs.* HPLC	$y = 0.992x + 14.52$	9	0.9961
Enzymic UV *vs.* Jaffé	$y = 1.130x + 16.28$	111	0.9922
Enzymic UV *vs.* enzymic PAP	$y = 0.957x - 5.91$	51	0.9817
Enzymic PAP *vs.* Jaffé	$y = 1.140x + 12.56$	52	0.9920

closer to the enzymic UV results than to the Jaffé and enzymic PAP results.

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