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REVIEW

CHROMATOGRAPHY AND ELECTROPHORESIS OF CREATININE AND OTHER GUANIDINO COMPOUNDS

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LIST OF ABBREVIATIONS

AARG	N-α-Acetylarginine
AGM	Agmatine
ARA	Argininic acid
ARG	Arginine
ASA	Argininosuccinic acid
CR	Creatine
CRN	Creatinine
G	Guanidine
GAA	Guanidinoacetic acid
GBA	Guanidinobutyric acid
GC	Gas chromatography
GCA	Guanidinocaproic acid
GGA	Guanidinoglutaric acid
GPA	Guanidinopropionic acid
GSA	Guanidinosuccinic acid
GVA	Guanidinovaleric acid
HARG	Homoarginine
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
MG	Methylguanidine
MS	Mass spectrometry
PG	Phenylguanidine (exogenous)
TAU	Taurocyamine
TCA	Trichloroacetic acid
TLC	Thin-layer chromatography

1. INTRODUCTION

Guanidino compounds in body fluids are important markers of renal diseases [1–10]. The following guanidino compounds have been demonstrated in human urine and plasma (serum) [11]: G, MG, GVA, GAA, GPA, GSA, ARG, AARG, HARG, TAU, ASA, CR and CRN. Among them, CRN appears to be most important clinically as its plasma (serum) level and renal clearance are routinely used for the diagnosis of renal disease and adjustment of dosage regimens in renal patients (see also refs. 79–81 and 110–112).

GSA, CRN, G, and MG are increased in the plasma and erythrocytes of the uremic patients [4,7,12–16]. Both GSA and MG are considered to be uremic toxins in the uremic syndrome as they gave rise to a complex of symptoms similar

to uremia [14,15]. Studies have also shown that GSA inhibits platelet aggregation and platelet factor III release [15,16] and, as with GBA, GAA and GPA [17], it may also be partly responsible for the uremic bleeding diathesis [15,17,18]. Injected MG was found to cause deaths in dogs from anemia (due to hemolysis and reduced red cell production and survival), gastric ulceration and hemorrhages, and peripheral neuropathy [14]. A persistent increase in G, the concentrations of which were directly proportional to CRN [19], was followed by anorexia, gastric discomfort, nausea, vomiting, pruritus, muscle fasciculations and peripheral paresthesias [13]. In vitro, CRN increased the spontaneous autohemolysis [20]. In rats, CRN induced a tranquilizing effect [21]. In dogs, CRN shortened the red cell survival [17]. In renal patients, oral administration of CRN induced an intolerance to carbohydrates [22]. Thus CRN might contribute to some uremic symptoms: hemolysis, carbohydrate intolerance and drowsiness [13].

TAU was suspected to be another uremic toxin as it induced convulsion in animals [23,24] and its levels increased in uremic rabbit brain [23].

In hyperargininemia (patients with a deficiency of arginase), the levels of ARG and ARG metabolites (GAA, AARG, ARA) and other unknown guanidino compounds increased in biological fluids [25]. Like TAU and MG, these arginine metabolites have been shown to be experimentally epileptogenic [26–31] and their levels may be correlated with the clinical symptoms of hyperargininemia: irritability, vomiting, episodic, hyperammonemia and epilepsy [25]. Thus evaluation of guanidino compounds could give useful information regarding pathophysiology and treatment for uremia and hyperargininemia.

2. ENDOGENOUS GUANIDINO COMPOUNDS

2.1. Liquid chromatographic methods

2.1.1. General

Guanidino compounds are similar in chemical properties. Their concentrations in normal human serum are generally too low to be quantified by conventional chromatographic methods: paper chromatography [32,33], thin-layer chromatography (TLC) [34,35], gas chromatography (GC) [36,37] and some ion-exchange liquid chromatographic (LC) methods with Sakaguchi or VogesProskauer color reactions [5,23,38–41]. Recently, more sensitive methods have been reported using high-performance liquid chromatography (HPLC) with fluorescence detection [10,42–48] and adapted for routine analysis [49]. Table 1 lists some of sensitive HPLC methods reported to data [42,44,48–53]. As serum or plasma levels of CRN and CRN clearance are used routinely for renal function diagnosis and for dosage adjustment, the assay sensitivity, specificity, accuracy and precision are critical. The assays of CRN are discussed separately in Section 3.

2.1.2. Sample work-up procedures

Conventional assays based on colorimetric methods require 5–20 ml and concentration of samples [54]. Methods using an amino acid analyzer require con-

TABLE 1

HPLC METHODS FOR ANALYSIS OF GUANIDINO COMPOUNDS

HPLC method	Column	Isocratic or gradient	Derivatization			Number of mobile phase components	Fluorescence (nm)		Approximate HPLC time (min)	Source[c]	Volume[b] (μl)
			Pre- or post-column	Reagent[a]	Reaction temperature (°C)		Ex.	Em.			
1	RP-C_8 (ion pair)	Isocratic	Post	NQS	65	2	355	505	10–25	Serum	100
2	RP-phenyl	Linear gradient	Pre	BZ		1	325	425	25	Serum	200
										Urine	50
3	RP-C_{18} (ion pair)	Isocratic	Post	PQ	70	3	375	460	35	Serum	50
4	Cation exchanger	Step gradient	Post	BZ	80	6	325	435	45	Serum	100
										Urine	100
5	Cation exchange	Step gradient	Post	NIN	50	8	395	500	37	Serum	100
6	Cation exchanger	Step gradient	Post	NIN	50	8	395	500	35	Plasma	200
7	Cation exchanger	Step gradient	Post	PQ	60	7	365	495	115	Plasma	1000
										Erythrocyte	1000
8	Cation exchanger	Step gradient	Post	PQ	50	6	365	460	100	Serum	100
										CSF	100

TABLE 1 (continued)

HPLC method	Deproteinization method	Compounds measured	Reported detection limit[d]	Reference
1	TCA	ARG, CRN, G, GAA, GBA, GPA, GSA, MG	20 ng	50
2	Ultrafiltration	GBA, GSA, MG, TAU	8–78 pmol/ml	51
3	Ultrafiltration	ARG, CRN, G, GAA, GBA, GPA, GSA, MG, TAU	1–60 ng per 50 µl	53
4	Perchloric acid	ARG, ARA, AARG, AGM, CR, CRN, G, GAA, GBA, GPA, GSA, MG, TAU	5–50 pmol (except CRN, CR: 1, 20 nmol)	52[e]
5	TCA	ARG, CR, CRN, G, GAA, GBA, GPA, GSA, MG, TAU	Not defined	49
6	TCA	ARG, CR, CRN, G, GAA, GBA, GPA, GSA, MG	1–5 pmol (except ARG, CRN: 50, 1000 pmol)	44
7	TCA TCA	AARG, ARG, CRN, G, GAA, GBA, GPA, GSA, MG, TAU	Not defined	12
8	Ultrafiltration	ARG, CRN, G, GAA, GBA, GPA, GSA, MG	1.4–78 pmol/ml (=0.11–8.3 µg/dl)	42

[a]NIN = ninhydrin; NQS = 1,2-naphthoquinone-4-sulfonate; PQ = 9,10-phenanthrene quinone; BZ = benzoin; TCA = trichloroacetic acid.
[b]Volumes of biological samples used for analysis.
[c]CSF = cerebrospinal fluid.
[d]Definition of detection limit: concentrations corresponding to twice the noise peak levels except ref. 53 (definition not given).
[e]This method used PG as an internal standard; all other methods used external standard method for quantification.

centration technique such as lyophilization [41]. As more sensitive detection systems become available, smaller amounts (100 μl–1 ml) of samples are required [42,44,48–53]. However, sample clean-up procedure remains essential.

Deproteinization is an important procedure for methods using either pre- or post-column derivatization and fluorescence detection. Loss of guadinino compounds during deproteinization is expected as several guanidino compounds are amphoteric; loss of amino acids (amphoteric) during deproteinization has been reported [12,55–57].

When using pre-column derivatization with benzoin, several oligopeptides with one or two arginyl residues tend to interfere with the assay. Ultrafiltration with a UMO5 membrane (Amicon Diaflo membrane, excluding substances > 500 daltons) effectively removes these interferences [51]. In this same study, deproteinization with perchloric acid did not remove the interference peaks from serum. Therefore, the authors concluded that deproteinization with acidic precipitating reagent such as perchloric acid was not suitable for sample clean-up for their HPLC method [51]. The ultrafiltration step was not necessary for urine unless from patients with proteinuria.

Other studies showed that besides ultrafiltration technique, deproteinization with trichloroacetic acid (TCA) effectively removed some interfering substances and gave good and reproducible recoveries [12,58]. Sulfosalicylic acid, perchloric acid and molybdenic tungstate were bad choices for deproteinization [12,58,59]. However, although TCA was a good deproteinization reagent, it eluted closely with TAU in some HPLC systems [60] and might affect the accurate determination of TAU [61] when short columns were used [62].

Appropriate sample handling and storage are also essential. An earlier study [63] showed that falsely high levels of GAA and MG were obtained when plasma samples were not frozen immediately. Adjusting the sample to a low pH (2–2.5) prior to analysis was found to be necessary for accurate determination of some guanidino compounds [59,63].

2.1.3. Selection of columns and column parameters

Ion-exchange columns have been used since the 1960s for isolation and purification of guanidino compounds [64–66]. Separation by cation-exchange columns prior to using a modified amino acid analyzer provides a sensitive method [67]. However, due to their chemical similarities, many guanidino compounds might elute together with amino acids [68,69]. Use of a strongly basic, quaternary ammonium anion-exchange resin in the OH^- form to separate some guanidino compounds from amino acids in urine has been reported [67]. However, during anion-exchange separation MG level was falsely increased possibly due to degradation of other guanidino compounds [67,68]. Similarly, the use of charcoal chromatography (adsorption onto charcoal in alkaline and elution with acidic ethanol) also tends to give a falsely high value of MG possibly due to partial conversion of CRN into monosubstituted guanidines during separation [4].

For pre-column derivatization using benzoin, reversed-phase column appears to be the column of choice [48]. Other types of columns are not suitable because benzoin derivatives fluoresce intensely in polar solvents (methanol, aqueous so-

lution) and because the derivatives are strongly retained (except for MG and PG) on silica gel and aluminum columns and not readily eluted from the column with methanol, ethyl acetate, tetrahydrofluran, acetonitrile, chloroform or hexane [48].

For post-column derivatization using ninhydrin, 1,2-naphthoquinone-4-sulfonate, 9,10-phenanthrene quinone and benzoin, both reversed-phase and cation-exchange columns have been used [42,44,49,50,52,53]. In general, ion-exchange columns require longer times for analysis and re-equilibration (35–100 min) as compared to the time (25–35 min) needed for analysis using reversed-phase columns [42,44,49,50,52,53].

Column temperature should be optimized for methods using post-column derivatization [42,44,49,50,52,53]. Usually, increase of the column temperature shortens retention and facilitates separation.

2.1.4. Selection of mobile phase

With ion-exchange columns, step gradient is required [42,44,49,52] necessitating the preparation of three to six different buffers with pH values of 3–11. The effects of pH and ionic strength on the capacity factors and the separation of guanidino compounds have been discussed earlier [42,44].

For reversed-phase column separation, linear gradient with an alkaline pH [48,51] or isocratic ion-pair elution [50,53] with a pH of 3.5–4 have been used. For post-column derivatization, two to four pumps are usually used to deliver three to four solvent mixtures including a mobile phase for column separation, an alkaline solution to mix with the column effluent, a solution containing the derivatizing reagent [12,42,44,49,52,53,67] and/or a less alkaline solution to provide an optimal medium for the reaction mixture to fluoresce [52]. A recently developed ion-pair reversed-phase HPLC procedure includes the derivatizing reagent, 1,2-naphthoquinone-4-sulfonate, in the mobile phase and only requires two pumps to deliver two solvent systems [50].

Figs. 1 and 2 show chromatograms of a standard mixture of guanidino compounds and of a serum sample, respectively, on a reversed-phase column [50].

2.1.5. Derivatization procedures

2.1.5.1. Pre-column derivatization. Benzoin is the only derivatizing reagent reported to date for both pre- and post-column derivatization [48,51,52]. Benzoin gives an intense fluorescence in aqueous potassium hydroxide solution of methylcellosolve. β-Mercaptoethanol is needed prior to heating in order to stabilize the fluorescent products and to prevent the degradation of products [70]. Sodium sulfite added before heating decreases the reagent blank [70,71]. One benzoin derivatization method could detect 40–170 pmol/ml guanidino compounds and was reported to be two to seven times more sensitive than the 9,10-phenanthrene quinone derivatization method and five to twenty-five times more sensitive than the ninhydrin derivatization method in one study [70].

2.1.5.2. Post-column derivatization. Derivatization of guanidino compounds with 9,10-phenanthrene quinone can only be done post-column as 9,10-phenanthrene quinone forms only one product with monosubstituted guanidines in al-

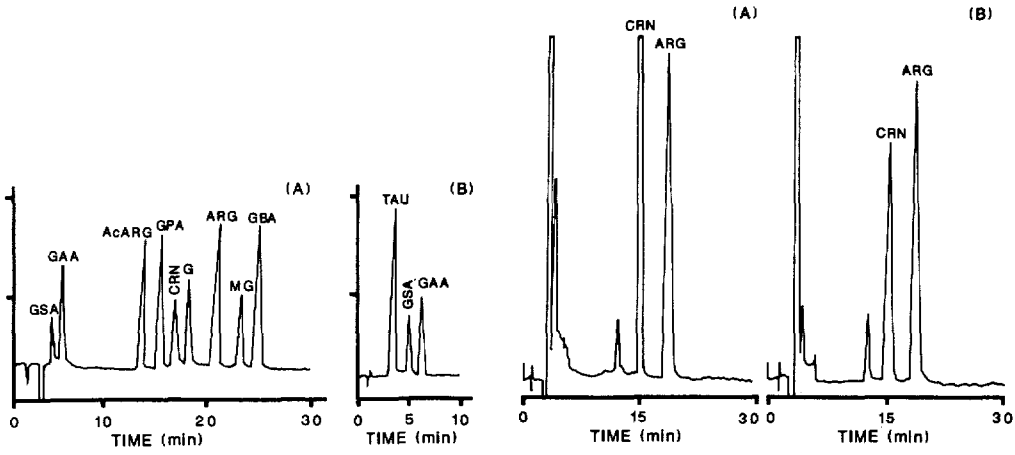

Fig. 1. Chromatograms of a standard mixture of guanidino compounds. For abbreviations and HPLC conditions, see text and Table 1, HPLC method 1 (ref. 50). (A) Mobile phase pH 4.0; (B) mobile phase pH 3.5. (Reprinted with permission from Anal. Biochem. Copyright Academic Press.)

Fig. 2. Chromatograms of a serum sample before (A) and after (B) hemodialysis therapy. For abbreviations and HPLC conditions, see text and Table 1, HPLC method 1 (ref. 50). (Reprinted with permission from Anal. Biochem. Copyright Academic Press.)

kaline medium [70,72]. 9,10-Phenanthrene quinone is not stable in alkaline solution and not soluble in water. 9,10-Phenanthrene quinone needs to be dissolved in methanol or dimethylsulfoxide and stored in refrigerator at 5°C and prevented from light exposure [42]. The fluorescent products are stabilized by β-mercaptoethanol [46].

As some sera of patients suffering from renal dysfunction show natural fluorescence at 330 and 425 nm, the excitation and emission maxima for benzoin derivatives, ninhydrin (excitation and emission at 395 and 500 nm) would be a better choice for derivatization for samples with natural fluorescence [49,73]. Ninhydrin is highly soluble in aqueous medium thus eliminating the problem of precipitation in the 9,10-phenanthrene quinone method [44,49]. Ninhydrin cleaves immediately in alkaline medium to produce O-carboxyphenylglyoxal [74], which can then combine with guanidines, monosubstituted guanidines and N,N-disubstituted guanidines but not with amino acids [75] to form highly fluorescent addition products [32,75,76].

1,2-Naphthoquinone-4-sulfonate also reacts with guanidino compounds in alkaline solution and yields a high-intensity fluorophore. The reaction is similar to 9,10-phenanthrene quinone reaction and is specific to guanidino compounds. However, as described earlier, 9,10-phenanthrene quinone is insoluble in water and needs to be dissolved in organic solvents (dimethylformamide, ethanol, etc.). As 1,2-naphthoquinone-4-sulfonate is not stable in alkali, it is added to the mobile phase [50] with a pH of 3.5 or 4.

2.1.6. Fluorescence detection

As discussed earlier and described in Table 1, the fluorescence detection wavelengths for these guanidino derivatives need to be optimized for both the maxi-

mum excitation and emission of the derivatives and the wavelengths of the interfering fluorescent substances from biological samples. The reaction temperature and pH for optimal fluorescence also need to be determined empirically.

2.2. Gas chromatographic methods

Mori [37] used GC with mass spectrometry (MS) for identification of less than 1 ng of GAA, GPA, GBA, ARG and HARG in rat and bovine brain tissues. Other researchers also used GC for simultaneous analysis of amino acids and some guanidino compounds [77]. However, GC methods [37,77] require lengthy sample preparation and derivatization procedures and might not be amenable for routine automatic analysis.

2.3. Other methods

An enzymatic method has been developed for MG [78]. This method uses two enzymes: methylguanidine aminohydrolase and methylamine oxidase to react with MG to form formaldehyde which is derivatized with methyl 3-aminocrotonate to form a fluorescent product. The method has been successfully applied to measuring MG levels in urine from both normal and renal patients [78] and is suitable in clinical monitoring.

3. METHODOLOGY FOR CREATININE ASSAY

3.1. General

As noted in Section 1, CRN is an important endogenous compound. Its plasma (or serum) concentration and renal clearance have been commonly used for the detection and/or quantification of renal impairment and also for dosage regimen adjustments in renal dysfunction. Accuracy and precision in measurements of CRN levels in plasma, serum and urine are, therefore, critical.

3.2. Methods involving alkaline picrate

The majority of assay methods for CRN are based on the Jaffe reaction in which CRN reacts with alkaline picrate to form an orange–red compound. The intensity of color is sensitive to assay conditions, such as working wavelength, composition of the Jaffe reagent, temperature and time. In addition, the Jaffe reaction has long been known to be non-specific when applied to the measurement of CRN in plasma. Many compounds are known to react with picric acid and to interfere with the assay [79–81]. Table 2 summarizes the various approaches that have been used to improve the specificity of the reaction [81]. However, it is generally believed that the alkaline reaction by itself is not specific for CRN and that deproteinization (and the various approaches listed in Table 2) along with careful control of reaction conditions are not adequate to eliminate the interferences [82]. Any compound that possesses an active methylene group

TABLE 2

SUMMARY OF SOME APPROACHES USED TO IMPROVE THE SPECIFICITY OF THE *JAFFE REACTION WITH CREATININE*

From ref. 81 with permission.

1.	Adsorption of creatinine by:
	(a) Kaolin
	(b) Lloyds reagent (Fuller's earth)
	(c) Ion-exchange resin
2.	Oxidation of interferents by:
	(a) Ceric sulphate
	(b) Iodine
3.	Solvent extraction of interferents
4.	pH Adjustment to decolorise the Jaffe-creatinine chromogen
5.	Continuous flow dialysis
6.	Kinetic measurement of reaction with:
	(a) Specific reaction interval measurement
	(b) Modified assay conditions

(such as keto–enol tautomer) has the potential for reacting with picrate [81]. A recent study [83] also indicates that the carbonyl group is the common structure for compounds reacting with picrate. The structures adjacent to the carbonyl group significantly affect the absorptivity and equilibrium constant of the reaction, but the steric hindrance is the major factor affecting the rate of reaction [83].

3.2.1. Enzymatic methods

Using CRN-decomposing enzymes by treating one portion of the sample with the enzyme systems and then comparing the difference in color produced by the Jaffe reaction in the enzyme-treated and untreated samples, one can attribute the difference to the true CRN content of the sample. Successful application of these methods, however, depends on the availability and purity of these CRN-decomposing enzymes [79–81].

3.3. Chromatographic methods

Recognizing the non-specificity associated with the alkaline picrate methods, many investigators have developed HPLC procedures for determination of CRN levels [84–99] (Table 2). These methods, in general, offer improved specificity and sensitivity.

3.3.1. General aspects of the chromatographic procedures used for the determination of creatinine in serum, plasma and/or urine

3.3.1.1. Sample work-up procedures. The method used for treating biological samples prior to their introduction onto an HPLC system generally fall into one of two categories: extraction or direct injection [100]. CRN is soluble in water and insoluble in most water-immiscible organic solvents [101]. Therefore, purification and isolation by the classical extraction method appears not to be effective [85]. Two recent studies have reported an approximately 60–70% extraction recovery for CRN [102,103].

The direct-injection is a simple and rapid method. However, it will result in a rapid increase in back-pressure and a deterioration of analytical column. To minimize this problem, several sample pre-treatment techniques have been described for removing proteins prior to the injection of samples. These include the use of guard or pre-column, ultrafiltration devices and various protein precipitants such as organic solvents and ionic salts. The efficacy of these deproteinizing methods have been reported [85,100]. All of the methods used effectively removed proteins from the biological samples. The differences among these methods are the complexity and time involved with the procedure [85,100]. With a cation-exchange column for sample treatment, in addition to the removal of proteins, other interfering compounds can also be removed with careful selection of pH and strength of the eluents [88,90,95]. Use of acetonitrile [85,87,100], methanol [84,100] or TCA solution [91,100] is easy and effective. However, these solvents may affect the baseline of the chromatogram in some assay conditions [92,104]. For ultrafiltration devices, one should verify that CRN is not adsorbed to the membrane used to ensure reproducible results [93,105].

Membranes of negligible adsorptive properties (such as YMB or YMT membranes from Amicon) have been recommended [93]. Meyer et al. [104] reported that deproteinizing serum or plasma samples with TCA or perchloric acid produced severe baseline depression not seen with deproteinization with acetonitrile. Achari et al. [92] reported that the supernatant, after precipitation of plasma proteins with acetonitrile, gave a blank value for CRN five to six times higher than those obtained from the plasma filtrate. However, acetonitrile, believed to be the simplest and easiest one to use among various solvents, has been shown to be adequate for routine analysis [87].

3.3.1.2. Selection of columns. Various analytical columns have been used for the separation of CRN from other constituents in biological samples (Table 3). Among over twenty HPLC methods reviewed, only three used normal-phase silica columns [99,102,103]. Patel and George [99] reported that no sign of deterioration of the silica column occurred after performing analyses over a period of twelve months. Fig. 3 illustrates typical chromatograms of creatinine separated on a silica column [99]. The ammonium hydroxide in the mobile phase does not etch the silica as one might expect. Ziemniak et al. [103] also reported that the silica column performance had been maintained with routine care for about five months without noticeable deterioration. The alkaline pH can be tolerated because of the large organic/aqueous ratio of the mobile phase (acetonitrile–methanol–water–ammonium hydroxide, 1000:50:50:2, v/v, at an apparent pH

TABLE 3

HPLC METHODS FOR ANALYSIS OF CREATININE

Author(s)	Reference(s)	Column	Composition of mobile phase	Detection	Sample pre-treatment	Source[a]	Other compounds assayed
Soldin and Hill	84	RP-C_{18} (paired ion, 30°C)	0.02 *M* Potassium phosphate (pH 5.1) containing 80 mg of sodium lauryl sulfate–methanol (76:24, v/v) (isocratic)	200 nm	Methanol	Serum, urine	
Chiou and co-workers	85–87	Cation exchanger	0.1 *M* Ammonium phosphate (pH 2.6)	215 nm	Acetonitrile	Plasma, urine	
			0.012 ~0.035 *M* Ammonium phosphate (pH 4.8) (isocratic)	254 nm (or 235 nm)	Acetonitrile, then methylene chloride	Saliva	
Lim et al.	88	RP-C_{18}	0.01 *M* Ammonium acetate (isocratic)	235 nm	Cation-exchange column	Serum, urine	
Krstulović et al.	89	RP-C_{18}	0.1 *M* Monopotassium phosphate (pH 2.5)	220 nm	Simple filtration or dilution	Amniotic fluid, urine, CSF	
			Acetonitrile–water (3:2, v/v) (gradient)	254 nm			
Spierto et al.	90	RP-C_{18}	0.01 *M* Ammonium acetate (pH 6.5–6.6) (isocratic)	236 nm	Ultra-filtration	Serum	Allopurinol (internal standard), uric acid
Ambrose et al.	91	Cation exchanger	0.02 *M* Lithium acetate (pH 4.68)	234 nm	10% TCA	Serum	
			0.075 *M* Lithium acetate (pH 7.1) (step gradient)				

Achari and co-workers	92,93	RP-C_{18} with guard column	0.05 *M* Sodium acetate (pH 6.5)–acetonitrile (98:2, v/v) (isocratic)	254 nm	Ultra-filtration	Plasma, urine	
Okuda et al.	94	RP-C_{18} at 50°C	0.1 m*M* Monopotassium phosphate–acetonitrile (4:1, v/v) (isocratic)	220 nm	Acetonitrile	Serum, urine	Phenacetin (internal standard)
Holmes et al.	95	RP-C_{18}	0.01 *M* Ammonium acetate (isocratic)	254 nm	Cation-exchange column	Serum	Allopurinol
Niklasson	96	RP-C_{18}	0.2 *M* Monopotassium phosphate (pH 6.6) (isocratic)	260 nm	Centri-fugation	CSF	Hypoxanthine, xanthine, urate
Zhiri et al.	97	RP-C_{18}	0.03 *M* Ammonium acetate–0.156 *M* methanol (pH 7.0) (isocratic)	235 nm	Five-fold dilution with mobile phase and precolumn	Plasma	Uric acid
Baranowski and Westenfelder	98	RP-C_{18}	0.05 *M* Ammonium acetate (pH 6.0)–acetonitrile (98:2, v/v) (isocratic)	236 nm	Ultra-filtration	Plasma, urine	*p*-Aminohippuric acid
Patel and George	99	Silica	200 ml Methanol, 800 ml acetonitrile, 10 ml concentrated ammonium hydroxide, (isocratic)	254 nm	Acetonitrile	Serum, urine	Quinine dihydrochloride monohydrate (internal standard)

[a]CSF = cerebrospinal fluid.

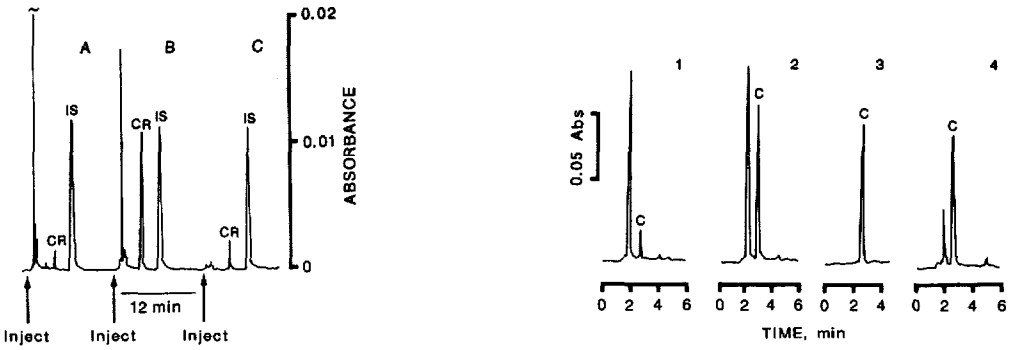

Fig. 3. Chromatogram of creatinine: (A) in serum, creatinine 9 mg/l; (B) in diluted urine, creatinine 55 mg/l; (C) in distilled water, creatinine 10 mg/l. Peaks: CR = creatinine; IS = internal standard. For HPLC conditions, see Table 3 (ref. 99). [Reprinted with permission from Anal. Chem. Copyright (1981) American Chemical Society.]

Fig. 4. Chromatograms of creatinine (C) obtained from various fluids: (1) plasma; (2) plasma to which was added 40 μg/ml creatinine; (3) water containing 40 μg/ml creatinine; (4) urine. The arrow indicates the time of injection. HPLC conditions: column, 5-μm Ultrasphere ODS, 25 cm × 4.6 mm I.D.; mobile phase, 0.05 *M* sodium acetate (pH 6.5)–acetonitrile (98:2, v/v); flow-rate, 1.0 ml/min; detection, 254 nm; sensitivity, 0.5 a.u.f.s. (ref. 92). (Reproduced from J. Chromatogr. Sci. by permission of Preston Publications, a division of Preston Industries.)

of 10.5). However, most reported HPLC methods used reversed-phase C_{18} columns for their robustness and reliable performance. Fig. 4 depicts separation of CRN on a reversed-phase column [92]. The performance of cation-exchange columns have been found to be practically unaltered after many months of usage [106].

3.3.1.3. Selection of mobile phase. Most of the HPLC methods for CRN assay used isocratic mobile phases. The composition of mobile phase is relatively simple: either buffer solution alone or buffer solution with some organic modifiers such as methanol or acetonitrile. The buffer salts used include ammonium acetate, ammonium phosphate, lithium acetate, potassium phosphate and sodium acetate (Table 2). In the paired-ion chromatography, Soldin and Hill [84] used sodium lauryl sulfate as the counter-ion to achieve the separation of CRN. In some mobile phase systems, the retention of CRN can easily be modified by adjusting the pH or the concentration of the buffer solution [85,96]. In modifying Chiou's method, Meyer et al. [104] also noticed that the stability of the chromatogram baseline can be enhanced by adding a small volume of acetonitrile in the mobile phase. They also observed that when analyzing the mouse plasma with very low CRN levels the resolution of the CRN peaks can be improved by the addition of sodium ions (i.e., adding 100 μl of 2 *M* sodium hydroxide per buffer, then adjusting the pH to 3.5 with phosphoric acid) [104]. In the ammonium acetate mobile phase system [88,90], however, the retention times for CRN and allopurinol (an internal standard) were not affected by the pH and the concentration of ammonium acetate of the mobile phase. Ambrose et al. [91] described cation-exchange chromatography with a simple-step gradient elution to achieve

the separation. A pH below the pK_a of CRN was used to make CRN sufficiently cationic to be retained by an appropriate cation-exchange resin. The pH was then increased to 7.1 to elute the neutral CRN molecule [91]. This mode of separation eliminates the interference from those molecules that are either uncharged or negatively charged at the lower pH [91]. The control of the eluent strength and pH is important for the retention and sharpness of the CRN peak [91]. Krstulović et al., [89] also described a gradient elution for the separation of CRN on a reversed-phase C_{18} system.

3.3.1.4. Detection and quantification method. CRN eluted from the analytical column is quantified by its UV absorption. Depending upon the assay conditions and the sensitivity required the following wavelengths have been used: 200, 215, 220, 234, 235, 236, 254 and 260 nm. Peak heights or areas are measured for quantification. Since the sample treatment procedures are simple and the recovery is complete, most HPLC procedures reported do not use an internal standard. The omission of internal standard, however, did not compromise the precision of the assay [90].

3.3.1.5. Methods for peak identification. To ascertain that the sample peak is entirely due to CRN is critical to the interpretation of assay results. The identity of chromatographic peaks can be deduced from evidence accumulated from (a) retention behavior, (b) chromatography with the reference compound, (c) absorbance ratios, (d) stopped-flow UV spectra and (e) enzymatic peak-shift techniques [89].

Absorbance ratios can be obtained by measuring the absorbances at different wavelengths [85,89]. From the computed ratios for the standards and the peaks of interest, one can estimate the purity of a chromatographic peak [89]. The enzymatic peak-shift technique utilizes the specificity of an enzyme in catalyzing a reaction. Due to the difference in absorption maxima of the substrate and/or products, as well as their retention behavior, incubation with enzyme can be used as a confirmatory test in conjunction with other identification methods [89]. The collection of all the evidence required for the identification of a chromatographic peak, therefore, relies on the instrumentation set-up in the laboratory and the availability of the specific enzyme system. The majority of the reported HPLC methods for CRN utilized the techniques of retention time comparison and co-chromatography with the reference CRN for identifying the CRN peak. Only a few methods also utilized absorbance ratio [85,89,104] and enzymatic peak-shift techniques [89,90,96].

4. SUMMARY

Chromatographic methods, in general, are considered as the definitive and reference procedure. The HPLC procedures reviewed all seem to provide improved and adequate specificity and sensitivity for CRN assay. The HPLC methods are relatively easy to use, have nearly complete recoveries ($\geq 95\%$) and have good precision. The within- and between-day variations are often less than 4% [85,87,90,94–97,99]. Most of the methods developed [84,85,90, 91,94,97,99] were tested for potential interference and the specificity for CRN was demonstrated.

One important advantage of the HPLC method [85] is that the presence of an interfering substance in the biological sample can most likely be detected by its influence on the sharpness and resolution of the CRN peak. It is understandable that there are possibilities that other constituents in the biological sample may also be co-eluting with CRN and the investigator should carefully examine the chromatogram obtained for any sign of interference. Ginman and Colliss [107] reported that azathioprine, cyclosporin A, cefotaxime and prednisolone used in patients after renal transplant interfered with the HPLC assay: the results are 40% higher than those by Jaffe method. These interferences, however, were corrected with a slight modification of the HPLC conditions [107].

Because of the non-specificity associated with the Jaffe reaction, the results obtained from Jaffe-based assays often exceed those from HPLC methods [95,108,109]. Such overestimation would be especially significant when the serum CRN is in the normal range where small changes may be indicative of a significant change in a patient's renal function [95,111].

It appears that HPLC methods are the methods of choice for simultaneous analysis of endogenous guanidino compounds. Depending on the purpose of analysis, one would choose a particular type of method, e.g., one among those representative methods listed in Table 1. If one is interested in measuring MG and GSA levels in serum or plasma, then depending on the sensitivity requirement and available instrumentations, one could choose many method listed in Table 1. However, if one needs to measure AARG, ARG and ARA, in addition to MG and GSA, then method 4 in Table 1 would be more appropriate. If TAU is the compound of interest, then one of the methods 2, 3, 4, 5, and 7 with an appropriate deproteinization procedure would be desirable.

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