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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF GUANIDINO COMPOUNDS USING NINHYDRIN REAGENT

II. GUANIDINO COMPOUNDS IN BLOOD OF PATIENTS ON HAEMODIALYSIS THERAPY

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SUMMARY

An automated high-performance liquid chromatographic method using alkali-ninhydrin reagent for the post-column derivatization of guanidines has been developed. This procedure was applied to measurements made before and after haemodialysis. Among the guanidino compounds found in human blood, methylguanidine showed the lowest removal rate. The removal rate of guanidinosuccinic acid correlated with the plasma α_1-globulin fraction. The removal rate of each guanidino compound decreased with the period of dialysis.

INTRODUCTION

Guanidino compounds in body fluids are important markers of renal disorder, and the measurement of these compounds gives useful information about renal function and the effect of haemodialysis [1—10].

As guanidino compounds are very similar in chemical properties, and their amounts in biological fluids are very small, complete separation and sensitive detection have been investigated using high-performance liquid chromatography (HPLC) coupled with fluorometric detection. The 9,10-phenanthrenequinone method [9—12] has been developed for this purpose, but the reagent is sparingly soluble in water, and large amounts of organic solvents are required in order to prevent precipitation. We have developed the ninhydrin method [13], and Kai and co-workers [14—16] have developed the benzoin method to overcome this problem.

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This paper describes a fully automated HPLC system for the analysis of guanidino compounds using the ninhydrin reagent. This system was applied to the determinination of guanidino compounds in the sera of haemodialysed patients at the stable stage before and after dialysis. Relationships between the amounts of guanidino compounds and the period of haemodialysis or the clinical chemical data are also discussed.

MATERIALS AND METHODS

Reagents and standards

A standard solution for chromatography was prepared by adding 2 ml of aqueous creatine solution (20.74 μg/ml) and 2 ml of 0.1 *M* hydrochloric acid to the standard mixture of guanidino compounds purchased from Wako (Tokyo, Japan). Ninhydrin, sodium hydroxide, sodium citrate and boric acid were of amino-acid-analysis grade. Reagent-grade sodium chloride and analytical-grade 60% perchloric acid were used. All the reagents were purchased from Wako.

Chromatography

Fig. 1 shows the flow diagram of the automated HPLC system for the determination of guanidino compounds. A sample was injected into the chromatograph from an autosampler, Model SIL-2A (Shimadzu, Kyoto, Japan) on which 48 samples can be loaded at one time. Stepwise gradient elution was begun as the sample was injected, and was carried out using the following mobile phases: elution buffer 1, 0.05 *M* trisodium citrate (pH 3.5); elution buffer 2, 0.12 *M* trisodium citrate (pH 5.0); elution buffer 3, 0.12 *M* trisodium citrate (pH 6.0); elution buffer 4, 0.12 *M* trisodium citrate solution containing 0.5 *M* sodium chloride and 0.1 *M* boric acid (pH 11.4). Eluents 5 and 6, for column-washing, were 0.2 *M* sodium hydroxide and redistilled water, respectively. The pH of eluents 1, 2 and 3 were adjusted with 60% perchloric acid, and that of eluent 4 was adjusted with 1.0 *M* sodium hydroxide. All the eluents were filtered with microfilter FM 22 (pore size, 0.22 μm; Fuji Film, Tokyo, Japan) prior to use. The flow-rate of the mobile phase was 0.7 ml/min. The gradient programme was as follows: eluent 1 for 7 min; eluent 2 for 7 min; eluent 3 for 3 min; eluent 4 for 15 min; eluent 5 for 2 min; eluent 6 for 3 min. The

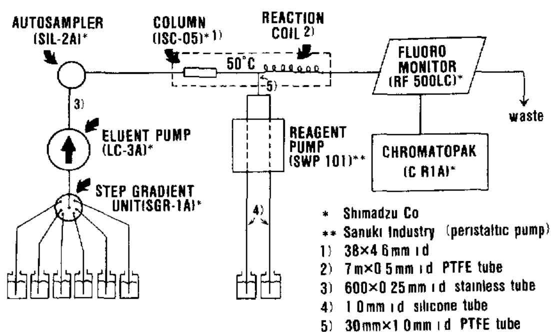

Fig. 1. Flow diagram of the HPLC system.

guanidino compounds were separated on an ion-exchange column ISC-05 (Shimadzu, Kyoto, Japan). Post-column derivatization was accomplished as follows. Aqueous 0.6% ninhydrin solution filtered with microfilter FM 22 and 1.5 *M* sodium hydroxide filtered with microfilter FR 20 (pore size, 0.20 μm; Fuji Film) were delivered with a peristaltic pump (Sanuki Industry, Tokyo, Japan) at a flow-rate of 0.4 ml/min and pre-mixed immediately before they were mixed with the eluate from the column. The resultant mixture was then heated in a reaction coil (7 m × 0.5 mm I.D.) placed in a water-bath kept at 50°C, and the fluorescence intensity was measured at 395 nm (excitation) and 500 nm (emission) with a fluorescence spectrophotometer RF-500-LC (Shimadzu). Quantitation was carried out with a data processor, Chromatopak C-R1A (Shimadzu). Retention times, peak height and the name and amount of each compound were recorded.

Samples

Sera of uraemic patients (31 male and 26 female) before and after haemodialysis were supplied from the Sagamidai Hospital. All haemodialysis was carried out with hollow fibres. Of the dialyses, 30% were by bicarbonate dialysis and the others by acetate dialysis. Hyland Q-PAK® Control serum I (Travenol, Tokyo, Japan) was used as a reference serum.

The sera were deproteinized prior to use as follows. To 100 μl of a serum were added 20 μl of 60% trichloroacetic acid. The resultant mixture was stirred then centrifuged at 3000 *g* for 5 min. To a 60-μl aliquot of the supernatant were added 25 μl of 0.8 *M* sodium hydroxide to bring the pH of the solution to between 2.0 and 2.5. The mixture was then chromatographed.

RESULTS AND DISCUSSION

Automated system for the determination of guanidino compounds

Ten guanidino compounds, taurocyamine (TAU), guanidino succinic acid (GSA), creatine (CT), guanidinoacetic acid (GAA), guanidinopropionic acid (GPA), creatinine (CTN), guanidinobutyric acid (GBA), arginine (ARG), guanidine (G), methylguanidine (MG), were separated in 37 min by the automated HPLC system shown in Fig. 1. Table I shows the day-to-day precision of the amount of guanidino compounds obtained from determination of the standard mixture. Coefficients of variation (n = 10) were in the range 0.7- 3.0%. Table II shows the recovery of guanidino compounds from serum when the standard mixture added to the control serum was analysed. The recoveries were in the range 93.85—107.97%, and the coefficients of variation ranged from 0.37 to 1.28%.

Analysis of guanidino compounds in the sera of patients

Guanidino compounds in the sera of 57 haemodialysed patients in the stable stages were determined using the automated system. The data were used to calculate the reduction rate of guanidines as follows:

$$\text{rate (\%)} = 100 \times \frac{[G]_b - [G]_a}{[G]_b} \qquad (1)$$

TABLE I

DAY-TO-DAY PRECISION OF THE PRESENT METHOD FOR STANDARD SOLUTIONS OF GUANIDINO COMPOUNDS

n = 10 (for ten days) in all cases.

Compound	Amount added (pmol)	Amount found (mean ± S.D.) (pmol)	C.V. (%)
TAU	893.3	876.8 ± 5.964	0.680
GSA	893.3	915.9 ± 18.31	1.999
CT	1242.1	1248.8 ± 20.27	1.623
GAA	446.6	429.5 ± 8.160	1.899
GPA	446.6	428.6 ± 8.221	1.918
CTN	4466.3	4428.6 ± 66 22	1.495
GBA	893.3	893.5 ± 17.36	1.943
ARG	1786.5	1732.1 ± 23.15	1.337
G	1786.5	1663.9 ± 40.52	2.435
MG	446.6	425.8 ± 12.70	2.983

TABLE II

RECOVERIES OF GUANIDINO COMPOUNDS FROM HUMAN SERUM AND THEIR PRECISIONS WITHIN DAY

n = 10 in all cases.

Compound	Amount added (pmol)	Percentage recovery (mean ± S.D.)	C.V. (%)
TAU	893.3	107.97 ± 0.852	0.79
GSA	893.3	97.21 ± 0.553	0.57
CT	1242.1	100.59 ± 1.016	1.01
GAA	446.6	97.57 ± 0.850	0.87
GPA	446.6	96.06 ± 0.338	0.37
CTN	4466.3	99.95 ± 0.728	0.73
GBA	893.3	96.77 ± 1.126	1.16
ARG	1786.5	93.85 ± 0.730	0.78
G	1786.5	99.00 ± 1.040	1.05
MG	446.6	99.82 ± 1.279	1.28

where $[G]_b$ and $[G]_a$ represent the concentration of a guanidino compound before and after haemodialysis, respectively. GSA showed the highest reduction rate, 78.2—82.4%, and MG the lowest, 37.1—46.0%.

Table III shows the relationship between the reduction rate of guanidino compounds and the period of haemodialysis. The reduction rates did not fluctuate during the first ten years. However, the rate decreased for the sera of patients who had been undergoing prolonged haemodialysis therapy. After haemodialysis for over ten years, the rate decreased by 20—55% compared with that at the first stage (one to two years) of dialysis. The degree of decrease in the rate varied among the guanidino compounds. The removal rate of GSA did not decrease after haemodialysis over ten years, whereas the rate of removal of MG apparently did decrease.

TABLE III

COMPARISON OF MEAN REDUCTION RATE OF SERUM GUANIDINO COMPOUNDS IN THE CASES OF FOUR GROUPS CLASSIFIED BY THE PERIOD OF HAEMODIALYSIS TREATMENT

The reduction rates were calculated with eqn 1

Compound	Reduction rate (%) (mean ± S.D.)			
	Period (years)			
	<1 (n=4)*	1–5 (n=25**)	5–10 (n=25***)	>10 (n=3 §)
TAU	51 24 ± 11 62	40 23 ± 14 97	38 93 ± 8 06	23 72 ± 15 97
GSA	79 94 ± 5.58	78 19 ± 8.72	79 83 ± 5 39	82.37 ± 2 55
CT	29.25 ± 23.87	31 93 ± 16 72	31 70 ± 25 51	15.55 ± 11 58
GAA	49.95 ± 19 07	46.77 ± 17.87	51 89 ± 9 86	36 82 ± 22 52
GPA	—	43 16 ± 6.53 (n=3)	45 64 ± 21.79 (n=6)	43.83 ± 19 72 (n=2)
CTN	55 67 ± 10 46	58.55 ± 7.60	59.15 ± 6.73	43 47 ± 22 40
GBA	41 25 (n=1)	46.31 ± 10 13 (n=7)	52.81 ± 19.05 (n=9)	32 65 (n=1)
ARG	65 56 ± 4 11	54 12 ± 8 96	57 19 ± 14 26	47 16 ± 20 70
G	47 82 ± 34 00	52 30 ± 10.35	49 05 ± 13 98	54 39 ± 12 10
MG	40 94 ± 9.14	45 95 ± 10 32	44.11 ± 9 41	37.13 ± 11 64

*One male, three females
**Ten males, fifteen females
***Eighteen males, seven females
§ Two males, one female.

TABLE IV

COMPARISON OF MEAN CONCENTRATION VALUES OF SERUM GUANIDINO COMPOUNDS IN THE CASES OF FOUR GROUPS CLASSIFIED BY THE PERIOD OF HAEMODIALYSIS TREATMENT

Compound	Concentration (μg/dl) (mean ± S D)			
	Period (years)			
	<1 (n=4*)	1–5 (n=25**)	5–10 (n=25***)	>10 (n=3 §)
TAU	252 4 ± 68 2	335 2 ± 74 2	375 9 ± 66 9	365 8 ± 151 4
GSA	415 9 ± 299 0	400 1 ± 233.5	465 6 ± 161 1	531 1 ± 145 6
CT	546 0 ± 227 1	560 3 ± 254 4	399 4 ± 164 1	423 4 ± 121 1
GAA	37 23 ± 10 42	36 90 ± 17 59	38 98 ± 16 93	39 56 ± 14 46
GPA	12 85 ± 2 80 (n=2)	15 32 ± 3 15 (n=17)	14 83 ± 10 31 (n=14)	20 90 ± 5 75 (n=2)
CTN §§	8 12 ± 6 59	12 72 ± 3 36	13 47 ± 2 07	13 27 ± 3 80
GBA	9 87 (n=1)	12 05 ± 5 81 (n=19)	10 72 ± 3 12	9 03 ± 0 72
ARG	2 210 ± 0 796	2 534 ± 0 821	2 288 ± 0 348	2 070 ± 0 223
G	13 26 ± 4 55	17 65 ± 6 15	23 03 ± 10 00	32 18 ± 17 75
MG	25 66 ± 20 64	48 97 ± 24 97	61 05 ± 15 20	58 00 ± 17 07

*One male, three females
**Ten males, fifteen females
***Eighteen males, seven females
§ Two males, one female
§§ Concentration of CTN in mg/dl

Table IV shows the relationship between the concentration of guanidino compounds in sera immediately before dialysis and the period of haemodialysis. The concentration increased with the period of haemodialysis. The rate of removal depends on several external factors, such as the method or equipment for dialysis.

The present study examined the relationship between the reduction rate of guanidino compounds and the clinical chemical data of the patients. Several chemical data that showed significant correlation coefficients ($p < 0.01$) are summarized in Table V. The α_1-globulin value showed a correlation with GSA

TABLE V

CORRELATION COEFFICIENTS OF VARIOUS CHEMICAL DATA

Dependent	Parameter	Correlation coefficient ($P < 0.01$)
TAU (%)	Urine volume (l per 24 h)	0.4272
GSA (%)	Age	−0.3583
	Haemodialysis (h/week)	0.4561
	Flow-rate	0.3150
	α_1-G	−0.6007
	P-lipid	−0.3404
	LDH	−0.3607
CT (%)	—	—
GAA (%)	α_1-G	−0.3341
	CPK	0.3403
GPA (%)	α_2-G	−0.3393
CTN (%)	Haemodialysis (h/week)	0.4944
	α_1-G	−0.3050 ($P < 0.05$)
GBA (%)	ALP	0.3055 ($P < 0.05$)
ARG (%)	—	—
G (%)	Urine volume (1 per 24 h)	−0.4106
	Haemodialysis (h/week)	0.3760
MG (%)	α_1-G	−0.3872

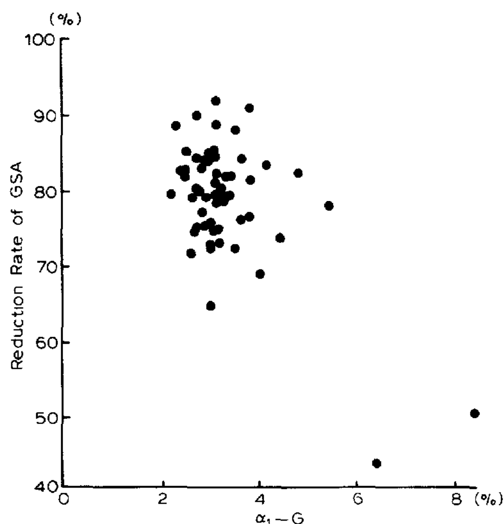

Fig. 2. Correlation between α_1-globulin fraction and reduction rate of GSA $y = -0.5042x$ ($\pm$ 1.813) + 95.28 ($\pm$ 6.255); $n = 57$, $r = 0.601$, S.D. = 6.755, $F = 31.36$ ($> F_0^{0.05} = 4.018$)

and MG (e.g. Fig. 2). This indicates that an increase in the amount of α_1-globulin in blood results in a reduction of the removal of GSA and MG from blood. A number of basic drugs have been shown to have a strong affinity for α_1-acid glycoprotein. α_1-Acid glycoprotein, one of the α_1-globulin fractions, is an acute phase reactant [18, 19], and this fraction, as well as albumin, may play an important role in the protein binding of guanidino compounds, which are strongly basic compounds.

Native fluorescence of sera of uraemic patients

Some of the sera of patients suffering from renal disfunction was found to show strong fluorescence [20], with excitation and emission maxima at 330 and 425 nm. We have examined the peak with native fluorescence by replacing the post-column derivatization reagent (ninhydrin and sodium hydroxide) with redistilled water. When the wavelengths of the fluorometer were set at 330 nm (excitation) and 425 nm (emission), many peaks were detected. These peaks may interfere with the analysis of guanidines with benzoin, the reaction product of which shows excitation and emission maxima at 325 and 425 nm. In contrast, these naturally fluorescent materials were found not to interfere with the determination of guanidino compounds with the ninhydrin reagent, because the reaction product showed the excitation and emission maxima at 395 and 500 nm. The use of ninhydrin reagent in the present system was thus shown to be effective for samples with natural fluorescence.

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