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HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY OF HUMAN URINE USING PERCHLORATE GRADIENT ELUTION SYSTEMS

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SUMMARY

A 100-µl volume of urine was chromatographed on a 50 × 0.4 cm I.D. column packed with a macroreticular anion-exchange resin. Elution was performed with a concave ammonium perchlorate gradient from 0 to 0.25 *M* at a flow-rate of 0.75 ml/min and a pressure of 7.5—11 MPa. With this perchlorate gradient, no baseline drift occurred in the detection at 254 nm, and even detection at 200 nm was possible. The effect of the addition of ethanol or acetonitrile to the ammonium perchlorate solution was investigated. For the assignment of peaks, ultraviolet spectra of the peaks were measured with stopped-flow scanning spectrophotometry.

INTRODUCTION

Several high-performance liquid chromatographic systems have been developed for the separation of ultraviolet (UV)-absorbing constituents of urine with anion-exchange resins [1—6] or reversed-phase packing material [7]. In these systems, chromatography using macroreticular anion-exchange resins revealed many advantageous characteristics for the separation of constituents of body fluids. For example, body fluids such as urine [4,6], blood plasma or serum [8], hemodialysate [8] and blood cells [9] could be analyzed by a

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standard analytical system using a macroreticular anion-exchange resin and a linear ammonium acetate gradient [6, 9]. However, under this chromatographic condition, the ammonium acetate gradient caused a baseline drift in the detection at 254 nm, and detection at shorter UV wavelengths was impossible because of absorbance of acetate ions. To improve the elution system, Miyagi et al. [5] proposed an elution method based on stepwise elution with ammonium chloride—acetonitrile as the mobile phase. Unfortunately, chloride ions in this mobile phase eroded the steel of the instrument, and the detection was not satisfactory in terms of wavelength limit. Thus, we developed new elution systems to eliminate the baseline drift and to detect the constituents of urine in the short UV-wavelength region.

EXPERIMENTAL

Chemicals

Analytical grade ammonium perchlorate, ethanol and acetonitrile were purchased from Wako (Tokyo, Japan). The reference compounds — creatine, creatinine, pyridoxine, uracil, histidine, theobromine, nicotinamide, hypoxanthine, adenosine, xanthine, phenylalanine, caffeine, tyrosine, tryptophan, theophylline, urocanic acid, uric acid, nicotinic acid, 4-aminohippuric acid, 4-hydroxy-3-methoxymandelic acid, 4-hydroxy-3-methoxyphenylacetic acid, *p*-aminobenzoic acid, hippuric acid, quinaldic acid, *p*-hydroxyphenylacetic acid, vanillic acid, kynurenic acid, *p*-hydroxyphenylpyruvic acid, benzoic acid, *p*-hydroxybenzoic acid, 3-hydroxyanthranilic acid, indoleacetic acid and indoleacrylic acid — were also purchased from Wako.

Resin

The strongly basic anion-exchange resin, Diaion CDR-10, is a macroreticular type having a particle size distribution of 5—7 μm. This resin was obtained from Mitsubishi Chemical Industries (Tokyo, Japan).

Apparatus

An Hitachi Model 634 high-performance liquid chromatograph was used for the urine analysis, and was coupled to a variable-wavelength photometer and 10-mV data recorder. Stopped-flow scanning spectrophotometry was performed with a scan speed of 60 nm/min and a slit width of 4.0 nm for scanning from 340 to 200 nm.

A 50 × 0.4 cm I.D. stainless-steel column was packed with Diaion CDR-10 using a high-pressure slurry technique [10] with 2.0 *M* ammonium perchlorate aqueous solution.

Sample preparation

The urine sample, usually a 24-h collection, was refrigerated until complete, then frozen and stored at −20°C. Before analysis, the sample was thawed and passed through a 0.22-μm Millipore filter to remove particulate matter.

Anion-exchange chromatography

A 100-μl urine sample was introduced onto the column. The urinary con-

stituents were then eluted with a concave ammonium perchlorate gradient at a flow-rate of 0.75 ml/min using the two-chamber gradient generator. The curvature of the gradient could be chosen by the curve coefficient (K) and total gradient volume (V). The curve coefficient is the ratio of the area of upper phase (V_1) against the area of lower phase (V_2) in the area of the rectangle (V_t) divided by the gradient curve. Then $K = V_1/V_2$. The concave perchlorate gradient was formed by placing 20 ml of water in the first chamber, and 20 ml of 0.25 M ammonium perchlorate solution in the second chamber, when $K = 3$ and $V = 20$ were selected. The column temperature was raised from 22 to 70°C over the first 30 min, then maintained at 70°C to the end of the run. Due to the increase in the column temperature, the inlet pressure changed from 11 to 7.5 MPa over the first 30 min. The ammonium perchlorate gradient did not affect the column inlet pressure.

Assignment of the chromatographic peaks

Peak assignments were carried out in three ways: (1) by comparing the retention time of a peak to those of the standard compounds; (2) by injecting the standard compounds along with the sample; and (3) by measuring the UV spectrum of a peak at the peak maximum by stopped-flow scanning spectrophotometry.

RESULTS AND DISCUSSION

Investigation of eluting ion for the separation and detection of UV-absorbing constituents of urine

The first purpose of this study was the elimination of the baseline drift caused by the acetate gradient. Some baseline compensation methods [11], such as series flow, dual columns and chemical compensation, were tested. However, these baseline compensation methods were complicated and not satisfactory for the routine method. Even if compensation is possible, detection at short UV wavelengths could not be performed. Therefore, a gradient elution system using other buffers or salt solutions, which do not absorb in the short UV-wavelength region, was necessary. Anions such as perchlorate, phosphate, sulfate, borate, nitrate, carbonate, sulfamate and chloride were tested. Of these, perchlorate and chloride were satisfactory for both the resolution and the detection of peaks. However, chloride ion was very harmful to the steel of the instrument. Therefore, perchlorate ion was chosen from many advantageous chromatographic results. Similarly, cations such as ammonium, sodium and lithium were tested, and it was found that the species of cation scarcely affected the separation and detection. Consequently, ammonium perchlorate solution was suitable for the separation and detection of UV-absorbing constituents of urine.

Separation of UV-absorbing constituents of urine with a linear or a concave ammonium perchlorate gradient

A series of chromatograms detected at several wavelengths is shown in Fig. 1. These chromatograms were run using the linear ammonium perchlorate gradient. In these chromatograms, baseline drift did not occur during detection at

254 nm, and even detection at 200 nm was possible. The final concentration of ammonium perchlorate solution (0.25 *M*) was sufficient to elute most of the UV-absorbing constituents of urine. However, when the linear ammonium perchlorate gradient was not satisfactory in resolving the peaks eluted at the beginning of the gradient, then the concave ($K = 3$) gradient was employed for high-resolution separation.

UV-absorbance chromatograms measured with the concave perchlorate gradient are shown in Fig. 2. These chromatograms were obtained using the same gradient curve coefficient ($K = 3$) but a different gradient volume (V). The gradient volume for the first (Fig. 2A), second (Fig. 2B) and third (Fig. 2C) chromatogram was 10, 15 and 20 ml, respectively. As Fig. 2C shows, the resolution at $V = 20$ gave excellent results and the resolution of peaks was improved. Also, the shape of the peaks eluted after hippuric acid was sharper than those obtained using the linear gradient elution system.

On the other hand, when the number of detectable peaks on the chromatograms was compared to that obtained with the acetate gradient elution system, the former was lower than the latter. Namely, the constituents strongly re-

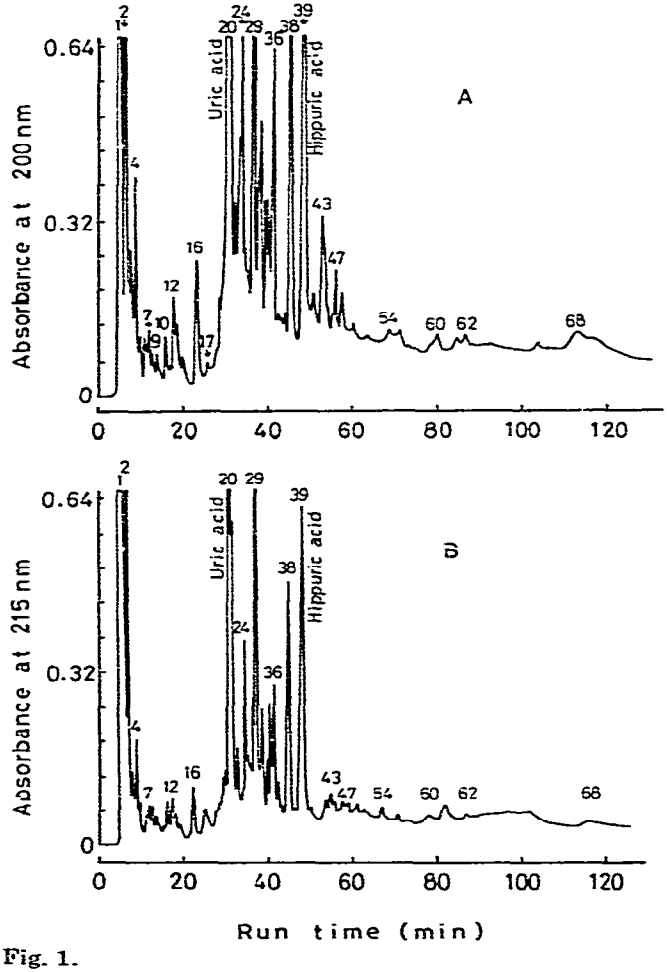

Fig. 1.

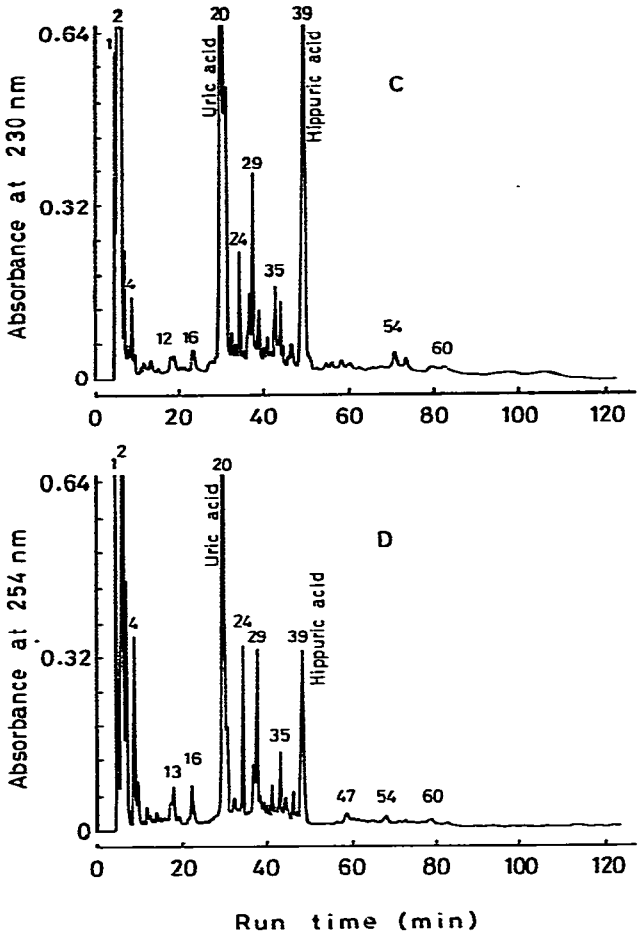

Fig. 1. Chromatograms of human urine detected at (A) 200 nm, (B) 215 nm, (C) 230 nm and (D) 254 nm, by a linear ammonium perchlorate gradient elution system. Conditions: column 50 × 0.4 cm I.D., packed with Diaion CDR-10; eluent, ammonium perchlorate solution (pH 5.5) varying in concentration from 0 to 0.25 *M* in a linear gradient; temperature, increasing from 22 to 70°C over the first 30 min, then 70°C to the end of the run; average flow-rate, 0.75 ml/min; average pressure, 8.5 MPa. For peak assignments, see Table I.

tained on the resin by non-ionic adsorption could not be eluted with the perchlorate gradient.

Concave gradient elution systems with ammonium perchlorate plus organic solvents

To elute the strongly retained constituents, the addition of ethanol or acetonitrile to the ammonium perchlorate solution (0.25 *M*) in the proportion of 15% was used. Two series of chromatograms obtained with ammonium perchlorate—ethanol and with ammonium perchlorate—acetonitrile are shown in Figs. 3 and 4, respectively. The effects of these additives on the retention times of standard compounds and of urinary constituents are seen in Table I. From

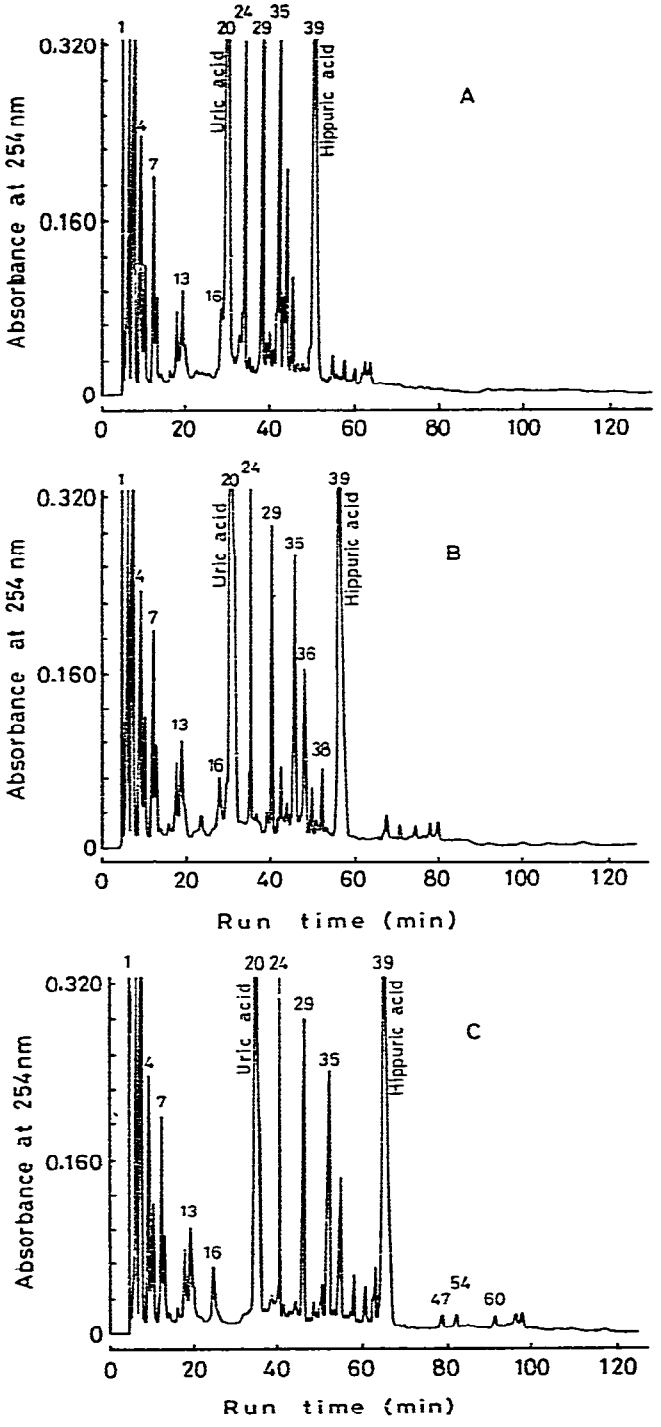

Fig. 2. Chromatograms of human urine measured by a concave ammonium perchlorate gradient elution system. Conditions were the same as in Fig. 1, except for gradient curve coefficient (K=3) and gradient volume (A=10, B=15 and C=20). For peak assignments, see Table I.

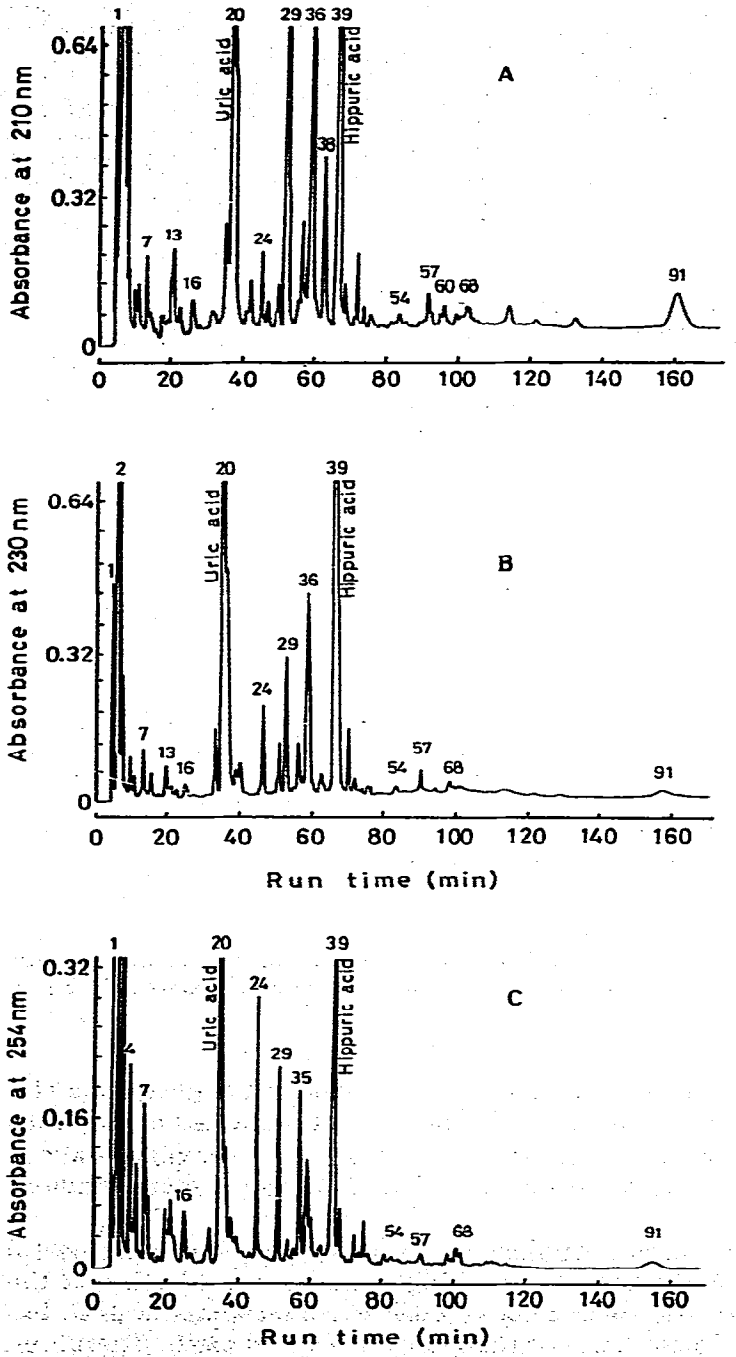

Fig. 3. Chromatograms of human urine measured by a concave gradient elution system with ammonium perchlorate solution plus ethanol. Run conditions were the same as in Fig. 2C. For peak assignments, see Table I.

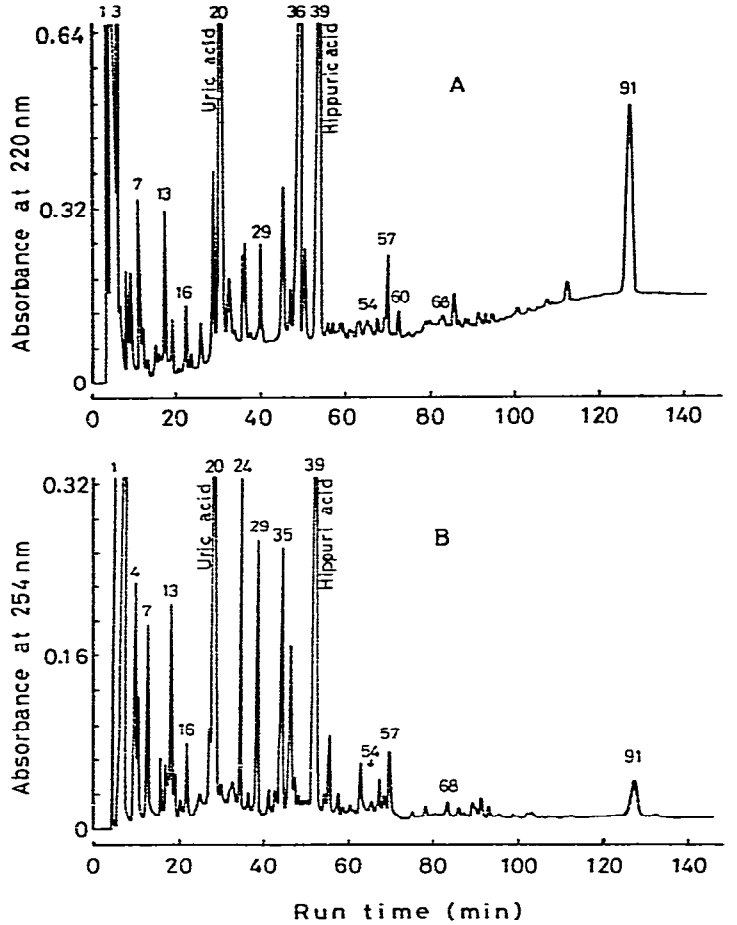

Fig. 4. Chromatograms of human urine measured by a concave gradient elution system with ammonium perchlorate solution and acetonitrile. Run conditions were the same as in Fig. 1 and Fig. 2C. For peak assignments, see Table I.

these chromatograms, it is evident that ethanol and acetonitrile gave good elution for the later-eluting compounds and for the strongly retained compounds on the resin. As a result, the resolution and the number of detectable peaks in these improved elution systems were equal to those of the acetate gradient elution system [4,6].

In these ammonium perchlorate gradient elution systems, the most useful advantage was the detectability of UV-absorbing constituents of urine at the short-wavelength region. As the figures show, each chromatographic peak has a characteristic variation in absorbance at the respective wavelength. Therefore, the assignment of peaks became easier by comparing the UV spectra of peaks to those of standard compounds. Many peaks could be measured in the UV spectra with stopped-flow scanning spectrophotometry. For example, the UV spectra of main peaks on the chromatograms are shown in Fig. 5. The assignment of main peaks was carried out by these methods. The results are listed in Table I. Also, the volume of the urine sample required to detect the main

TABLE I

RETENTION TIMES OF STANDARD COMPOUNDS AND ASSIGNMENT OF MAIN PEAKS ON THE CHROMATOGRAMS

No.	Compound	Peak No.	Retention time (min)			
			A*	B*	C*	D*
1	Creatine	2	6	6	6	6
2	Creatinine	2	6	6	6	6
3	Histidine	3	7	7	7	7
4	Pyridoxine	4	8	8	8	8
5	Uracil	5	9	9	9	9
6	Phenylalanine	6	10	10	10	10
7	Hypoxanthine	13	18	19	18	18
8	Xanthine	16	22	26	22	21
9	Adenosine	17	22	26	23	22
10	Nicotinamide	17	22	26	24	23
11	Caffeine	17	23	26	24	23
12	Theophylline	18	23	28	26	24
13	Tyrosine	18	24	28	26	25
14	Tryptophan	18	25	28	26	26
15	Urocanic acid	19	26	33	33	29
16	Uric acid	20	28	35	35	30
17	Nicotinic acid	23	30	38	36	32
18	4-Hydroxy-3-methoxymandelic acid	26	32	40	39	34
19	Homovanillic acid	27	41	51	49	36
20	*p*-Hydroxyphenylacetic acid	28	43	53	51	38
21	*p*-Aminobenzoic acid	34	45	56	54	42
22	Vanillic acid	36	47	58	56	46
23	Hippuric acid	39	49	65	64	53
24	*p*-Hydroxyphenylpyruvic acid	41	52	75	72	56
25	Kynurenic acid	57	70	85	91	69
26	*p*-Hydroxybenzoic acid	58	75	87	93	72
27	3-Hydroxyanthranilic acid	59	76	89	95	75
28	Quinaldic acid	60	79	92	98	78
29	Indoleacetic acid	68	—	—	101	83
30	Indoleacrylic acid	91	—	—	155	127

*A: Linear perchlorate gradient elution system (Fig. 1). B: Concave perchlorate gradient elution system (Fig. 2C). C: Concave gradient of perchlorate and ethanol (Fig. 3). D: Concave gradient of perchlorate and acetonitrile (Fig. 4).

peaks at short UV wavelengths such as 200 nm was much smaller. Furthermore, not only aromatic compounds, which do not have a high molecular absorption coefficient at 254 nm, but also organic compounds, have more or less absorptivity in the short UV-wavelength region. The possibility of separating and detecting these compounds arose.

CONCLUSION

The purpose of this study was to modify the anion-exchange chromatographic system using the acetate gradient for the detection of UV-absorbing constituents in the short-wavelength region. The benefits of the perchlorate gra-

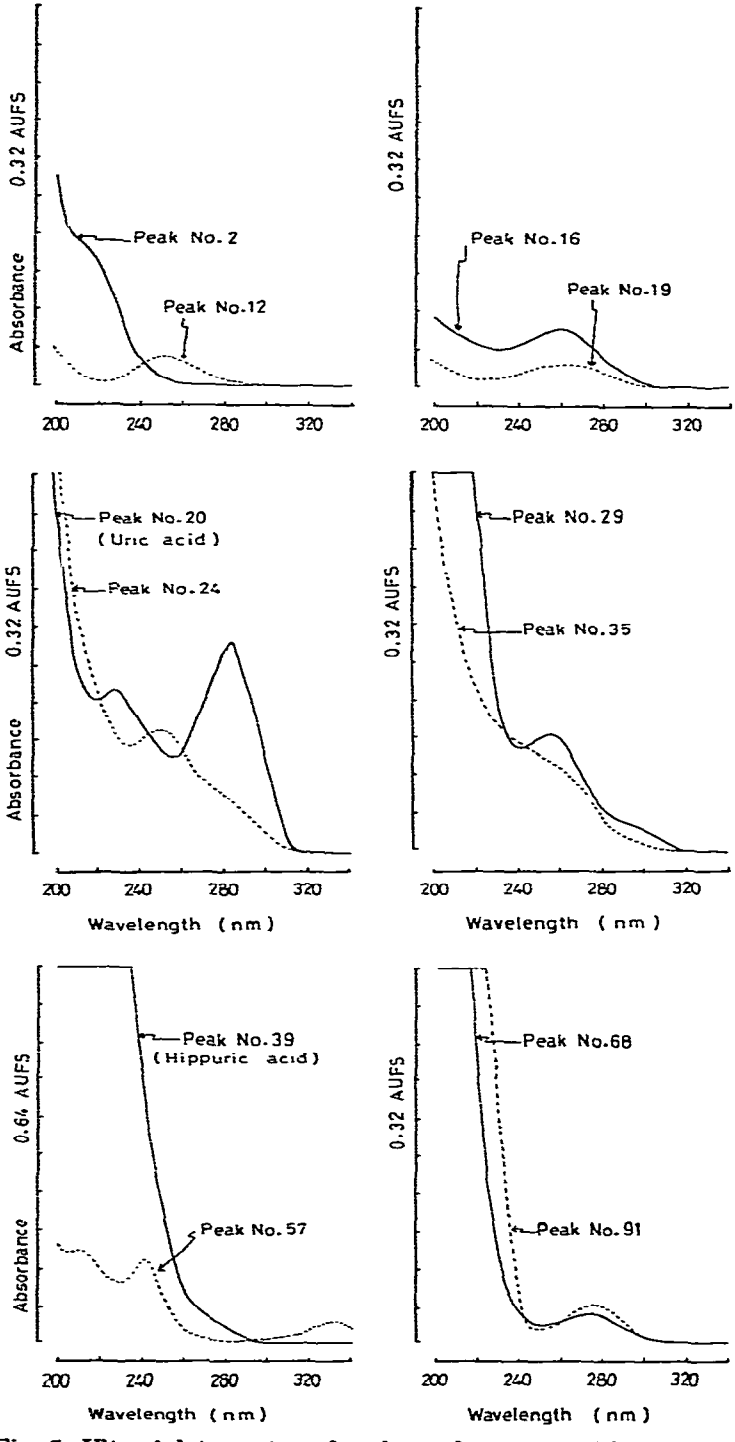

Fig. 5. Ultraviolet spectra of main peaks measured by stopped-flow scanning spectrophotometry. The stopped-flow scanning spectrophotometry was performed under the conditions 60 nm/min scan speed and 4.0 nm of slit width, with scanning from 340 to 200 nm. For peak numbers, see Table I.

dient elution systems include elimination of baseline drift, and an increase in sensitivity of detection. As well as these ammonium perchlorate gradient elution systems being useful as independent systems for the separation and detection of many organic compounds, the systems can supplement the defects of the ammonium acetate gradient elution system. Using these acetate and perchlorate gradient elution systems, the chromatographic investigation of the constituents of body fluids should be facilitated.

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