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# SIMULTANEOUS DETERMINATION OF SERUM UREMIC TOXINS, CATIONS AND ANIONS AND UREA DETERMINATION BY POST-COLUMN COLORIMETRY USING IMMOBILIZED ENZYME

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## SUMMARY

A column-switching method which combines ion-exchange and ion-pair reversed-phase chromatography was developed for the determination of serum uremic toxins, cations and anions. Serum urea, which was poorly separated by reversed-phase chromatography, was analyzed using an immobilized-urease column and detected by post-column colorimetry. Apart from the simultaneous analysis, anion analysis using ion-pair reversed-phase chromatography on an ODS column was also developed. The origin of the system peak observed in anion analysis with an eluent containing phthalate has been clarified.

## INTRODUCTION

Shintani and Ube [1] have previously reported the simultaneous analysis of serum cationic and anionic compounds using a column-switching method combined with serum urea (U) analysis.

Described in the present study is the investigation of the simultaneous analysis of serum uremic toxins, cations and anions using a column-switching method. The uremic toxins were analyzed using a modified ion-pair reversed-phase liquid chromatographic (LC) method [2], which was combined with post-column colorimetric determination of serum U using a column with immobilized enzyme.

Methyl guanidine (MG), a trace serum component, was analyzed using an endcapped ODS column and UV detection at 200–210 nm. Previous papers [1,2] have described the analysis of serum creatinine (CR) and MG using a cation-exchange column or a non-endcapped ODS column (Zorbax ODS). Using these methods, serum MG could not be detected owing to low detection

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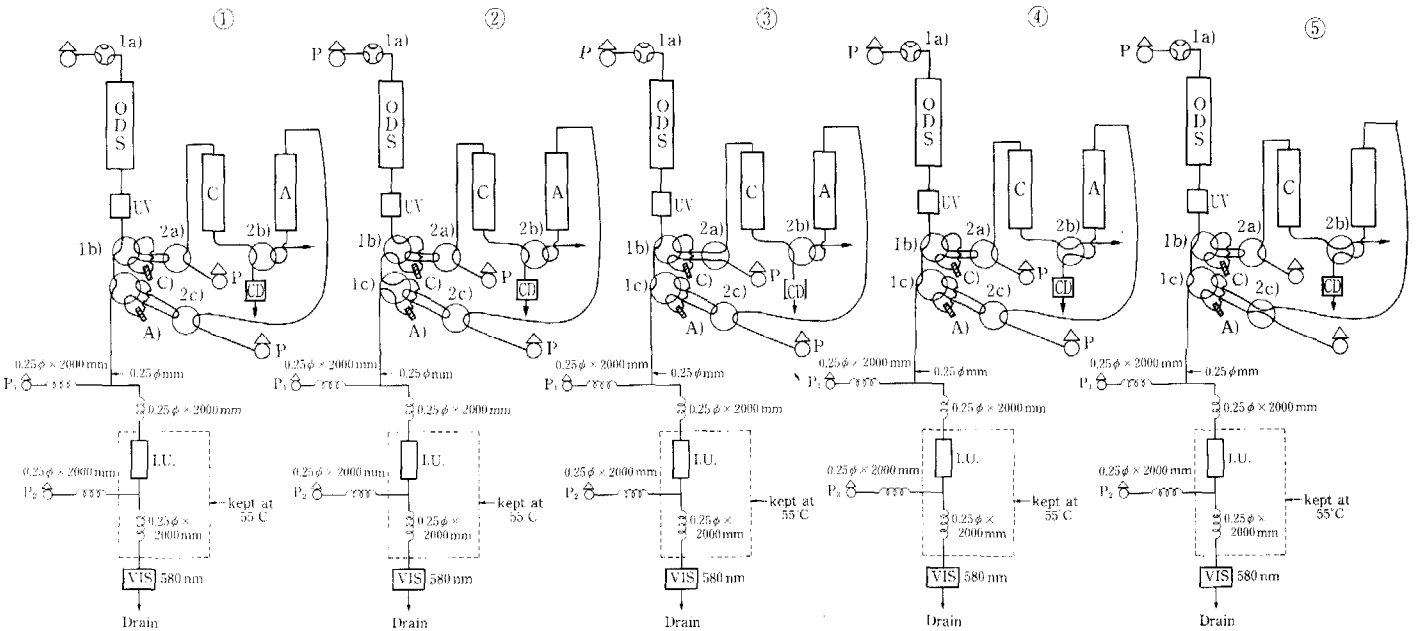


Fig. 1. Equipment for the simultaneous determination of uremic toxins, cations and anions analyzed by both ion-pair reversed-phase LC and ion-exchange chromatography. Abbreviations: P = pump; ODS = ODS column; UV = UV detector; CD = conductometric detector; C = cation-exchange column; C) = cation-exchange pre-column; A = anion-exchange column; A) = anion-exchange pre-column; 1a), 1b) and 1c) = six-position valves; 2a), 2b) and 2c) = four-position valves, $P_1$ = pump for buffer solution; $P_2$ = pump for coloring reagent solution; I.U. = immobilized-urease column, 50 mm × 4.6 mm I.D. The tubing indicated as a spiral is PTFE.

sensitivity and CR and MG peaks showed tailing. These drawbacks can now be improved by using the proposed ion-pair reversed-phase method. In addition, this method also enables the analysis of serum mono- and divalent cations; this was not possible with the previous method [1,3].

Anion-exchange chromatography of serum anions such as chloride and bicarbonate for determining the anion gap [4] has been reported previously [1]. This method is now used for the simultaneous analysis. Although anion analysis by ion-pair reversed-phase LC was more rapid and more sensitive than anion-exchange chromatography, it could not be used for the simultaneous analysis owing to poor retention of serum anions on the ODS pre-column.

Finally, the origin of the system peak is also described.

## EXPERIMENTAL

### *Reagents and materials*

Reagents and materials used were the same as previously reported [1–3]. Reagents used were of the highest purity commercially available.

### *Apparatus*

The pump (P) shown in Fig. 1 used for ion-pair reversed-phase LC was a Shimadzu Model LC-3A (Shimadzu, Kyoto, Japan). The pumps used for cation and anion chromatography (P, Fig. 1) were Model SP-8750 (Spectra-Physics, San Jose, CA, U.S.A.). The pumps used for post-column colorimetry ($P_1$, $P_2$, Fig. 1) were Model RP-203 (Yanagimoto, Kyoto, Japan). The UV—visible detector was a UVILOG-7 (Oyobunko, Tokyo, Japan) and the post-column reaction system was a UVILOG RS-300 (Oyobunko). Ultrafiltration was carried out using an MPS-3 filter (Amicon, Danvers, MA, U.S.A.). Other equipment used is described in previous reports [1–3].

### *Procedures*

The conditions for the simultaneous column-switching analysis of serum U, uric acid (UA), CR and MG, cations (Na, $NH_4^+$, K, Mg and Ca) and anions (Cl and bicarbonate) are given in the legends to Figs. 1, 2, 3, 5 and 7, and in the text. The preparation of the immobilized-enzyme column for serum U analysis is described in a previous report [1].

## RESULTS AND DISCUSSION

### *Ion-pair reversed-phase LC analysis of serum uremic toxins*

The analytical conditions are given in the legend to Fig. 2. An endcapped ODS column (SSC-ODS-L-1202, 200 mm × 4.6 mm I.D., 5 $\mu$m particle size, Senshukagaku, Tokyo, Japan) with 10% carbon loading was used. When other columns, such as Zorbax ODS, Nucleosil 5 $C_{18}$ [2] and a cation-exchange column for ion-pair chromatography [1,2] were used, CR and MG peaks showed tailing, CR, MG and UA were not sufficiently separated and serum MG was not detected [1,2].

Using an ODS-L-1202 column and a mobile phase containing 0.2–1 m*M* barium hexanesulphonate as a counter ion at pH 2.7, CR and MG peaks

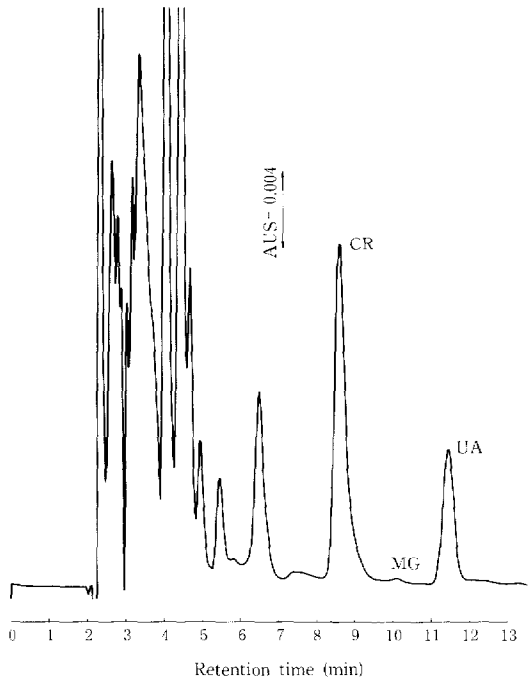


Fig. 2. Chromatogram of uremic toxins in serum analyzed by ion-pair reversed-phase LC. Sample size of serum ultrafiltrate, 10 $\mu$l; column, Senshupak ODS-SSC-L-1202 (200 mm × 4.6 mm I.D.; temperature, ambient; eluent, 1 m*M* barium hexanesulphonate with 30 m*M* barium perchlorate; the mixture was acidified to pH 2.7 with phosphoric acid; flow-rate, 1 ml/min; UV detection at 210 nm. CR and UA were also detected at 234 nm. Sensitivity, 0.04 a.u.f.s.; chart-speed, 10 mm/min. The amounts determined are shown in Table I. Peaks: CR = creatinine; MG = methylguanidine; UA = uric acid.

exhibited tailing, though CR, MG and UA were well separated from each other. Serum MG was not detected owing to low detection sensitivity (the standard MG peak was asymmetric). By adding barium perchlorate (30 m*M*) to a 1 m*M* solution of barium hexanesulfonate, peak tailing of MG and CR was eliminated, the analysis was shortened and the detection sensitivity for MG enhanced. The exchange capacity of the cation and anion pre-columns used for the simultaneous analysis using the column-switching method were not exceeded, and the compounds of interest in serum were well resolved without tailing (Fig. 2).

MG was detected at 210 nm, while CR and UA were detected at 234 nm (Fig. 2). The detection sensitivities of UA and CR at 234 nm were 30% higher and 30% lower, respectively, than those at 210 nm. The relationship between the concentrations of barium perchlorate and barium hexanesulphonate and the logarithm of the retention times ($t_R$) of CR, MG and UA were linear. While the $t_R$ of UA was little affected, that of MG varied sharply with the concentration [2,5] of barium perchlorate because strongly basic MG formed ion pairs. When the endcapped ODS column with 20% carbon loading (Senshupak ODS-H-1201, 200 mm × 4.6 mm I.D., 5 $\mu$m particle size) was used under the conditions given in the legend to Fig. 2, serum MG was not detected owing to excessively long retention, and CR was insufficiently separated from serum constituents.

TABLE I

SERUM CONCENTRATIONS DETERMINED AND DETECTION LIMITS

For analytical conditions, see legends to Figs. 1—3, 5 and 7. In all cases, $n=10$ and the coefficient of variation = 2%; signal-to-noise ratio = 2. Amount of sulfate determined: 102 mg/l (coefficient of variation = 1.2%, $n=5$).

| | Mean values of detection limit (ng per 10 μl) | | | Serum concentration (mg/l) |
|---|---|---|---|---|
| | Conductometry | UV | Visible | |
| Sodium | 2.1 | | | 2972 |
| Ammonium | 2.5 | | | 3.7 |
| Potassium | 2.6 | | | 152 |
| Magnesium | 51.0 | | | 15* |
| Calcium | 45.8 | | | 106* |
| Urea | | | 150.0** | 228 |
| Uric acid | | 1.15 | | 6.7 |
| Creatinine | | 0.54 | | 12.5 |
| Methylguanidine | | 6.26 | | 0.5 |
| Chloride | 20.0 | | | 3543 |
| Bicarbonate | 120.2 | | | 703 |

*The amount of total magnesium and calcium in serum.

**The detection limits by conductometric and UV detection (210 nm) are 7.78 and 500 mg per 10 μl, respectively.

The detection limits and serum levels of these compounds are listed in Table I. The quantitative results are similar to those reported in previous papers [1,2,6]. Serum MG, which was not detected by the previous methods [1,2], could be monitored using this method owing to improved detection sensitivity. The amount of serum MG shown in Table I agrees with that reported by Baker and Marshall [7]. Serum U was poorly retained on the ODS column under the conditions shown in Fig. 2 and was eluted close to the void volume. Serum U was detected specifically and selectively by post-column colorimetry. The serum cations and anions eluted in the void volume under these conditions were trapped and washed in the respective pre-columns, as shown in Fig. 1, and simultaneously analyzed using the column-switching method.

*Determination of serum urea by post-column indophenol colorimetry using an immobilized-urease column*

Serum U was converted to $NH_4^+$ in the immobilized-urease column and detected colorimetrically by the indophenol method [8]. In the simultaneous analysis, serum $NH_4^+$ eluted before U when using an ODS column and was successively determined using a cation-exchange column. Therefore, endogenous serum $NH_4^+$ did not interfere with the serum U determination.

The conditions for post-column colorimetry are shown in Figs. 1 and 3. The acidic eluent from the ODS column was neutralized with a Good's buffer solution. The pH range of 6.4—7.6 was optimal for the activity of urease and colour development by the indophenol method [9]. Next, the colour-developing reagent was introduced and the reaction mixture detected at 580 nm, as shown in Fig. 3. The immobilized-urease column was thermostated at 55°C [9]. At

temperatures above 55°C, deactivation of the enzyme took place. The serum concentration and the detection limit of U are shown in Table I and the separation is illustrated in Fig. 3. The results are almost the same as those obtained using the immobilized-urease column placed before the analytical column and injection port, combined with conductometric detection [1]. The sensitivity of the conductometric detector [1] for U was clearly superior to post-column indophenol colorimetry (Table I), which in turn was superior to the previous method of direct detection at 210 nm using ion-pair reversed-phase LC on the non-endcapped ODS column [2] (Table I). The peak broadening of serum U determined by post-column colorimetry was reduced by using 0.25 mm diameter tubing for the buffer solution, colour-developing reagents, a PTFE tube for heating and a stainless-steel tube from the UV section to the buffer solution path section [10] (Fig. 1), by using a quick-return pump, Model RP-203 and T-shaped mixing equipment (LEE visco mixer AAEX0320000A, Lee, Westbrook, CT, U.S.A.). The results of this method are superior to those obtained by post-column fluorometry using the immobilized-urease column [11].

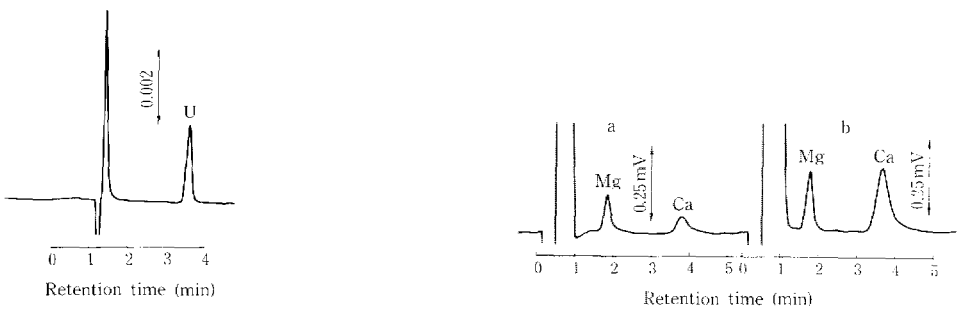


Fig. 3. Chromatogram of serum urea (U) analyzed by post-column indophenol colorimetry. The flow-rate of buffer solution and coloring reagent solution, 0.5 ml/min; detection at 580 nm, 0.02 a.u.f.s.; Good's buffer solution, 50 m*M* N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid—8 m*M* sodium nitroprusside—80 m*M* sodium salicylate; coloring reagent solution, 800 mg/l sodium hypochlorite. Other analytical conditions are given in the text. The amount of serum urea is given in Table I.

Fig. 4. Chromatograms of serum magnesium and calcium analyzed by cation-exchange chromatography. (a) Obtained when serum ultrafiltrate was applied. The amounts determined represent free serum magnesium and calcium, which were 0.012 and 0.056 mg/ml, respectively. The coefficient of variation ($n=10$) was 1.2%. (b) Obtained when acidified serum ultrafiltrate at pH 3.3 was applied. The amounts determined represent the sum of free and bound serum magnesium and calcium, which were 0.015 and 0.106 mg/ml, respectively. The coefficient of variation ($n=10$) was 1.3%. Sample size, 20 μl; column, Oyobunko cation-exchange column (ASC-4000); temperature, ambient; eluent, 0.6 m*M* ethylenediamine adjusted to pH 6.1 with phosphoric acid; flow-rate, 2.3 ml/min; detector, conductometer; sensitivity, range × 10, coarse × 3; chart-speed, 10 mm/min.

*Analysis of serum cations using cation-exchange chromatography*

Ca and Mg were analyzed simultaneously using an Oyobunko pellicular cation-exchange column (ASC-4000, base: styrene divinylbenzene (SDVB), 0.03 mequiv./g, 250 mm × 4.6 mm I.D.). The mobile phase consisted of 0.6 m*M* ethylenediamine, pH 6.1. The Mg peak was separated from the serum constituents and the water dip, and Ca peak showed less tailing. However, below pH 6.1, Mg was not resolved from the serum constituents and the water

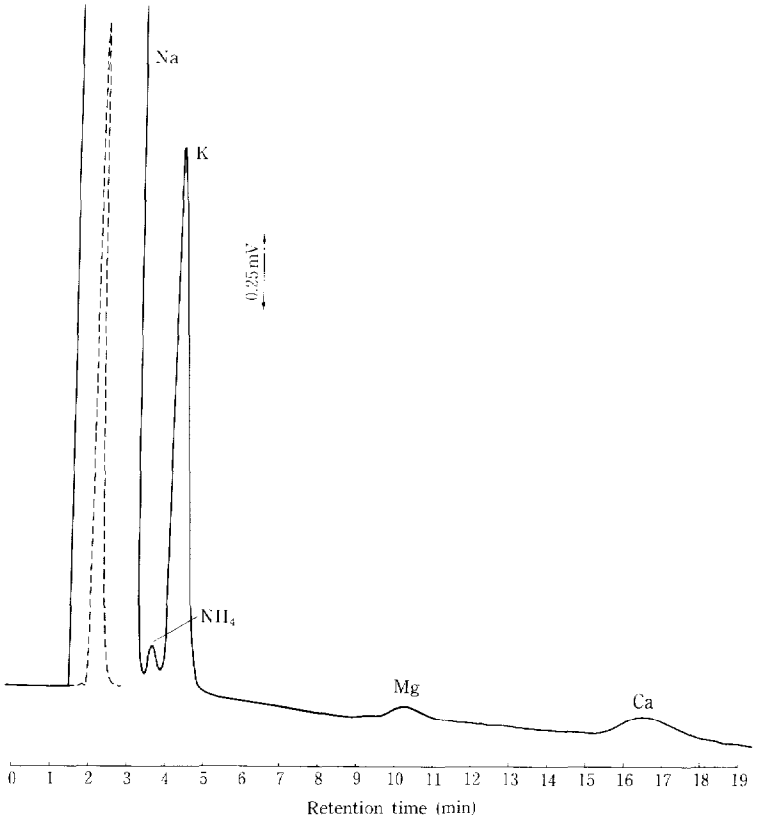


Fig. 5. Chromatogram of serum cations analyzed by cation-exchange chromatography. The chromatogram of sodium, shown by the broken line, was obtained by twenty-fold dilution of serum ultrafiltrate. The amounts determined are shown in Table I. Sample size of acidified serum ultrafiltrate at pH 3.3 with and without dilution, 20 $\mu$l; column, Toadenpa cation-exchange column 301, 250 mm × 4.6 mm I.D.; temperature, ambient; eluent, 4 m*M* copper sulphate; flow-rate, 1 ml/min; detector, conductometer; sensitivity, range × 10, coarse × 3; chart-speed, 10 mm/min.

dip. The chromatogram of a serum sample and the quantitative results are shown in Fig. 4 and Table I, respectively.

The conditions for the simultaneous analysis of the mono- and divalent cations were also established (Fig. 5). This method was used for the simultaneous analysis using the column-switching method. However, the detection sensitivities of Ca and Mg were higher when they were analyzed separately from the monovalent cations.

Using the proposed method for the simultaneous analysis of the mono- and divalent cations, serum Na, $NH_4^+$ and K were clearly separated and serum Ca showed no tailing (Fig. 5). The quantitative results and the chromatogram of a serum sample are shown in Table I and Fig. 5, respectively.

*Analysis of cationic compounds by cation-exchange chromatography using m-phenylenediamine eluent*

Serum Na, $NH_4^+$, K and CR were analyzed using 0.37 m*M* *m*-phenylenediamine as the eluent and detected by conductometry. The quantitative results

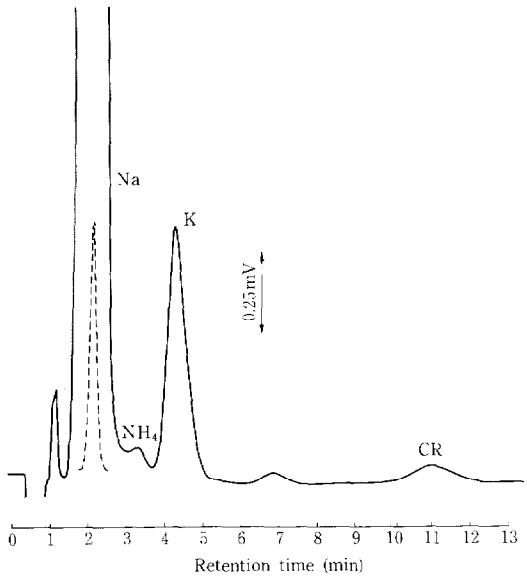


Fig. 6. Chromatogram of serum sodium, ammonium, potassium and creatinine (CR) analyzed by cation-exchange chromatography. Chromatogram of sodium, shown by the broken line, was obtained by 100-fold dilution of serum ultrafiltrate. Sample size of serum ultrafiltrate with and without dilution, 20 $\mu$l; column, Wescan cation-exchange column 269-004, 250 mm × 4.6 mm I.D.; temperature, ambient; eluent, 0.37 m*M* *m*-phenylenediamine; flow-rate, 2 ml/min; detector, conductometer; sensitivity, range × 10, coarse × 3; chart-speed, 10 mm/min.

are almost the same as with the pH 2.03 phosphoric acid eluent [1] (Fig. 6). Using phosphoric acid [1], the CR peak showed tailing and was insufficiently detected by conductometry. On the contrary, using *m*-phenylenediamine, the CR peak showed no tailing and could be detected with sufficient sensitivity even by conductometric detection. Similar results were obtained with *o*- or *p*-phenylenediamine instead of *m*-phenylenediamine. However, these substances were not used in routine analysis because substituted phenylenediamines are prone to photo decomposition. The relationship between the logarithm of $t_R$ and the concentration of substituted phenylenediamines and ethylenediamine was linear.

*Analysis of serum anions by anion-exchange chromatography*

The anion analysis using a Wescan anion-exchange column (269-001, base: silica, 0.02 mequiv./g, 250 mm × 4.6 mm I.D.) shown in Fig. 7 was used for the simultaneous analysis. The pH of the mobile phase was adjusted to 4.5, since the anions were better separated from serum constituents at this pH. The sensitivity for Cl and bicarbonate ions was lowered when the pH decreased, owing to changes in the extent of dissociation of both bicarbonate and phthalate in the mobile phase [12]. The quantitative results and the detection limit of serum Cl and bicarbonate are shown in Table I. They are practically the same as those given in a previous report [1]. Both conductometric detection and the more sensitive indirect photometric detection method were used. However, since other serum components eluting close to the bicarbonate peak were

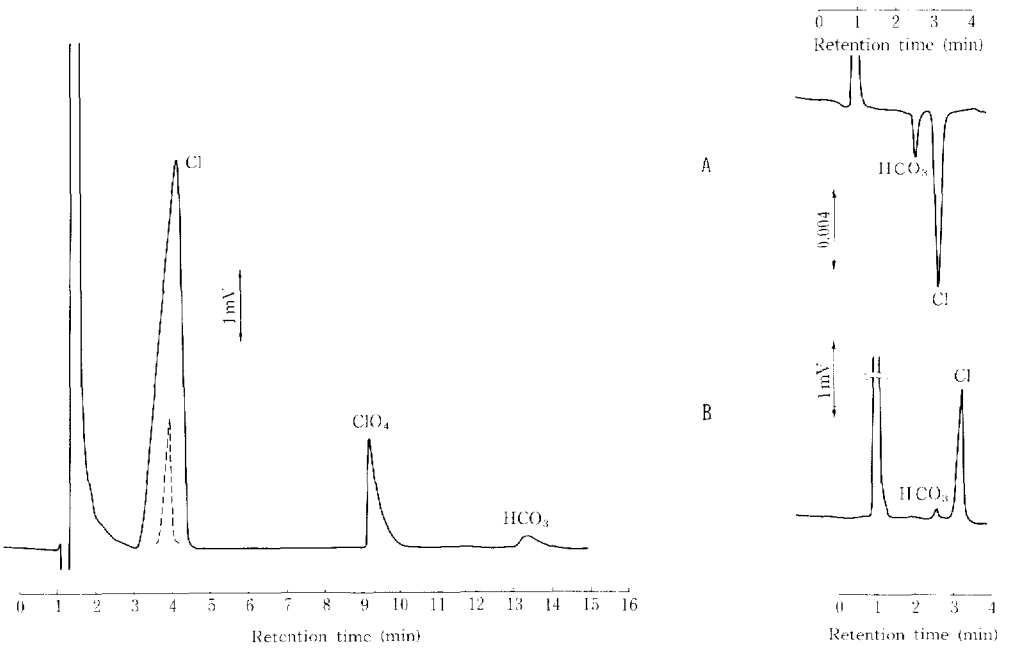


Fig. 7. Chromatogram of serum anions analyzed by anion-exchange chromatography. Chromatogram of chloride, shown by the broken line, was obtained by ten-fold dilution of ultrafiltered serum. Sample size of serum ultrafiltrate with and without dilution, 20 $\mu$l; column, Wescan anion-exchange column 269-001, 250 mm × 4.6 mm I.D.; temperature, ambient; eluent, 4 m*M* KHP adjusted to pH 4.5 with potassium hydroxide; flow-rate, 2 ml/min; detector, conductometer; sensitivity, range × 10, coarse × 3; chart-speed, 10 mm/min. The elution of bicarbonate preceded that of hexanesulphonate used as the counter ion by ion-pair reversed-phase LC, as shown in Fig. 2. Counter ion and perchlorate used for fast elution by ion-pair reversed-phase LC in simultaneous analysis did not interfere with the anion analysis.

Fig. 8. Chromatograms of serum anions analyzed by ion-pair reversed-phase LC combined with (A) indirect photometric detection at 265 nm, 0.04 a.u.f.s. and (B) conductometric detection using TBAOH counter ion eluent with range × 10, coarse × 3. Sample size of ten-fold diluted ultrafiltered serum, 10 $\mu$l; column, Nucleosil 5 $C_{18}$ 150 mm × 4.6 mm I.D.; temperature, ambient; eluent, 0.5 m*M* KHP with 10% TBAOH, 5.33 ml/l; this mixture was adjusted to pH 7 with phosphoric acid; flow-rate, 2 ml/min; chart-speed, 10 mm/min.

not detected by conductometric detection, this method was more suitable for serum bicarbonate analysis (Fig. 7).

*Analysis of serum anions by ion-pair reversed-phase LC*

Serum anions were also analyzed by ion-pair reversed-phase LC using an ODS column. This method could not be used for the simultaneous analysis using the column-switching method since the anions were not sufficiently retained on the ODS pre-column. However, this method afforded higher sensitivity and faster analysis for serum Cl and bicarbonate than anion-exchange chromatography (Fig. 7). The chromatogram and the quantitative serum levels of Cl and bicarbonate are shown in Fig. 8 and Table I, respectively.

Using the endcapped ODS columns (Toadenpa 101 and ODS columns),

Cl was insufficiently separated from bicarbonate and the water dip. With a Showadenko reversed-phase column (ICI-613, base: S-DVB, Tokyo, Japan), serum bicarbonate was insufficiently separated from the serum constituents. The best results were obtained using a non-endcapped Nucleosil 5 $C_{18}$ column. The eluent should contain a counter ion, e.g., tetramethylammonium hydroxide (TMAOH), tetramethylammonium iodide (TMAI), tetraethylammonium hydroxide (TEAOH), tetraethylammonium iodide (TEAI), tetrapropylammonium hydroxide (TPAOH), tetrapropylammonium iodide (TPAI), tetrabutylammonium hydroxide (TBAOH), tetrabutylammonium iodide (TBAI), tetrapentylammonium hydroxide (TPenAOH) or tetrapentylammonium iodide (TPenAI), and potassium hydrogen phthalate (KHP). The mixture was adjusted to pH 3—8 with phosphoric acid or potassium hydroxide.

When the mobile phase contained a counter ion without KHP, the Cl peak exhibited considerable leading. When phthalic acid (PA) was used instead of KHP, Cl and bicarbonate peaks could not be measured, because they eluted close to the void volume owing to low eluent pH. When KHP was used instead of PA, Cl peak leading disappeared but its separation from bicarbonate and the water dip was insufficient except when TBAOH and TBAI were used. When TPAOH was used, bicarbonate produced a negative peak with respect to Cl. Using TPenAOH, the bicarbonate peak was sufficiently retained on the column,

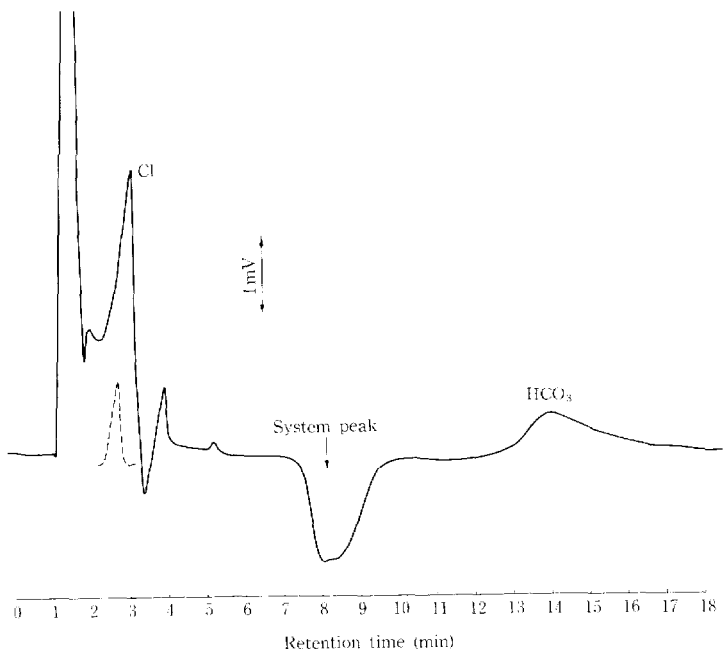


Fig. 9. Chromatogram of serum anions analyzed by ion-pair reversed-phase LC using TBAI counter ion eluent. Chromatogram of chloride, shown by the broken line, was obtained by ten-fold dilution of ultrafiltered serum. Sample size of serum ultrafiltrate with and without dilution, 20 $\mu$l; eluent, 0.27 m*M* TBAI with 0.5 m*M* KHP—acetonitrile (4:1); flow-rate, 1.5 ml/min; detector, conductometer; sensitivity, range ×10, coarse ×3; chart-speed, 10 mm/min. Other analytical conditions were the same as in Fig. 8. Chloride and bicarbonate peaks exhibited leading and tailing, respectively. The elution of the system peak preceded that of bicarbonate peak.

but the peak showed tailing. Furthermore, TPenAOH and TPenAI precipitated on the ODS column owing to their low water solubility. When 20—30% acetonitrile was added to the solution of KHP and TBAI or TPenAI, Cl was clearly separated from bicarbonate, but the Cl peak showed leading (Fig. 9). A large negative system peak preceded the bicarbonate peak, as shown in Fig. 9.

When TBAOH was used, Cl was clearly separated from bicarbonate and the water dip (Fig. 8) provided the pH was maintained between 6 and 7. This method afforded higher sensitivity and faster analysis than anion-exchange chromatography (Fig. 7). Since the mobile phase contained KHP, both indirect photometric detection (UV, 265 nm) and conductometric detection were used simultaneously. The sensitivity of the indirect photometric detection method was superior to that of the conductometric detection, especially for bicarbonate. The $t_R$ of bicarbonate was at a maximum in the vicinity of pH 4.5 (Fig. 8) and it decreased at pH values below and above 4.5. The sensitivity lowered when the pH decreased owing to poorly ionized bicarbonate. The $t_R$ of Cl increased when the pH decreased. Since these results were almost the same as those obtained using the anion-exchange column, the cause was thought to be the same.

As described in a previous paper [1], ion-pair reversed-phase LC is not suited for cation analysis.

*Simultaneous analysis using a column-switching method*

The simultaneous analysis of serum uremic toxins, cations and anions was carried out using the conditions given in the legends to Figs. 1, 2, 3, 5 and 7 combined with a column-switching method and post-column colorimetry. Undiluted serum ultrafiltrate was injected into the LC system (Fig. 1) for the determination of components other than serum Na and Cl for which a 10- to 100-fold diluted ultrafiltrate was used. The chromatograms obtained by dilution of the serum are shown by broken lines in Figs. 5—7 and 9. The analytical conditions and the chromatograms obtained by the simultaneous analyses are shown in Figs. 2, 3, 5 and 7.

The simultaneous analysis of mono- and divalent cations was impossible; anions were insufficiently separated from the water dip and could not be analyzed using a single column, as reported previously [13—17]. Jones and coworkers [15,17,18] reported an improved method. However, while the separation of the cations was improved, that of the anions remained insufficient.

The simultaneous analysis of mono- and divalent cations, anions and uremic toxins was successfully achieved using the method proposed in this paper. Serum uremic toxins in step 1 were analyzed under the conditions shown in Fig. 2. The mobile phase was drained out 2.1 min after injection, as shown in step 1. The selector valve was switched to trap the inorganic serum cations and anions eluted in the void volume in Fig. 2 which were then chromatographed in respective cation and anion pre-columns in step 2. Then, the selector valve was switched from step 2 to 1, and UA, CR and MG were analyzed under the conditions shown in Fig. 2. U was analyzed by post-column colorimetry as shown in Fig. 3. Meanwhile, the pre-columns and the sample loops (volume, 15 $\mu$l each) were washed with approximately 10 ml of distilled water in order to bring the pH to ca. 7. The anion and cation precolumns were connected

in the opposite direction to prevent peak broadening. The ions, trapped and enriched at the inlet of each pre-column, were eluted first without significant peak broadening. The selector valve was switched to step 3 in order to elute the cations trapped on the cation-exchange pre-column. The analysis was carried out as described in Fig. 5. When Ca was completely eluted, the selector valve was switched to step 4 for a period of 10 min to divert the flow towards the conductometric detector and to wash out the eluent used for cation analysis. Finally, the selector valve was switched to step 5, the anions were eluted from the anion exchange pre-column and analyzed as in Fig. 7.

The analyses were not perturbed by manual valve switching, as observed previously [1]. The recoveries from each pre-column were nearly 100%. Barium (from barium hexanesulphonate and barium perchlorate) was eluted after Ca (Fig. 5), and therefore it did not interfere with the cation analysis. Since the columns were continuously washed with the mobile phase after the analysis of each component, as shown in Fig. 1, the added compounds did not interfere. In anion analysis, perchlorate was clearly separated and eluted prior to bicarbonate (Fig. 7) and hexanesulphonate was eluted after bicarbonate.

*Simultaneous analysis of divalent cations and Cl and bicarbonate anions by anion-exchange chromatography*

This analysis is illustrated in Fig. 10. The quantitative results of the free and total serum Mg and Ca, and Cl and bicarbonate were almost the same as those in Table I and Fig. 4. Serum Cl was sufficiently separated from the water dip using a mixture of KHP and EDTA instead of EDTA alone [19], while these methods were unsuitable for monovalent cations, they were useful for the anion analysis shown in Fig. 7.

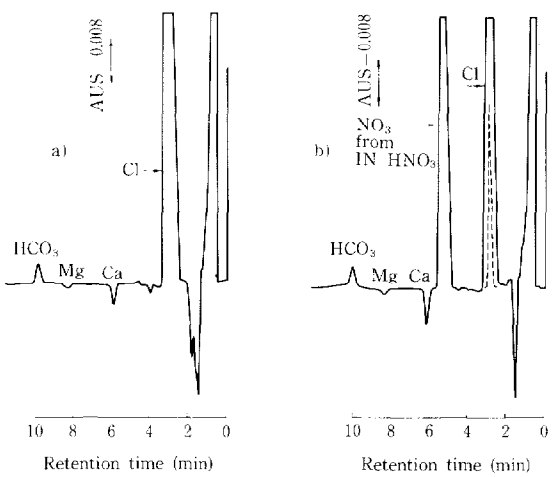


Fig. 10. Chromatograms of serum divalent cations and anions analyzed using a single anion-exchange column. (a) Obtained when serum ultrafiltrate was applied; (b) obtained when acidified serum ultrafiltrate at pH 3.3 was applied. Serum amounts determined are almost the same as in Fig. 4 and Table I. The chloride peak, shown by the broken line, was obtained by ten-fold dilution of ultrafiltered serum. Sample size, 20 $\mu$l; column, Toadenpa anion-exchange column 202; temperature, ambient; eluent, 2 m*M* KHP with 0.2 m*M* EDTA. This mixture was adjusted to pH 6.7 with potassium hydroxide; flow-rate, 2 ml/min; indirect photometric detection at 280 nm, 0.08 a.u.f.s.; chart-speed, 5 mm/min.

The Toadenpa anion-exchange column (202) was found to be most appropriate for serum Cl separation from the water dip. Using Oyobunko (ASA-4000) and Wescan (269-001) anion-exchange columns, the Cl peak overlapped with the water dip. Bicarbonate was well retained on either column, the elution order being Ca, Mg, bicarbonate. When KHP was used in the eluent, the indirect photometric detection method (at 280 nm) offered higher sensitivity than conductometric detection. No interferences with serum constituents were observed in the indirect photometric detection method.

*Origin of the system peak in anion analysis when using KHP as an eluent*

In anion analysis using KHP in the eluent and anion-exchange or ODS columns with either conductometric or indirect photometric detection, a negative or a positive peak was observed at a position other than the water dip or other dips (Fig. 9). This deflection was called a system peak [20]. The elimination of the system peak was necessary since it interfered with the analysis. It should be noted that even the injection of water produced a system peak. This peak was either positive or negative. Since the $t_R$ of the KHP peak coincided with that of the system peak, it was concluded that the system peak was due to phthalate. Positive and negative deflections were caused by a change in phthalate concentration in the mobile phase, depending on the pH difference between the sample and the mobile phase. When the sample pH was lower than that of the mobile phase, a negative system peak was observed owing to a decrease in the concentration of charged phthalate. Conversely, when the sample pH was higher than that of the mobile phase, a positive system peak was observed. The retention time of the system peak was inversely proportional to the pH of the eluent. The same was observed with the anion-exchange and ODS columns. In order to inhibit the development of the system peak, the pH of the mobile phase should therefore be the same as that of the sample. This was achieved by adding to the sample a buffer solution of the same pH as that of the mobile phase, and composed of ions different from those to be determined.

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