Journal of Chromatography, 344 (1985) 145—156
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2731

SIMULTANEOUS DETERMINATION OF SERUM CATIONS, ANIONS AND UREMIC TOXINS BY ION CHROMATOGRAPHY USING AN IMMOBILIZED ENZYME

HIDEHARU SHINTANI*

Department of Medical Devices, National Institute of Hygienic Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158 (Japan)

and

SHOZO UBE

Senshu Science Co. Ltd., 31-10, 3-chome, Igusa, Suginami-ku, Tokyo 167 (Japan)

(First received February 12th, 1985; revised manuscript received May 28th, 1985)

SUMMARY

A procedure is described for the analysis of serum cations and uremic toxins by simultaneous conductivity (CD) and ultraviolet (UV) photometric detection and using phosphoric acid as the eluent. In addition, studies were made on the determination of urea in serum using immobilized urease, and on the simultaneous determination of cationic and anionic compounds. The ammonium ion produced from urea by immobilized urease was determined by CD. No significant differences in amounts were found between the present method and conventional colorimetric methods. There was no significant reduction in immobilized urease activity after two months of continuous use. Simultaneous analysis of cationic and anionic compounds in serum was performed by switching valves in order to change the eluents and to change the channel to the analytical column. No marked baseline variation was observed and satisfactory recovery of anions was achieved.

INTRODUCTION

In previous papers [1, 2] we have described a method for the quantitative analysis of cations in serum by ion chromatography using conductivity detection (CD) as well as a serum pretreatment. We have also reported the quantitative analysis of uremic toxins such as urea (U), uric acid (UA), creatinine (CR), methylguanidine (MG), sodium and chloride in serum by reversed-phase ion-pair chromatography using an ODS column and an ultraviolet (UV) photometric detector [3]. Ion-pair chromatography placed some restrictions

0378-4347/85/$03.30

on the mobile phase for the separation of urea from serum admixtures, it also showed a low sensitivity to urea in serum.

To improve these deficiencies, we have now used ion chromatography and CD for the analysis of serum urea in the form of ammonium ions produced by reaction with immobilized urease contained in a column. In addition, a serum pretreatment by means of ultrafiltration [1] was employed. Sodium and potassium using CD, and creatinine using UV detection, were analyzed simultaneously in serum under identical conditions.

We have also simultaneously analyzed anions and mono- and divalent cations in serum by switching connecting valves, mobile phases and flow channels.

EXPERIMENTAL

Reagents and materials

Equine serum was obtained from Flow Labs. (McLean, VA, U.S.A.), the support for the column of immobilized urease, Eupergit C, from Roehm Pharma (Weiterstadt, F.R.G.) and urease (EC 3.5.1.5, Type IV from Jack Beans) from Sigma (St. Louis, MO, U.S.A.). Other reagents used were high grade and commercially available.

Instruments and equipment

The high-performance liquid chromatograph LC-3A (Shimadzu, Kyoto, Japan) was equipped with a non-suppressor type conductivity detector (Wescan Instruments, Santa Clara, CA, U.S.A.), an UV detector SPD-2A (Shimadzu), a sample injector with Rheodyne loop valves (7125 and 7010, each 10 μl; Alltech Assoc., Arlington Heights, IL, U.S.A.), the latter being used for the injection into the column of immobilized enzyme (50 × 4.6 mm I.D.), high-pressure four- and six-position valves (SHV-5000-4M and SHV-5000-6M, each with a

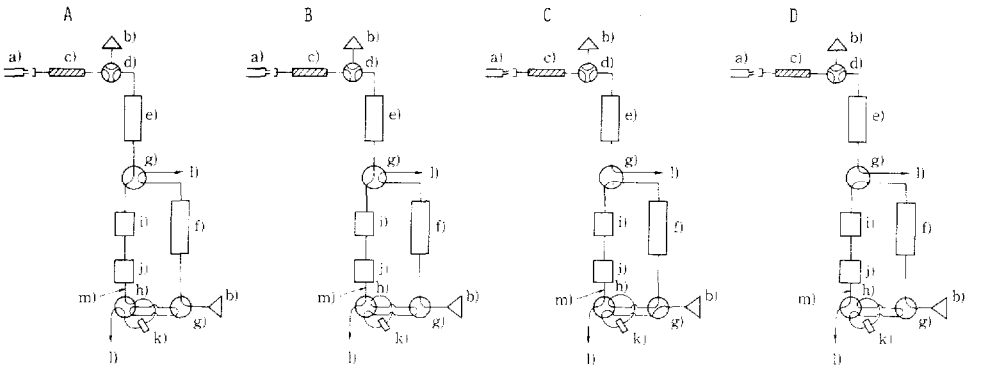

Fig. 1. Equipment for simultaneous determination of cations, anions and uremic toxins by ion chromatography using a column of immobilized urease. A—D are different positions of the apparatus (see text). a = Syringe (1 ml) for injection; b = pump; c = column of immobilized urease; d = injection port and valve with 10-μl loop; e = cation-exchange column; f = anion-exchange column; g = four-position injection valve; h = six-position injection valve with 15-μl loop; i = UV detector; j = conductivity detector; k = anion-exchange precolumn; l = drain; m = PTFE tubing (0.25 mm I.D.).

15-μl loop; Senshu Science, Tokyo, Japan). The anion-exclusion column 269-006 was obtained from Wescan, anion-exchange columns Wescan 269-001, HPX-72S from Bio-Rad Labs. (Richmond, CA, U.S.A.) and Zipax SAX from Mitsubishikasei (Tokyo, Japan). A precolumn for anion analysis (Wescan 269-003), cation-exchange columns (Shimadzu ISC-05 and Wescan 269-004) and gel permeation columns (Q-801, Q-802 and B-803; Showa Denko, Tokyo, Japan) were also employed. Colorimetry for serum urea was carried out with BUN Test Sanwa (Sanwa Chemical Research, Nagoya, Japan), Ureanase Nissui (Nissui Pharmaceutical, Tokyo, Japan) and Amitest-N (Chugai Pharmaceutical, Tokyo, Japan) apparatus. Other instruments and equipment were as used previously [1—3].

TABLE I

ANALYTICAL CONDITIONS FOR CATIONS AND UREMIC TOXINS

	Sodium	Urea (ammonium)	Potassium	Creatinine	Methylguanidine
Injection volume (μl)	10	10	10	10—50	10—50
Column	Wescan cation-exchange column 269-004, 250 × 4.6 mm I.D.				
Temperature	Ambient in all cases				
Mobile phase	Phosphoric acid (pH 2.03) except in the case of urea (pH 2.28)				
Flow-rate (ml/min)	1.0 in all cases				
Detector	Wescan non-suppressor type conductivity detector (range ×10, coarse × 3) except in the case of creatinine and methylguanidine, which were detected at 234 and 210 nm, respectively, using a Shimadzu SPD-2A UV detector. Output voltage from conductivity detector and input full-scale voltage: 10 mV in each case				
Serum pretreatment	Undiluted ultrafiltered serum except in the case of sodium determination, which was carried out using 100 times diluted ultrafiltered serum. Serum urea was treated with immobilized urease contained in a column preceding the injection port				

TABLE II

ANALYTICAL CONDITIONS FOR ANIONS

	Chloride	Bicarbonate
Injection volume (μl)	10	10
Column	Wescan anion-exchange column 269-001, 250 × 4.6 mm I.D.	
Temperature	Ambient in both cases	
Mobile phase	4 m*M* Potassium hydrogen phthalate adjusted to pH 4.5 with potassium hydroxide	
Flow-rate (ml/min)	2.0 in both cases	
Detector	CD as in Table I	
Serum pretreatment	Ultrafiltered serum diluted 2 and 50 times for serum bicarbonate and chloride, respectively	
Retention time (min)	3.8	17.6

Procedures

The apparatus for the analysis of cations and anions in serum is shown in Fig. 1. The column-switching method for simultaneous cation and anion analysis is described in the text. Fig. 1 includes the ion chromatographic system used in combination with the column of immobilized enzyme. The conditions employed for analyzing cationic and anionic compounds in serum are shown in Tables I and II, respectively. Urease was immobilized with Eupergit as described [4], suspended in distilled water and packed into a column (50 × 4.6 mm) using the conventional slurry method. Water flowing through this column at a rate of 1 ml/min caused little increase in pressure.

RESULTS AND DISCUSSION

Eluent conditions for analyzing uremic toxins in serum

For chromatography for cations, phosphoric acid (pH 2—2.5) was found to be the only eluent that resulted in the simultaneous detection of monovalent cations and cationic uremic toxins by CD and UV. Nitric acid (pH ca. 2), which is generally used as the eluent in ion chromatography with CD, was unsuitable for UV detection. Sulphonic acid (pH ca. 2) was unsuitable for CD. Perchloric acid (pH ca. 2) was suitable for both detectors but resulted in a poor reproducibility of retention time, t_R.

Mono- and divalent cations in serum were first analyzed by ion-pair chromatography (counter ions: 1—2 m*M* each of sodium hexanesulphonate, sodium heptanesulphonate, sodium octanesulphonate, sodium dodecanesulphonate and sodium octadecanesulphonate) using reversed-phase columns (C_8 and C_{18}) and CD. Phosphoric acid (pH 2.1) containing 0—20% acetonitrile was the eluent. The chromatograms thus obtained exhibited broad peaks with leading phenomena for divalent cations and insufficient separation for monovalent cations. A similar result was reported by Molnár et al. [5]. However, the situation was improved by the use of a mobile phase of phosphoric acid (pH ca. 2) and a Wescan 269-004 cation-exchange column (capacity around 0.04 mequiv./g) as shown in Table I. A linear relationship was found between the pH (1.9—2.3) of an aqueous solution of phosphoric acid used as the eluent and the

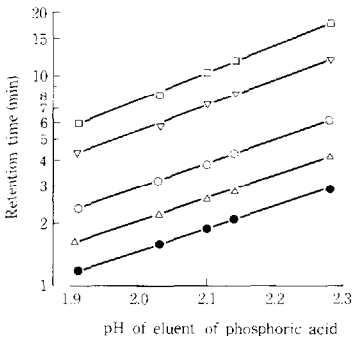

Fig. 2. Relationship between the logarithm of the retention time and the eluent pH: (•) sodium; (△) ammonium; (○) potassium; (▽) creatinine; (□) methylguanidine.

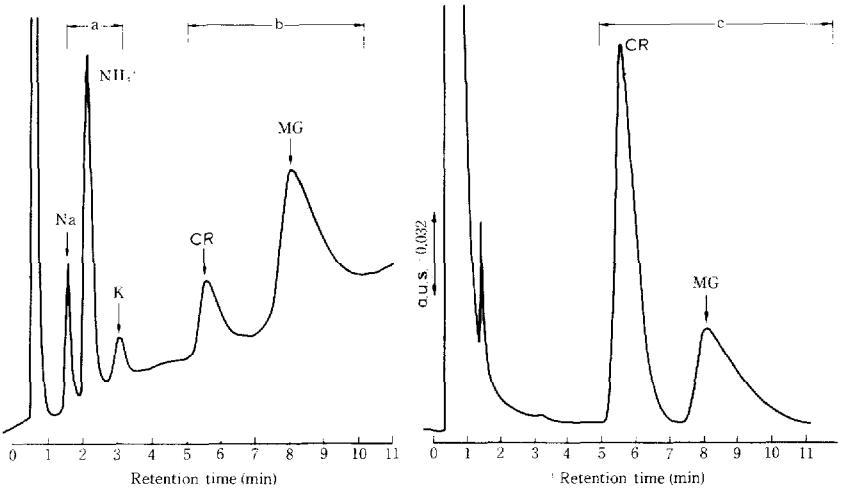

Fig. 3. Chromatograms of a standard aqueous solution of sodium, ammonium (NH_4^+), potassium, creatinine (CR) and methylguanidine (MG) at 3.02, 9.86, 3.54, 6.00 and 15.30 mg/dl, respectively. Volumes of 10 μl were applied for Na^+, NH_4^+ and K, 50 μl for CR and MG. Phosphoric acid (pH 2.03) was used as the mobile phase, and detection was carried out by CD for Na^+, NH_4^+, K^+, CR and MG (a for Na^+, NH_4^+ and K^+ and b for the remainder), and by UV (210 nm, a.u.f.s. = 0.32) for CR and MG (c). The retention times for Na^+, NH_4^+, K^+, CR and MG were 1.6, 2.2, 3.2, 5.7 and 8.2 min, respectively. For other analytical conditions see Table I.

logarithm of t_R for Na^+, NH_4^+, K^+, CR and MG (Fig. 2). In the anion analyses (Table II), similar linearity (Fig. 2) was found between the logarithm of t_R for Cl^- and bicarbonate and the pH of the mobile phase which contained a fixed concentration of potassium hydrogen phthalate. A linear relationship was also found between the logarithm of t_R of Cl^- and bicarbonate and the concentration of potassium hydrogen phthalate, at fixed mobile phase pH [6, 7]. A chromatogram of a standard aqueous solution is shown in Fig. 3.

Methods of determining serum uremic toxins, particularly U, and of injecting serum sample in the column of immobilized enzyme

Our attempts to analyze simultaneously UA and U in serum without urease treatment, using a Shimadzu high-capacity ISC-05 cation-exchange column or a Wescan 269-004, were unsuccessful due to insufficient separation of these compounds from the water dip. U and UA were eluted around the void volume due to poor retention on the cation-exchange column. The analysis of serum urea, in the form of uronium, was achieved using an anion-exclusion column Wescan 269-006 combined with phosphoric acid (pH 2.1) as eluent, but showed low sensitivity to CD. The analysis of other uremic toxins, in particular UA, was unsuccessful because of insufficient separation from serum admixtures. The use of an anion-exchange column Bio-Rad HPX-72S or gel permeation column Q-801, Q-802 or B-803 resulted in sufficient separation of U, UA, CR and MG in a standard aqueous solution but there was insufficient separation, except of UA from serum admixtures, when using the serum sample. Urea exhibited the poorest separation from serum admixtures. UA had the greatest t_R when using both techniques, and therefore had sufficient separation from

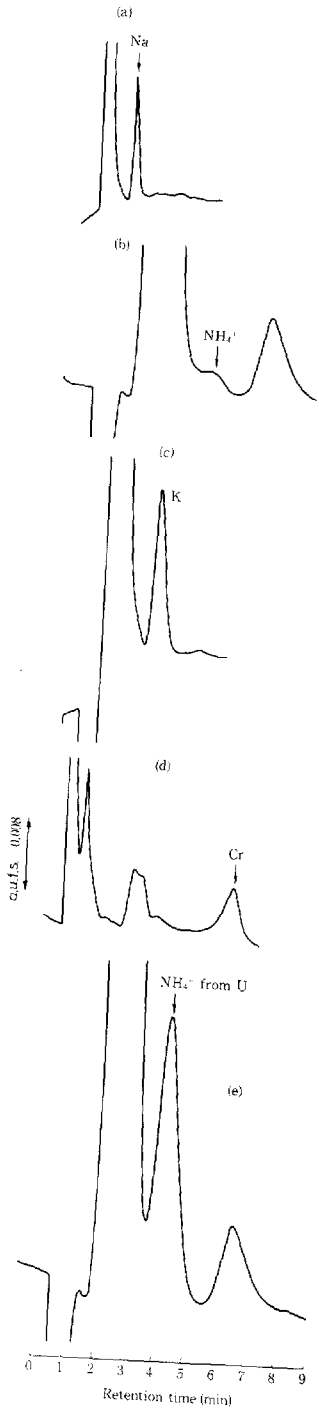
(a)
Na
(b)
NH4
(c)
K
(d)
Cr
a.u.f.s. 0.008
NH4 from U
(e)
0
1
2
3
4
5
6
7
8
9
Retention time (min)

TABLE III

LINEARITY RANGE AND DETECTION LIMIT

The linearity range in serum was from the detection limit to around 10 times the amount of potassium, creatinine and bicarbonate and to around 3 times the amount of sodium, ammonium (urea) and chloride and to around 100 times the amount of methylguanidine.

Ion	Mean detection limit (n=10) (ng)	
	CD	UV
Sodium	3.86*	—**
Ammonium	7.78*	—***
Potassium	25.10*	—**
Creatinine	93.82§	20.50§§
Methylguanidine	119.56§	45.52§§§
Chloride	48.36†	—**
Bicarbonate	417.56†	—**

*10 μl were applied. For other analytical conditions see Table I.
**Inactive to UV.
***The detection limit for urea at 210 nm (a.u.f.s. 0.04) was 500 ng (10-μl injection), indicated previously [3].
§50 μl were applied. For other analytical conditions see Table I. The amount of creatinine was almost the same as the detection limit by CD, therefore UV detection was preferred.
§§50 μl were applied. A.u.f.s. 0.32 detected at 210 nm. Other analytical conditions as in Table I.
§§§50 μl were applied. A.u.f.s. 0.08 at 210 nm. Other analytical conditions as in Table I.
†10 μl were applied. For other analytical conditions see Table II.

serum admixtures. To obtain sufficient separation of U from serum admixtures, CD, phosphoric acid (pH ca. 2) as the eluent and a low-capacity cation-exchange column combined with the column of immobilized enzyme were used, markedly improving the detection limits when compared with reversed-phase ion-pair chromatography (Table III) [3].

It was found that when the column of immobilized enzyme was placed after the injection port but before the analytical column, it came into contact with the acidic mobile phase and rapidly deteriorated. In order to prevent

Fig. 4. Chromatograms of cations and uremic toxins in serum. A 10-μl volume of 100-times diluted ultrafiltered serum was applied for sodium determination (a) and of non-diluted ultrafiltered serum for ammonium (b), potassium (c), creatinine (d) and urea (e) determination. These compounds were detected by CD except for creatinine which was detected at 210 nm (a.u.f.s. = 0.08) by UV. Urea was determined by CD using immobilized urease. The treated serum was then ultrafiltered and injected without dilution for HPLC. The mobile phase and retention times were as in Fig. 3 except for ammonium and urea (urea was transformed into ammonium with the use of immobilized urease). Phosphoric acid (pH 2.28) was used as the mobile phase for ammonium and the retention time was 4.2 min. For other analytical conditions see Table I. The concentrations of sodium, ammonium, potassium, creatinine and urea in these chromatograms were 2.87, 0.31, 13.65, 1.20 and 20.68 mg/dl, respectively.

this and improve the conversion of U into NH_4^+, the column of immobilized enzyme was placed before the injection port. Also, this column had a dead volume of about 400 μl when packed with urease, therefore, a 500-μl portion of the undiluted ultrafiltrate of serum was applied and the column was allowed to stand at room temperature for 10—15 min in order fully to convert serum urea into NH_4^+. Then another 100 μl of the serum ultrafiltrate were applied so that the sample loop was filled only with 10 μl of the urease-treated serum. Finally, the contents of the sample loop were transferred to the analytical column. This resulted in about 100% conversion of urea into NH_4^+ (Fig. 4). When the ultrafiltrate was allowed to stand in the column for at least 8 min during the early stage of the immobilized column usage, a 100% conversion rate was attained. When the ultrafiltrate was transferred rapidly from the column of immobilized enzyme to the analytical column without standing, the extent of conversion was about 45%. Therefore, the extent of conversion increased in proportion to the period of standing up to 8 min after addition to the column of immobilized enzyme. The lifetime of this column was prolonged by placing it before the injection port, washing with phosphate buffer (pH 7) after use and by storing it in a refrigerator. Data for urea in serum revealed that the within-experiment, day-to-day and month-to-month changes in the coefficient of variation (C.V.) were mostly 1.5% or less (n=10 in each case). When the column of immobilized enzyme was used continuously for two months, the detection limit was reduced by about 20% compared with that at the beginning, due to the decrease in urease activity. For this reason, a calibration curve was prepared for each experiment. However, when the period of standing was more than 15 min after two months of continuous use, for instance around 20 min, the conversion attained was still 100%.

Urea in serum was analyzed by the immobilized-urease method and colorimetry (BUN Test Sanwa [8], Ureanase Nissui [9] and Amitest-N [10]), and the results were compared. The sensitivity of the immobilized-urease method combined with CD was found to be superior to that of colorimetry and reversed-phase ion-pair chromatography with UV detection as described previous-

TABLE IV

AMOUNTS DETERMINED BY CD AND UV METHODS

Indicated amounts are mean values (mequiv./l or mg/dl) ± coefficient of variation (%). n=10. Serum pretreatment as in Tables I and II. Only creatinine was determined using UV detection. The amount of methylguanidine in serum was less than the detection limit by UV.

Ion	Mean value	Coefficient of variation (%)
Sodium	124.7* ± 0.6	0.6
Potassium	3.5* ± 0.5	0.5
Creatinine	1.2 ± 0.6	0.6
Ammonium	0.3 ± 0.5	0.5
Urea	20.7 ± 0.4	0.4
Chloride	98.3* ± 0.7	0.7
Bicarbonate	67.5 ± 1.0	1.0

*In mequiv./l; all other values in mg/dl.

ly [3]. The results were comparable, the C.V. being about 1.5% or less (n=10). The amounts of urea in serum were determined by the immobilized-urease method (Table IV), and also by a urease-batch method. In the latter, 50 μl of urease (10.7 mg/ml) were added to 1 ml of serum and incubated at 37°C for 15 and 30 min (with no difference in results for the two incubation periods). The amount of urea found with each method was practically the same (C.V. = 0.5%, n=10).

Amount of each compound in serum

The ultrafiltrate of serum obtained as described previously [1, 2] was used undiluted for the quantitative analysis of serum NH_4^+, K^+ and urea using CD, undiluted for CR and MG using UV detection, but diluted 2-, 50- and 100-fold for the analysis of bicarbonate, Cl^- and Na^+, respectively using CD. The amounts found and the chromatograms showing the separation of uremic toxins from serum admixtures are shown in Table IV and Fig. 4, respectively. The amount of CR in serum was almost the same as the detection limit for CR by CD, therefore UV detection was preferred (Table IV). The amount of MG in normal serum was less than the detection limit by UV.

Simultaneous determination of cationic and anionic compounds in serum

Serum was ultrafiltered and the ultrafiltrate used both in diluted and undiluted form for HPLC [1, 2], see Fig. 1 and Tables I and II. The cations were analyzed first and then the anions. In Fig. 1, the analytical column placed near the injection port is the cation-exchange column. After elution of the solvent front (Fig. 1B), the six-position valve was switched to collect the anionic compounds eluted around the solvent front (Fig. 1A). The broadening of anion peaks was prevented by trapping and subsequent concentration of the anionic compounds using a precolumn placed in a loop of small volume in order to minimize the dead volume, and the eluent excess was drained. Subsequently, the six-position valve was switched and cationic compounds were analyzed for 7 min (Fig. 1B). The upper four-position valve was then switched (Fig. 1D) for 10 min in order to replace the mobile phase in the channel to the detector by the mobile phase for the anion analysis. During this period, the precolumn for anion analysis and the contents of the loop were washed with 5 ml water to return the pH to around 4—5 (suitable for further anion analysis). This procedure was carried out to prevent baseline variation in further anion analysis. It did not remove previously trapped and concentrated anions in the precolumn as is indicated by the satisfactory recovery. The lower four-position valve was then switched (Fig. 1C) for the analysis of serum anions previously condensed in the precolumn. The channel from the conductivity detector to the six-position valve comprised PTFE tubing (0.25 mm I.D.) in order to prevent damage to the CD cell.

The baseline showed little variation when the mobile phase for the analysis of cations was changed to that for the analysis of anions, because each mobile phase flowed at a constant rate in each analytical column, the mobile phase in the channel to the detector was replaced (Fig. 1D) and the precolumn was washed with water to assure a suitable pH for further anion analysis. Broadening of peaks was not observed, since anionic compounds were previously con-

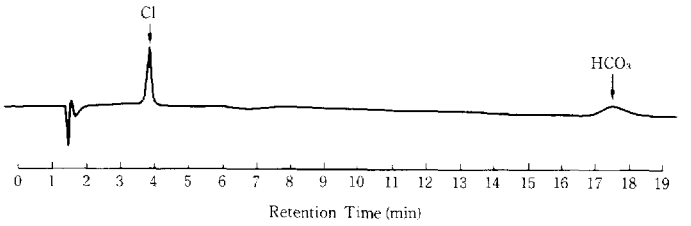

Fig. 5. Chromatogram of a standard aqueous solution of chloride and bicarbonate, 15.5 and 42.1 mg /dl, respectively. The retention times of chloride and bicarbonate were 3.8 and 17.6 min, respectively. Cation analysis was carried out from injection to 7 min. After a further 10 min, anion analysis was carried out under the conditions in Table II. For other analytical conditions see Table II. The chromatogram showing the cations has been omitted as it closely resembles that shown in Fig. 3a and b (CD) and 3c (UV detection).

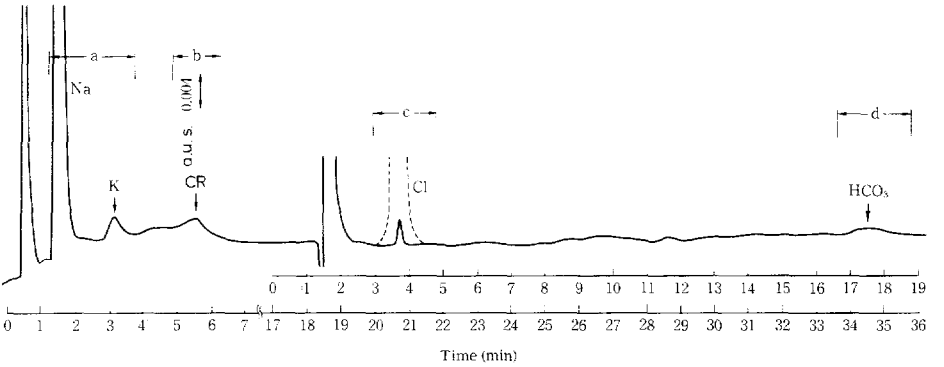

Fig. 6. Chromatogram of cations and anions in serum. A 10-μl volume of 10-times diluted ultrafiltered serum was applied for sodium (a) and potassium (a) determination using CD and for creatinine determination (b) using UV detection (210 nm, a.u.f.s. = 0.04). The concentrations of sodium, potassium and creatinine were 28.68, 1.37 and 0.12 mg/dl, respectively. Other analytical conditions as in Table I. A 10-μl volume of 2- and 50-times diluted ultrafiltered serum were applied for bicarbonate (d) and chloride (c) determination, respectively, using CD. The peak indicated by a broken line is for chloride, when 2-times diluted ultrafiltered serum was applied. The concentrations of chloride and bicarbonate in this chromatogram were 6.98 and 33.77 mg/dl, respectively. Other analytical conditions for anions as in Table II.

densed in the precolumn. An almost 100% recovery of serum anionic compounds was attained from the precolumn. The chromatograms of a standard aqueous solution and that of a serum sample are shown in Figs. 5 and 6, respectively. The analysis of divalent cations in serum was performed under the same conditions as for the analysis of monovalent cations, except for the mobile phase. Divalent cations did not interfere with the analysis of monovalent cations and uraemic toxins because the former were not eluted by the diluted phosphoric acid used in this experiment. The mobile phase for the analysis of divalent cations was as described previously [2] using ethylenediamine adjusted to pH 6.1. Other conditions are shown in Fig. 1 and Table I. Clinically significant anions, Cl^- and bicarbonate, were determined in order to estimate the anion gap [11]. Table IV indicates the amounts determined by the present method.

Indirect photometric ion chromatography [2, 6, 7] using 0.5—1.0 m*M* copper(II) sulphate as the eluent and UV detection for the simultaneous determination of cationic and anionic compounds caused no significant baseline variation when the mobile phase was changed as in the above procedure. It was found to be superior to the CD method [1] as regards the simultaneous determination of mono- and divalent cations. This indirect method was, however, considerably inferior to the present method using CD, as regards the separation of NH_4^+ from Na^+ and K^+ in serum [2] as well as that of bicarbonate from serum admixtures, due to the elution of bicarbonate around the solvent front when using the previously described procedure [7]: eluent, 0.5 m*M* disodium phthalate; column, Zipax SAX (500 × 2.1 mm I.D.); UV detection, 240 nm. Therefore, indirect photometric ion chromatography was considered to be inadequate for the analysis of these serum compounds, due to insufficient separation.

One previously published study that determined cations and anions simultaneously, used a chelating agent to complex the divalent cations, which were then separated and detected as anions along with the uncomplexed anions [12, 13]. Another method for simultaneous analysis of cations and anions using as eluent a mixture of lithium carbonate and lithium acetate dihydrate and an electrochemical detector has been reported [14]. These methods, however, were found to be inferior to the present method owing to the inability to analyze monovalent cations in the former method [12, 13] and divalent cations in the latter [14]. In these methods, the kinds of anions which can be eluted and analyzed are restricted owing to the limitation of the kinds of eluents for anion analysis. The indirect photometric method reported by Small and Miller [6] also has limitations in the simultaneous analysis of cations and anions. Therefore, the present method is believed to be superior due to its success in analyzing both mono- and divalent cations and anions.

CONCLUSIONS

We have examined various eluents for ion chromatography and found that phosphoric acid (pH ca. 2) is the only suitable eluent for simultaneous detection by both UV and CD. The use of a column of immobilized enzyme, preceding the injection port, made it possible to determine simultaneously uremic toxins such as U, CR, NH_4^+, Na^+ and K^+. Moreover, cationic and anionic compounds in serum were determined simultaneously by appropriate switching of valves, switching the cation- and anion-exchange columns and by the use of a column of immobilized enzyme.

ACKNOWLEDGEMENT

The authors are indebted to Dr. Maureen H. Higgin for her kindness in revising this manuscript.

REFERENCES

1 H. Shintani, K. Tsuji and T. Oba, Bunseki Kagaku, 33 (1984) 347.
2 H. Shintani, J. Chromatogr., 341 (1985) 53.
3 H. Shintani, K. Tsuji and T. Oba, Eisei Kagaku, 30 (1984) 1.
4 H. Huck, A. Schelter-Graf, J. Danzer, P. Kirch and H.L. Schmidt, Analyst (London), 109 (1984) 147.
5 I. Molnár, H. Knauer and D. Wilk, J. Chromatogr., 201 (1980) 225.
6 H. Small and T.E. Miller, Jr., Anal. Chem., 54 (1982) 462.
7 K. Hayakawa, H. Hirai and M. Miyazaki, Bunseki Kagaku, 32 (1983) 504.
8 C.J. Hallet and J.G.H. Cook, Clin. Chim. Acta, 35 (1971) 33.
9 D. Glick, Methods Biochem. Anal., 17 (1969) 311.
10 Y. Yajima, Shinyaku Rinsho, 30 (1981) 525.
11 P.H. Lolekha and S. Lolekha, Clin. Chem., 29 (1983) 279.
12 M. Yamamoto, H. Yamamoto and Y. Yamamoto, Anal. Chem., 56 (1984) 832.
13 S. Matsushita, J. Chromatogr., 312 (1984) 327.
14 V.K. Jones and J.G. Tarter, J. Chromatogr., 312 (1984) 456.