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## Simultaneous Determination of the Most Abundant Cationic Species in Human Urine by Capillary Isotachopheresis

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**Summary:** Analytical isotachopheresis has proved to be an excellent method for separation and quantitative measurement of ionic species in biologic fluids. Isotachopheretic separation of cations in presence of a complex-forming nonionic detergent (e. g. Triton X-100) in 98% methanol enables simultaneous quantitative determination of ammonium, sodium, potassium, magnesium, and protonated creatinine in urine samples. For calcium determination in urine, lead acetate was used to precipitate anions that form complexes with calcium.

The mean deviation from 100% recovery of ammonium, sodium, potassium, magnesium, calcium and creatinine added to urine samples is 3.1%. The proposed method shows good agreement with chemical (routine) methods.

### Introduction

Quantitative determinations of components in urine samples is of limited value for two main reasons:

1. The concentration of components varies strongly with diuresis. A 24-hour urine collection is therefore necessary, in order to calculate daily excretion. There are, however, many factors in the collection, storage and handling of urine samples which influence the final analytical result.
2. The daily excretion rate is strongly dependent on intake of nutrients and water.

Nevertheless, a number of clinical situations require quantitative urine analysis for adequate patient management. Patients depending on haemodialysis or undergoing an immunosuppressive therapy and patients depending on parenteral nutrition present such situations. Another clinical situation is given by the diagnosis of metabolic disorders leading to urolithiasis. Hesse (1) has estimated normal values for the amount of a series of cations and anions excreted daily in order to estimate the risk for urolithiasis.

Some other indications for urinary electrolyte determinations are given by Eastham (2) and Durborski (3).

Our own experience shows that determination of urinary sodium, potassium, magnesium, calcium and creatinine accounts for about 50% of all routine chemical urine analyses. Hirst (4) has used a multi-channel urine analyser of the continuous flow type for the simultaneous determination of sodium, potassium, calcium, phosphate, creatinine and urea. He has encountered problems with the high dynamic range that is required in urine analysis, as opposed to the analysis of blood serum or plasma.

Isotachopheresis is extremely suitable for the simultaneous determination of either cations or anions. We recently reported a method for the separation and measurement of anionic components (5). While anionic components have also been studied by ion-chromatography (6), no alternative method is known for the simultaneous analysis of the cationic components in the same sample or specimen. Several methods of isotachopheretic separations of cation mixtures

have been proposed (14, 15) and most of them have been reviewed by *Bocek & Foret* in 1984 (16). However, none of these methods can be used for the simultaneous determination of the most abundant cationic species (e.g.  $\text{NH}_4^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and creatinine- $\text{H}^+$ ) in human urine. The isotachopheretic procedure offers the big advantage of high dynamic range. From the practical standpoint, it is limited only by the time that can reasonably be spent on an analysis.

## Materials and Methods

### Urine samples

The 24-h urine specimens were collected, stored at 4 °C during the sampling period, and acidified with conc. HCl.

### Reagents

All chemicals used were of analytical grade. Electrolyte systems and other operational conditions are summarized in table 1.

The separation capillary was occasionally treated overnight with a 0.4 g/l aqueous solution of hydroxypropylmethylcellulose (HPMC). This procedure improved the performance by lowering the minimal detectable amount and by sharpening the boundaries between zones.

A reference mixture of seven cations was prepared, containing creatinine and chloride salts of ammonium, caesium, sodium, potassium, magnesium and calcium respectively. The concentration of each component was 1.0 mmol/l. The standards of calibration for the quantitative determination of these cations in urine were prepared with four different concentrations (3.0–80 mmol/l). Caesium chloride with a final concentration of 10 mmol/l was used as an internal standard. For calcium determination a precipitating solution containing a mixture of 15 mmol/l caesium chloride and 85 mmol/l lead acetate was used to eliminate interfering anions (e.g. sulphate, phosphate, oxalate) from native urine.

### Instruments

To compare isotachopheretic results with routine methods we used a flame photometer (Model IL 553, Instrumentation Laboratory) for the determination of sodium and potassium, and a polarized Zeeman atomic absorption spectrophotometer (Model 180-80, Hitachi, Ltd. Tokyo, Japan) for the estimation of magnesium and calcium. Ammonium was determined on a Cobas Bio centrifugal analyser. The ammonium assay kit (Procedure No. 170-UV) was purchased from Sigma Diagnostics, St. Louis, MO, USA. For the determination of creatinine Beckman Creatinine Analyser 2 was used.

A Shimadzu IP-2A isotachopheretic analyser, equipped with a potential gradient detector, a UV-detector, a 200 mm FEP capillary (0.5 mm I.D.) and a 40 mm PTFE capillary (1.0 mm I.D.) as the precolumn was used for the separation and measurement of the standard and sample solutions. The apparatus was also equipped with a current program. Microliter samples were injected with a Hamilton syringe. The isotachopheretic patterns for these samples were recorded at a chart speed of 4.0 cm/min. The data processor used was the isotachopac I-E1B (Shimadzu Corporation, Kyoto, Japan), which calculated the intervals between peaks (zone length), potential gradient values and time between two arbitrary potential values.

### Procedures

Human urine or standard solution was routinely diluted to 1/3 of initial concentration with caesium chloride 15 mmol/l (internal standard) for the simultaneous determination of ammonium, sodium, potassium, magnesium and creatinine to yield a solution containing 10 mmol/l of CsCl. Without further pretreatment a volume of 1.0  $\mu\text{l}$  was injected into the isotachopheretic analyser. For calcium determinations urine samples and standards were diluted 1 + 2 with the precipitating solution, vortex-mixed and centrifuged. A 1  $\mu\text{l}$  aliquot of supernatant fluid was injected. Since ions having different mobilities travel at an identical speed under isotachopheretic condition, a potential gradient corresponding to the mobility is formed in each zone. The potential gradient is inversely proportional to the mobility. For practical reasons it is often converted into P.U. (potential unit) values, which are general qualitative indices. They are calculated using the following equation:

$$\text{P.U. value} = (P_X - P_L) / (P_T - P_L), \text{ where}$$

$P_X$ ,  $P_L$  and  $P_T$  are potential gradient values of sample ion X, leading ion and terminating ion, respectively. From the equation follows that  $0 < \text{P.U.} < 1$ .

## Results

Two different procedures were compared for the determination of cations.

Figure 1 shows the isotachopherogram of the cation reference mixture illustrating the characteristic zone heights of seven cationic components. In this procedure (System B, table 1 and 2) the poor separation between  $\text{K}^+$  and  $\text{NH}_4^+$  complicated the quantitative determination of the ions in urine samples. The effect of Triton X-100 (System A, table 1 and 2) on the separation of a reference mixture of cations is shown in figure 2. Addition of 8 g/l Triton X-100 to the leading electrolyte results in decreased potassium mobility (tab. 2) and well separated zones of potassium and ammonium. Figure 2 also shows the UV-absorbances of ammonium and alkali metal complexes.

In table 2 the P.U. values (qualitative index) for some cations obtained in the operational systems are summarized. This proves that alkali metal ions and ammonium ion formed complexes with Triton X-100 in methanol and accordingly changed their mobilities. A typical example of the resulting isotachopherogram of a urine sample is shown in figure 3.

### Calibration and quantification

Calibration curves were established for ammonium, sodium, potassium, magnesium, calcium and creatinine standards dissolved in both water and pooled urine. For each component the ratio of the zone length to the zone length of the internal standard caesium was calculated and this ratio ( $M/\text{Cs}$ ) was plotted against the concentration (mmol/l) of the component in the standard solution which had been injected. The

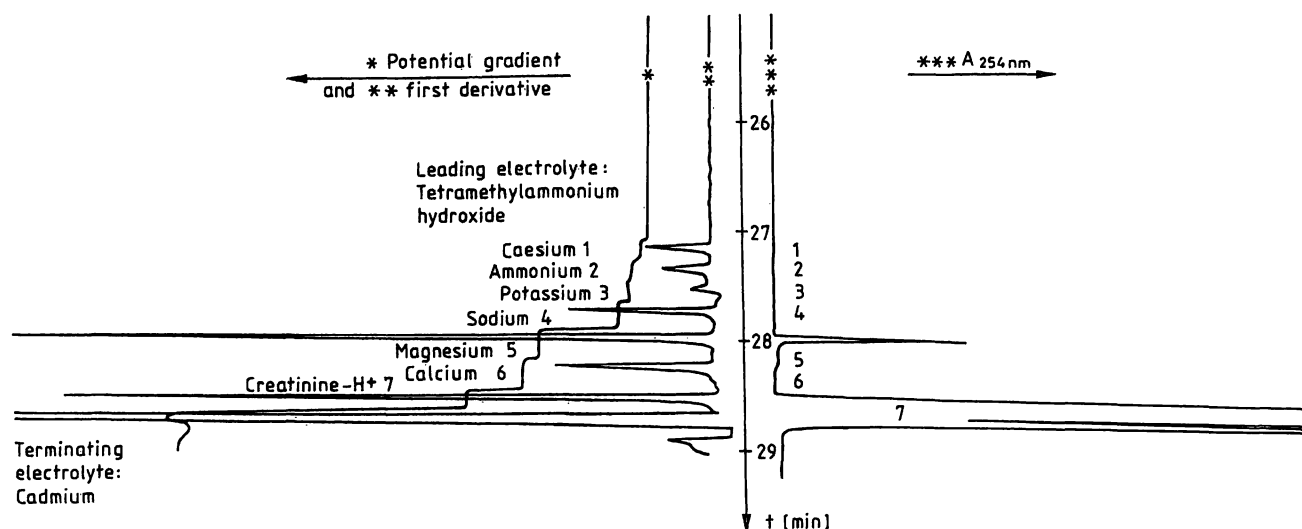


Fig. 1. Isotachopherogram of a standard mixture of seven cations.  
volume injected: 5.0  $\mu$ l (1 mmol/l of each component) Electrolyte system B was used.

Tab. 1. Operational systems.

	System A	System B
Solvent	980 ml/l methanol	980 ml/l methanol
Leading electrolyte	10 mmol/l tetramethylammonium hydroxide 12 ml/l acetic acid 0.12 g/l hydroxypropylmethylcellulose 8 ml/l Triton X-100 pH = 5.62	10 mmol/l tetramethylammonium hydroxide 12 ml/l acetic acid 0.12 g/l hydroxypropylmethylcellulose none pH = 5.57
Terminating electrolyte	30 mmol/l cadmium nitrate pH = 6.0	30 mmol/l cadmium nitrate pH = 6.0
Migration current	200 $\mu$ A (7 min) $\rightarrow$ 75 $\mu$ A	200 $\mu$ A (7 min) $\rightarrow$ 75 $\mu$ A
Bath temperature	20 $^{\circ}$ C	20 $^{\circ}$ C

Tab. 2. PU values (qualitative index) for some cations obtained in the operational systems.

Cation	System A (n = 13)		System B (n = 7)	
	PU	$\pm$ SD	PU	$\pm$ SD
Tetramethylammonium	0	—	0	—
Caesium	0.137	0.0060	0.016	0.0008
Ammonium	0.053	0.0027	0.038	0.0022
Potassium	0.168	0.0103	0.044	0.0033
Sodium	0.102	0.0048	0.063	0.0026
Lithium	0.090*	0.0033	0.098*	0.0013
Magnesium	0.225	0.0061	0.226	0.0039
Calcium	0.263	0.0071	0.262	0.0034
Creatinine	0.383	0.0114	0.380	0.0046
Creatine	0.537*	0.0088	0.560*	0.0050
Zinc	0.705*	0.0052	0.694*	0.0037
Lead	0.575*	0.0150	0.888*	0.0046
Cadmium	1.000	—	1.000	—

\* n = 4

relationships between concentration (X) of components and the zone length ratio M/Cs (Y) obtained from isotachopherograms are presented in table 3. The calibration plots shown in figures 4, 5 and 6 for the six components were linear over a range of concentration up to 32 mmol/l for ammonium and creatinine, 80 mmol/l for sodium and potassium, 16 mmol/l for magnesium and 7.5 mmol/l for calcium with a sample volume of 1  $\mu$ l. No mixed zones were observed throughout the whole concentration range studied.

Correlation coefficients ranged from 0.995 to 1.000. Every calibration standard was determined in triplicate. The imprecision was very low. The coefficients of variation were 0.85%, 4.72%, 1.70%, 1.40%, 1.77% and 1.36% for ammonium, sodium, potassium, magnesium, calcium and creatinine, respectively.

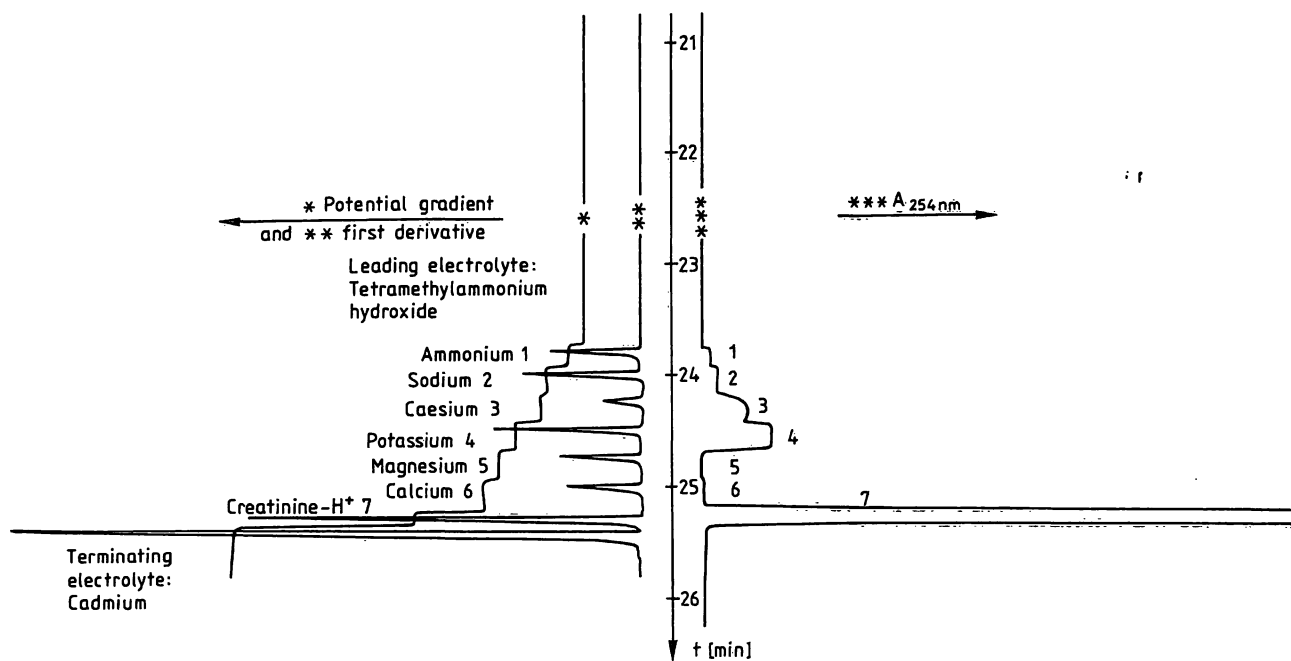


Fig. 2. Isotachopherogram with the same conditions as in figure 1. Electrolyte system A was used.

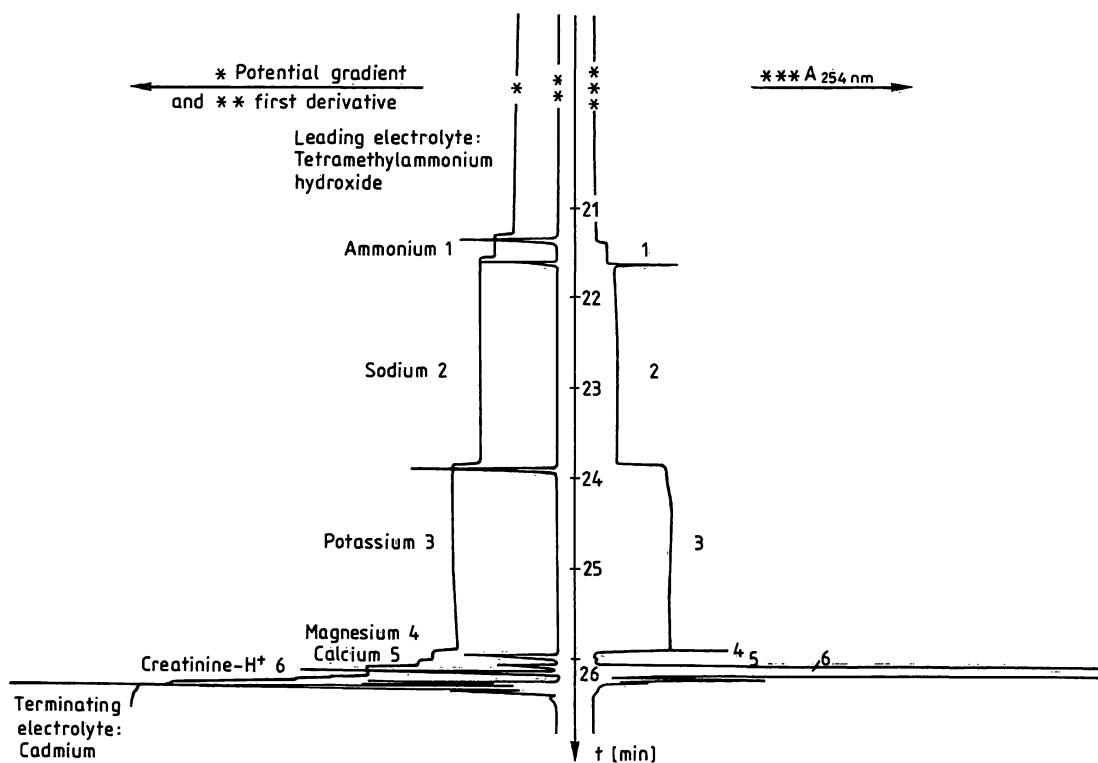


Fig. 3. Isotachopherogram of a pooled urine.  
Volume injected: 1.0  $\mu$ l  
Components identified are indicated in figure 2.

Tab. 3. Linearity of relation between zone length ratio, M/Cs (y) and concentration (x) in water or pooled urine  
 M = zone length of cation  
 r = correlation coefficient  
 Cs = zone length of caesium (internal standard)

Cation	Calibration curve of standard solution	Calibration curve after standard addition in pooled urine
Ammonium	$y = 0.0242x + 0.0155$ $r = 1.000$	$y = 0.0256x + 0.3490$ $r = 1.000$
Sodium	$y = 0.0285x + 0.0485$ $r = 0.998$	$y = 0.0261x + 3.290$ $r = 0.995$
Potassium	$y = 0.0348x + 0.0345$ $r = 0.998$	$y = 0.0369x + 1.5160$ $r = 1.000$
Magnesium	$y = 0.0329x + 0.0097$ $r = 0.999$	$y = 0.0316x + 0.1165$ $r = 0.999$
Calcium	$y = 0.0312x + 0.0124$ $r = 0.999$	$y = 0.0328x + 0.0786$ $r = 0.998$
Creatinine	$y = 0.0248x - 0.002$ $r = 1.000$	$y = 0.0254x + 0.003$ $r = 1.000$

Precision and accuracy

The within-day variation (CV) obtained by assaying three different pooled urine samples 12 times each in one run, ranged from 0.92–4.95%.

The between-day variations for the same samples assayed once each in 6 different runs ranged from 1.00–7.41% (tab. 4). Different known amounts of creatinine and chloride salts of ammonium, sodium, potassium, magnesium and calcium, respectively, were added to a pooled urine sample. Data obtained are summarized in table 5. The mean deviation from 100% recovery of these components added to urine samples is 3.1%.

Method comparison

The proposed method was directly compared with alternative routine methods. Figures 7 through 12 show the correlation between isotachophoretic determinations with routine methods. The corresponding techniques were an enzymatic method for ammonium (7), flame photometry for sodium and potassium (8), atomic absorption spectrometry for magnesium and calcium (9) and a colorimetric assay for creatinine (10). Isotachophoretic results were plotted against the results of the routine methods. The set of data were statistically evaluated by linear regression, calculation of correlation and by the procedure of *Passing & Bablok* (11). The characteristics of the straight lines (slope, intercept) are well within the confidence limits for the methods. The results are summarized in table 6. The isotachophoretic method shows a good correlation with the routine methods.

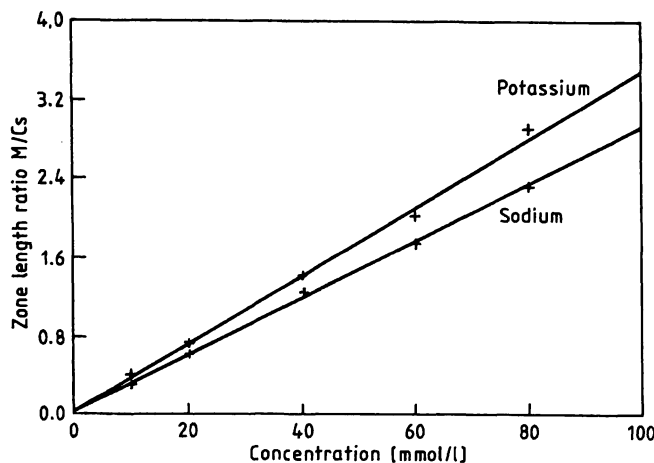


Fig. 4. Calibration curves for sodium and potassium standards. Zone length ratios M/Cs are plotted against concentrations of sodium and potassium (mmol/l). M = Zone length of cation, Cs = Zone length of caesium

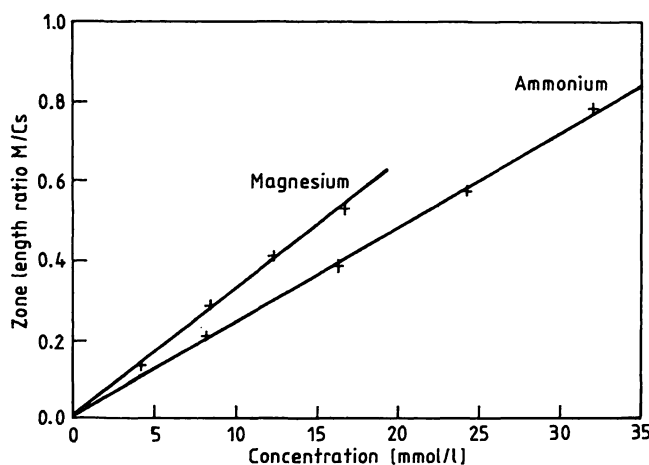


Fig. 5. Calibration curves for ammonium and magnesium standards.

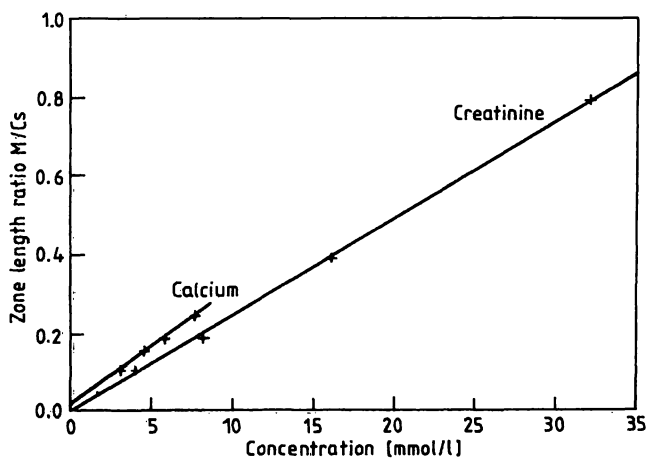


Fig. 6. Calibration curves for creatinine and calcium standards.

Tab. 4. Precision of analysis of urinary cationic compounds

Results are mean  $\pm$  SD of determinations with three pooled urine samples. 1  $\mu$ l of the pooled urine samples after 3-fold dilution with internal standard solution (1 + 2) were analysed repeatedly.

Cation	Within-day (n = 12)		Between-day (n = 6)	
	c, mmol/l	CV, %	c, mmol/l	CV, %
Ammonium	15.3 $\pm$ 0.27	1.76	14.7 $\pm$ 1.09	7.41
	16.8 $\pm$ 0.24	1.43	16.7 $\pm$ 0.33	1.98
	14.9 $\pm$ 0.21	1.41	15.1 $\pm$ 0.82	5.43
Sodium	90.8 $\pm$ 1.15	1.27	91.3 $\pm$ 1.85	2.03
	88.8 $\pm$ 0.95	1.07	86.7 $\pm$ 0.87	1.00
	202 $\pm$ 5.55	2.75	204 $\pm$ 7.08	3.47
Potassium	64.0 $\pm$ 0.59	0.92	64.4 $\pm$ 1.43	2.22
	34.6 $\pm$ 0.26	0.75	34.6 $\pm$ 0.55	1.59
	118 $\pm$ 4.23	3.58	120 $\pm$ 5.58	4.65
Magnesium	3.49 $\pm$ 0.14	4.01	3.57 $\pm$ 0.10	2.80
	3.84 $\pm$ 0.15	3.91	3.67 $\pm$ 0.10	2.72
	14.6 $\pm$ 0.18	1.23	14.8 $\pm$ 0.92	6.22
Calcium	4.91 $\pm$ 0.20	4.07	5.01 $\pm$ 0.19	3.79
	3.95 $\pm$ 0.14	3.54	3.91 $\pm$ 0.11	2.81
	5.02 $\pm$ 0.14	2.79	5.28 $\pm$ 0.20	3.79
Creatinine	5.91 $\pm$ 0.20	3.38	5.48 $\pm$ 0.34	6.20
	7.41 $\pm$ 0.16	2.24	7.10 $\pm$ 0.33	4.65
	21.4 $\pm$ 1.06	4.95	22.4 $\pm$ 0.97	4.33

Tab. 5. Analytical recovery of ammonium, sodium, potassium, magnesium, calcium and creatinine from pooled urine.

Cation	Added mmol/l	Measured mmol/l	Theoretical mmol/l	Recovery %
Ammonium	0	12.39	—	—
	3.00	15.49	15.39	103
	6.00	18.59	18.39	103
	9.00	21.54	21.39	102
	12.00	24.57	24.39	102
Sodium	0	109.2	—	—
	15.0	124.9	124.2	105
	30.0	139.3	139.2	100
	45.0	150.6	154.2	92
	60.0	164.9	169.2	93
Potassium	0	42.5	—	—
	15.0	58.2	57.5	105
	30.0	72.5	72.5	100
	45.0	89.6	87.5	105
	60.0	104.4	102.5	103
Magnesium	0	1.93	—	—
	1.00	3.00	2.93	107
	2.00	3.95	3.93	101
	3.00	4.91	4.93	99
	4.00	6.02	5.93	102
Calcium	0	2.11	—	—
	1.21	3.45	3.32	111
	2.42	4.54	4.53	100
	3.63	6.10	5.74	110
	4.83	7.12	6.94	104
Creatinine	0	7.89	—	—
	2.00	9.90	9.89	100
	4.00	11.99	11.89	102
	6.00	13.65	13.89	96
	8.00	16.12	15.89	103

## Discussion

Separation and quantification of six cationic species in aqueous solution or, especially in human urine, presents a formidable problem. Alkali and alkaline earth ions are chemically rather inert or undergo reactions which are not very specific. Physical methods like atomic emission or atomic absorption photometry are therefore used routinely to determine their concentration. For the electrophoretic separation according to effective ionic mobilities conditions had to be found that alter the relative mobilities in aqueous solution to afford practically measurable differences. Although very small differences in potential gradient can in principle be detected, a rather long analysis time is required for the separation of mixed zones. Also, a leading electrolyte had to be found which moves faster than any of the ions to be determined by the procedure. Tetramethylammonium in methanolic solution was finally adopted as the solution to this problem. However, the effective mobilities of ammonium and potassium were very close to one another. Using Triton X-100 as a non-ionic complex-forming agent, the appropriate changes in ionic mobilities were established. From the UV-absorbance we conclude that complex formation increases in the order ammonium, sodium, caesium, potassium. One of the many advantages of the isotachopheretic method is the high degree of freedom offered by a great number of parameters that can independently be cho-

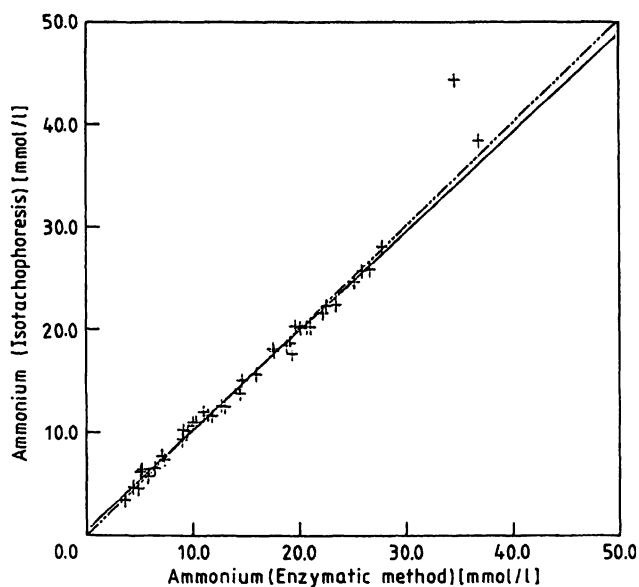


Fig. 7. Correlation between present method and enzymatic method for determination of ammonium in 43 urine samples.

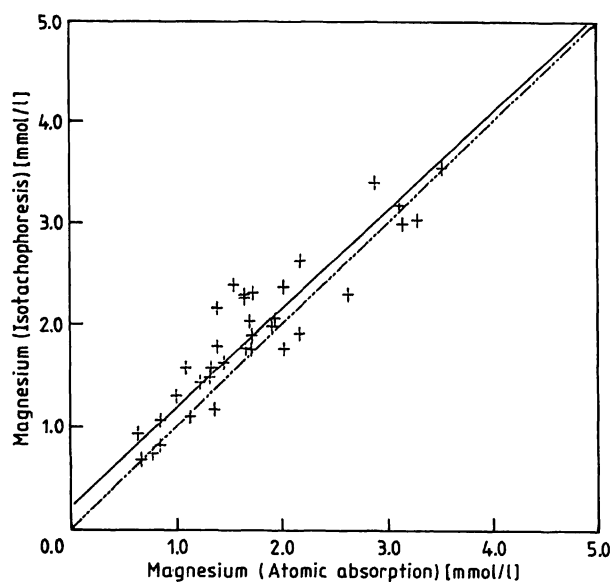


Fig. 10. Correlation between present method and atomic absorption spectrometry for determination of magnesium in 37 urine samples.

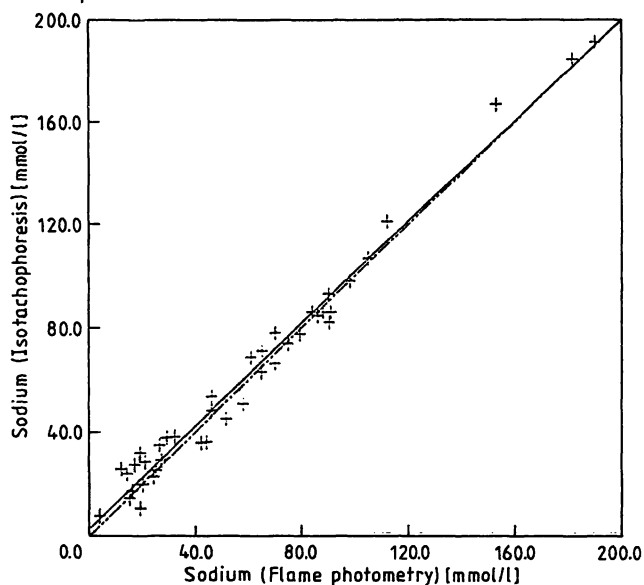


Fig. 8. Correlation between present method and flame photometry for determination of sodium in 42 urine samples.

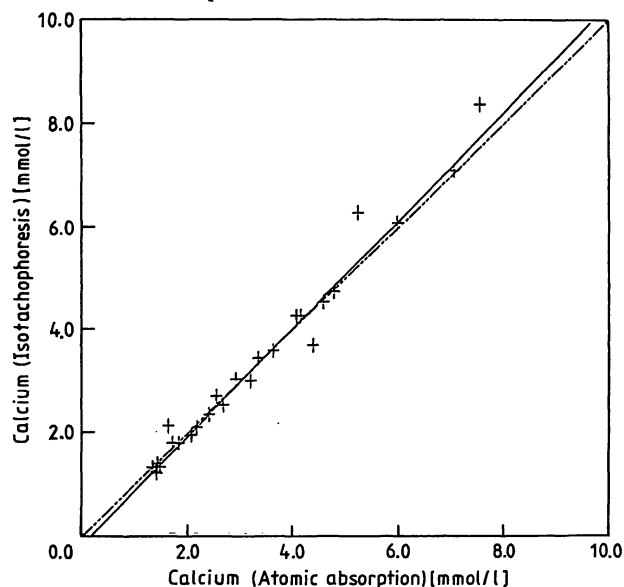


Fig. 11. Correlation between present method and atomic absorption spectrometry for determination of calcium in 26 urine samples.

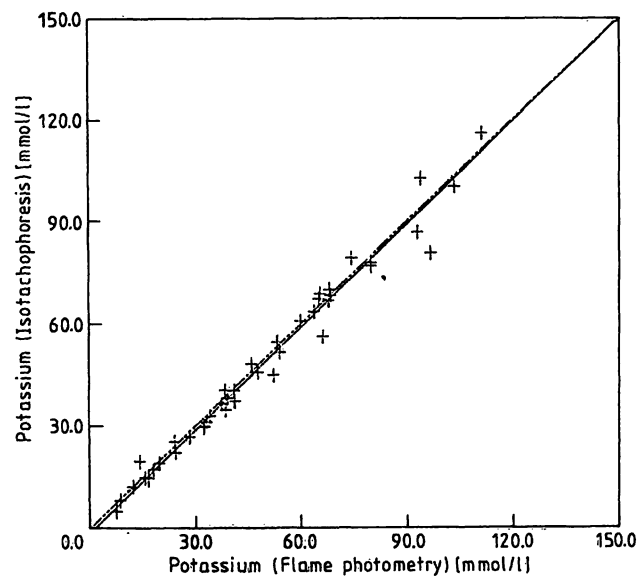


Fig. 9. Correlation between present method and flame photometry for determination of potassium in 42 urine samples.

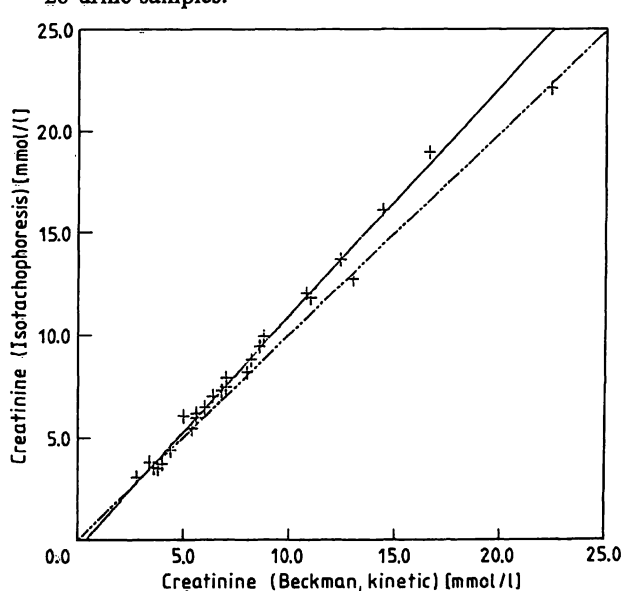


Fig. 12. Correlation between present method and colorimetric method for determination of creatinine in 26 urine samples.

Tab. 6. Comparison of methods  
 $r$  = correlation coefficient  $b$  = slope  $a$  = intercept (mmol/l)

Cation	$n$	$\bar{x}$	$\bar{y}$	$r$	$a$	$b$
Ammonium (mmol/l) y = Isotachophoresis x = Enzymatic method	43	15.217	15.442	0.984	- 0.685	1.060
Sodium (mmol/l) y = Isotachophoresis x = Flame photometry	42	59.819	62.941	0.989	+ 2.144	1.000
Potassium (mmol/l) y = Isotachophoresis x = Flame photometry	42	48.900	47.992	0.992	- 1.252	1.002
Magnesium (mmol/l) y = Isotachophoresis x = Atomic absorption	37	2.017	2.178	0.985	+ 0.354	0.905
Calcium (mmol/l) y = Isotachophoresis x = Atomic absorption	16	3.672	3.794	0.984	- 0.126	1.068
Creatinine (mmol/l) y = Isotachophoresis x = Jaffe reaction, kinetic	26	8.115	8.654	0.992	+ 0.018	1.065

sen. These include the type, concentration and pH of leading and terminating electrolyte, the solvent, the diameter and length of the capillary, the current, the complexing agents (non-ionic or anionic). It is therefore almost always possible to find suitable conditions for a certain problem. The zone length for a given concentration of one ionic species depends only on the diameter of the capillary in the detector (which is constant) and the concentration of the leading electrolyte. Highly reproducible results can therefore be obtained. Imprecision due to the variation of the injected volume can be eliminated using an internal standard.

This standard, or a second standard, can also be used to avoid errors in determining the volume of the 24-hour urine collections. *Ronquist* (12) proposed the addition of a constant, determined volume of LiCl of constant concentration to the empty collecting container. The final concentration of lithium, as determined, for example, by isotachophoresis would then also permit calculation of the total 24-hour volume.

Lithium is an alkali metal ion which appears only occasionally in the urine of patients on lithium therapy. It can easily be detected between sodium and magnesium using System B (tab. 1 and tab. 2). In System A, however, the effective mobilities of lithium and sodium are rather close to one another (tab. 1

and tab. 2). The presence of lithium is indicated by the appearance of a small peak in the first derivative of the potential gradient over time curve.

The only cation that cannot be measured in the untreated urine is the calcium ion. Due to the presence of a number of complexing anions like sulphate, phosphate and oxalate the amount of calcium in native urine is always underestimated by the isotachophoretic method. In addition, the amount of "free" calcium varies considerably with the concentration of these anions. If calcium is to be determined, the interfering anions must first be eliminated. We found that precipitation of these anions by lead ions, which have a lower mobility than creatinine (tab. 2), is suitable for this purpose.

The main disadvantage of isotachophoresis is the time of analysis, which is comparable with that of chromatographic procedures. It is hoped that further development of the method and construction of more sophisticated equipment with multielectrode arrays as described by *Schuhmacher* (13) will finally increase the practicability of this procedure.

#### Acknowledgement

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