RESEARCH ARTICLE

An improved reference method for serum cations measurement by ion chromatography

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Background: To improve the accuracy of the routine methods in laboratory medicine, ion chromatography with a simple sample treatment procedure, which can completely remove the proteins and/or organics in human serum, has been developed for the determination of serum cations.

Methods: Chromatographic conditions for the separate and simultaneous determination of K, Na, Ca, and Mg were investigated. Furthermore, various factors influencing the mineralization of human serum, such as the selection and amount of oxidant, were also examined systematically and optimized.

Results: The optimized experimental conditions are as follows: 1.0 mL of serum specimen digested with 2 mL nitric acid (120°C) followed by 2 mL hydrogen peroxide (80°C). The specimens were then redissolved and determined by ion chromatography under the optimum eluent concentration of 32 mmol/L methanesulfonic acids. The measurement accuracy and precision are less than 1.0% for all the analytes by analyzing NIST certified reference materials, IFCC-RELA specimens and serum specimens. The results were also comparable with the reference values obtained by the inductively coupled plasma mass spectrometry (ICP-MS), which were found to be in good agreement.

Conclusions: Ion chromatography with a simple sample treatment procedure for the determination of cations in human serum with high sensitivity and specificity was developed. The proposed method could be recommended as a candidate reference method for the determination of serum cations.

KEYWORDS

candidate reference method, ion chromatography, serum cations

1 | INTRODUCTION

Serum cations containing sodium, potassium, magnesium, and calcium play a central role in numerous processes in the human body, such as volume and osmotic regulation, myocardial rhythm, blood coagulation, and neuromuscular excitability.¹ The reference ranges in serum of normal individuals are in a range of 135–145 mmol/L for

sodium, 3.4–5.0 mmol/L for potassium, 0.65–1.0 mmol/L for magnesium and 2.15–2.65 mmol/L for calcium, respectively.² The diagnosis of many metabolism disorders, such as diabetes, advanced renal disease, polydipsia, aldosterone deficiency or excess, cardiac disorders, and alcoholism, was determined with the abnormal concentration values of these cations.¹ Therefore, it is very important for the determination of those cations in laboratory medicine.

To improve the accuracy of the routine methods in laboratory medicine, the reference methods, which could directly be calibrated

Zou and Shen contributed equally to this work.

with primary standards, were used for accuracy assessment of routine methods with a representative panel of patient specimens.^{3,4} According to the list of reference measurement methods/procedures for electrolytes from Joint Committee for Traceability in Laboratory Medicine (JCLTM), the traditional reference methods were flame atomic emission spectrometry (FAES) for serum sodium and potassium^{5,6} and flame atomic absorption spectrometry (FAAS) for magnesium and calcium,^{7,8} respectively. The primary measurement procedures for sodium, potassium, magnesium, and calcium were inductively coupled plasma mass spectrometry (ICP-MS).⁹⁻¹² However, those methods were normally very elaborate and time-consuming and not suitable for every metrological task. Thienpont et al¹³ first reported that the ion chromatography could be recommended as reference method for the determination of serum cations in clinical chemistry. However, according to their previous work, the serum specimens were simply treated by acidic dilution to dilute the sample matrix and release the doubly charged cations from the proteins. Those proteins still existed and would cause suppressor injury and column contamination, which was not suitable for long-term application with these types of samples.

Herein, wet digestion was used for the complete mineralization of human serum specimens. A simple sample treatment procedure with simple, inexpensive equipment and less subject to interferences has been developed for the determination of serum sodium, potassium, magnesium, and calcium by ion chromatography. Experimental parameters such chromatographic conditions and wet digestion methods were studied and optimized, and the proposed method was applied to the determination of sodium, potassium, magnesium, and calcium in human serum specimens.

2 | EXPERIMENTAL

2.1 | Instrumentation

Ion chromatography was carried out with a model Dionex-1100 ion chromatography from Thermo (Thermo Fisher Scientific Inc. USA) equipped with a 25 μ L injection loop. For chromatographic separation, an IonPac CG 16 guard column (50 mm $\times$ 5 mm i.d.) coupled to an IonPac CS 16 analytical column (250 mm $\times$ 5 mm i.d.) was used. Electronic suppression was achieved with a Cation Self-Regenerating Suppressor system (CSRS 300, 4 mm) used in the auto-suppression recycle mode. Detection was based on conductivity. The chromatographic signals were performed with a chameleon chromatography workstation (Chromeleon 7.1.2). All the above-mentioned equipment were purchased from Thermo Fisher. A water purification system (Millipore, USA) was used to provide Milli-Q water of ultrapure quality (18.2 M Ω). A hot plate (IKA, Germany) was used to remove the proteins and/or organics in human serum by wet digestion. A Shimadzu TOC-5050 total organic carbon analyzer (Shimadzu, Germany) was used for the determination of the residual organic carbon content. Weighings were made with a model of XS205 Dual Range electronic balance (Mettler Toledo, Switzerland), which has a readability of 0.01 mg. A rotator PTR-35 (Grant-bio, UK)

was used to redissolve the lyophilized serum such as RELA specimens. Pipetting was done using 5-mL and 1-mL pipette tips from Eppendorf (Eppendorf AG, Germany).

2.2 | Materials and reagents

The standard reference materials were obtained from National Institute of Standards and Technology (NIST): NaCl (SRM 919b), KCl (SRM 918b), CaCO₃ (SRM 915b), and Mg-gluconate (SRM 929a). Methanesulfonic acid (purity > 99.5%), nitric acid (purity > 99.999%), hydrogen peroxide ($\geq 30\%$, for trace analysis), and perchloric acid (99.999%) were purchased from Sigma (Sigma-Aldrich Co. LLC., USA). Filtrations were carried out with Millex-LH syringe filters (0.22 μ m pore size, Hydrophilic, PTFE, 13 mm, IC-certified) from Millipore (USA). For filtration and injection, 1-mL plastipak syringes (purchased from Zhejiang Yusheng Medical Instruments Co., Ltd. China) were used. The stock solution of Na, K, Ca, and Mg was prepared by dissolving the standard reference materials in 0.2% HNO₃ solution using a PFA volumetric flask (Vitalab, Germany). Working standard solution was prepared fresh daily by stepwise dilution of the stock with 0.2% HNO₃ solution. The working solutions containing internal standard and specimen were prepared by gravimetric method, and the density measurement was similar to Zhang's report.¹⁴ To exclude the external contamination at any stage of the IC analysis, no glassware (except the conical flask) was used and hand contact of vials was avoided. The conical flasks used in this study were soaking in 10% HNO₃ solution for at least 24 hours before rinsing thoroughly with Milli-Q water.

2.3 | Sample preparation procedure

Three types of wet digestion of sample preparation procedures were applied to determine metals contents in human serum samples: HNO₃, HNO₃-H₂O₂, and HNO₃-HClO₄. To calculate precision, 5 replicate measurements were performed for all sample preparation procedures.

2.3.1 | HNO₃ wet digestion

Five replicate samples (1.0 mL) of each human serum variety were accurately pipetted in 50-mL conical flasks. About 2.0 mL of nitric acid was added to each flask and kept for 30 minutes at room temperature. Then, the samples were heated on a hot plate at 120°C until dryness and cooled down to room temperature. For blank determination, 5 conical flasks had the serum replaced by Milli-Q water. Then, the samples were redissolved in 25 mL of HNO₃ solution (0.2%), filtered through a 0.22 μ m microporous membrane and determined by ion chromatography.

2.3.2 | HNO₃-H₂O₂ wet digestion

1.0 mL of sample was pipetted in 50-mL conical flasks, and 2.0 mL of nitric acid was added. The mixture was kept for 30 minutes at

room temperature, heated on a hot plate at 120°C until close to dryness and then approximately 2.0 mL of 30% (m/m) H_2O_2 was added to each conical flask. The temperature was decreased to 80°C until dryness, and white powders were obtained. Finally, the white powders were redissolved in 25 mL of HNO_3 solution (0.2%), filtered through a 0.22 μm microporous membrane and determined by ion chromatography.

2.3.3 | $\text{HNO}_3\text{-HClO}_4$ wet digestion

1.0 mL of sample was pipetted in 50-mL conical flasks, and 2.5 mL of $\text{HNO}_3\text{-HClO}_4$ (4:1, v/v) was added. The mixture was kept for 30 minutes at room temperature, heated on a hot plate at 120°C for 2 hours, and enhanced the temperature to 150°C until dryness. Then, the samples were redissolved in 25 mL of HNO_3 solution (0.2%), filtered through a 0.22 μm microporous membrane, and determined by ion chromatography.

2.4 | Ion chromatography optimization

In this study, Na, K, Ca, and Mg were simultaneously separated using the isocratic conditions with an IonPac CS16 analytical column (250 mm $\times$ 5 mm) and detected with suppressed conductivity detection. The eluent was 32 mmol/L methanesulfonic acid with a flow rate of 1.0 mL/min. The guard column and analytical column were maintained at 40°C in a column heater.

3 | RESULTS AND DISCUSSION

3.1 | Optimization of ion chromatography

As previous literature description,¹⁵ ion chromatography shows a great advantage for the determination of the cations in human serum. All elements, including the internal standard (rubidium, Rb), can be determined in a single measurement within 30 minutes, as depicted in Figure 1. In the chromatogram, the peaks are complete

FIGURE 1 Serum chromatogram after complete digestion

baseline separated from each other, although the concentrations of the cations differ by more than 2 orders of magnitude, which are similar to Schiel's report.¹³ However, according to the results of our experiments, the IonPac CS16 analytical column may have more advantages for the separation of alkaline earth metal and ammonium than IonPac CS12 or IonPac CS12A analytical column which is chosen for disparating concentration ratios of adjacent eluting cations such as sodium and ammonium in diverse sample matrices. Besides, no impurity peak can be observed in the serum chromatogram, indicating the presence of other cations has no influence on the determination of Na, K, Ca, and Mg under the selected condition.

It is known that the eluent concentration plays an important role in the separation of cations. Therefore, to obtain a high chromatographic resolution (R), the effect of methanesulfonic acid concentration on the separation of cations was studied in the range of 30–34 mmol/L. As shown in Figure 2, the optimum eluent concentration for separation of cations was 32 mmol/L methanesulfonic acids, at which the chromatographic resolutions were at least 2.7. Except in this concentration, the chromatographic resolutions were decreased.

3.2 | Optimization of the sample digestion procedure

Ion chromatography was very suitable for the separation and analysis of inorganic ions in aqueous solution as the micromembrane suppressor could be injured by organic solvents, organic macromolecules, heavy metal ions, etc. and the analytical column could be contaminated by organic macromolecules such as proteins, which would cause instability baseline, low chromatographic resolution, low precision and accuracy, and so on. However, the human serum specimens contain large amounts of organics; for instance,

FIGURE 2 Effect of eluent concentration on the separation of cations

proteins with the concentration up to 80 g/L.¹⁶ Therefore, the serum specimens should be treated before injecting to the ion chromatography system for separation and analysis. Thienpont and coworkers have done a lot of work in the determination of serum cations by ion chromatography.^{13,17-19} According to their previous work, the serum specimens were simply treated by acidic dilution to dilute the sample matrix and release the doubly charged cations from the proteins. While, the proteins were still existed and would cause suppressor injury and column contamination, which was not suitable for long-term application with these types of samples. Later, Schiel and coworkers¹⁵ have reported a microwave digestion to completely remove the proteins and/or organics in human serum. This new ion chromatography measurement procedure has been applied to the determination of cations in serum samples with satisfactory results.

Based on the above research work, it seems that the serum specimens should remove the organic matter prior to its determination by ion chromatography. The use of nitric acid and nitric acid with hydrogen peroxide or perchloric acid for organic matrix digestion is the most common approach used for sample pretreatment during the analysis of metal elements.²⁰ Herein, to establish an efficient sample preparation procedure with simple, inexpensive equipment and less subject to interferences, 3 types of wet digestion methods were investigated for the determination of metals contents in human serum samples.

The 3 types of wet digestion methods (HNO_3 , $\text{HNO}_3\text{-H}_2\text{O}_2$, and $\text{HNO}_3\text{-HClO}_4$) were evaluated by comparing the residual carbon content, the residual of the digests, and the element recoveries by analyzing the RELA specimens. The degree of dissolution varied with the treatment that was used. In general, the digestion solutions appeared to be light yellow suspension when the HNO_3 or $\text{HNO}_3\text{-HClO}_4$ was used. With 2.0 mL HNO_3 wet digestion, bulk materials with the color of yellow were obtained, which could attribute to the amino acids containing benzene ring in proteins reacted with HNO_3 according to previous research.²¹ The UV-Vis spectra of serum specimen after digestion with HNO_3 are shown in Figure S1 in the ESI, and the residual carbon contents were more than 90% based on the TOC results. Despite the good recovery and precision of the analytes, the low mineralization rate showed that this digestion procedure was not appropriate. Those organics still existed and would cause suppressor injury and column contamination, which was not suitable for long-term application with these types of samples. After the serum specimens digested with 2.0 mL HNO_3 (120°C) followed

by 2.0 mL H_2O_2 (80°C), serum samples were almost completely mineralized into inorganic salts based on the UV-Vis spectra (See Figure S2 in the ESI) and TOC results. The absorption peaks at 216 nm and 303 nm were related to the characteristic of $\pi\text{-}\pi^*$ and $n\text{-}\pi^*$ electronic transitions in nitrate molecular orbitals. With 2.0 mL HNO_3 and 0.5 mL HClO_4 wet digestion, the white powders of inorganic salts were also obtained. However, because of the high boiling point of HClO_4 , the residual of the digests exists even the heat time was extended to 5 hours at 150°C. Besides, the prepared serum specimens contain large amount of NH_4ClO_4 as shown in Figure S3 in the ESI, which could reduce the chromatographic resolution for Na and NH_4 . The satisfying recoveries of the analytes were obtained from which the serum samples were digested with $\text{HNO}_3\text{-H}_2\text{O}_2$ or $\text{HNO}_3\text{-HClO}_4$.

Based on the above experimental results and discussions, the best results were obtained with the composition ratio of 1:1 $\text{HNO}_3/\text{H}_2\text{O}_2$ (2.0 mL/2.0 mL) for wet digestion. Increase in H_2O_2 amount from 1:1 to 1:2 did not influence the mineralization of serum sample for wet digestion. The use of diluted solutions of 0.2% HNO_3 solution improved the performance of the ion chromatography, avoiding the wear of the equipment components that made contact with the digested samples. However, it is not advocated the concentration of HNO_3 solution over 0.5% because this results in a low chromatographic resolution.

3.3 | Analytical performance

Based on the above experimental results, the optimal conditions for the proposed method were as follows: 1.0 mL of serum specimen digested with 2 mL nitric acid (120°C), followed by 2 mL hydrogen peroxide (80°C), redissolved in 25 mL of HNO_3 solution (0.2%), filtered through a 0.45 μm microporous membrane and determined by ion chromatography. The eluent was 32 mmol/L methanesulfonic acid with a flow rate of 1.0 mL/min, and the guard column and analytical column were maintained at 40°C in a column heater. Under the optimal conditions, the analytical performance of the proposed method, including linear range, calibration curve, and the limits of the detection (LODs), was evaluated, and the results are summarized in Table 1. The LODs, calculated by signal-to-noise ratio of 3, were 0.50 $\mu\text{g/L}$ for Na, 2.14 $\mu\text{g/L}$ for K, 1.16 $\mu\text{g/L}$ for Mg, and 2.42 $\mu\text{g/L}$ for Ca, respectively. The limits of quantifications (LOQs), calculated by signal-to-noise ratio of 10, were in the range of 0.93-3.67 $\mu\text{g/L}$.

TABLE 1 Analytical performance of ion chromatography with a simple sample treatment procedure for the determination of serum cations

Cations	Linear range (mg/L)	Calibration curve	Coefficient of determination (r^2)	LODs ($\mu\text{g/L}$)	CV (%) (n = 6)
Na	0-150	$Y = 0.2684X + 0.0164$	1.0000	0.50	0.27
K	0-20	$Y = 0.1696X - 0.0073$	1.0000	2.14	0.37
Mg	0-2.5	$Y = 0.4960X + 0.0001$	1.0000	1.16	0.60
Ca	0-10	$Y = 0.3116X + 0.0210$	0.9999	2.42	0.65

Method precision was determined in terms of repeatability and quantified by the coefficient of variation (CV) of the replicate measurements. In this work, 6 replicate serum specimens were digested and detected under the optimal conditions. CV was determined to be 0.27%

for Na, 0.37% for K, 0.60% for Mg, and 0.65% for Ca, respectively. A comparison of CV obtained by the proposed method with that obtained by other approaches followed by ion chromatography analysis was carried out, and the results were similar to the reference methods.

TABLE 2 Inaccuracy of the proposed method for lyophilized and liquid serum specimens^a (mmol/L, n = 10)

Cations	Specimen	IC	CV (%)	Reference value	Bias (%)
Na	Serum-I	132.79	0.36	133.0 ^b	-0.16
	Serum-II	148.54	0.20	149.0 ^b	-0.31
	2015 RELA-A	140.40	0.18	139.59 ^c	0.58
	2015 RELA-B	154.20	0.13	154.20 ^c	0.00
	2016 RELA-A	130.78	0.20	131.18 ^c	-0.29
	2016 RELA-B	141.55	0.24	142.08 ^c	-0.34
K	Serum-I	3.444	0.30	3.46 ^b	-0.46
	Serum-II	5.360	0.15	5.36 ^b	0.02
	2015 RELA-A	5.880	0.19	5.860 ^c	0.34
	2015 RELA-B	3.130	0.95	3.133 ^c	-0.09
	2016 RELA-A	4.917	0.32	4.931 ^c	-0.28
	2016 RELA-B	6.807	0.30	6.829 ^c	-0.32
Mg	Serum-I	0.732	0.39	0.73 ^b	0.27
	Serum-II	0.980	1.00	0.97 ^b	1.03
	2015 RELA-A	1.943	0.97	1.928 ^c	0.80
	2015 RELA-B	0.911	0.51	0.908 ^c	0.28
	2016 RELA-A	0.732	0.44	0.722 ^c	1.33
	2016 RELA-B	1.468	0.59	1.454 ^c	0.99
Ca	Serum-I	2.037	0.83	2.03 ^b	0.34
	Serum-II	3.009	0.96	2.99 ^b	0.64
	2015 RELA-A	3.029	0.42	3.026 ^c	0.11
	2015 RELA-B	1.992	0.80	2.001 ^c	-0.46
	2016 RELA-A	3.464	0.32	3.481 ^c	-0.48
	2016 RELA-B	2.513	0.45	2.526 ^c	-0.52

^aLimits of equivalence of IFCC-RELA: Na $\pm$ 1.25%; K $\pm$ 2.00%; Mg $\pm$ 3.75%; Ca $\pm$ 2.50%.

^bThe reference value certified by National Center for Clinical Laboratories using ICP-MS.

^cMean of all participants

TABLE 3 Inaccuracy of the proposed method for NIST certified reference materials (mmol/L)

Cations	Specimen	IC	Target Value ^a	Bias (%)
Na	NIST SRM 956d L1	119.6	120.0 $\pm$ 0.7	-0.32
	NIST SRM 956d L2	138.6	139.3 $\pm$ 0.9	-0.53
	NIST SRM 956d L3	158.4	158.7 $\pm$ 1.1	-0.22
K	NIST SRM 956d L1	5.782	5.752 $\pm$ 0.049	+0.53
	NIST SRM 956d L2	3.724	3.730 $\pm$ 0.035	-0.16
	NIST SRM 956d L3	1.625	1.611 $\pm$ 0.014	+0.87
Mg	NIST SRM 956d L1	1.478	1.471 $\pm$ 0.010	+0.45
	NIST SRM 956d L2	0.956	0.961 $\pm$ 0.007	-0.47
	NIST SRM 956d L3	0.432	0.434 $\pm$ 0.003	-0.39
Ca	NIST SRM 956d L1	3.418	3.407 $\pm$ 0.030	+0.33
	NIST SRM 956d L2	2.858	2.857 $\pm$ 0.025	+0.05
	NIST SRM 956d L3	2.279	2.282 $\pm$ 0.018	-0.12

^aTarget value certified by NIST.

TABLE 4 The calibration and measurement capability in our laboratory

Analytes	Measurement range (mmol/L)	Expanded uncertainty (CMC) ($k = 2$)
Sodium (Na)	110-170	$U_{rel} = 0.5\%-0.3\%$
Potassium (K)	3.0-6.7	$U_{rel} = 0.8\%-0.4\%$
Magnesium (Mg)	0.5-2.0	$U_{rel} = 1.4\%-0.7\%$
Calcium (Ca)	2.0-3.0	$U_{rel} = 1.2\%-0.9\%$

3.4 | Analytical applications

The trueness of the proposed method was demonstrated using IFCC-RELA specimens, by comparison with the mean values of 10 measurements obtained by the proposed method with the reference values from all the participant results of IFCC-RELA intercomparison studies (Referenzinstitut für Bioanalytik [RfB], Bonn, Germany). The results are given in Table 2. As shown in Table 2, the deviation of the proposed method's results from the IFCC-RELA group mean ranged between -0.34% and 0.58% for Na with a CV of $\leq 0.24\%$, between -0.32% and 0.34% for K with a CV of $\leq 0.95\%$, between 0.28% and 1.33% for Mg with a CV of $\leq 0.97\%$, and between -0.48% and 0.64% for Ca with a CV of $\leq 0.80\%$, respectively. The results obtained by the proposed method from the fresh serum specimens were compared with those results obtained using a Joint Committee for Traceability in Laboratory Medicine (JCTLM) listed method as primary measurement procedures. The results were found to be in good agreement, showing that there exists no distinctive difference between the determined values and reference values.

The trueness of the proposed method was further demonstrated by analyzing the NIST certified reference materials. Each level of the material was measured 3 times, and the measurement results are shown in Table 3. The obtained results were all in good agreement with the certified values.

The measurement uncertainty of the proposed method is strictly evaluated according to the evaluation procedure of measurement uncertainty, which was established based on Guide to the Expression of Uncertainty in Measurement (GUM, 1999). When evaluating the calibration and measurement capability (CMC), the different mole concentrations of samples were detected, and their measurement uncertainty was evaluated. With the mole concentration from low to high, the uncertainty of measurement was descending. According to the China National Accreditation Service for Conformity Assessment standard CNAS-GL 37 "Guidance on Expression of Calibration and Measurement Capability (CMC)," the range of values could be used to represent the calibration and measurement capability, and the results are shown in Table 4.

4 | CONCLUSIONS

Ion chromatography with a simple sample treatment procedure for the determination of cations in human serum specimens

was developed. This proposed method could be recommended as a reference measurement procedure in clinical chemistry. Compared with most of the analytical methods for the determination of serum cations, the advantages of the proposed method can be summarized as follows: (1) simultaneous determination of serum sodium, potassium, magnesium, and calcium; (2) this wet digestion method could completely remove the organics in serum, avoiding the risk of suppressor injury and column contamination, which was not suitable for long-term application with these types of samples; and (3) the use of IonPac CS 16 analytical column could separate the cations more effectively compared to the other analytical column such as IonPac CS 12 or IonPac CS 12A. Furthermore, this simple sample treatment procedure could be combined with other methods of analysis, such as ICP-AES and ICP-MS.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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