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On-line deproteinization by adsorption of proteins on a polyethylene powder pre-column for the determination of Na, K, Mg and Ca in human serum by high-performance liquid chromatography

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Abstract

Deproteinization of human serum was carried out on-line using a polyethylene powder cartridge as pre-column. The serum sample, after dilution, was injected into the chromatograph, and when passing through the cartridge the proteins were adsorbed by the polyethylene. A protein-free eluate was carried to the analytical column while the pre-column was washed with methanol and water to elute the adsorbed proteins by changing the pump channels. After washing, the pre-column was conditioned with the eluent to receive the next sample. Deproteinization was evaluated off- and on-line using pooled serum, and testing the protein residue after passing through a polyethylene cartridge with the Comassie Brilliant Blue reaction. Parameters such as serum volume, eluent and washing solution were investigated. Sodium, potassium, magnesium and calcium determinations were performed by high-performance liquid chromatography with conductimetric detection. The proposed method is suitable for the determination of these cations in serum samples without further treatments. The total analysis time was about 20 min and a linear range from 0.3 to 1.2 mg/l for sodium, potassium, magnesium and calcium was observed. The method was characterized by a precision of about 95% and recoveries from spiked samples were between 96 and 102%. The results for serum samples analysed by the proposed method were compared with photometry and atomic absorption spectrometry.

Keywords: Proteins; Sodium; Potassium; Magnesium; Calcium; Polyethylene

1. Introduction

The determination of sodium, potassium, magnesium and calcium in human blood is one of the most common analyses in clinical laboratories. Normally these electrolytes are analysed separately by flame emission, spectrophotometry, ion sensitive electrodes or atomic absorption spectrometry [1,2]. The possibility of their simultaneous determination by HPLC is very attractive and many papers have dealt with this [3–6], but sample pretreatment is always necessary to eliminate or reduce the proteins.

For the HPLC analysis of blood samples, the removal of proteins is the most important cleanup step because they can precipitate when in contact with solvents or buffer salts commonly used in mobile phases and thereby block tubing, causing

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increases in back pressure or deterioration of column performance. To avoid this problem, a number of sample preparation techniques have been described for removing proteins prior to injection of the sample. For the determination of sodium, potassium, magnesium and calcium in human blood, serum or plasma, ultrafiltration [4,6] and acidification with trichloroacetic acid followed by centrifugation are techniques normally employed [7,8].

Polyethylene (PE) powder has already been used as a stationary phase for chromatographic separations [9]. In previous works we used PE powder as sorbent for the preconcentration of metallic traces in the form of complexes [10–12], and for the separation of cyanide from blood plasma by retention of the nickelcyano complex on a methylene blue impregnated PE column [13].

Studies have shown that proteins such as albumin, collagen and fibrinogen are able to be adsorbed by polymeric materials such as PE [14–16] and have attempted to use this polymer as a biomaterial.

In this work we used PE powder as an adsorbent for serum proteins to clean up the sample prior to chromatographic analysis. This analysis was the simultaneous determination of serum cations, sodium, potassium, calcium and magnesium. By introducing a pre-column filled with PE in the chromatographic system, the cleanup step was carried out on-line with separation.

2. Experimental

2.1. Apparatus

The chromatographic equipment consisted of a DX-300 gradient chromatography system (Dionex, Sunnyvale, CA, USA) with a conductivity detector (CDM-3) and a cation self-regenerating suppressor (Dionex CSRS). A C-R6A data processor (Shimadzu, Kyoto, Japan) and a second chromatographic pump LC-IOAS (Shimadzu) were used.

A Lambda 16 UV–Vis spectrophotometer (Perkin-Elmer, Überlingen, Germany), an AA-200 atomic absorption spectrometer (Varian, Melbourne, Australia), a Digital DM 20 pH meter (Digimed, São Paulo, Brazil), and a MS-Reglo peristaltic pump (Ismatec, Switzerland) were used.

2.2. Reagents

Sodium, potassium, magnesium and calcium stock standard solutions (1000 mg/l) were prepared in water from NaCl, KCl, $MgCl_2 \cdot 2H_2O$ and $CaCl_2 \cdot 4H_2O$ (Merck, Darmstadt, Germany). Working solutions of the individual cations and in mixture were prepared by appropriate dilution.

The Coomassie Brilliant Blue reagent for protein quantification consisted of a solution containing 100 mg Coomassie Brilliant Blue (Sigma, St. Louis, MO, USA), 50 ml 95% ethanol and 100 ml 85% (m/v) H_3PO_4 dissolved in water and diluted to 1000 ml.

The regenerant was a 100 mmol/l solution of tetrabutylammonium-hydroxide (TBAOH) (Riedel-de Haën) 40% (v/v) in water. A solution of 6.0 mmol/l of DL-2,3-diaminopropionic acid mono-hydrochloride (Sigma) in 60 mmol/l HCl (Merck) was used as eluent.

The water used throughout was distilled, de-ionised and further purified using a Milli-Q high-purity water device (Millipore, Bedford, MA, USA).

2.3. Serum samples

All experiments were performed with a single lot of pooled human serum collected at the Santa Maria University Hospital. All tests were performed with samples diluted 1:100 with water. If not stated otherwise, the samples referred to in the text have this dilution factor. For the determination of serum Na the dilution was 1:2000.

2.4. Retention of the proteins on PE powder

Preliminary experiments in an off-line system were made to define the conditions for the retention and elution of the proteins on and from PE.

PE columns were prepared with plastic pipette tips of 1000 μl, packed with about 400 mg PE powder (60 mesh) furnished by PPH (Pólo Petroquímico de Triunfo, Brazil) and fixed in the column with small pieces of glass wool.

2.4.1. Retention of proteins

A system with a peristaltic pump, silicon tube (1 mm) and the PE column was used to aspirate eluent plus sample through the column at a flow-rate of 1.0

ml/min. Aliquots of 100, 200, 500 and 1000 μl of serum were dissolved in the eluent to a total volume of 2.0 ml. The 2.0 ml of each solution was pumped trough a PE column and the effluents assayed for proteins with the Coomassie Brilliant Blue test; 100 μl of sample was mixed with 2.5 ml of the reagent and the spectrum recorded from 400 to 800 nm. The spectrum of a reference solution, 100 μl serum sample in 2.0 ml eluent, was recorded directly.

2.4.2. Elution of proteins

A home-made injector with a 100 μl loop and a six-port valve to change the solvents were introduced into the system used to investigate the retention of proteins. The systems started with 3.0 ml eluent into which 100 μl serum sample was injected. This was followed by 5.0 ml methanol, 5.0 ml water and 3.0 ml eluent. All these fractions were collected separately and assayed for proteins as described above. The reference spectrum was that of a solution of 100 μl serum in 3.0 ml eluent.

2.4.3. Retention/elution of proteins in the chromatographic system

The PE chromatographic pre-column was packed with 400 mg of PE powder (60 mesh) and a 50×4.6 mm empty stainless steel column. The column was attached to the chromatograph without the cationic column, a 100 μl sample was injected and the pump program described in Table 1 was performed. The following fractions were collected: 2.5 ml eluent, 5.0 ml methanol, 5.0 ml water and 3.0 ml eluent, respectively, and the spectra were obtained as above to confirm the presence of proteins. In this case the reference spectrum was that of 100 μl serum in 5.0 ml methanol.

Table 1
Dionex program pump for chromatographic on-line deproteinization and serum cation determination. After the eluent, methanol and water were passed through the PE pre-column to wash and condition the column for the next sample injection

Time (min)	Eluent	Flow (ml/min)	Valve injection	Four-port valve
0.0	Water	0.5	Off	On
0.1	Water	0.5	On	On
0.7	Water	0.5	Off	On
5.0	Methanol	1.0	Off	Off
10.0	Water	1.0	Off	Off
15.0	Water	0.5	Off	Off
17.0	DAP-HCl	0.5	Off	Off
20.0	Water	0.5	Off	On

2.5. Chromatographic separation

Separation was performed on an Ion Pac CS-10 (250×4 mm) column. DL-2,3-Diaminopropionic acid monohydrochloride (DAP-HCl) (6 mmol/l) in 60 mmol/l HCl was used as eluent with a flow-rate of 0.5 ml/min, and methanol and water were used to wash the PE column. The regenerant was a 100 mmol/l solution of tetrabutylammonium-hydroxide (TBAOH). By means of the program described in Table 1, while the ions are being separated on the cationic column, the PE column is washed with methanol followed by water and finally by the eluent to be conditioned for the next sample injection. The eluent is always pumped by an auxiliary pump (Shimadzu); at the first time it flows through the PE and separator columns, and the sample is injected into the carrier by means of a four-way valve (Dionex pump); 5 min after the sample is injected, the eluent is lead to the separator column while methanol is pumped through the PE column (Dionex pump), followed by water and eluent. This process occurs at the same time that the ions are being separated on the separator column. Fig. 1 shows all parts of the system.

2.6. Methods for comparison

Flame atomic absorption spectrometry (FAAS) and photometry were used as comparison methods. Sodium and potassium were determined by FAAS following the standard parameters (wavelength, gas) for these elements programmed in the instrument. Serum samples were diluted 1:50 for sodium determination and 1:5 for potassium determination. Calcium and magnesium were measured by means of photometric reagents (kit test) furnished by Bioclin (Belo Horizonte, Brazil); calcium was determined by the Cresolftaleine-complexone method and magnesium by the Magon sulfonate method. Calcium was determined by adding 50 μl of sample to 2.5 ml reagent and measuring the absorbance of the solution at 550 nm. Magnesium was determined by adding 20 μl of sample to 2.0 ml reagent and measuring the

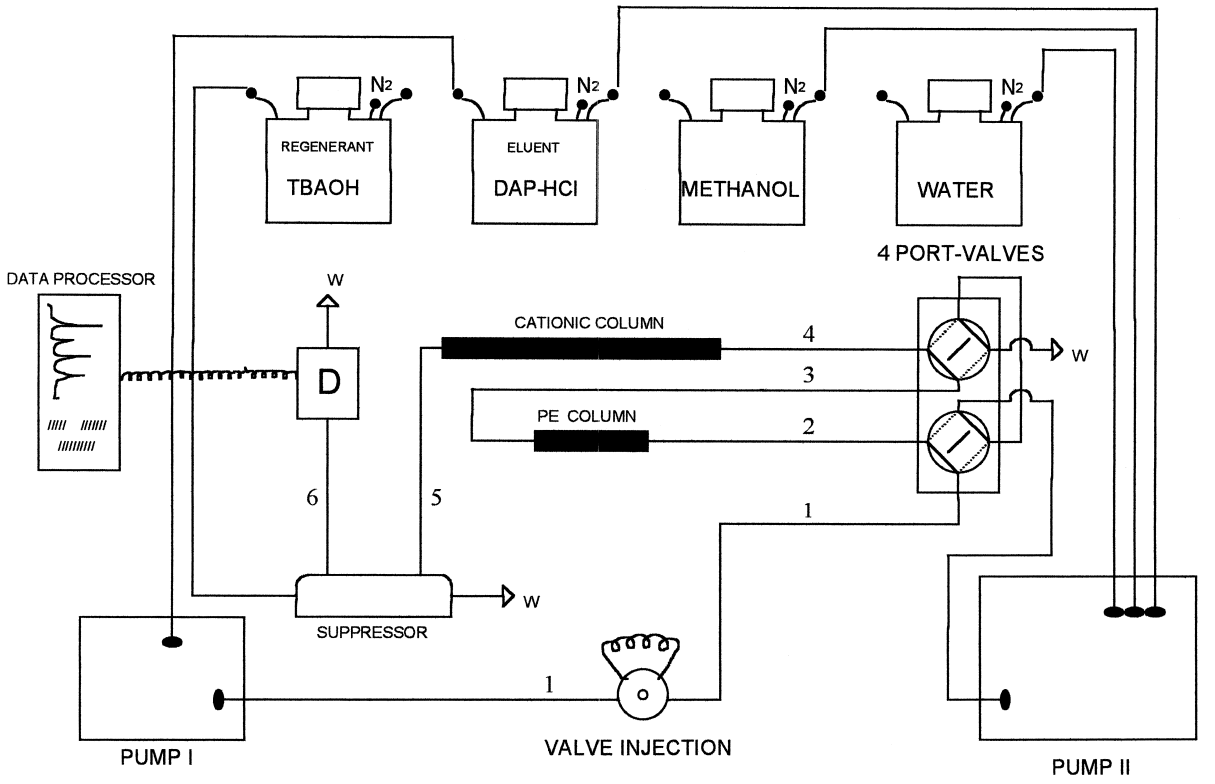

Fig. 1. Chromatographic system for the determination of Na, K, Ca and Mg in serum with conductimetric detection, after on-line deproteinization on a PE powder pre-column. The sample is injected (1) and through a four-way valve (2) reaches the PE column where the proteins are adsorbed. The deproteinized sample flows through (3) the four-way valve and (4) reaches the cationic column where the ions are separated. According to the pump program shown in Table 1, 5 min after injection, the sample reaches the cationic column and the four-way valve changes its position to pump methanol and water through the PE column. The PE column is washed with 5 ml of each solvent, and the proteins are desorbed and wasted through the valve. The PE column is reconditioned with 3 ml eluent, the valve returns back to its original position and the system is prepared to receive the next sample injection. Total analysis time 20 min.

absorbance of the solution at 505 nm. For both metals, analytical curves were obtained in the same way, adding the standard solutions to the same reagent volume (2.5 ml for Ca and 2.0 ml for Mg) to give curves in the range 10–30 mg/l for magnesium and 50–150 mg/l for calcium.

3. Results and discussion

3.1. Retention of proteins on polyethylene powder

The retention of proteins on PE was tested by dissolving different volumes of serum in a final volume of 2.0 ml eluent, and passing these solutions through the PE column. The collected effluents were assayed for proteins with the Coomassie test. Fig. 2 shows the absorption spectra of these samples (curves 1 to 4) as well as the spectrum of the original serum (100 μl) also diluted to 2.0 ml with eluent (curve 5). As the samples have the same final dilution, the difference between each spectrum and spectrum 5 shows the retention of the proteins for each investigated serum volume. The proteins retained from each solution were not calculated, but from Fig. 2 it can be seen that, when 100 μl serum is present, no proteins are found in the effluent. Even when the sample contains 200 μl serum, practically all proteins are retained, because curve 2 shows a small absorbance at 610 nm; 200 μl serum should give a peak double the height of the peak of curve 5.

Fig. 3 shows the spectra of the washing solvents

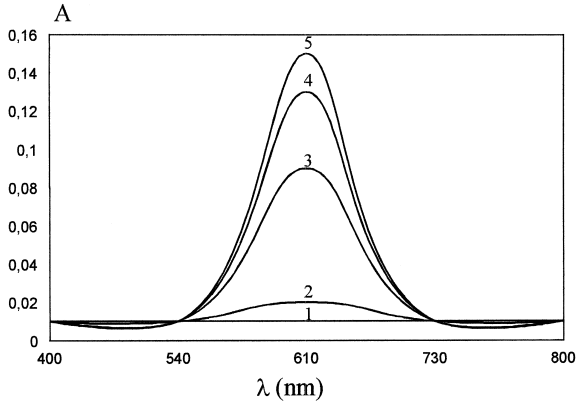

Fig. 2. Spectra of different serum volumes in 2.0 ml final volume eluent, assayed by the Coomassie Brilliant Blue test for proteins, after passing through a PE column. Curves 1, 2, 3 and 4 correspond to 100, 200, 500 and 1000 μl serum, respectively. Curve 5 is a reference sample: 100 μl serum in 2.0 ml without passing through the column.

(methanol, water and eluent) obtained by passing these solvents through the column after retention of the proteins in 100 μl serum. In this case, serum was injected into the eluent stream and after that eluent was changed to methanol (5.0 ml) (curve 1) followed by water (5.0 ml) (curve 2) and by eluent again (3.0 ml) (curve 3). The curves show qualitatively that the amount of protein in 100 μl serum, diluted in 5 ml methanol (curve 4), is distributed in 10 ml of

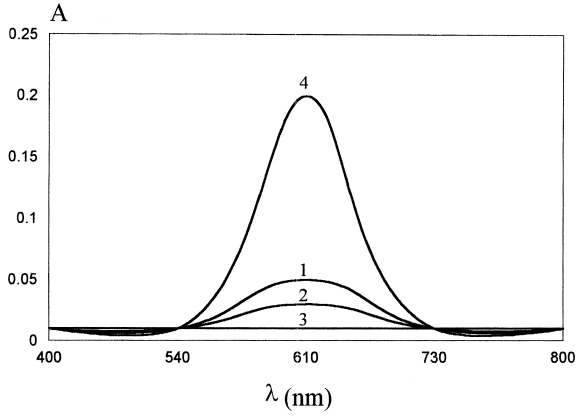

Fig. 3. Spectra of the washing solvents (assayed for proteins by the Coomassie Brilliant Blue test) after passing through a PE column where the proteins of 100 μl serum were adsorbed. Curve 1, 5.0 ml methanol; curve 2, 5.0 ml water; curve 3, 3.0 ml eluent. Curve 4 is the reference: 100 μl serum in 5.0 ml methanol.

washing solvents: 5 ml methanol (curve 1) and 5 ml water (curve 2).

The results show that 5 ml methanol is sufficient to elute all adsorbed proteins from the PE surface. The use of water following methanol attempts to condition the PE column for receiving the eluent (aqueous solution) of the next injection; if the PE was still wet by methanol the proteins would probably not be adsorbed.

3.2. Efficiency of protein retention/elution in the chromatographic system

Based on the results of the interaction of proteins with PE (adsorption/desorption) in the off-line system, the chromatographic system was set up to allow total retention of the proteins before the sample reached the cationic column and also to allow complete washing of the PE column while the cations were being separated on the cationic column. A chromatographic column (50×4.6 mm) filled with about 400 mg PE powder was used to guarantee the retention of proteins from a 100 μl sample volume. The Dionex pump was programmed (Table 1) to allow passage through the PE column of 5.0 ml methanol, 5.0 ml water and 3.0 ml eluent to wash the column and to prepare it for the next sample injection.

In order to evaluate the efficiency of the chromatographic system with regard to on-line protein retention, it was mounted without the cationic column and after sample injection, eluent (first portion), methanol and water fractions were collected and the presence of proteins assayed by the Coomassie Brilliant Blue test. Fig. 4 shows the spectrum of each solvent and of the reference (100 μl serum in 5.0 ml eluent). As the proteins appear in the methanol fraction and practically in the same concentration as in the reference, the on-line retention and elution of proteins were also efficient.

3.3. Chromatographic separation

The system used for cation separation is described in the Dionex Product Selection Guide (1997–98) for water analysis. DL-2,3-Diaminopropionic acid

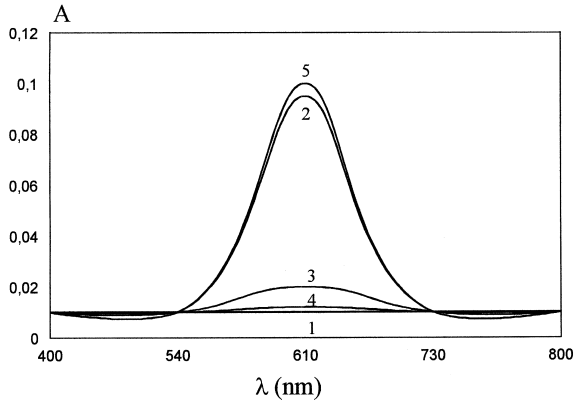

Fig. 4. Spectra of the fractions of eluent (2.5 ml), methanol (5.0 ml), water (5.0 ml) and eluent again (3.0 ml) obtained in the chromatographic system using the pump program described in Table 1 and injecting a 100 μl serum sample. Curve 1, eluent; curve 2, methanol; curve 3, water; curve 4, eluent. Curve 5 is the reference sample: 100 μl serum in 5.0 ml eluent.

Table 2
Retention time, calibration range, and sample dilution for the determination of each ion

Cation	Retention time (min)		Calibration range (mg/l)	Sample dilution
	Without PE column	With PE column		
Na^+	3.6	3.7	0.3–1.2	1:2000
K^+	4.7	4.7	0.3–1.2	1:100
Mg^{2+}	8.0	8.7	0.3–1.2	1:100
Ca^{2+}	14.6	15.6	0.3–1.2	1:100

monohydrochloride as eluent permits the simultaneous determination of alkaline and alkaline earth metals. Other mixtures were tested as eluent, however either the alkaline metals were not separated, the peaks appeared too close to the sample injection peak but calcium and magnesium were well separated, or sodium and potassium showed good separation but calcium and magnesium were not eluted.

Cation separation was first optimised with standard solutions and without the PE column. The chromatographic data related to each metal are shown in Table 2. After introduction of the PE column into the system, cation separation from standard solutions was carried out again to check its influence on metal recovery. The results (Table 2) show that the presence of the PE column does not interfere with cation separation.

We attempted to investigate protein separation and cation recovery using albumin solution spiked with metals, but albumin showed a high concentration of Na ions. Therefore, tests were performed directly with the pooled serum spiked with the metals. The recoveries of the spiked samples are shown in Table 3. The results show that the ions are not retained on the PE surface and the peaks observed for the serum correspond to those of the ions. Fig. 5 shows the chromatograms of standards and serum analyses. There is no difference between the signals obtained from standards and serum, showing again that the PE

Table 3
Recoveries from serum samples spiked with metals

Sample	Na^+			K^+		
	No addition (mg/dl)	Plus 100 mg/dl	Recovery (%)	No addition (mg/dl)	Plus 10 mg/dl	Recovery (%)
A	312.5	410.5	98	14.5	24.4	99
B	323.4	425.4	102	18.4	28.4	100
C	328.3	425.3	97	20.1	30.0	99
D	334.8	435.8	101	19.6	29.4	98
	Mg^{2+}			Ca^{2+}		
	No addition (mg/dl)	Plus 1 mg/dl	Recovery (%)	No addition (mg/dl)	Plus 5 mg/dl	Recovery (%)
A	1.8	2.8	96	13.5	18.6	102
B	2.1	3.1	97	9.4	14.4	100
C	1.6	2.6	102	10.3	15.1	96
D	1.4	2.4	102	11.9	17.0	102

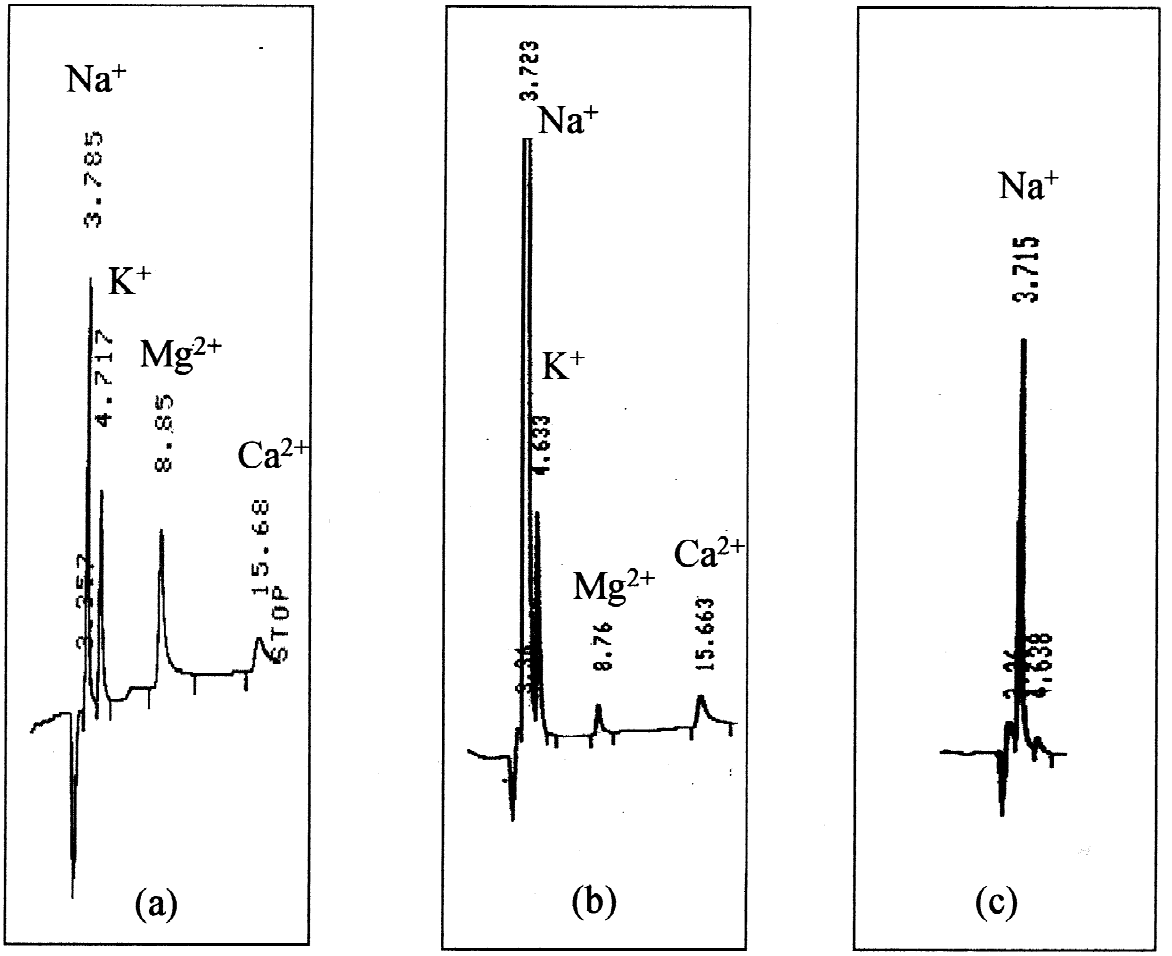

Fig. 5. Chromatographic peaks of Na, K, Ca and Mg obtained with the system shown in Fig. 4. (a) Injection of a standard with 0.6 mg/l of each cation. (b) Injection of a serum sample (dilution 1:100). (c) Injection of a serum sample diluted 1:2000. Sample volume 100 μl.

column causes no interferences in metal determination.

3.4. Comparison methods

Two methods were used for comparison with the proposed chromatographic method. Serum samples from different donors were analysed by the proposed method and by FAAS for sodium and potassium. Calcium and magnesium were determined photometrically using a kit test. The results are shown in Table 4 and application of Student's *t*-test indicates that there are no significant differences between the procedures at the 95% confidence level.

4. Conclusion

Polyethylene is able to adsorb proteins from serum and therefore to promote serum deproteinization. The process is reversible and the proteins are desorbed with methanol. The use of PE powder for the simple, rapid and micro-sample deproteinization of human serum seems very useful for the determination of

Table 4
Comparison between the results obtained with the proposed method and FAAS and photometry in the analysis of serum samples from different donors ($n = 3$)

Sample	Proposed method HPLC (mg/dl)	FAAS (mg/dl)	Photometry (mg/dl)	Deviation (mg/dl) (%)
Sodium				
1	310.6±15.2	309.8±5.2	–	+0.3
2	337.2±16.4	344.6±6.5	–	−2.1
3	346.8±13.8	329.7±4.5	–	+5.2
4	335.3±17.0	317.8±4.5	–	+5.5
5	343.9±11.5	323.8±6.6	–	+6.2
6	321.8±12.0	312.6±5.4	–	+2.9
7	328.9±16.1	332.6±6.3	–	−1.1
8	330.9±13.5	318.8±7.4	–	+3.8
Potassium				
1	13.1±0.7	13.6±0.2	–	−3.7
2	11.5±0.6	12.1±0.2	–	−5.0
3	20.5±0.9	20.0±0.4	–	+2.5
4	14.3±0.7	15.3±0.3	–	−6.5
5	27.5±0.9	24.5±0.6	–	+12.2
6	14.1±0.7	14.5±0.3	–	−2.8
7	17.5±0.8	21.1±0.3	–	−17.1
8	33.3±1.2	28.8±0.6	–	+15.6
Magnesium				
1	2.4±0.1	–	2.0±0.05	+20.0
2	2.8±0.1	–	2.6±0.05	+7.7
3	1.5±0.08	–	1.6±0.03	−6.3
4	1.7±0.09	–	1.9±0.04	−10.5
5	2.0±0.1	–	1.9±0.04	+5.3
6	2.2±0.1	–	2.2±0.05	0
7	2.4±0.1	–	2.4±0.05	0
8	1.7±0.09	–	2.2±0.03	−22.7
Calcium				
1	12.4±0.6	–	10.4±0.2	+19.2
2	15.9±0.5	–	12.0±0.3	+32.5
3	7.2±0.4	–	7.5±0.2	−4.0
4	10.3±0.5	–	8.9±0.1	−15.7
5	10.6±0.6	–	10.0±0.1	+6.0
6	11.4±0.6	–	10.4±0.1	+9.6
7	9.1±0.5	–	8.0±0.08	+13.8
8	10.7±0.5	–	9.6±0.1	+11.5

serum cations by HPLC with conductimetric detection.

Using a PE pre-column, serum could be deproteinized on-line. After serum was passed through the PE pre-column, a protein-free sample was obtained and directly introduced into the chromatographic column without problems or interferences.

The analysis is carried out in 20 min, including deproteinization and cation separation. The time is limited by the retention time of the cations, as the washing and conditioning of the PE pre-column is done during cation separation on the chromatographic column.

The proposed method shows a precision of about 95% and results comparable to those obtained by FAAS and photometry, but it is simpler and more convenient considering the four cations are determined simultaneously and no further sample treatment, such as addition of deproteinizing agents and centrifugation, is necessary. The proposed method

can be applied to the automated routine determination of sodium, potassium, calcium and magnesium in human serum.

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