Evaluation of Select Variables in the Ion Chromatographic Determination of F^-, Cl^-, Br^-, NO_3^-, SO_4^{-2}, and PO_4^{-3} in Serum Samples

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Abstract

A full experimental design at two levels is applied for the estimation of the significance of select factors that may influence the ion chromatography (IC) determination of F^-, Cl^-, Br^-, NO_3^-, SO_4^{-2}, and PO_4^{-3} in serum samples. The factors studied are various sample deproteinization procedures, eluent composition, and flow rates. Deproteinization using either acetonitrile–NaOH or ultrafiltration can be used in order to obtain a significant protein removal before IC analysis; however, the former is recommended because it is less time-consuming and cheaper. Better resolution is obtained when a sodium hydroxide solution is used as the eluent. There is no influence of the sample's deproteinization procedures on the chromatographic resolution.

Introduction

The ability to determine the concentration of physiologically and pathologically related anions with a single sample treatment and technique is of great importance. Ion chromatography has become one of the most powerful tools for the quantitative analysis of anions in a wide variety of matrices. One of the problems associated with serum anion determination by this technique is the lack of similar sensitivity for all the anions to be determined simultaneously. Of all the inorganic anions present in human serum, chloride, bicarbonate, and phosphate are the most routinely determined. However, the demand for sulfate, bromide, nitrate, and fluoride serum analysis is increasing because of the clinical information derived from the levels in serum. The importance in human physiology of the inorganic anions sulfate (1), bromide (2–4), nitrate (5,6), and chloride (7) have been described.

The biochemical analysis of metabolites in whole blood and serum requires initial deproteinization to remove hemoglobin and other proteins that may interfere with the assays. Different agents have been used. One of them is perchloric acid (8,9); however, it has the disadvantage that percholorate inhibits some enzyme reactions considerably and it has to be removed by precipitation with potassium hydroxide. A simple deproteinization procedure has been reported by Khan et al. (10) involving the use of sulfosalycylic acid (SSA). However, the SSA agent is often contaminated with sulfate. Tricholoroacetic acid has been reported (11) as a time-consuming procedure and interferes with the anion elution profile. The precipitation method based on acetonitrile (ACN) (12) has not been successful because proteins are not quantitatively precipitated by this method, which limits the lifetime of the separator column to a maximum of two months. Centrifugation or ultracentrifugation has been reported (11) as an efficient method for the deproteinization of serum samples.

A number of studies have reported the determination of some inorganic anions in serum by ion chromatography. These have included the simultaneous determination of inorganic phosphate, bromide, nitrate, and sulfate in human serum (1) and the determination of thiocyanate (13), bromide (14), and sulfate (15). In measuring systems in which the signal is linearly related to the component of interest, matrix components, and instrumental parameters, factorial analysis is a convenient tool to apply. Factorial approaches to experimental designs contrast with simplex approaches in that several experiments can be performed simultaneously and are used to calculate the main effects and the interaction effects of several factors. Full factorial designs at two levels of variation for the input factors are often used in analytical chemistry (16,17).

This research deals with the optimization of a method for the simultaneous analysis of chloride, fluoride, nitrate, bromide, sulfate, and phosphate in human serum by isocratic ion chromatography. A factorial design at two levels was applied in order to estimate the magnitude of the main effects and various two-factor interactions under the experimental conditions of interest. It further allowed for a better interpretation of the results obtained. The evaluation of parameter significance is a very important step in the optimization procedure. The selection criterion for choosing the factors involved in this design was dictated by

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variables that may influence the resolution such as deproteinization treatment, eluent composition, and flow rate.

Experimental

Apparatus and reagents

The ion chromatographic equipment used was a Dionex (Sunnyvale, CA) DX 500 system with a 100-µL injection loop, an Ion Pac AS11 analytical column (4 × 250 mm), and an Ion Pac AG11 Guard column (4 × 50 mm) (Dionex). The column temperature was 25°C. The apparatus was equipped with an ion fiber suppressor ASRS-11-4 mm (Dionex Anion self regenerating) that was continuously regenerated by water and a conductimetric detector (Dionex) PeakNet Chromatography Workstation.

All chemicals were of analytical-reagent grade. Sodium hydroxide was obtained from Merck (Darmstadt, Germany), and Na_2CO_3, $NaHCO_3$, NaCl, $NaNO_3$, NaF, Na_2SO_4, Na_3PO_4, and KBr (> 99.9% pure) were from Aldrich (St. Louis, MO). Milli-Q deionized water was used throughout (Milli-Q water purification system, Millipore, MA) as well as HPLC-grade ACN (EM Science, NJ). Ultrafree-Cl low binding cellulose 10,000 nominal molecular weight limit filters were used (Millipore) as well as 0.45- and 0.47-µm nylon filters (Alltech, Deerfield, IL).

A protein assay was performed with the Coomassie Plus Protein assay reagent kit (Pierce, Rockford, IL).

Serum samples

A pooled serum sample was obtained from the Medical Center at our research institute. Each deproteinization method was performed on an aliquot of this pooled sample.

Sample treatment

A preliminary study was carried out in order to assess the efficiency of protein removal by different methods. For this reason, three deproteinization treatments that have been reported in the literature were tested: ACN (18,19), ACN–NaOH (20), and ultrafiltration (19). Protein quantitation was carried out on an aliquot of the same serum sample after each deproteinization treatment, measuring the absorbance at 595 nm.

The ACN treatment consisted of mixing 0.2 mL of serum sample with an equal volume of ACN, then centrifuging at 2000 × g for 5 min.

For the ACN–NaOH treatment, 500 µL of a serum sample, 50 µL of NaOH (2M), and 150 µL of deionized water were added and shaken for a few seconds. Then, 1 mL of ACN was added and vortex mixed for 10 s. The resulting mixture was centrifuged for 5 min at 755 × g. Finally, 1 mL of the supernatant solution was diluted with 5 mL of deionized water and injected into the chromatograph.

For the ultrafiltration, a 1:10 diluted serum sample was filtered for 30 min and injected.

Results and Discussion

Protein removal

The sample preparation for ion chromatographic analysis of serum is much more elaborate than for other physiological fluids. Proteins that are present in high concentration must be removed before the sample is injected, because they negatively affect the separation efficiency of the ion-exchange columns.

The principal objective of this study was to find an efficient, fast, and reliable deproteinization method that yields a solution suitable for subsequent ion chromatographic analysis. The three deproteinization methods described in the Experimental section were applied, and the resulting percentages of protein removal were 21.5%, 93.3%, and 98.6% for the ACN, ACN–NaOH, and ultracentrifugation treatments, respectively.

After the efficiency of these methods was verified, it was decided to choose the ACN–NaOH mixture and ultrafiltration deproteinization procedures as variables to be considered in the experimental design in order to observe their influence on the chromatographic resolution.

Optimization

In order to obtain proper information on the significance of the factors mentioned, a full 2^4 factorial design at two levels was applied. This type of design involved sixteen experiments. Four factors were examined in this design: a sodium hydroxide solution (X_1), a sodium carbonate–sodium bicarbonate mixture (X_2),

Table I. Experimental Variables Considered in the Application of the 2^4 Full Factorial

Natural variable	Coded variable	Level (+1)	Level (–1)
NaOH	X_1	12mM	6mM
Na_2CO_3–$NaHCO_3$	X_2	3mM,2.4mM	0
Flow rate	X_3	1.5 mL/min	1.0 mL/min
Deproteinization procedure	X_4	ultrafiltration	NaOH–ACN

Table II. 2^4 Experimental Design*

Experiment	X_1	X_2	X_3	X_4	Resolution criterion
1	–1	–1	–1	–1	11.36
2	1	–1	–1	–1	10.62
3	–1	1	–1	–1	12.30
4	1	1	–1	–1	7.41
5	–1	–1	1	–1	11.03
6	1	–1	1	–1	10.99
7	–1	1	1	–1	11.50
8	1	1	1	–1	7.14
9	–1	–1	–1	1	10.04
10	1	–1	–1	1	10.84
11	–1	1	–1	1	12.69
12	1	1	–1	1	7.56
13	–1	–1	1	1	10.52
14	1	–1	1	1	11.07
15	–1	1	1	1	11.32
16	1	1	1	1	9.26

* Resolution taken as the response.

the flow rate (X_3), and a deproteinization procedure (X_4) (Table I). Two levels were chosen associated with the –1 and +1 levels of the corresponding coded variables. These were selected on the basis of the literature review on this topic, and the deproteinization procedures were selected according to the results obtained and described in the previous section.

The selected experimental response was a criteria of peak resolution (R_s) and the peak area. This criterion of resolution consisted in the addition of the resolution values of all consecutive peaks plus the number of peaks that appears in the chromatogram (21). Table II shows the results.

The significance was evaluated through the application of the student t-test with a 0.05 significance level. Table III shows these results. The variables of X_1 at the –1 level (6mM), X_2 at the –1 level, and the interaction of X_1 and X_2 resulted as being significant at the 95% level. The influence of X_1 revealed that a better resolution was obtained when this sodium hydroxide concentration was used. The use of the Na_2CO_3–$NaHCO_3$ mixture did not influence the resolution, and this agreed with the result of the significance test. The X_1–X_2 interaction revealed that an improvement on the resolution was obtained when both mixtures were used.

Table III. Calculation of the Effects

Effect	Estimated value	Standard deviation	Experimental t-value	Probability level
b_0	10.42	0.1613	64.58	0.0000
b_1	–1.0574	0.1613	–6.55	0.0012*
b_2	–0.4545	0.1538	–2.95	0.0317*
b_3	–0.0619	0.1613	–0.10	0.7039
b_4	0.1256	0.1613	0.77	0.4714
b_{12}	–1.0655	0.1538	–6.92	0.0012*
b_{13}	0.2638	0.1945	1.35	0.2331
b_{14}	0.1955	0.1613	1.21	0.2797
b_{23}	–0.0945	0.1538	–0.61	0.5659
b_{24}	0.2514	0.1538	1.63	0.1630
b_{34}	0.0621	0.1613	0.38	0.7158

* Significant effect.

Identification

Figure 1 shows the chromatograms of an aqueous solution run at an eluent composition of 6 and 12mM NaOH (Figures 1A and 1B) and a serum sample using isocratic conditions (Figures 1C and 1D). Peak identification was based on retention times.

As it can be seen from these chromatograms, in general, the aqueous standard and the serum samples exhibited a well-defined resolution and symmetrical peaks (not broadened) in less than 16 min. Shorter retention times, mainly for the monovalent and divalent anions, were observed when changing the concentration of the eluent mixture (12mM). Furthermore, higher signals were obtained at this condition. Observed in the chromatogram of the serum sample was the appearance of a second peak (probably acetate), which was coeluted with fluoride.

Figures 1C and 2 show the effect on the resolution from the deproteinization treatments. It can be observed that there was not a significant change between the results obtained. Therefore, either treatment (deproteinization by using ACN–NaOH or by ultrafiltration) could be used. This result was consistent with that obtained in the experimental design in which the deproteiniza-

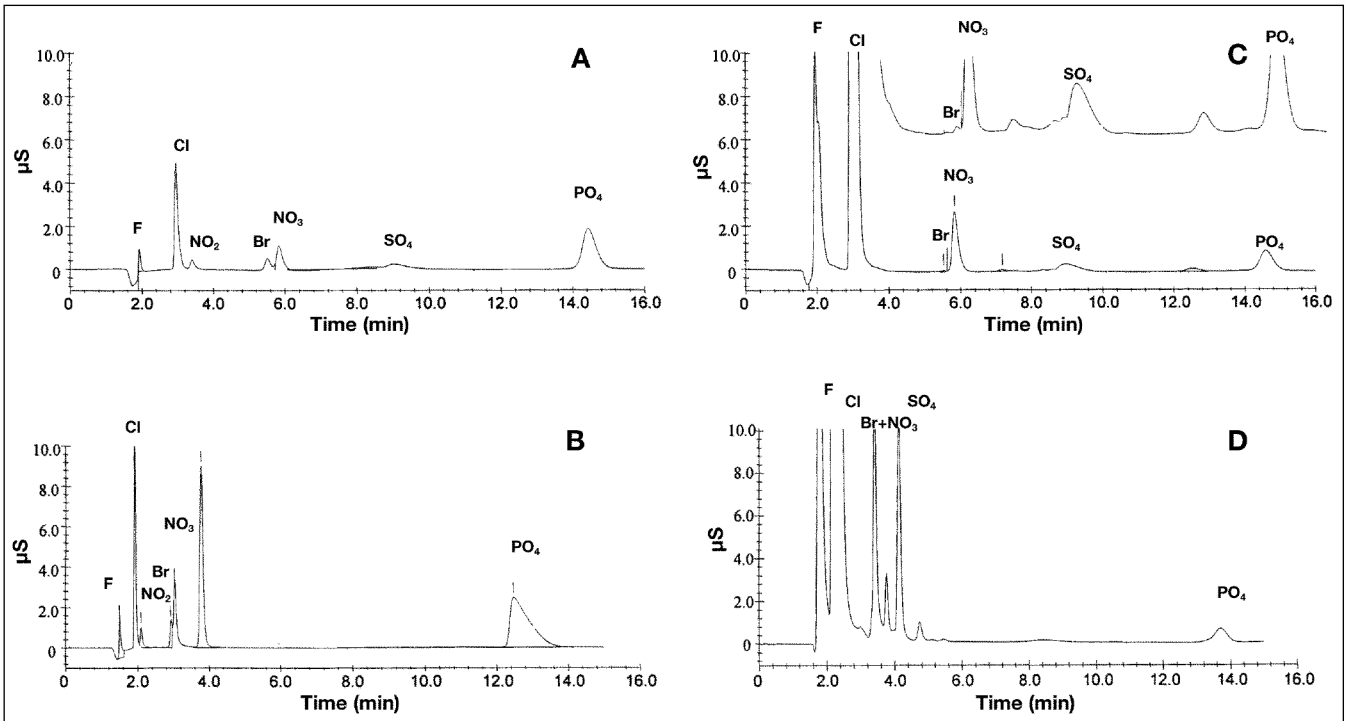

Figure 1. Chromatograms of an aqueous solution run at two different eluent compositions (A and B) and a serum sample using two different isocratic conditions (C and D): (A) an aqueous standard of 6mM NaOH eluent and 1-mL/min flow rate; (B) an aqueous standard of 12mM NaOH eluent and 1-mL/min flow rate; (C) a serum sample of 6mM NaOH eluent, ACN–NaOH deproteinization, and 1-mL/min flow rate; and (D) a serum sample of 12mM NaOH eluent, ACN–NaOH deproteinization, and 1-mL/min flow rate.

tion treatment did not have any statistical significance. However, we recommend the ACN–NaOH mixture because it is less time-consuming (the sample can be deproteinized in approximately 5 min compared with the 30 min that it takes for the ultrafiltration procedure).

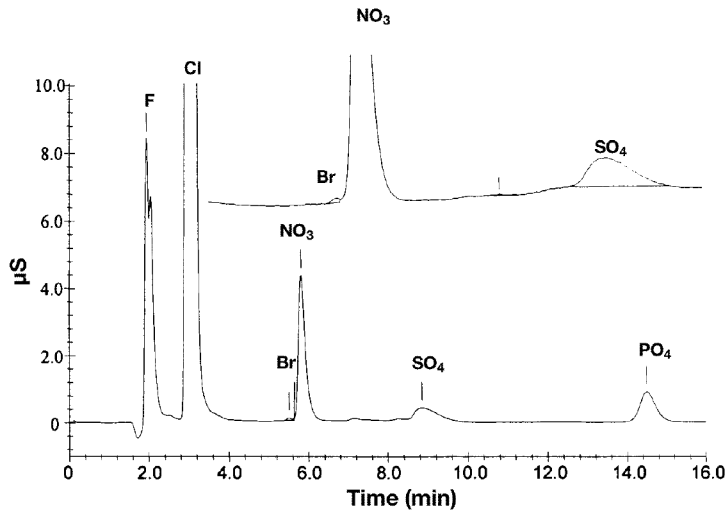

Figure 2. Chromatogram of a serum sample with ultrafiltration: 6mM NaOH eluent and 1-mL/min flow rate.

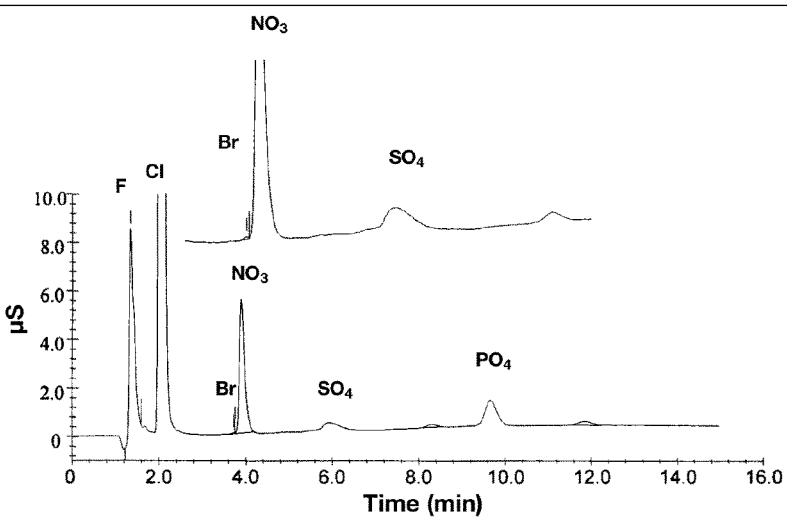

Figure 3. Chromatogram of a serum sample with ACN–NaOH: 6mM NaOH eluent and 1.5-mL/min flow rate.

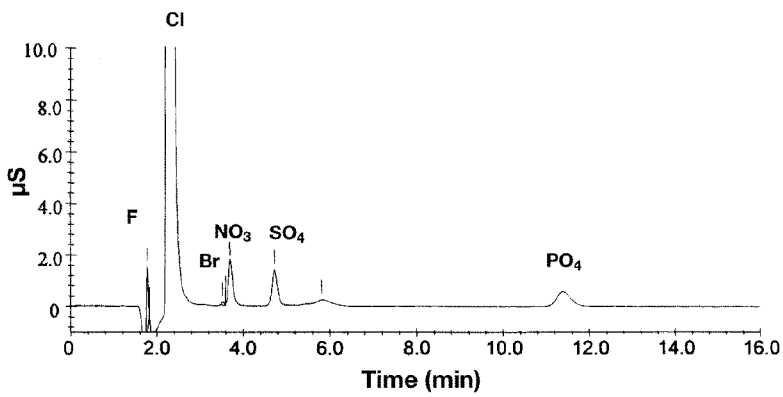

Figure 4. Chromatogram of a serum sample with ultrafiltration: 6mM NaOH–Na_2CO_3–$NaHCO_3$ eluent and 1-mL/min flow rate.

Even when the flow rate was not significant, according to the results from the significance test a flow rate of 1.5 mL/min (+1 level, X_3) is more convenient if analysis time is considered important (as shown in Figures 1C and 3).

The significance of the b_{12} interaction was verified by the use of the interaction diagrams (22). The b_{12} interaction at the –1 level revealed that the resolution was improved when the NaOH (6mM) and Na_2CO_3–$NaHCO_3$ mixtures were used. The chromatogram resulting from this experimental condition is shown in Figure 4. However, this condition is not recommended when fluoride has to be determined, because its peak appeared within the water dip.

In order to further evaluate the efficiency of the deproteinization procedures, an evaluation of the signal sensitivity was carried out for each anion by taking into account the peak area as a measurement of the response in the experimental design. The result of this study (not shown) led to the conclusion that the treatment procedure does not have any significant effect on the signal sensitivity.

Because of the complexity of the serum matrix, the column efficiency was checked in order to see if any modification (probably occasioned by the matrix) could have affected it. For this reason, a control solution containing all the anions considered in this study was passed through the column before and after running the serum samples for their analysis (shown in Figure 5). In order to evaluate column efficiency, the theoretical plates were calculated for each anion before and after the set of experiments involved in this research, and no significant change was observed. No change in the signal sensitivity and resolution was obtained. Therefore, it can be said that the serum matrix did not alter the column's original characteristics with it being able to analyze the inorganic anions in this matrix under the conditions developed in this work.

Conclusion

It has been shown in this work that the simultaneous determination of six important physiological anions in human serum is possible using ion chromatography under isocratic conditions without altering the column's original conditions and thus its efficiency. This is an important aspect, taking into account the complexity of the serum matrix. Scarce information has been given about column integrity after this type of analysis.

The application of the experimental design to

this particular application for evaluating the significance of input parameters for analytical determinations allows for the obtaining of important information on the variables that most influence the determination of the anions in this matrix and on the interactions between chemical and instrumental parameters.

There was no influence of the sample's deproteinization procedures on the chromatographic resolution.

Table IV. Data for the Anions in Figure 5A

Anion	Retention time (min)	Area
Fluoride	1.82	71263
Chloride	2.30	674127
Nitrite	2.52	42713
Bromide	3.48	91697
Nitrate	3.60	294013
Sulfate	4.48	1065847

Table V. Data for the Anions in Figure 5B

Anion	Retention time (min)	Area
Fluoride	1.82	71282
Chloride	2.27	666512
Nitrite	2.48	37662
Bromide	3.43	107169
Nitrate	3.55	284737
Sulfate	4.25	1074038

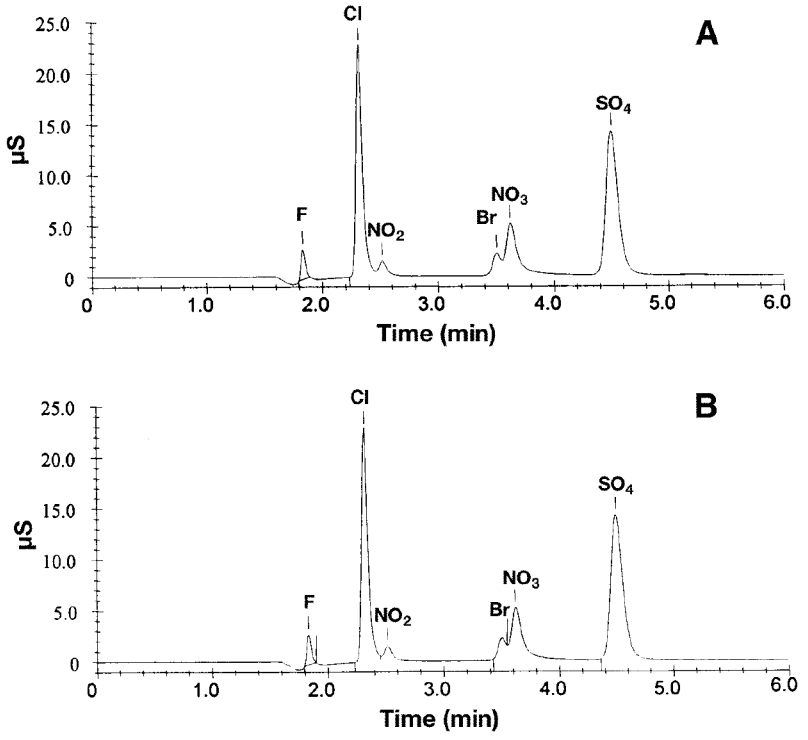

Figure 5. Column performance (A) before and (B) after 40 injections of serum sample. Data for A and B are shown in Tables IV and V, respectively.

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