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Short communication

Optimization and validation of an ion chromatographic method for the simultaneous determination of sodium, ammonium and potassium in exhaled breath condensate

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Abstract

An ion chromatographic method with conductivity detection for the simultaneous quantification of sodium, ammonium and potassium in exhaled breath condensate (EBC) was developed and validated. A factorial design was used to optimize the chromatographic conditions, which resulted in baseline separations of the cations within 6 min. The method requires no pre-treatment of EBC samples. The optimized method was used for the intra-individual screening of cations in EBC of 10 healthy volunteers. The LOQs were low (0.3, 0.1 and 0.2 μM for sodium, ammonium and potassium, respectively), compared with levels detected in healthy volunteers. The responses were linear with good precision, and samples could be stored for at least 10 weeks at refrigerating conditions.

Keywords: Ion chromatography; Exhaled breath condensate; Sodium; Ammonium; Potassium; Chemometrics

1. Introduction

Airway inflammation is often assessed by invasive methods such as bronchoscopy, bronchoalveolar lavage and blood sampling. A non-invasive but indirect method of studying biological markers of airway inflammation is the exhaled breath condensate (EBC) method [1–3]. By cooling the exhaled air from a subject, a condensed solution consisting of water and airway lining fluid is obtained. This EBC is then used in various assays depending on the biological marker studied, for example, hydrogen peroxide [4], malondialdehyde [5] and 8-isoprostane [6]. However, a methodological problem is that the non-volatile biological markers originating from the airway lining fluid are diluted by the water vapour in EBC. This dilution might be one of the causes of the rather large inter- and intra-individual variation seen in various biomarkers assessed in EBC [7,8]. One possibility to avoid this problem is to use sodium and potassium as a dilution marker and to report the ratio between the measured biomarkers and these cations in the EBC [9].

The determination of cations in aqueous samples and human body fluids can be made by several techniques, for example, atomic absorption and ion chromatography [10,11]. Ion chromatography is a sensitive and well-tested separation technique, and no sample preparation is needed for condensate samples. In previous studies, ion-selective electrodes (ISE) have been used for the determination of sodium and potassium in EBC [8,12]. A great advantage of ion chromatography is that cations can be separated and quantified simultaneously, which would make ion chromatography a good technique if the determination of cations in EBC were

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to be standardized. With ion chromatography it is also possible to determine ammonium, which may be valuable in the determination of other biomarkers in EBC, for example, pH.

The aim of this investigation has been to optimize and validate a chromatographic method for the determination of sodium and potassium in EBC. The optimization was made by experimental design and multivariate analysis [13–16]. The resulting chromatographic method was applied to the determination of sodium and potassium in the EBC of 10 healthy volunteers.

2. Experimental

2.1. Equipment

The chromatographic system consisted of a Varian 2510 HPLC pump (Varian, Walnut Creek, CA, USA), an HP1100 ALS autosampler (Agilent, Little Falls, DE, USA) equipped with a rheodyne valve and a variable loop, and a Waters 430 conductivity detector (Waters Assoc., Milford, MA, USA). Since the mobile phase has a higher conductivity than the sample ions, the polarity was switched in order to reverse the output signal. The separation column was a Hamilton PRP-X200, 250 mm × 4.6 mm i.d., 10 μm particle size, packed with a poly(styrene-divinylbenzene)sulfonate cation exchanger (Hamilton, Reno, NV, USA). In connection with the instrumentation described, a water bath for controlling the mobile phase temperature was available. The eluent flow-rate was 2 ml/min in accordance with the recommendation from the column manufacturer. The injection volume was 30 μl. Data acquisition was performed using the HP Chemstation software version A.06.03 (Agilent Technologies, Palo Alto, CA, USA). In order to avoid contamination, all equipment involved in the treatment of samples and standard solutions was made of polypropylene.

2.2. Chemicals

Methanol, acetonitrile and nitric acid were purchased from Merck (Darmstadt, Germany). Tartaric acid, 2-propanol and picolinic acid were from Sigma–Aldrich. All reagents were of analytical grade. The reference standards sodium, ammonium, potassium and lithium were obtained from Analytical Standards AB (Mölnlycke, Sweden). Water was purified by an Alpha-Q water purification system (Millipore, Bedford, MA, USA).

Stock solutions were made for each cation by diluting the reference substance with purified water. Standards were prepared by diluting and mixing the stock solutions with purified water to give the multi-cation solutions needed.

2.3. Sampling of EBC

The EBC samples were collected with an EcoScreen breath condenser purchased from Jaeger (Wurtsberg, Germany). Subjects were told to rinse their mouths with purified water for 30 s prior to sampling. A nose clip was used in order to facilitate mouth breathing. Subjects breathed into the condenser with normal frequency for 10 min. The breath condenser used in the present study was equipped with a polypropylene mouthpiece attached to a polypropylene non-rebreathing valve. This valve was coupled to a lamellar condenser tube (made of Teflon-coated aluminium) with a sample container made of polyoxymethylene. Saliva contamination was avoided by the use of a saliva trap. In a previous study where the same equipment was used it was observed that EBC samples did not exhibit amylase activity [17]. The lamellar was cooled to −20 °C in order to condense the exhaled breath. Aliquots of EBC were transferred to 0.6 ml polypropylene vials (Chromacol, Trumbull, CT, USA) with Teflon sealings prior to stability testing. Pooled EBC samples from six healthy volunteers were used for method development and validation.

2.4. Chromatographic responses

The chromatographic responses used in this work were capacity (k'), selectivity (α), symmetry (Asf) and number of theoretical plates (N). All were the mean values from duplicate injections. The dead time, t_0, was defined as the time at which the first disturbance of the baseline appeared after injection and in accordance with calculations of the column volume. The retention times of the solutes were measured from the time of injection.

2.5. Statistical methods

The experimental variables were evaluated using statistical multivariate regression. The central composite facial design (CCF design [18–20]) and analysis of chromatographic data were performed using Modde 6.0 software (Umetri, Umeå, Sweden). With three experimental variables, the design led to 14 runs under different conditions. In addition, three replicates were fulfilled in order to estimate the reproducibility [18]. The design was made to resolve linear, cross-term and quadratic behaviour of the experimental variables with low uncertainty, that is, a small confidence interval for the regression variables. All runs were carried out in random order. The partial least squares (PLS) [21,22] method was used to calculate the regression coefficients. Cross-validation was performed in order to evaluate the quality of the resulting statistical model. The capacity factors were transformed logarithmically in order to achieve normally distributed data.

3. Results and discussion

3.1. Screening of effects of mobile phase temperature, different modifiers and acids

A preliminary screening of the variables was made with univariate conditions to study the effect of different mod-

ifiers and acids on retention and selectivity of the cations. Acetonitrile, 2-propanol and methanol, with a content of 30%, were tested as modifiers (concentration and temperature were kept constant at 4 mM HNO_3 and 20 °C, respectively). However, methanol was the only modifier that gave acceptable retention times and selectivity. Picolinic acid, tartaric acid and nitric acid were investigated as mobile phase additives (concentration of acid, methanol and temperature were kept constant at 4 mM, 30% and 20 °C, respectively), all resulting in different background conductivity levels. Even though nitric acid has high background conductivity, the chromatographic peak shape and retention was better using nitric acid as the mobile phase additive. The screening of the variables resulted in the ranges of the design; nitric acid: 1–10 mM, methanol: 20–40% (v/v) and column temperature: 20–40 °C.

3.2. Statistical experimental design and evaluation of the statistical model

A statistical model was sought that was well fitted to the data and also had a good predictive capability. The fraction of data that could be explained by the model (R^2) and the capacity to predict new data within the experimental domain (Q^2) were used as criteria of model quality. In the first model, linear, quadratic and interaction terms were included as well as all the responses. Nonsignificant terms and responses that were difficult to model were excluded until an optimal model was fulfilled, comprising linear and quadratic terms as well as selectivity and log-transformed capacity and factors as responses (Table 1). The optimal model was used to predict experimental conditions that gave as high separation factors and as low capacity factors as possible. The resulting mobile phase consisted of 5.5 mM HNO_3 and 40% methanol and had a mobile phase temperature of 30 °C. The chromatographic run-time was less than 6 min. These experimental conditions were validated and thereafter used to assess storage stability as well as to determine cations in the EBC from healthy volunteers.

Table 1
Obtained values for R^2 and Q^2 depending on terms included in the model

Model terms	R^2	Q^2
k', α, Asf, N		
Linear, interaction, quadratic terms	0.66–0.99	0–0.79
Linear, quadratic terms	0.33–0.99	0–0.95
Linear terms	0.07–0.98	0–0.93
k', α		
Linear, interaction, quadratic terms	0.99–1.00	0.83–0.90
Linear, quadratic terms	0.96–0.99	0.87–0.94
Linear terms	0.90–0.98	0.79–0.94

Model terms: capacity factor (k'), selectivity factor (α), asymmetry factor (Asf) and column efficiency (N). R^2: variation explained by the model; Q^2: fraction of variation explained by the model.

Table 2
Calibration data for ion chromatographic determination of sodium, ammonium and potassium

Parameter	Sodium	Ammonium	Potassium
Concentration range (mg/l)	0–20	0–20	0–20
Slope (l/mg)	88.446	107.65	50.394
Intercept	8.3057	10.867	4.6296
Correlation coefficient (R^2)	0.9999	0.9997	0.9998

3.3. Validation of the optimized method

Validation of the method was performed using the chromatographic conditions set by the optimization, that is, with a mobile phase containing 40% methanol and 5.5 mM HNO_3 (pH 1.97), and with a temperature of 30 °C. The characteristics observed were as follows: precision, linearity, limit of detection (LOD) and limit of quantification (LOQ).

3.3.1. Effect of sample matrix

The standard addition method was used to examine the influence of the sample condensate matrix on the cation determinations. The standard addition curve was constructed by the triplicate injections of a pooled condensate sample to which had been added a known concentration of each of the assayed cations [23]. In addition, triplicate injections were made on the pooled condensate blank. The calibration curve was made as above but with a matrix of purified water instead of condensate. The sample concentrations for all solutes calculated from the calibration curve based on purified water agreed with the concentrations calculated from the standard addition curve within the 95% confidence interval. Thus, the sample matrix does not influence the cation determinations and water can be used for calibration, resulting in more time-efficient analyses.

3.3.2. Calibration and linearity

Standard multi-cation solutions were prepared at six concentration levels within a concentration range from 0 to 20 mg/ml. Duplicate samples were injected. The resulting calibration curves were linear in the considered concentration intervals and with high correlation factors for all assayed cations. The statistical data are shown in Table 2.

3.3.3. Limit of detection and limit of quantification

The approach that has been made in this work for estimating the LOD and the LOQ is based on the standard deviation of response and slope.

$$\mathrm{LOD} = \frac{3.3\mathrm{S.D.}}{S}$$

$$\mathrm{LOQ} = \frac{10\mathrm{S.D.}}{S}$$

Here S.D. is the standard deviation of response and was imputed from the analyses of 10 standard solutions with the concentration of 0.4 mg/l for sodium and ammonium and

Table 3
LOD and LOQ for ion chromatographic determination of sodium, ammonium and potassium

Cation	S.D. (mg/l)	LOD (mg/l)	LOQ (mg/l)
Sodium	0.068	0.002	0.007
Ammonium	0.024	0.001	0.002
Potassium	0.046	0.003	0.009

0.8 mg/l for potassium, while *S* is the slope of the calibration curve. The results are shown in Table 3. The resulting LODs were 0.002, 0.001 and 0.003 mg/l for sodium, ammonium and potassium, respectively, which correspond to 87, 56 and 77 nM.

A way of lowering the LODs would be to use eluent suppression [24] that reduces the chromatographic baseline noise. This was, however, not performed in the present study, as the levels of cations in the EBC of healthy volunteers were significantly higher than the calculated levels of detection.

3.3.4. Precision

Within-day precision of the analytical method was estimated by triplicate injections of three different concentration levels (0.5, 1.2 and 1.6 mg/l) of the multi-cation solution on the same day. The within-day precision explained as a percentage coefficient of variation (CV) for the concentration levels ranged from 1 to 4% for sodium and from 3 to 4% for ammonium. The within-day precision for potassium was lower; the range for the CV was 8–15%.

Between-day precision was performed on three subsequent days by triplicate injections of standard solutions (0.5, 1.2 and 1.6 mg/l). The CVs were found to be 3–12, 3–6 and 10–23% for sodium, ammonium and potassium, respectively.

3.3.5. Selectivity

A standard solution made of sodium, ammonium, potassium and lithium (2 mg/l for all, which corresponds to Na^+: 0.09 mM, NH_4^+: 0.11 mM and K^+: 0.05 mM) was analysed. The optimized conditions resulted in baseline separation for all solutes, as shown in Fig. 1A.

3.3.6. Sample stability

The EBC samples were collected, pooled and analyzed immediately or stored at approximately −20, −80 °C, or refrigerating conditions (approximately +6 °C), respectively. The stored samples were analyzed after 1, 4 and 10 weeks of storage. Duplicate samples were analyzed and double injections from each sample were made. The recovery data are presented in Table 4. The levels of sodium and ammonium are stable at all occasions, but potassium tends to increase after 4 and 10 weeks at all storage temperatures. The reason for this might be that the levels of potassium were low in the present pooled sample and this might contribute to a lower repeatability and reproducibility.

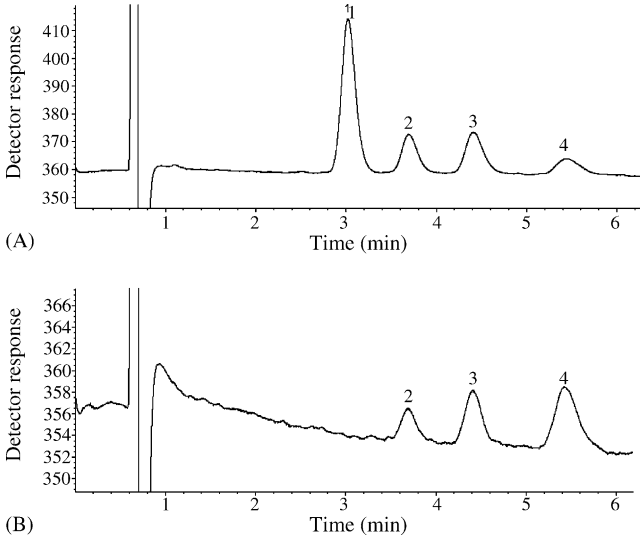

Fig. 1. Ion chromatogram showing (A) the separation of a standard multi-cation solution with lithium, sodium, ammonium and potassium (Na^+: 0.09 mM, NH_4^+: 0.11 mM, K^+: 0.05 mM); and (B) an authentic EBC sample from a healthy volunteer (Na^+: 0.02 mM, NH_4^+: 0.04 mM, K^+: 0.04 mM). Solutes: (1) lithium; (2) sodium; (3) ammonium; (4) potassium.

3.4. EBC cations in healthy volunteers

The optimized method was used to assess sodium, ammonium and potassium in the EBC of 10 healthy volunteers (six females, mean age 32 years, and four males, mean age 41 years). For each volunteer, the EBC was collected during a single day on five occasions, each 2 h apart. The collection time for each occasion was 10 min, resulting in 1000 ± 300 μl of collected EBC, which was stored at −80 °C prior to analysis. Results are presented in Table 5, and a typical ion chromatogram is shown in Fig. 1B.The levels of sodium tended to decrease during the day while levels of ammonium remained constant and potassium concentrations

Table 4
Recovery (%) of sodium, ammonium and potassium in the pooled breath condensate ($n = 6$) after storage

Storage temperature (°C)	Storage time								
	1 week			4 weeks			10 weeks		
	Na^+	NH_4^+	K^+	Na^+	NH_4^+	K^+	Na^+	NH_4^+	K^+
+6	100	98	97	93	98	126	96	95	126
−20	96	97	111	94	97	124	98	95	135
−80	96	97	97	96	100	128	99	94	132

Table 5
Median concentrations (mM) of sodium, ammonium and potassium in EBC in 10 healthy volunteers, collected on five occasions during 1 day

Collection	Sodium (mM)	Ammonium (mM)	Potassium (mM)
1	0.09 (0.06–0.14)	0.11 (0.09–0.15)	0.23 (0.19–0.38)
2	0.11 (0.06–0.13)	0.11 (0.08–0.16)	0.33 (0.25–0.46)
3	0.08 (0.03–0.11)	0.10 (0.06–0.15)	0.29 (0.25–0.36)
4	0.04 (0.02–0.05)	0.12 (0.08–0.17)	0.15 (0.05–0.42)
5	0.04 (0.03–0.07)	0.13 (0.09–0.22)	0.24 (0.12–0.31)

Intermediate ranges in brackets.

fluctuated. The levels of sodium, ammonium and potassium in EBC obtained in the present study agree with the average levels in the study performed by Effros et al.: 0.24, 0.23 and 0.08 mM for sodium, ammonium and potassium, respectively [8].

4. Conclusions

An ion chromatographic method with conductivity detection has been developed for the simultaneous determination of sodium, ammonium and potassium in EBC. Three ion chromatographic parameters were optimized: temperature of the eluent, concentration of nitric acid in eluent and concentration of methanol in eluent.

The resulting analysis time was less than 6 min. The LOQs were well below the concentrations of cations that normally are present in EBC.

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