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Determination of hydrogen peroxide in exhaled breath condensate by flow injection analysis with fluorescence detection

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Abstract

A method for the determination of hydrogen peroxide in exhaled breath condensate (EBC) by automated flow injection analysis (FIA) with fluorescence detection was developed and validated. In the enzymatic assay a fluorescent dimer of *para*-hydroxyphenyl acetic acid (HPAA) was formed by the redox coupling reaction between hydrogen peroxide and horseradish peroxidase (HRP). The calibration curve of hydrogen peroxide was linear over a range of 40–5000 nM. The coefficient of variation (CV) for within-day precision was 1–3%; for between-day precision, it was 2–5% over the validated range. The assay requires a small sample aliquot (150 μl) and no incubation time, and has an analytical runtime of <2 min. It is therefore suitable for larger studies. The method was used to detect hydrogen peroxide in EBC of asthmatic patients and healthy volunteers. A statistically significant difference was found between patients with asthma (n = 19) and control subjects without asthma (n = 19), 780 nM versus 480 nM (P = 0.03).

Keywords: Hydrogen peroxide; Exhaled breath condensate

1. Introduction

Inflammatory cells such as macrophages and neutrophils release hydrogen peroxide, which can be detected in exhaled breath condensate (EBC). Elevated levels of hydrogen peroxide have been found in a number of respiratory disorders, including cystic fibrosis [1], chronic obstructive pulmonary disorder [1–4] and asthma [5–9]. Thus, hydrogen peroxide is considered to be a possible biomarker of airway inflammation.

Sampling EBC enables the biomonitoring of hydrogen peroxide. The EBC sampling technique is non-invasive, rapid and simple [9,10]. It also reduces the number of interfering substances such as blood, urine and sputum compared with more complicated matrices. Methods for the determination of hydrogen peroxide at low levels are based on colorimetric [8,11], chemiluminescent [12] or fluorimetric [4–6,13–15] detection techniques.

The aim of this study was to develop and validate a method for the determination of hydrogen peroxide in EBC, based on a derivatization procedure with HRP or HPAA, followed by fluorescence detection. To increase the sample throughput, a modified flow injection analysis (FIA) was used. The method was used to quantify hydrogen peroxide in 38 subjects, of whom 19 had asthma and 19 did not.

2. Experimental

2.1. Chemicals and reagents

Hydrogen peroxide (30% in water solution) and methanol (high-performance liquid chromatography (HPLC)

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grade) were obtained from Merck (Darmstadt, Germany). Horseradish peroxidase (HRP), *para*-hydroxyphenyl acetic acid (HPAA) and homovanillic acid (HVA) were obtained from Sigma (Steinheim, Germany). Unless otherwise stated, all stock solutions and working standards were prepared in purified water supplied by the Millipore Alpha-Q water system (Millipore, Bedford, MA, USA). The enzyme was stored at −20 °C, and all prepared solutions were kept refrigerated until use.

2.2. Derivatization procedure

Horseradish peroxidase was dissolved to an activity of 2.5 U/ml and HPAA and HVA to a concentration of 1.5 mM. The working solution consisted of a mixture of 400 μl HRP enzyme solution and 1000 μl of either HPAA or HVA substrate. Stock solutions of enzyme and substrate were prepared weekly, and the working solution was prepared daily.

An aliquot of 150 μl EBC was transferred to a 0.6 ml polypropylene vial (Chromacol, Trumbull, CT, USA) with Teflon sealing. The working solution of enzyme and substrate (15 μl) was added to the sample, which was then swirled vigorously at ambient temperature. Standards and samples were derivatized on the same day and were either analyzed directly after sampling or kept on dry ice before storage in a −80 °C freezer for a maximum period of 2 weeks until analysis.

2.3. Analytical system

Measurements of hydrogen peroxide in EBC were taken with a modified flow injection analysis (FIA) system equipped with a PU-980 pump and an FP-920 fluorescence detector (Jasco Corporation, Tokyo, Japan). To avoid air bubbles in the optical cell, a stainless steel capillary with a diameter of 0.5 mm was introduced after the detector in order to increase back pressure. The excitation and emission wavelengths were 285 and 400 nm, respectively. The mobile phase consisted of water-methanol (56:44, v/v) and had a flow-rate of 1 ml/min at ambient temperature.

An ASTED 233XL laboratory robot equipped with a 30 μl Rheodyne 7010 injection valve (Gilson, Villiers-le-Bel, France) was utilized to inject samples into the flow stream. The injection volume was 30 μl. Peak heights were registered with HPChemstation software, version A.06.03 (Agilent Technologies, Palo Alto, CA, USA).

2.4. Evaluation of the assay

Determination of hydrogen peroxide in EBC samples was accomplished with a three-point calibration curve based on one blank and three measurements at higher concentration levels (200, 500 and 1000 nM hydrogen peroxide dissolved in purified water). Standards were prepared each sampling day and were treated as authentic EBC samples.

The possibility of using water instead of EBC as a matrix for the calibration curves was investigated. This was performed by comparing regression equations based on standard addition as well as on a standard curve. Standards were prepared by spiking either EBC or purified water to yield concentrations of 50, 100, 150, 200, 250, 300 and 5000 nM hydrogen peroxide.

2.5. Study population

Exhaled breath condensate was collected from patients with physician-diagnosed asthma (n = 19) visiting the outpatient clinic at the Department of Respiratory Medicine and Allergology, Sahlgrenska University Hospital (Göteborg, Sweden). The patient group was divided into two subgroups of patients with and without current asthmatic symptoms. Respiratorily healthy members of our staff (n = 9) and patients visiting the outpatient clinic for non-respiratory conditions (e.g. gastroenterological allergies or eczemas) (n = 10) were used as controls (n = 19). Exhaled breath condensate from members of our staff was used for method development and validation.

The study was approved by the Ethics Committee of Göteborg University, Göteborg, and all participants gave their informed consent prior to being included in the study.

2.6. Sample collection

Exhaled breath condensate samples were collected with an EcoScreen breath condenser purchased from Jaeger (Würtzberg, Germany). All subjects were asked to rinse their mouths with purified water for 30 s prior to collection. A nose clip was used to prevent nose breathing during sampling. All participants in the study were asked to breathe tidally with normal frequency for 4 min (study population) and for 10 min (method validation). The breath condenser included a polypropylene mouthpiece attached to a two-way non-rebreathing valve, also made of polypropylene, which was connected to Teflon-coated aluminum condensor tube. The EBC was collected in a polyoxymethylene sample container connected to the tube. A saliva trap was also connected in order to avoid contamination from hydrogen peroxide in saliva. Salivary contamination was not checked by amylase determination, but according to Gessner et al. EBC samples do not exhibit amylase activity [16]. The temperature in the tube was between −25 and −20 °C. Prior to gravimetrical determination of the volume, the collected sample was centrifuged at 1200 rpm for 5 min. Sampling was performed both in the morning and in the afternoon, and was followed by immediate addition of the enzyme–substrate mixture and freezing of the treated samples. Duration from the end of sampling until the derivatized sample was frozen was 10 min when HPAA substrate was used and 70 min when HVA substrate was used. For evaluation of storage conditions, a −20 °C and a −80 °C freezer were used.

2.7. Statistical evaluation

Evaluation of the effect of the sample matrix was performed with a paired samples *t*-test. Coefficients of variation (CV) were used for the evaluation of precision. The non-parametric Kruskal–Wallis test was used for comparing groups within the study population. All methods are included in SAS software package, version 8.0 (SAS Institute, Cary, NC, USA). Mean values and *P*-values are presented. A *P*-value < 0.05 was considered significant.

3. Results and discussion

3.1. Choice of substrate

Hydrogen peroxide lacks a chromophore and cannot be determined by fluorescence detection without a derivatization procedure.This procedure involves an enzymatic reaction with horseradish peroxidase and a substrate, either HVA or HPAA [17–20]. The formation of the precursor to the fluorescent compound is due to a reduction–oxidation reaction between hydrogen peroxide and the enzyme. These highly reactive precursors then dimerise to yield a highly fluorescent end product, Fig. 1.

Heinmöller et al. evaluated the selectivity of HRP for hydrogen peroxide and higher *n*-alkyl hydroperoxides by reversed-phase chromatography and fluorescence detection. They found HRP in combination with the substrate HPAA to be almost selective to hydrogen peroxide and higher *n*-alkyl hydroperoxides [21]. Furthermore, to our knowledge the presence of higher hydroperoxides in EBC has not previously been investigated.

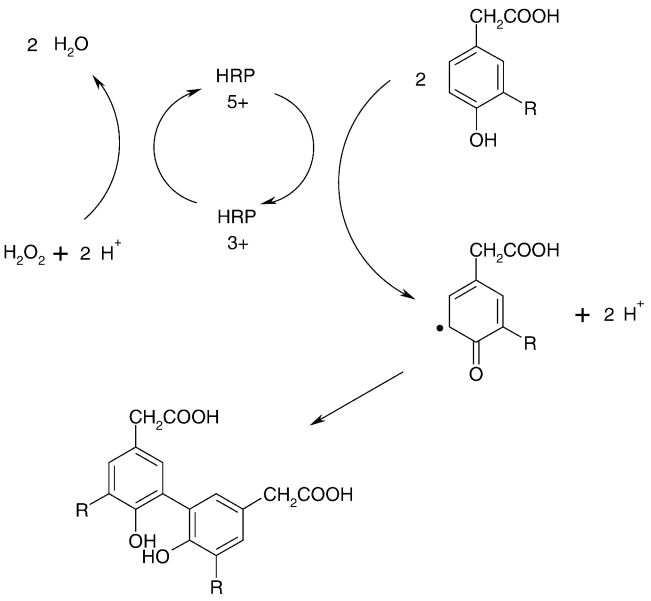

Fig. 1. Chemical formulae of reagents and reaction products: R—OCH_3 (homovanillic acid); R—H (*para*-hydroxyphenylacetic acid).

We found HPAA to be a more suitable substrate than HVA as the reproducibility of the assay of an 800 nM hydrogen peroxide standard solution was higher with HPAA than with HVA (4% versus 8% CV, $n = 5$). Another advantage to using HPAA is that the derivatization procedure required to form the fluorescent reaction product is less time consuming. The reaction is rapid with HPAA but requires 60 min of incubation with HVA [14]. Thus, the incubation time for the HPAA substrate was studied in order to assure that the derivatization reaction was complete. Aliquots of a hydrogen peroxide standard were injected into the analytical system every 5 min after addition of the enzyme–substrate mixture until 30 min had passed. From the first to the last injection, there was no significant change in the fluorescence signal. Therefore, the reaction was considered to be complete within 5 min and the derivative could be placed in an autoinjector at ambient temperature without loss of hydrogen peroxide. Consequently, HPAA was used throughout the study.

3.2. Flow injection analysis

The typical recorder output of the derivatization product, dimer 2,2′-dihydroxybiphenyl-5,5′diacetate, is shown in Fig. 2. Because of peak tailing, we used peak height for quantification instead of peak area. However, a recent study that compared hydrogen peroxide levels using both peak height and peak area found no significant difference in detection limits [15]. The analytical runtime was less than 2 min.

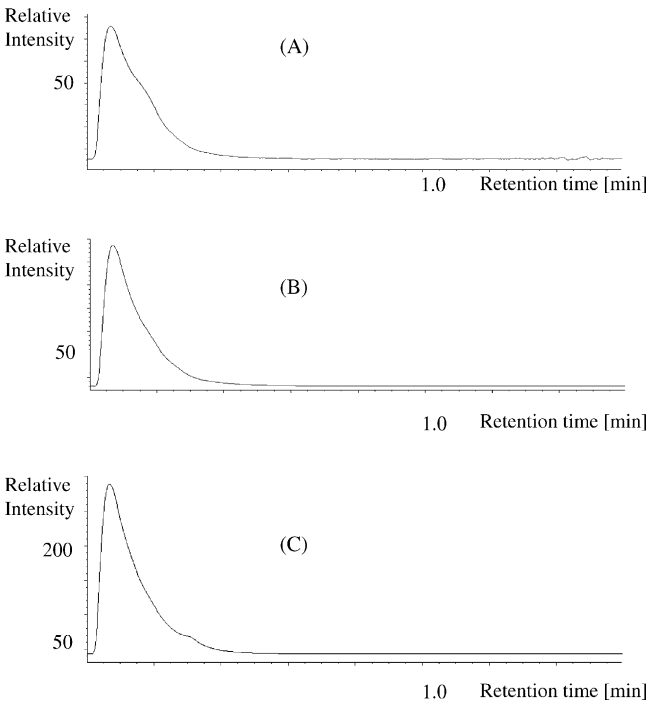

Fig. 2. Flow injection analysis of the hydrogen peroxide derivatization product dimer 2,2′-dihydroxybiphenyl-5,5′diacetate. (A) Blank based on purified water; (B) 200 nM hydrogen peroxide standard based on purified water; (C) authentic exhaled breath condensate (EBC) sample containing hydrogen peroxide (285 nM).

Table 1
Influence of standard addition to a pooled sample. Comparison of concentrations of hydrogen peroxide calculated by (A) calibrator diluted in purified water and (B) standard addition of calibrator to pooled exhaled breath condensate (EBC)

Spiked H_2O_2 (nM)	H_2O_2 determined by standard addition (nM)	H_2O_2 determined by standard curve (nM)
50	40	40
100	100	90
150	140	130
200	200	180
250	270	270
300	320	360
500	510	550

3.3. Method validation

3.3.1. Effect of sample matrix

To determine authentic EBC samples, calibration curves can be prepared either with an EBC matrix or with purified water. Standard addition curves are more time consuming to prepare than standard curves as it is necessary to collect extra EBC material for calibration. Therefore, the calibration curves based on the two different matrices were compared. Since HRP enzyme activity is dependent on many factors, including temperature and storage time, it is necessary to prepare new standards for calibration each day. In the present study, samples and standards were treated the same way, with the same enzyme–substrate mixture used for both samples and standards. The standard addition curve was prepared with pooled EBC, while the standard curve was based on purified water. Hydrogen peroxide was spiked at six concentration levels for both matrices, in duplicates, and the resulting hydrogen peroxide concentration was calculated. As the difference in resulting hydrogen peroxide levels between the calibration modes was not significant at the 95% confidence interval (see Table 1), the calibration curves used in the present work were based on purified water in order to simplify calibration.

3.3.2. Linearity and detection limits

Linearity was assessed by duplicate injections of hydrogen peroxide standards including six concentration levels, ranging from blank to 5000 nM. The calibration curve was linear (R^2 = 0.9988) over the studied range. The limit of detection (LOD) was 40 nM and was calculated from 10 separate blank measurements using the equation LOD = 3.3 × S.D./S, where S.D. is the standard deviation of the blanks and S is the slope of the calibration curve. The limit of quantification (LOQ) was calculated as LOQ = 10 × S.D./S and determined to be 130 nM. Recently, another study showed a detection limit of 20 nM using a fluorimetric method and flow injection analysis [15]. The detection limit of the present investigation was somewhat higher, but well below the levels of non-asthmatic subjects (480 nM, n = 19), as shown in Table 2.

Table 2
Mean values and standard deviations of hydrogen peroxide in asthmatic and non-asthmatic patients and in healthy controls

Group	n	Hydrogen peroxide (nM)
Healthy controls	9	510 ± 260
Patients with non-respiratory conditions	10	460 ± 370
Total without asthma[a]	19	480 ± 320
Asthmatic patients with symptoms	9	850 ± 570
Asthmatic patients without symptoms	10	720 ± 320
Total with asthma[a]	19	780 ± 430

[a] P = 0.03 when comparing hydrogen peroxide levels 'Total with asthma' with 'Total without asthma'.

3.3.3. Precision

Precision of the developed method was determined by analysis of standards containing 500, 1500 and 4500 nM hydrogen peroxide. By injecting three different replicates of each concentration on the same day, we were able to assess within-day variation. By injecting three more standard samples of each concentration on a subsequent day (using a new calibration curve) we could also determine between-day variation. The within-day variation was found to be 1–3% CV at different concentrations, while the between-day variation was determined to be 2–5% CV.

3.4. Stability of frozen samples

In order to determine the stability of EBC samples during storage, samples were derivatized and stored together with derivatized standards in either −20 or −80 °C freezers. Samples were stored in 0.6 ml polypropylene tubes with Teflon sealing, and analyzed after 1, 2 and 4 weeks of storage. The concentration of hydrogen peroxide levels in authentic samples stored at −80 °C increased (0.5%, n = 13) over 4 weeks' storage. This insignificant increase may have been due to random errors. In contrast, 4 weeks' storage at −20 °C resulted in a nearly 10% decrease in the concentration of hydrogen peroxide levels (n = 9). Consequently, we recommend storage at −80 °C.

3.5. Hydrogen peroxide in asthmatic patients

The analytical method was used to screen 19 asthmatic patients and 10 patients with non-respiratory conditions visiting the Department of Respiratory Medicine and Allergology, Sahlgrenska University Hospital, as well as nine healthy controls. The collected volume of EBC in the studied group was on average 530 ± 130 μl for sampling over 4 min. It was possible to discriminate between asthmatic patients, non-asthmatic patients and non-asthmatic healthy controls (780 versus 480 nM, P = 0.03), as shown in Table 2. The levels obtained from the asthmatic patients with symptoms were higher than those obtained from asthmatic patients without symptoms. This may be explained by the fact that asthmatic patients take anti-inflammatory medication such as

corticosteroids to avoid symptoms, which decreases their levels of hydrogen peroxide [22]. The results obtained in the present study are higher than those reported by Emelyanov et al. [8]. With a colorimetric assay for 70 asthmatics and 17 healthy controls, the levels obtained in that study were 127 nM versus 24 nM [8]. This difference in concentration levels may be explained by differences in sampling. We used a commercially available breath condenser designed for tidal breathing at normal frequency, with a short sampling duration, as hydrogen peroxide is a volatile compound. The variation within our studied groups may be explained by diurnal variation as sampling was performed both in the morning and in the afternoon [20]. The influence of expiratory flow-rate on hydrogen peroxide concentrations is also a source of variation [23]. A further source of variation might be the dilution of biomarkers by water vapour in EBC during sampling [24,25]. However, another study of 50 healthy subjects, in which HVA was used as a substrate instead of HPAA, obtained a mean hydrogen peroxide level of 450 nM, which is similar to our results [20].

4. Conclusion

A method for the determination of hydrogen peroxide in EBC has been developed and validated. The sampling is non-invasive and rapid, and the analytical method proved to be sensitive, accurate and easy to perform. With this method, high-throughput analyses can be achieved.

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