An efficient and reproducible method for measuring hydrogen peroxide in exhaled breath condensate

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Abstract
We investigated the sensitivity and reproducibility of a test procedure for measuring hydrogen peroxide (H₂O₂) in exhaled breath condensate and the effect of storage of the condensate on the H₂O₂ concentration, and compared the results to previous studies. Twenty stable COPD patients breathed into our collecting device twice for a period of 10 min. The total exhaled air volume (EAV) and condensate volume were measured both times and the H₂O₂ concentration of the condensate was determined fluorimetrically. The concentration was measured again after freezing the reaction product at −70 °C for a period of 10, 20 and 40 days. We collected 2–5 ml condensate in 10 min. The EAV and condensate volumes were strongly correlated. There was no significant difference between the mean H₂O₂ concentration of the first and second test. We obtained a detection limit for the H₂O₂ concentration of 0·02 μmol l⁻¹. The H₂O₂ concentration appeared to remain stable for a period up to 40 days of freezing. Compared to previous studies we developed a more efficient breath condensate collecting device and obtained a lower H₂O₂ detection limit. The measurement of exhaled H₂O₂ was reproducible. In addition, storage of the samples up to 40 days showed no changes in H₂O₂ concentration.

INTRODUCTION

Oxidative stress plays an important role in the development of chronic obstructive pulmonary disease (COPD). Oxidants may damage different cells and enzyme systems in the lungs (1,2). Oxidative stress is increased in COPD patients compared to healthy subjects, due to (increased) exposure to exogenous oxidants such as inhaled cigarette smoke and air pollution, or enhanced endogenous production of hydrogen peroxide (H₂O₂) due to neutrophilic inflammation and due to a relatively impaired antioxidant system (3,4).

The degree of oxidative stress can be determined by measuring the H₂O₂ concentration in exhaled breath condensate. This is a relatively easy and non-invasive method and reflects the oxidative status in the lungs of these patients. However, there are several methodological issues at present, which limits its application in large and prospective trials.

Firstly, there is no standardized method for collecting breath condensate. The various collecting devices described in literature collect variable amounts of breath condensate, ranging from 1 to 5 ml in 15–20 min (3–13). For the analysis at least 1 ml is needed. If a more efficient collecting device could be found, the collecting time could be considerably reduced, which is less inconvenient for the patient. As such an efficient collecting device is a prerequisite.

Secondly, the H₂O₂ detection limit varies from study to study; most investigators achieved a detection limit around 0·1 μmol l⁻¹. It is important to obtain a low detection limit because the H₂O₂ concentrations measured in previous studies were low (< 1·0 μmol l⁻¹) in selected patient groups as well as in healthy control groups. In a number of cases this was even below the detection limit (3–13). We have tried to lower the detection limit.

Thirdly, there are limited findings on the stability of H₂O₂ concentration during storage of the breath condensate before analysis. The period of storage by freezing the breath condensate varies from 6 h up to 1 month (3–6). Detailed data on the actual stability of H₂O₂ concentrations are not provided in most studies. The advantage of storage of the breath condensates is that...
condensates need not be analysed immediately, which is a time-consuming procedure. Furthermore all measurements can be performed using one calibration curve, which is more reliable.

In view of the above mentioned problems, we conducted this study with the following research questions. Firstly, can we adapt the breath condensate collecting devices described in literature in order to develop a more efficient device? Secondly, are we able to improve the existing analytical methods to establish a lower detection limit? Thirdly, is the procedure reproducible in a group of COPD patients? Finally, is the H$_2$O$_2$ concentration influenced by freezing the condensate–reagent mixture at $-70\, ^\circ\mathrm{C}$?

**MATERIALS AND METHODS**

**Patients**

We performed this study in a population of COPD patients, because in most healthy persons the H$_2$O$_2$ concentration is below the H$_2$O$_2$ detection limit. The H$_2$O$_2$ concentration is significantly higher in COPD patients (3,13). Twenty stable COPD patients were recruited from the outpatient clinic of a large non-academic teaching hospital. Inclusion criteria were: (1) a diagnosis of COPD according to the ERS criteria (14); (2) smokers or ex-smokers with at least 10 pack-years; (3) age between 40 and 85 years; (4) a forced expiratory volume in 1 sec (FEV$_1$) < 60% of predicted and FEV$_1$ vital capacity (VC) ratio < 60%, with a reversibility of less than 12% of predicted after taking a bronchodilator. Patients used their own bronchodilators, but any inhaled corticosteroids and/or acetylcystein were discontinued for at least 4 weeks prior to the study. The study was approved by the local ethics committee. All patients gave informed consent.

Twenty stable COPD patients (19 male, mean age 65 years, mean FEV$_1$ 48% predicted) from the outpatient clinic were included in the study. Eight patients were current smokers and 12 were ex-smokers. Patient characteristics are shown in Table I.

**Collecting device**

The collecting device consisted of a mouthpiece connected to a valve system. The expiration valve was connected to the top of a 0.4 m high glass bulb cooler with an inside air volume of 155 ml, which was constantly cooled by a reverse stream of ice water (0 °C). At the base of the bulb cooler the condensate is collected in a glass container. To the side opening of the T-glass tube at the base of the bulb cooler, an air volume meter (Ohmeda type 5410) is attached. To avoid condensation in the valve system, the system is heated by a fan producing a diverging stream of hot air with a minimum temperature of 42 °C.

**Table I.** Characteristics of study population (n=20)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years, mean ± sd)</td>
<td>65 ± 6.8</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>19/1</td>
</tr>
<tr>
<td>Smoking status (smoker/ex-smoker)</td>
<td>8/12</td>
</tr>
<tr>
<td>Pack-years (PY, mean ± sd)</td>
<td>39 ± 12.5</td>
</tr>
<tr>
<td>FEV$_1$ (% of predicted, mean ± sd)</td>
<td>48 ± 7.25</td>
</tr>
<tr>
<td>FEV$_1$/FVC (%, mean ± sd)</td>
<td>49 ± 8</td>
</tr>
</tbody>
</table>

**Fig. 1.** Collecting device: a mouthpiece is connected to a valve system and the expiration valve is connected to the top of a 0.4 m high glass bulb cooler, which is constantly cooled by a reverse stream of ice water (0 °C). At the base of the bulb cooler the condensate is collected in a glass container. To the side opening of the T-glass tube at the base of the bulb cooler, an air volume meter (Ohmeda type 5410) is attached. To avoid condensation in the valve system, the system is heated by a fan producing a diverging stream of hot air with a minimum temperature of 42 °C.
obtained breath condensate and change the measured H$_2$O$_2$ concentration, we also investigated this effect by performing the following tests.

The bulbcooler was flushed with physiologic saline (0.9%) before performing a breath test. The condensate was obtained in fractions of 0.3 ml and the sodium concentration of these fractions was measured by means of a selective sodium electrode. Using this method we could determine how much condensate had to be collected in order to wash out all traces of the saline left behind on the glass wall after flushing the bulbcooler with saline. By comparing the subsequent sodium concentrations, it was affirmed that the waterfilm on the inside wall of the glass bulbcooler was replaced for 95% after the formation of 1.8 ml of condensate during the breath test. The actual collected condensate volumes varied in our patients from 1.85 to 4.75 ml. This meant that the waterfilm, formed after flushing the cooler, was almost completely replaced by breath condensate at the end of each test and that the H$_2$O$_2$ concentration of the condensate had to be corrected for the dilution with the volume of the waterfilm, which was formed by flushing the cooler before each test.

To determine the degree of dilution, the volume of the waterfilm (a ml) formed in the cooler after flushing had to be measured. We determined this by flushing the cooler again with a NaCl containing solution (1500 mmol l$^{-1}$), followed by flushing the cooler with a fixed amount of distilled water (5 ml). Subsequently, the sodium concentration of the solution collected at the base of the bulbcooler was measured (b mmol l$^{-1}$) and compared to the initial concentration of the NaCl. The volume of the film in the bulbcooler could be calculated by: 

$$a = b \times 5/1500.$$ 

With this method the volume of the waterfilm on the inside of the bulbcooler was determined to be 0.35 ml. This was used for correcting the H$_2$O$_2$ concentration of the breath condensates (condensate volume c + 0.35/c × measured H$_2$O$_2$ concentration).

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The condensate of three random patients was checked for amylase in order to exclude contamination of the breath condensate by saliva. An enzymatic colorimetric assay for the quantitative determination of $\alpha$-amylase in human serum, plasma and urine, according to the recommendations of the IFCC, was used.

After collection of the condensate, its volume was measured. Subsequently, to 250 $\mu$l of breath condensate, 10 $\mu$l p-hydroxyphenylacetic acid and 10 $\mu$l 15 U l$^{-1}$ horse-radish peroxidase were added within 30 min after collection (15).

The fluorescence of the reaction product (dimer 2,2'-dihydroxydiphenyl-5,5'-diacetate) was measured with an automated sampler, flow injection and scanning fluorescence detector from Waters, Millipore Corporation (Milford, MA, U.S.A.), with an excitation wave length of 295 nm and an emission wave length of 405 nm. The reaction product was injected into the fluorimeter which produced a curve and the fluorescence was presented by the area under the curve. Besides a blank measurement, several standard concentrations of H$_2$O$_2$ were measured, using concentrations of up to 3·0·$\mu$mol l$^{-1}$. Additionally, the detection limit was determined by measuring several times the standard H$_2$O$_2$ concentration of 0·0·$\mu$mol l$^{-1}$ and calculating the mean and standard deviation of the samples, with the detection limit being 3 × σ. Each collected condensate was analysed in duplicate and mean values were used for further analysis.

To determine the specificity of the assay, in a pilot study of 17 condensate samples, catalase was added to convert H$_2$O$_2$ to water, and the H$_2$O$_2$ concentration was measured again.

**Procedure of the breath test**

All patients performed the breath tests in the morning, after they had taken their medication. They refrained from smoking after midnight of the preceding day, since cigarette smoke contains oxidants. Before the test, patients rinsed their mouth with tap water to avoid contamination of the breath condensate. They breathed in the collecting device twice for a period of 10 min, while wearing a nose-clip. After both tests the condensate volumes and total exhaled volume (EAV) were measured.

To test the reproducibility of the whole procedure, the H$_2$O$_2$ concentration measured during the first test was compared to the concentration measured during the second test.

**Analysis of H$_2$O$_2$ in the condensate**

P-hydroxyphenylacetic acid 1·0·mmol l$^{-1}$ from Fluka AG (Bormen, Belgium) was used for the analysis, together with horse radish peroxidase 15 U ml$^{-1}$ and catalase 160 U ml$^{-1}$ from Sigma Chemical Co (St Louis, MO, U.S.A.). Distilled water TKF7114 was obtained from Baxter BV (Utrecht, The Netherlands) and the hydrogen peroxide 1072091000 from Merck KgaA (Darmstadt, Germany). Several condensate-reagent mixtures were frozen at $-70$°C. In a pilot study of 34 condensates, the stability of the condensate-reagent mixture was tested (the condensates were also collected in stable COPD patients not included in the study population). The fluorescence of the reaction product was measured directly and 10, 20 and 40 days after storage at $-70$°C.
Statistical methods

Results are expressed as mean ± SEM. The paired student t-test was used to compare the results obtained from the first and second test, with \( \alpha = 0.05 \). The relation between the mean values of the first and second test and the difference between the two tests was presented in a Bland–Altman plot. The relation between the EAV and condensate volume was expressed as correlation coefficient. The mean \( \text{H}_2\text{O}_2 \) concentration of the samples measured at day 0 was compared with the concentration at day 10, day 20 and day 40 using the paired student t-test (\( \alpha < 0.05 \)).

RESULTS

Collecting device

The mean condensate volume collected in 10 min was 2.8 ± 0.14 ml after the first test and 3.2 ± 0.15 ml after the second test. The volume was significantly higher the second time (\( P < 0.01 \)). Three condensates were checked for the presence of amylase; no amylase was found.

Validation of the analysis of \( \text{H}_2\text{O}_2 \) in the condensate

A calibration curve was constructed, with a range up to 3.0 \( \mu \text{mol l}^{-1} \) (Fig. 2). The detection limit was determined to be 0.02 \( \mu \text{mol l}^{-1} \). After adding catalase to 17 separate condensates, the mean \( \text{H}_2\text{O}_2 \) concentration decreased from 0.25 \( \mu \text{mol l}^{-1} \) to below the detection limit.

Reproducibility of the breath test

The \( \text{H}_2\text{O}_2 \) concentration measured during the first breath test was 0.22 ± 0.03 \( \mu \text{mol l}^{-1} \) and during the second test 0.21 ± 0.03 \( \mu \text{mol l}^{-1} \). There was no significant difference between both measurements (\( P = 0.7 \)). The reproducibility is also shown in a Bland–Altman plot (Fig. 3). There was no significant difference in \( \text{H}_2\text{O}_2 \) concentration between current smokers and ex-smokers.

The mean EAV was 108.66 ± 58 l after the first test and 103.4 ± 6.32 l after the second test. The difference was significant (\( P = 0.04 \)). The correlation (\( r \)) between the EAV and the volume of the collected condensate was 0.90 (\( P < 0.01 \)) during the first test and 0.95 (\( P < 0.01 \)) during the second test (Fig. 4).
Storage of the reaction product

The H$_2$O$_2$ concentration in 15 frozen reaction products was measured immediately and after a period of 10 days [Fig. 5(a)]. Twelve reaction products were measured directly and 20 days later [Fig. 5(b)], and seven were repeated after 40 days [Fig. 5(c)]. The H$_2$O$_2$ concentration appeared to remain stable over all these time periods. There was no significant difference in mean H$_2$O$_2$ concentration between day 0 (0.44 ± 0.19 μmol l$^{-1}$) and day 10 (0.43 ± 0.18 μmol l$^{-1}$; $P = 0.56$), on day 0 (0.48 ± 0.13 μmol l$^{-1}$) and day 20 (0.47 ± 0.14 μmol l$^{-1}$), or day 0 (0.39 ± 0.16 μmol l$^{-1}$) and day 40 (0.38 ± 0.17 μmol l$^{-1}$).

**DISCUSSION**

The present study shows that the collecting device used in this procedure was able to collect a sufficient amount of condensate within a smaller time period. A lower detection limit for measuring H$_2$O$_2$ concentration in breath condensate was obtained compared to other studies. The reproducibility of the whole procedure was acceptable; there was no significant difference in H$_2$O$_2$ concentration between tests 1 and 2. Finally, the H$_2$O$_2$ concentration did not appear to be influenced by storage of the reaction product at -70°C for a period up to 40 days.

Initially, we used other constructions for collecting breath condensate that have been described in literature (3,4,7). A condense chamber and subsequently a sphere were cooled with solid carbon dioxide, but the volume of the obtained condensate was very variable. This was probably caused by the inability to establish sufficient contact time between the exhaled air and the cooling system. Therefore the amount of condensate collected was never more than 1 ml after 15 min of breathing. Antczak (9) and Nowak (4) used a glass tube cooled by a package of ice and were able to collect 2–5 ml condensate in 20 min. With our current device we collected 2–5 ml condensate in only 10 min by means of a glass bulb-cooler cooled by a reverse stream of ice water, which leads to better condensation.

In accordance with other investigators the patients were instructed to rinse their mouth before each breath test to avoid contamination of the breath condensate by saliva. Unlike others, we did not use a separate saliva trap, because the device was constructed in such a way that the exhaled air went up through the valve system before entering the cooled bulb-cooler. Indeed, no amylase was found.

Recently, Schleiss *et al.* showed that exhaled H$_2$O$_2$ depends on expiratory flow rates (18). In our study we did not control expiratory flow rates. There was a significant, but small difference in EAV between the first (108 ± 61) and second test (103 ± 61). The mean flow rate during the first test was 181 ml sec$^{-1}$ and during the second test was 171 ml sec$^{-1}$. However, H$_2$O$_2$ concentrations were similar in the two tests (0.21 and 0.22 μmol l$^{-1}$, respectively).

To ensure that no H$_2$O$_2$ was left behind from the previous patients, the bulb-cooler was cleaned before and after each breath test. We cleaned the cooler with distilled water.

No tap water was used in order to avoid contamination with particles that could influence the fluorescence. Flushing the cooler with distilled water before each test caused dilution of the obtained condensate by distilled water from the waterfilm, so we corrected the H$_2$O$_2$ concentration for this dilution. The correcting factor could have been avoided by heating the collecting device to make all H$_2$O$_2$ disappear. However, the heating and
cooling down of the collecting device would be time consuming and thus unpractical, since we wanted to measure several patients on the same day with an interval of only 30 min.

Looking at the method of analysis described in literature to determine the H₂O₂ concentration in the breath condensate, two methods have been used in general; the spectrophotometrical method according to Gallati and Pracht (16) and the fluorimetal method according to Hyslop and Sklar (15) or Ruch (17). Sznajder (5), Loukides et al. (16) and Pracht (16) and the fluorimetal method according to Gallati and condensate, two methods have been used in general; the spectrophotometrical method according to Gallati and Pracht (16) and the fluorimetal method according to Hyslop and Sklar, and were able to establish a detection limit of 0·1 μmol l⁻¹. Others, using a fluorimetal method, found detection limits of 0·083–0·1 μmol l⁻¹ (4, 6, 8,9). We performed the analysis of the breath condensate according to Hyslop and Sklar, and were able to establish a detection limit of 0·02 μmol l⁻¹.

This is lower than the detection limits described by others using the same method. This could be explained by the fact that the fluorimeter reads the degree of fluorescence of the condensate—reagens mixture and this is converted into a concentration. Usually a cuvet containing the condensate—reagens mixture is placed into the fluorimeter. We chose to use flow injection of the mixture with fluorimetal detection instead, which may be more sensitive (although comparative data are lacking).

Our test procedure for measuring exhaled H₂O₂ concentration proved to be reproducible. There was no significant difference between the H₂O₂ concentration in the first and second test. When we consider the Bland–Altman plot of these tests, all but one patient showed a difference between the two tests of less than 2 SD from the mean difference. In this patient the relatively great difference might be explained by a delay between the collection of the condensate and its analysis.

In this study we found a mean H₂O₂ concentration of 0·21 μmol l⁻¹ in stable COPD patients. Dekhuijzen et al. found a similar mean H₂O₂ concentration in stable COPD patients (3). The concentration measured by Nowak et al. was somewhat higher: 0·48 μmol l⁻¹ (4). Other investigators measured the H₂O₂ concentration in asthma patients; in these patients the H₂O₂ concentration varied from 0·6 to 0·8 μmol l⁻¹ (6, 9, 12).

There was a difference in EAV and condensate volume during the first and second test, which can be explained by variation within the patients themselves. It proved to be difficult to perform several breath tests with exactly the same tidal volume and frequency. The mean EAV was lower after the second test, while the condensate volume was higher. A possible explanation is that different breathing patterns cause different V₀/V₉ ratios (dead space volume/tidal volume). The humidity of the V₀ is lower than that of the V₉, which could lead to a difference in humidity of the exhaled air. However, EAV and condensate volume were strongly correlated both times.

Finally, storage of the reaction product for at least 10 days at −70 °C was possible without any changes in H₂O₂ concentration and appeared to have virtually no influence on the measured H₂O₂ concentration up to 40 days (Fig. 4). Others have studied the possibilities of storage of the breath condensate and found that the H₂O₂ concentration in the condensate remained stable for only a few hours to several days. This could be explained by the fact that they froze the breath condensate instead of the reaction product, as we did in the present study.

In conclusion, we were able to develop a more efficient breath condensate collecting device, by adapting collecting devices described in literature. By adapting the fluorimetal method of analysis we could lower the detection limit. The test procedure proved to be reproducible and easy to perform. In addition, the H₂O₂ concentration after storage of the breath condensate appeared to remain stable for a period up to 40 days. These findings may facilitate the application of this method in future prospective and multi-centre studies.

Acknowledgements

This study was sponsored by 3M Pharmaceuticals. The authors would like to thank Peter Brands for his help with the construction of the collecting device. We would also like to thank Theo de Boo, MSc (Department of Medical Statistics, University Medical Centre, Nijmegen, The Netherlands) for his help with the statistical procedures. Finally we thank the research nurses of the Catharina Hospital, Marit van Albada and Jeannette Bertram, for their help and support.

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