Title: Validation of Assays for Inflammatory Mediators in Exhaled Breath Condensate

Short title: Validation of Assays in Exhaled Breath Condensate

Keywords: Exhaled Breath Condensate, Interleukin 8, Leukotriene B₄, Secretory leukoprotease inhibitor, Alpha-1-Antitrypsin, Myeloperoxidase.

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Abstract

The use of exhaled breath condensate as a tool for non-invasive assessment of lung inflammation is becoming commonplace. Many authors use commercial ELISA kits to measure inflammatory mediators within EBC. However, the very low concentrations of mediators within EBC are often below the commercially validated concentration range of the relevant ELISA and crucially the linear part of the sigmoid standard curve. This study seeks to validate a series of assays for use in EBC and to compare the results in EBC with those from matched sol-phase sputum samples.

The following mediators: Leukotriene B₄, interleukin 8, secretory leukoprotease inhibitor, alpha-1-antitrypsin were measured by ELISA. Myeloperoxidase was measured by chromogenic substrate assay.

Mediator concentrations reached lower limit of quantification in only one assay (alpha-1-antitrypsin) in 19.6% of subjects, whilst mediator concentrations reached lower limit of detection in three assays (leukotriene B₄, interleukin 8 and alpha-1-antitrypsin in 31%, 6.5% and 61% of subjects respectively). No significant correlations were present between any mediators in exhaled breath condensate and sol-phase sputum.

These results indicate that care must be exercised when interpreting mediator measurements in exhaled breath condensate and that assays must be validated at concentrations relevant to those found within the biological fluid.
Introduction

Recently, there has been widespread interest in the use of exhaled breath condensate (EBC) as a fully non-invasive methodology for the assessment of inflammation in a variety of lung diseases including chronic obstructive pulmonary disease (COPD), cystic fibrosis, bronchiectasis, primary ciliary diskinesia and asthma. Analysis of EBC has used a variety of sensitive methods including ELISA [1], multiplex ELISAs [1] and more recently mass spectroscopy [2].

However, most investigators have used commercial ELISAs to determine mediator concentrations as they have the advantage of easy availability, simple methodologies, good reproducibility and come “ready validated”. Assay validation relates to a number of performance criteria such as lower limit of detection (LLD), reproducibility as determined by the coefficient of variation (both intra and inter-assay), linearity, spike return, performance in the media being assessed and specificity. However, commercial validation procedures do not describe the performance aspects of the assay within the relevant biological samples for differing disease states. Furthermore, it does not define lower limit of quantification (LLQ), which will also vary according to the biological fluid, and the research requirements. The very low mediator concentrations commonly reported in EBC may therefore require a sensitivity not appreciated in the validation of a conventional commercial ELISA. This is specifically important as the conventional validation of ELISA reproducibility is often only undertaken at concentrations on the linear part of the typical
sigmoid curve for mediator concentration to signal. The variability of measurements increases greatly outside this linear portion and is particularly relevant for measurements at or around the LLD.

A variety of inflammatory mediators have previously been assessed in EBC in a range of lung diseases. Most interest has been focused upon the eicoinasoid leukotriene B₄ (LTB₄); typically measured using a commercial ELISA from Cayman Chemical Company (Ann Arbor, MI USA). Concentrations reported in stable COPD range from a median value of 10.6 pg/ml [3] to 100.6 pg/ml [1]. Izqueirdo et al [4] reported LTB₄ concentrations at 1.1 pg/ml and below, despite suggesting that the samples below the LLD were recorded as undetectable (the LLD specified by the manufacturers for this assay is 13 pg/ml). Little data is available concerning assay validation at these low levels although Leung et al [5] has suggested that the assay is not reproducible at concentrations found in EBC. Measurement of LTB₄ by mass spectroscopy found that LTB₄ could be detected in patients with asthma who were not on therapy (LLQ given as lowest calibrator; 100pg/ml). Conversely, Panchaud et al [3] suggest that the calibration curve for the assay is linear between 165 pg/ml and 990 pg/ml and thus quantification should be less robust outside this range.

The assessment of interleukin 8 (IL-8) in EBC has received less attention; Izquierdo et al [4] described lower concentrations of IL-8 in EBC from emphysematous subjects (0.34 pg/ml), compared to chronic bronchitics (2.32 pg/ml) and controls (3.32 pg/ml). Zihlif et al suggested that IL-8
concentrations were undetectable in both children with primary ciliary
dyskinesia and controls [6]. Simpson et al [7] described detectable IL-8 in
smokers but questioned the validity of the ELISA, on the other hand Sack et al
[1] were able to measure IL-8 in EBC using a multiplex system. However,
very little information is available concerning other proteins such as alpha-1-
antitrypsin (AAT), secretory leukoprotease inhibitor (SLPI) or myeloperoxidase
(MPO) or other inflammatory cytokines in EBC.

This study sought to determine if it was possible to validate a series of assays
(Commercial ELISAs LTB₄; (GE Bioscience, Amersham, U.K. and Cayman
Chemical Company Ann Arbor, MI USA.): IL-8 and SLPI (R&D Systems
Europe Ltd, Abingdon, U.K.): an in-house ELISA for AAT and a chromogenic
activity assay for MPO for use in the measurement of mediators in EBC. In
addition, we wished to compare results in EBC with those found in matched
spontaneous sol-phase sputum samples from the same patient on the same
day to determine if the EBC level reflected the results of airway secretions.
Methods

Study subjects

Sixty-one subjects were recruited for the study; 12 patients with bronchiectasis (confirmed on high-resolution CT scan), 19 patients with COPD, and 30 normal subjects with normal spirometry and no history of lung disease. All subjects were assessed in a stable state (no antibiotics or oral corticosteroids for ≥2 months). Twelve of the nineteen COPD patients fulfilled standard criteria for the diagnosis of chronic bronchitis (daily sputum production for at least 3 months of 2 consecutive years) [8]. Patients with underlying immune deficiency, allergic bronchopulmonary aspergillosis or cystic fibrosis were excluded from the study.

Assessment and investigations

Exhaled breath condensate was obtained by the use of an Rtube apparatus (Respiratory Research Charlottesville VA USA) for a collection period of 20 minutes at a temperature of −40°C and was stored at −70°C until analysed. Collection was conducted with reference to the recommendations set out by Harvath et al [9]. Matched spontaneous sputum sol-phase was obtained from 9 of the subjects with COPD and chronic bronchitis and 9 of the subjects with bronchiectasis. Sputum was collected over a four hour period in the morning from rising, into sterile containers. The sputum was ultracentrifuged at
50000×g for 90 min at 4°C and the sol phase was removed and stored at -70°C until analysed.

Following EBC collection, all subjects underwent spirometry with reversibility (twenty minutes before and after inhalation of 400μg of salbutamol.) [10] Post bronchodilator forced expiratory volume in the first second (FEV1) is expressed as percentages of the predicted normal reference values [11].

**Assays on sputum sol phase and Exhaled Breath Condensate**

Mediator quantification was determined by interpolation of the signal from a standard curve of known concentrations. The intra and inter-assay coefficient of variation was determined for a pooled sample of EBC and samples prepared from the mediator standard measured on six occasions. In addition, recovery was determined in both EBC and standard buffer by comparing the assay result with that expected from a known mediator spike [12]. Briefly single samples or pure mediators were assayed on six occasions to obtain the intra-assay coefficient of variation. At this point, a known quantity of the pure mediator was added to the sample, which was re assayed. The result was compared to the standard curve and the new value obtained by interpolation. This second value was subtracted from the predicted value to obtain the proportion “recovered”.

The lower limit of detection (LLD) for an assay was defined as two standard deviations above the mean signal for twenty sample blanks. Lower limit of quantification (LLQ) as defined as the point at which both intra and inter-assay coefficient of variation and spike recovery became acceptable for each assay
(<12%). This concept is demonstrated for the SLPI ELISA in Figure 1. All EBC samples were analysed without dilution.

The methods for LTB₄ (GE Bioscience, Amersham UK), SLPI and MPO have been described in detail previously [12]. The second commercial LTB4 ELISA (Cayman Chemical Company) was used according to the manufacturers instructions.

IL-8 was measured by ELISA using a commercially available kit (R&D Systems Abingdon U.K.) The sol-phase intra-assay coefficient of variation was less than 8.5% throughout the working range of the assay. Sputum samples spiked with IL-8 resulted in greater than 88% recovery.

AAT was measured by an in-house ELISA relative to a commercially available serum standard (The Binding Site Limited, Birmingham, U.K.). In brief, two hundred microlitres of antihuman AAT (The Binding Site, Birmingham, U.K.) in 0.05M sodium carbonate/bicarbonate pH 9.6 was added to a Nunc MAXISORP (Loughborough, U.K.) microtitre plate and incubated overnight (4°C). The plate was then washed three times with PBS containing 1% (v/v) Tween 20 (Sigma-Aldrich Company Limited. Poole, Dorset, U.K) and 0.5%(w/v) dried skimmed milk (PBS-T). Two hundred microlitres of standard or sample were added to the plate followed by a 1h incubation at 37°C. The plate was again washed three times with PBS-T. Two hundred microlitres of antihuman AAT peroxidase conjugate (The Binding Site Limited, Birmingham, U.K.) in PBS-T was then added to each well, and incubated for 60 minutes at
37°C, followed by three more washes with PBS-T. Two hundred microlitres of 3,3',5,5' tetramethylbenzidine ELISA substrate solution (Sigma-Aldrich Company Limited, Poole, Dorset, U.K) was then added to each well followed by an incubation for 10 minutes at 25°C. The reaction was then stopped with 50µl of 0.1M H₂SO₄ acid and the plate was read at 450nm with a 570nm correction and the AAT concentration obtained by interpolation from the standard curve. Sol phase intra-assay coefficient of variation was less than 10.2% throughout the working range of the assay and sputum samples spiked with AAT gave greater than 85% recovery.

Statistics

Statistical analyses were performed using SPSS version 12 (SPSS Inc, Chicago, IL, US). Normally distributed data are expressed as mean (SD), and the patient groups were compared using one way analysis of variance (ANOVA) with Bonferroni correction being used if significant differences were detected. Correlations between matched sol-phase sputum and EBC samples were assessed by Spearman rank correlation. Statistical significance was accepted at p<0.05.
Results

Baseline characteristics

Baseline characteristics are shown in table 1. The mean age (±SD) of COPD and bronchiectasis patients was similar (COPD 67.1±7.6 years; bronchiectasis 67.7±5.3 years; p=1). Normal subjects were significantly younger (50±8 years) than both COPD (p<0.001) and bronchiectasis (p<0.001) groups. Patients with COPD had a significantly lower FEV1% predicted (48.9±14.7%) compared to both normal subjects (107±13.2%; p<0.001) and patients with bronchiectasis (70.3±25.8%; p=0.007). Patients with bronchiectasis had worse FEV1 compared to normal subjects (p<0.001). The majority of COPD patients were either GOLD (Global Initiative for Chronic Obstructive Lung Disease) [13] stage 2 (42.1%) or stage 3 (47.4%). The remaining 2 patients (10.5%) were stage 4. All patients in the bronchiectasis group had an idiopathic disease (no evidence of causative /susceptibility factors such as AAT or immunoglobulin deficiencies, allergic bronchopulmonary aspergillosis, ciliary dyskinesia etc)

Assays on sputum sol phase and Exhaled Breath Condensate

All assays had an intra and inter-assay CV of <15% in the linear part of the standard curve and >85% spike recovery.
The LLD for the LTB_4 ELISA (GE Bioscience, Amersham UK) was 7 pg/ml, which is also consistent with the manufacturer’s value of approximately 6 pg/ml. Quantification was acceptable at 20 pg/ml when the intra-assay CV became less than 12% and spike recovery reached 77%. At values less than 20 pg/ml the intra-assay coefficient of variation ranged from 21.4% at 12 pg/ml to 111.4% at 1.6 pg/ml with spike recoveries of between 81.8% at 12 pg/ml and 12.7% at 1.6 pg/ml. Samples from only nineteen subjects (twelve controls, three COPD and CB, and four bronchiectatic patients) of the total subjects (sixty-one) had values that exceeded the lower limit of detection of the assay. However, no sample values exceeded the LLQ of the assay (Figure 2).

We also assessed a second LTB_4 assay (Cayman Chemical Company), the LLD for this assay was 9.2 pg/ml which is consistent with the value (approximately 13 pg/ml) reported by the manufacturer. Quantification however, only became acceptable at 30 pg/ml where the intra-assay coefficient of variation was less than 12% and spike recovery was 86%. At values less than 20 pg/ml the intra-assay coefficient of variation ranged from 21.4% at 16 pg/ml to 68.6% at 2.5 pg/ml.
Interleukin 8

The LLD for the IL-8 ELISA was consistent with the manufacturer value of approximately 10pg/ml. Quantification became acceptable at 40 pg/ml, where the intra-assay coefficient of variation was less than 12% and spike recovery exceeded 88%. Below 40 pg/ml the intra-assay coefficient of variation ranged from 13.9 % at 30 pg/ml to 90.7% at 8 pg/ml, with spike recoveries of between 57.6% at 31 pg/ml and 6.1% at 3.9 pg/ml. Samples from only four subjects (two controls, one COPD, and one bronchiectatic individual) out of the sixty-one subjects exceeded the lower limit of detection of the assay, but no sample reached the assay LLQ (Figure 2).

SLPI

The LLD for the SLPI ELISA (25.5 pg/ml) was consistent with the manufacturer value of approximately 25 pg/ml. Quantification became acceptable at 100 pg/ml where the intra-assay coefficient of variation was less than 12% and spike recovery was 102.8%. At values less than 100 pg/ml intra-assay coefficient of variation ranged from 10.2 (at 62 pg/ml) to 56.2% (at 8 pg/ml), whilst spike return varied from 78% at 62 pg/ml to –60% at 8 pg/ml. The relationship between the assay CV and the linear part of the ELISA sigmoid curve is shown in Figure 1. No samples from the forty-six subject samples where this was assayed reached either the LLD or the LLQ (Figure 2).
**Alpha-1-Antitrypsin**

The in house AAT ELISA had an LLD of 0.8 ng/ml. Quantification was acceptable at 2 ng/ml, when the intra-assay coefficient of variation was less than 12% and spike recovery exceeded 77%. At values less than 2 ng/ml the intra-assay CV ranged from to 55.5% at 0.5 ng/ml to 119.2% at 0.03 ng/ml, whilst spike recovery ranged from 75.5% at 1.75 ng/ml to 54.4% at 0.2 ng/ml. Forty-six samples were assayed. Samples from twenty-eight of the subjects (thirteen controls, five COPD and CB, six COPD and four bronchiectatic individual) reached the LLD of the assay. Samples from nine subjects (three controls, five COPD with CB and one COPD patient) exceeded the LLQ of the assay (Figure 2).

**MPO**

The LLD for the MPO activity assay was 0.015 units/ml. Quantification became acceptable at 0.05 units/ml at which point intra-assay CV was less than 4.59% and spike recovery was 103.34%. Samples from fifty-two subjects were assayed. No samples reached either LLD or LLQ (Figure 2).

**Correlation with Sputum**

If the issues raised above are not appreciated, interpolation of the signal from the standard dose response curve would provide apparent “values” in the detectable range. For instance the LTB₄ levels when above the LLD would be 11.3 pg/ml on average by interpolation using the linear part of the standard
curve (similar to those reported in the literature). However, even when these derived values were related to the sputum value for the same patient sample there was no significant direct correlation for any mediator (when corrected for the effects of multiple analyses (Table 2)).
Discussion

The collection of EBC is thought to provide a valuable non-invasive technique to measure inflammatory mediators within the airways. However, the assays described here show that extreme care needs to be taken when interpreting mediator results in this fluid. All of the ELISAs described confirm poor reproducibility for values derived from below the linear part of the sigmoid curve of standard quantity to signal described by an ELISA. This is understandable as small variations in the optical density at this point can result in large changes in the value derived by interpolation and confirmed by variable and unreliable spike recovery results. The most accepted definition of LLQ is the lowest concentration that can be measured with a definite level [14]. The LLQ described here is the value above which the intra-assay CV is shown to be less than 12% and spiked mediator recovery exceeds 80% and assay characteristics are clearly linear. Only samples from 19.6 percent of the subjects in our study were within these criteria and for only one assay (AAT).

There has been very little published data regarding the validation of assays used in EBC at very low concentrations. SLPI, AAT and MPO activity measurements have not previously been reported in EBC.

We have chosen sol-phase of spontaneous sputum as a surrogate for mediator concentrations found within the lungs. Currently, no obvious “gold standard” exists to which we might reference EBC mediator concentrations
with those found within the lungs [15]. Exhaled breath condensate reflects air from the whole bronchial tree. However, airway secretions (in particular spontaneous sputum) contain high levels of mediators. As we collected EBC and sputum from the same patients we felt this to be the best comparison. In addition the data by Biernacki et al demonstrated that EBC LTB₄ is increased in exacerbation and falls upon resolution [16]. Since exacerbations of COPD are events affecting the bronchial tree and since similar changes occur in sputum, this is the most appropriate sample for comparative purpose [17].

It is possible that EBC also reflects changes in the distal airways and hence comparison with bronchioalveolar lavage (BAL) would be appropriate, even in light of the lack of relationship between BAL demonstrated by Jackson et al [15]. However it seems inappropriate to assess this at present until valid assays are identified for EBC.

In order to enable reliable mediator determination it might be argued that concentrating the EBC is necessary; although such procedures may also prove inaccurate due to protein loss [18] and other confounding factors such as unknown stability of LTB₄ in EBC when lyophilised [9]. The results described in the current study suggest that EBC cannot be usefully applied to determine concentrations either within a study or as a diagnostic tool, for the assays and collection techniques we have used.

Despite the current results questioning the validity and conclusions drawn from studies involving the mediators assessed here; EBC may still prove a
useful tool. Assays such as Interleukin 6 may detect quantifiable levels in EBC and other biomarkers may prove measurable by current methodologies.

The use of other collection equipment or the application of coatings to the collection equipment to prevent binding of mediators may be necessary. For example, Rosías et al [19] have shown that glass and silicone were superior to aluminium, polypropylene and Teflon when measuring 8-isoprostane and albumin levels in EBC. Liu et al demonstrated that total protein levels in EBC were higher when using Ecoscreen® when compared to glass and Rtube in collection devices might prevent mediator binding [20], whilst Leung et al have demonstrated that cysteinyl leukotrienes and LTB₄ were poorly correlated when collected using Ecoscreen® and Rtube [5]. However, the effect of differing collection devices upon specific protein inflammatory mediators is unknown. Alternatively, it might be possible to prevent absorption of mediators upon the collection equipment by pre blocking with agents such as bovine serum albumin although this might in itself cause reproducibility issues.

The presence of proteinases, receptors, antibodies and other interfering proteins, along with variations in pH and protein concentration ensure that sputum is a challenging matrix for mediator measurement. However, the high mediator concentrations present often enable deleterious effects upon ELISAs to be substantially negated by dilution as has been shown for secretory proteinase inhibitor in the presence of neutrophil elastase [21]. Critically this is not possible in EBC at present.
The alternatives are to develop more sensitive ELISA assays, proteomics or mass spectroscopy measurements. However, with more sensitive assays a balance between the signal/noise ratio will need to be taken into account [9].

The results indicate performance of any assay must be fully characterised to interpret the results. This study investigated both ELISA and chromogenic substrate techniques, however the validation methodologies are also critical for all methodologies used for the assessment of mediators within EBC.
References


Table 1  Demographic data for subjects and patients.  Values are mean with standard deviation in parenthesis unless stated otherwise.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>COPD</th>
<th>Bronchiectasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (female)</td>
<td>30 (11)</td>
<td>19(8)</td>
<td>12(6)</td>
</tr>
<tr>
<td>Age</td>
<td>50 (8)</td>
<td>67(5.3)</td>
<td>67(7.6)</td>
</tr>
<tr>
<td>FEV1%pred</td>
<td>107(13.2)</td>
<td>48.9(14.7)</td>
<td>70.3(25.8)</td>
</tr>
<tr>
<td>Height [m]</td>
<td>1.69 (0.09)</td>
<td>1.68 (0.05)</td>
<td>1.66 (0.09)</td>
</tr>
<tr>
<td>Smoking History</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smokers</td>
<td>9</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>3</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>Non-smokers</td>
<td>18</td>
<td>0</td>
<td>9</td>
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</table>
Table 2  Spearman rank correlations between sol-phase and EBC mediators.

<table>
<thead>
<tr>
<th></th>
<th>p</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>LTB4</td>
<td>0.185</td>
<td>18</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.440</td>
<td>18</td>
</tr>
<tr>
<td>SLPI</td>
<td>0.140</td>
<td>15</td>
</tr>
<tr>
<td>AAT</td>
<td>0.211</td>
<td>18</td>
</tr>
<tr>
<td>MPO</td>
<td>0.324</td>
<td>18</td>
</tr>
</tbody>
</table>

One tailed p values are shown a p value <0.05 was considered significant.

Spearman rank correlation did not reach significance for any mediator.