Published in final edited form as:

J Breath Res. 2010 December ; 4(4): 046002. doi:10.1088/1752-7155/4/4/046002.

Effects of sample processing, time and storage condition on cysteinyl leukotrienes in exhaled breath condensate

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Abstract

Cysteinyl leukotrienes (CysLTs) can be measured in exhaled breath condensate (EBC); however, there is considerable variation in reported EBC CysLT concentrations from asthmatic MTSY and healthy subjects between published studies, which may be partially explained by CysLT degradation during processing and storage. We assessed CysLT stability over 6 months in EBC from healthy subjects stored at -80°C , layered with argon and then stored at -80°C or stored in 0.2% formic acid in methanol at -80°C following solid-phase extraction (SPE). We found significant CysLT degradation over time in both spiked and unspiked EBC samples stored at -80°C or layered with argon. CysLT recovery was significantly greater after storage of 6 months in 0.2% formic acid in methanol following SPE; however, there was substantial variability in endogenous CysLT recovery over time, possibly attributable to inter- and intra-assay variability at the low end of the CysLT assay range. Despite the greater recovery of CysLTs in EBC stored in methanol following SPE, the degree of variability introduced by this method appears unacceptably high. We believe that the development of more sensitive and less variable methods for quantifying CysLTs in EBC are required before CysLTs can reliably be utilized as biomarkers in exhaled breath. Sample processing and storage, as well as inter- and intra-assay variability, should be carefully considered in the design of clinical studies that include assessments of EBC constituents as biomarkers.

Introduction

The analysis of exhaled breath condensate (EBC) constituents holds the promise of a noninvasive method to allow for the measurement of a variety of inflammatory mediators in the airways that may further facilitate understanding of the pathobiology of airway diseases, lead to new diagnostic approaches and serve as biomarkers that could be used to assess therapeutic interventions [1, 2]. The concentration of nitric oxide in exhaled breath has been studied in enough detail to allow for the development of guidelines for its measurement for research and clinical purposes [3]. In contrast to exhaled nitric oxide analysis, the measurement of potential biomarkers of disease in EBC is an immature field. Although a variety of volatile and non-volatile compounds have been detected in EBC by many

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different investigators, few studies have assessed the sensitivity and reproducibility of EBC methodologies needed to effectively apply this technique to clinical and research questions.

A Task Force report by the American Thoracic Society (ATS) and European Respiratory Society (ERS) identified the need for studies that assess methodological issues involving EBC collection and analysis before the widespread introduction of EBC measures into clinical practice or as biological outcome measures in clinical studies [4]. Published studies reporting the impact of degradation of inflammatory mediators during EBC sample storage are lacking. Since lipid and protein mediators measured in EBC are most commonly analyzed using enzyme-linked immunoassays, clinical samples are typically stored for a period of weeks to months so that they can be batched with other samples. Therefore, the stability of potential biomarkers in EBC and standardization of procedures to maximize sample stability are important issues that must be addressed in order for EBC constituents to be accepted as validated biomarkers for use in larger human trials or as clinical tools.

Cysteinyl leukotrienes (CysLTs), LTC₄, LTD₄ and LTE₄, are important pro-inflammatory mediators in asthma [5]. Although CysLTs can be measured in EBC, there is considerable variation between published studies in the concentration of CysLTs in EBC from asthmatic and healthy subjects [6–9], and EBC CysLT concentrations are below the detection limit of enzyme immunoassays (EIA) in many asthmatic and/or healthy subjects [9–12]. These results may be partially explained by differences in EBC sample processing and storage. Published studies reporting the impact of degradation of inflammatory mediators during EBC sample storage are lacking.

CysLT degradation during sample storage may hamper accurate assessment of CysLT concentrations in EBC, as these polyunsaturated fatty acids are subject to lipid peroxidation and autolysis [13, 14]. The storage stability of CysLTs in EBC and the ideal storage environment to prevent CysLT degradation has not been ascertained. We recently reported that concentration of samples using a solid phase extraction (SPE) method significantly improves the detection of CysLTs in EBC [15]. The final step of this SPE method is sample elution with 0.2% formic acid in methanol. Although CysLTs are more stable in methanol than in biological fluids or aqueous media [16, 17], most investigators who have reported the concentrations of CysLT in breath condensate have simply stored EBC samples at –70 to –80 °C for variable periods of time prior to CysLT measurement. Our aims were to determine the storage stability of CysLTs in untreated EBC samples stored at –80 °C, samples layered with argon gas to prevent sample oxidation and then stored at –80 °C and samples stored in 0.2% formic acid in methanol at –80 °C following an eicosanoid SPE protocol [15]. We compared the effects of these three strategies on CysLT stability over the course of 6 months in EBC spiked with a known concentration of LTC₄. We then investigated the effects of these strategies on the stability of endogenous CysLTs in unspiked EBC from several healthy subjects over the course of 6 months. Some of these results were previously reported in abstract form [18].

Materials and methods

Subjects

EBC was collected from three healthy adult volunteers for experiments involving spiking the EBC with exogenous LTC₄. For experiments monitoring the stability of endogenous CysLT, EBC was collected from an additional four healthy adult volunteers. The study was approved by the Institutional Review Board of Seattle Children's Hospital, and informed consent was obtained from all participants.

Experimental design

Spiked EBC experiments—Twelve milliliters of EBC was collected from each of three healthy volunteers (36 mL total) using the R-Tube[®] system (Respiratory Research, Charlottesville, VA) during up to 60 min of tidal breathing while wearing a nose clip. The R-Tube[®] condenser was cooled on dry ice prior to EBC collection. To ensure that the condenser was always <0 °C, every 15 min during EBC collection the R-Tube[®] condenser was replaced with a fresh condenser cooled on dry ice. Immediately following collection EBC was frozen at –80 °C. Subsequently the EBC samples from these three subjects were thawed, pooled and vortexed. Twelve milliliters of pooled EBC was used for each processing/storage condition. EBC was subdivided into 4 mL aliquots for each of the three processing/storage conditions. Each 4 mL aliquot of EBC was spiked using Cayman[®] LTC₄ standard provided in the Cayman EIA (Cayman[®] catalog# 520501) to a target concentration of 120 pg mL⁻¹. The Cayman LTC₄ comprises 0.001% LTC₄ in 99.999% ethyl alcohol without preservatives or other contaminants. Each aliquot was then further divided into four storage time points (baseline, 1 month, 3 months and 6 months), each with five replicate samples of 0.2 mL each. Calculation of CysLT recovery for each time point was performed by dividing the measured CysLT concentration at a given storage interval by the baseline CysLT concentration under the same processing/storage condition.

The three processing/storage conditions were: (1) EBC processed using an eicosanoid SPE protocol and then stored in 0.2% formic acid in methanol at –80 °C, (2) EBC layered with argon and then stored at –80 °C, and (3) EBC stored at –80 °C without any modification.

Unspiked EBC experiments—A total of 7.5 mL of EBC was collected from each of four healthy adults during up to 40 min of tidal breathing. EBC from each subject was divided equally into aliquots for each processing/storage condition. For each condition, unspiked EBC was divided into samples of 0.8 mL for each of three time points (baseline, 3 months, 6 months). Samples processed/stored using SPE were eluted with 0.4 mL of 0.2% formic acid in methanol and then stored at –80 °C. Prior to CysLT assay, samples were evaporated to dryness under argon and then re-suspended with 0.2 mL of the EIA buffer solution to achieve a fourfold sample concentration factor. Unspiked EBC samples stored either after argon overlay or without any modification were also concentrated fourfold immediately prior to CysLT assay using the above SPE method.

Solid-phase extraction—Sample aliquots processed by SPE were first acidified to a pH <4 and then loaded onto Oasis[®] HLB cartridges (Waters, Milford, MA) that had been

preconditioned with ultrapure water. Cartridges were centrifuged at 300 rpm until all samples had entered the cartridge matrix, and then 0.03% formic acid in water was added to each cartridge. The cartridges were then centrifuged again at 300 rpm for 5 min. Then 0.03% formic acid in ultrapure water and absolute ethanol was added to each cartridge, followed by centrifugation at 300 rpm for 5 min. Hexane was then added to each cartridge followed by centrifugation at 700 rpm for 8 min. Sample was then eluted from each cartridge using 0.2 mL of 0.2% formic acid in methanol. The samples were then stored at -80°C in 0.2% formic acid in methanol until CysLT assay. Immediately prior to CysLT assay, the samples were evaporated to dryness under a stream of argon and then re-suspended with the Cayman[®] EIA buffer. Baseline CysLT concentrations were measured on the day EBC was spiked and initially processed.

All EBC samples were processed and stored using either silanized glass or polypropylene tubes to prevent loss of leukotrienes due to absorption by tube surfaces.

Measurement of cysteinyl leukotrienes

CysLT concentrations were measured using the Cayman[®] EIA (catalog# 520501). All samples were analyzed in duplicate. Baseline concentrations were measured on the day EBC was initially processed. The lower limit of detection based on the Cayman[®] EIA standard curve is 7.8 pg mL^{-1} . Cayman[®] reports that the CysLT EIA cross-reactivity to arachidonic acid and glutathione is negligible.

Statistical analyses

Data were analyzed using two-way repeated measure ANOVA to determine the independent effects of time and storage condition on EBC CysLT recovery rates. Unpaired *t*-tests were used to compare EBC CysLT recovery rates between storage conditions at each time point. A *p*-value <0.05 was considered statistically significant. Confidence intervals (CI) (95%) were calculated for recovery rates for each storage condition and time point.

Results

The mean baseline concentration of CysLT in EBC samples spiked with LTC₄ standard was 123 pg mL^{-1} (s.d. 13.6) for samples stored at -80°C , 116 pg mL^{-1} (s.d. 19.2) for samples layered with argon prior to storage at -80°C and 107 pg mL^{-1} (s.d. 22.2) for samples processed with SPE prior to storage in 0.2% formic acid in methanol (table 1). The mean coefficient of variation (CV%) of EIA sample duplicates for EBC spiked with LTC₄ was 13.7% (s.d. 9.6). The mean CysLT recovery rate in spiked EBC samples stored at -80°C was similar at 1 and 3 months, 87% (95% CI: 68–106%) and 93% (95% CI: 68–101%), respectively. However, after 6 months of storage the CysLT recovery rate was only 71% (95% CI: 60–81%). The recovery of CysLTs from EBC samples that had been layered with argon prior to storage at -80°C was not significantly different from the recovery of CysLTs from EBC samples that were simply stored at -80°C at any time point (table 1 and figure 1). However, the stability of CysLTs in EBC samples processed using a SPE method and then stored in 0.2% formic acid in methanol was excellent at all time points, with recovery rates of 91% (95% CI: 79–103%) at 1 month, 115% (95% CI: 101–129%) at 3 months and

97% (95% CI: 82–113%) at 6 months. Furthermore, the recovery of CysLTs stored in 0.2% formic acid in methanol was significantly greater than that for EBC samples simply frozen at -80°C after 3 ($p = 0.008$) or 6 months ($p = 0.005$) of storage, or for samples layered with argon prior to storage at -80°C for 3 ($p = 0.02$) or 6 months ($p = 0.05$) (figure 1).

Baseline concentrations of endogenous CysLTs in unspiked EBC from our four healthy subjects were 5.2, 7.2, 11.7 and 50.2 pg mL^{-1} . The mean CV% for EIA sample duplicates of endogenous CysLT EBC samples was 19.6% (s.d. 15.9). The stability of endogenous CysLT in EBC samples frozen at -80°C over time was poor, with CysLT levels measured at 3 months being only 39.5% of baseline, and levels measured at 6 months only 19.5% of baseline (figure 2). The stability of CysLT in EBC layered with argon prior to storage at -80°C was fair after 3 months of storage, with measured CysLT levels of 77.3% of baseline, but was poor after 6 months of EBC storage with measured CysLT levels of only 14.6% of baseline. Sample storage in 0.2% formic acid in methanol following SPE resulted in significant improvement in the stability of endogenous CysLTs as compared to both storage at -80°C and storage at -80°C after treatment with argon (figure 2; $p = 0.04$), with mean CysLT level recovery rates of 145% of baseline after 3 months of storage and 136% of baseline after 6 months of storage. However, there was substantial variability in CysLT recovery rates between subjects, ranging from 66 to 247% after 3 months of storage and from 78 to 271% after 6 months of storage.

Discussion

We found that in human EBC samples spiked with LTC_4 there was significant degradation over time when stored at -80°C with or without argon overlay, particularly when the storage duration was greater than 3 months. However, when samples were first processed using a SPE method and then stored in 0.2% formic acid in methanol, CysLT recovery was excellent even for samples that had been stored for 6 months, albeit associated with high variability. Furthermore, we found that the stability of endogenous CysLTs in human EBC samples that were simply stored at -80°C was poor after 3–6 months, with less than 20% of the baseline concentration of CysLT detected after storage for 6 months. Processing EBC samples via a SPE method followed by storage in 0.2% formic acid in methanol dramatically increased the recovery of endogenous CysLTs in EBC samples; however, the variability in recovery of endogenous CysLTs was very high with mean concentrations markedly greater than 100%, raising concern that improvements in storage stability following SPE are associated with excessive variability.

Several incidental findings from this investigation illustrate important methodological pitfalls in the measurement of inflammatory mediators in EBC. We and others have previously demonstrated that concentrations of CysLTs in EBC samples are frequently below the detection limit of currently available EIA [9–12, 15]. We reported a method to concentrate samples using a SPE protocol that allows detection of CysLTs in EBC samples from healthy and asthmatic subjects. In this study baseline endogenous CysLT levels from three out of four healthy subjects would have been below the EIA detection limit if they had not been first concentrated fourfold, illustrating the importance of sample concentration when measuring CysLTs in EBC.

A second important methodological problem in the measurement of biomarkers in EBC samples is the variability in EIA results at the low end of assay detection ranges. Even after concentrating EBC samples fourfold, endogenous levels of CysLTs from three of our four subjects were at the low end of the Cayman[®] CysLT EIA standard curve (raw concentration range of 21–47 pg mL⁻¹). Cayman's CysLT EIA product information advises caution when interpreting CysLT levels ≤ 30 pg mL⁻¹, due to intra-assay variation of up to 43%, and inter-assay variation of up to 65%, at the low end of the assay range. Among our endogenous CysLT EBC samples that would have been below the EIA detection limit if they had not been concentrated prior to assay, we found a mean intra-assay CV% for sample duplicates of 24%. We believe that intra- and inter-assay variation at the low end of the EIA range was likely a factor that contributed to our findings of mean CysLT recovery rates of $>100\%$ and substantial inter-subject variation in the recovery rates observed in the samples stored in methanol following SPE. Another possible explanation for our finding of CysLT recovery significantly $>100\%$ for endogenous CysLTs following SPE is that sample storage in methanol might alter conversion of arachidonic acid metabolites not detected by the Cayman[®] ELISA to LTC₄/D₄/E₄, thereby increasing the total detected concentration of CysLTs detected by the Cayman[®] ELISA. A limitation of this study is the lack of experiments to investigate such possible mechanisms.

EIA inter-assay variability is an important methodological challenge in any clinical study wherein an EBC biomarker is used as an endpoint, even if samples are concentrated sufficiently to ensure that biomarker levels are in the most reliable range of the assay used. Cayman reports inter-assay variation of approximately 10–20% for CysLT levels between 62.5 and 125 pg mL⁻¹. Although CysLT levels in this range are at the upper limit of what would be expected in most human EBC samples, we deliberately chose a target LTC₄ spike concentration of 120 pg mL⁻¹ to minimize the potential confounding effects of inter-assay variability on our ability to determine the effects of storage conditions on EBC CysLT stability. A LTC₄ spike concentration of 120 pg mL⁻¹ also reduced potential confounding of our results by endogenous CysLT content in our spiked EBC samples. In our LTC₄ spiked EBC samples the effect of inter-assay variability is illustrated by the finding that CysLT recovery increased between 1 and 3 months under all three storage conditions (figure 1). It is more likely that levels of CysLTs in samples analyzed after 3 months of storage was overestimated by the assay used at that time point by 10–20% due to the expected inter-assay variation of the Cayman[®] EIA. Therefore, we suspect that the true recovery rate of CysLT in EBC samples simply stored at -80°C for 3 months is approximately 75–85%.

Given that most clinical trials are conducted over periods of months to sometimes several years, EBC samples from different patients would typically be assessed on different EIA plates after variable durations of sample storage. The issue of inter-assay variation becomes even more problematic in longitudinal studies wherein EBC CysLT levels may be monitored repeatedly to assess changes in airway inflammation over time or in response to a therapeutic intervention, requiring multiple CysLT assay kits likely from different manufacturer lot numbers. Regardless of the EBC sample storage condition utilized, the estimates of CysLT recovery after 1–6 months of sample storage that we report here should assist other investigators to design future studies with adequate sample power to detect changes in EBC CysLT levels in response to therapeutic interventions.

There are several potential limitations inherent in our study design. Because we used the Cayman[®] EIA to measure CysLT in EBC we do not know the relative proportions of LTC₄, LTD₄ and LTE₄ in our unspiked EBC samples. Cayman[®] reports that their EIA has specificities for LTC₄, LTD₄ and LTE₄ of 100%, 100% and 67%, respectively [19]. Because *in vivo* LTC₄ is rapidly metabolized to LTD₄ and LTE₄ [13] our estimates of CysLT recovery may not fully account for LTE₄. It is possible that the improved stability of CysLTs that we observed in samples processed with SPE and stored in 0.2% formic acid in methanol reflects an arrest in metabolism of LTD₄ to LTE₄. Despite this limitation we believe our results are relevant because the majority of studies that have reported CysLTs in EBC have used the Cayman[®] CysLT EIA, and because it is not practical to separately measure LTC₄, LTD₄ and LTE₄ in EBC samples. Another limitation of our study design that resulted from limited EBC sample volume is that we only concentrated samples 4-fold to measure endogenous CysLTs, in contrast to our prior study where we concentrated samples 6.5-fold. However, most investigators have not concentrated EBC samples prior to measuring CysLTs, partially explaining results from studies that have reported a substantial percentage of EBC samples wherein CysLTs were not detectable.

There are several sources of variability inherent in our study design, and in any study addressing concentrations of biomarkers in EBC, including: (1) the EBC collection method (in this case the R-tube[®] system), (2) the EBC sample processing methodology (one factor we tested in this study), (3) sample storage duration (the other factor we tested) and (4) the inter and intra-assay variability of the biomarker assay used (in this case the Cayman[®] ELISA). All of these issues need to be taken into consideration when interpreting the results of investigations of biomarkers in EBC. Most published EBC studies have not rigorously attempted to study these factors. Despite the limitations of our study design, these results highlight the need for further research to improve and standardize methods for the measurement of biomarkers in clinical breath condensate samples.

In conclusion, we found that CysLTs spiked into human EBC samples at a target concentration of 120 pg mL⁻¹ degraded significantly after storage at -80 °C for more than 3 months. Degradation of endogenous CysLT stored at -80 °C with or without argon overlay for 3–6 months was even more striking. However, EBC CysLT recovery after storage for 3–6 months was significantly greater when samples were first processed using a SPE method, followed by storage in methanol. Despite the superior CysLT recovery rate we observed in samples processed with SPE and stored in 0.2% formic acid in methanol, we found substantial variability in CysLT levels over time in spiked EBC samples that is attributable to EIA inter-assay variability. We also observed extremely high inter-subject variability in the recovery of endogenous EBC CysLTs over time that may be at least partially attributable to the variability of the Cayman[®] EIA at the low end of the assay range. Despite the greater recovery of CysLTs after long-term storage following SPE, the degree of variability introduced by this method appears unacceptably high. We believe that the development of more sensitive and less variable methods for quantifying CysLTs in EBC are required before CysLTs can reliably be utilized as biomarkers in exhaled breath. In the meantime, to improve the sensitivity of CysLT detection in EBC we suggest that investigators consider concentrating an aliquot of their EBC samples using SPE and storing samples in methanol

for no more than 6 months prior to performing CysLT assays. Furthermore, it is imperative that CysLT EIA inter-assay variability, as well as the variability of CysLT measurements at the low end of the assay range, be considered in sample size calculations during the design of studies utilizing EBC CysLT measurements to ensure adequate study power.

Acknowledgment

This work was supported by NIH/NHLBI grant K23HL077626.

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Figure 1. Effects of time and storage condition on cysteinyl leukotriene (CysLT) recovery in LTC₄ spiked exhaled breath condensate samples. Open bars represent samples stored at −80 °C; striped bars represent samples layered with argon and then stored at −80 °C; solid bars represent samples processed via SPE and then stored in 0.2% formic acid in methanol at −80 °C.

Figure 2. Effects of time and storage condition on endogenous cysteinyl leukotriene (CysLT) stability in exhaled breath condensate samples. Open bars represent samples stored at -80°C ; striped bars represent samples layered with argon and then stored at -80°C ; solid bars represent samples processed via SPE and then stored in 0.2% formic acid in methanol at -80°C .

Table 1

Effects of time and processing/storage condition on EBC CysLT stability.

	Storage at -80 °C				Layered with argon then storage at -80 °C				SPE/0.2% formic acid in methanol storage at -80 °C			
	1 month	3 months	6 months	1 month	3 months	6 months	1 month	3 months	6 months	1 month	3 months	6 months
Baseline CysLT (pg mL⁻¹) ± s.d.	123.2 ± 13.6				116.1 ± 19.2				107.3 ± 22.2			
Storage time												
Mean CysLT (pg mL ⁻¹) ± s.d.	107.5 ± 41.8	114.7 ± 17.5	87.2 ± 26.2	98.2 ± 35.0	109.2 ± 23.9	94.7 ± 13.7	97.6 ± 22.8	123.0 ± 27.4	104.3 ± 29.1			
% CysLT recovery	87%	93%	71% *	85%	94%	82% *	91%	115% **	97% **			
95% CI for % CysLT recovery	68–106%	85–101%	60–81%	68–101%	83–105%	75–88%	79–103%	101–129%	82–113%			

CysLT: cysteinyl leukotrienes.

* Independent effect of time on the CysLT recovery rate $p = 0.004$ (analyzed using two-way ANOVA).

** Independent effect of the storage condition on the CysLT recovery rate $p = 0.015$ (analyzed using two-way ANOVA).