Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 823–830

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Immunoassay-based measurement of clinical biomarkers for monitoring changes in nasal cavity

Yan J. Zhang*, Sherri Luroe, Frank Schieber, Joan Kelsey, Fizal Nabbie, Giovanni Rizzi, Penny Richards, Russell Weiner, Paul W. Rhyne

Biomarker and Bioanalytical Sciences, Pharmaceutical Candidate Optimization, Bristol-Myers Squibb Research and Development, Princeton, NJ 08543, United States

ARTICLE INFO

Article history:
Received 17 March 2009
Received in revised form 17 June 2009
Accepted 23 June 2009
Available online 2 July 2009

Keywords:
Biomarker
Nasal lavage
ELISA

ABSTRACT

Background: Many drugs for treatment of allergies, migraine headaches, inflammation, and other indications are administered into the nasal cavity providing access to the immune and central nervous systems. One of the concerns for using this route of administration is potential damage to the nasal epithelium and mucosal regions. We assembled a panel of clinical biomarkers that can be used to monitor changes in the nasal epithelium, mucosa, and olfactory regions in preparation for clinical trials involving drugs administered via intranasal route. These biomarkers included albumin, elastase, IL-6, IL-8, lactoferrin, myeloperoxidase and nerve growth factor.

Methods: Immunoassays were developed and used to measure changes in these biomarkers in nasal lavage samples collected twice daily from 30 assumed-healthy volunteers over a 2-day period. Various statistical methods including analysis of variance (ANOVA), paired *t*-test and Pearson's product–moment correlation were used to evaluate the data.

Results: Although the basal levels of these biomarkers were varied among subjects, the data show that the concentrations of albumin, elastase and IL-8 were significantly higher in samples collected in the morning compared to samples collected later during the day. Pre-washing nasal cavity prior to collecting nasal lavage samples did alter the measurement of elastase and albumin, but did not influence the levels of the other biomarkers.

Conclusions: These data show that this panel of biomarkers can be used to monitor changes in the nasal cavity including those affected by diurnal fluctuations. These results also provide useful baseline values and sources of variability for each biomarker that could be used to help design clinical trials.

1. Introduction

Delivery of drug molecules targeted to the central nervous system (CNS) is challenging mainly because of the impenetrable nature of the blood–brain barrier (BBB) that protects the CNS. There are several routes in which molecules can gain entry into the CNS such as direct diffusion across the BBB for small (<600 kDa) lipid soluble molecules, polycationic interactions between the molecule and the negatively charged BBB endothelium, and transcellular routes across the nasal epithelium [1]. Transcellular routes include receptor mediated endocytosis, pinocytosis, traversal across the tight junctions between the sustentacular cells and olfactory neurons, and direct entry into olfactory neurons followed by axonal transport to olfactory bulbs [1,2]. Thus, the main advantages of administering the delivery of CNS targeted drugs through the nasal epithelium include the broad range of CNS access pathways and convenience of intranasal delivery.

While delivery of drugs to the CNS via intranasal route is advantageous, there is still a concern for potential side effects on the epithelium, mucosa, and olfactory regions. One approach is to use clinical biomarkers to help monitor the condition of the nasal cavity. Several studies have been published where clinical biomarkers were used to monitor changes in the nasal epithelium and mucosa [3–7]. These studies include allergic reactions, respiratory tract diseases, respiratory disorders, infections, and exposure to pollutants. Different sets of biomarkers allow the condition of the nasal cavity to be monitored. Changes in albumin [4,8], urea [5,9], and histamine [10,11] have been used to monitor changes in vascular permeability. Elastase has been used to assess neutrophil activation [12], lactoferrin for changes in glandular secretion [13,14], and neuronal growth factor (NGF) for mast cell presence and neurochemical changes [15,16]. Many other biomarkers that have been used include substance P [17], MMP-9 [18], eotaxin [19], tryptase [20], eosinophil cationic protein [20], and a variety of cytokines including IL-6 and IL-8 [21].

* Corresponding author. Tel.: +1 609 252 4902; fax: +1 609 252 6171.
E-mail address: yan.j.zhang@bms.com (Y.J. Zhang).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.06.043

One of the challenges for monitoring the condition of the nasal cavity is in obtaining a clinical sample to perform the analysis. Some studies have collected nasal secretions using filter paper or other similar devices [4,7,22,23], whereas others have collected nasal lavages obtained through the use of physiological appropriate solutions [13,22–27]. The inconsistent collection of clinical samples between these studies has contributed to variability in biomarker levels. Another factor that influences biomarker levels is diurnal fluctuations which are known to occur in the nasal mucosa [28]. Biomarkers, such as albumin and secretory IgA (sIgA) [29,30], were shown to rise and fall at different times of the day. Thus it is important to consider the timing of sample collection in regards to this cycle. Numerous studies have measured biomarkers from a single time point at approximately the same time of the day in which diurnal fluctuation was not a factor [5,25]. Other studies measured biomarkers without accounting for diurnal fluctuations [3,4,7,27,31–34], which could influence the interpretation of the results.

In this study, we assessed a panel of biomarkers that could be used to monitor the condition of the nasal cavity for early clinical studies. The panel of biomarkers should be able to detect changes in the nasal epithelium, mucosa, and olfactory regions. We also wanted to assess if these biomarkers are influenced by diurnal changes in the nasal cycle, as samples at multiple clinical time points would likely be collected in future studies. Thus, a small pilot study was conducted using healthy subjects in which nasal lavage samples were taken twice a day over a 2-day time period. Immunoassays were developed to measure these biomarkers in the collected nasal lavage samples. We found that some of the biomarkers were in fact influenced by diurnal cycles while others were not. The data described in this study demonstrates that the selected biomarkers are easily measured and are useful to monitor changes in the nasal cavity.

2. Materials and methods

2.1. Study population and clinical design

Thirty assumed-healthy subjects aged 18–45 were selected for this study. These subjects had no clinically significant deviation in medical history, physical examination, anterior nasal examination, or ECGs. All subjects had a body mass index (BMI) between the ranges of 18 and 32 kg/m^2. All subjects gave their informed consents and the study was approved by the local Ethics Committee.

The nasal lavage procedure was initiated between 8:00 and 10:00 am (categorized as the morning time point). The afternoon lavage time point was initiated 6 h after the morning time point. The schedule was repeated on the following day using matched clock times for each subject. All subjects underwent a nasal pre-wash before each scheduled nasal lavage, with a 10-min delay between the pre-wash and the nasal lavage. The samples from the pre-washes were collected and processed in the same procedure as that used for nasal lavage.

2.2. Nasal lavage fluid collection and processing

Subjects were administered 2.5 mL of Nasaline® solution (Camexco, Inc., Bridgewater, NJ) slowly into each nostril (a total of 5 mL per subject) for approximately 20–30 s. The subjects were instructed to hold their breath without swallowing and then expel the lavage into a sterile specimen cup by bending their heads forward and gently blowing the nostrils. The contents of the dish were transferred into a sterile test tube, shaken vigorously for approximately 10 s, and stored on ice until processed.

The lavage samples were processed by gentle mixing followed by centrifugation at 3000 × *g* for 15 min at 4 °C using an Eppendorf Centrifuge 5804 R (Brinkmann Instruments, Inc., Westbury, NY). The supernatant was mixed with 1/100 volume of protease inhibitor cocktail (Sigma, St. Louis, MO), pipetted, and stored at −70 °C until ready for analysis.

Prior to analysis, the thawed lavage samples were mixed with an equal volume of phosphate buffered saline (PBS). The diluted samples were filtered using a 96 well filter plate with pore size of 10 μM (Millipore, Billerica, MA). The supernatants were diluted to the final appropriate dilution using phosphate buffered saline containing 0.05% Tween®-20 (PBST) for each assay as shown in Table 2.

2.3. Biomarker immunoassays

2.3.1. Albumin ELISA

A double-antibody sandwich ELISA was developed to measure human albumin in lavage samples. 100 μl/well of goat polyclonal anti-human albumin (Bethyl, Inc., Montgomery, TX) at 10 μg/mL in PBS was passively coated onto Nunc ImmunoPlate Maxi Sorp microtiter plates (Nalge Nunc International, Rochester, NY) at room temperature (RT) for 1 h. The plate was washed three times with PBST (phosphate buffered saline containing 0.05% (v/v) Tween® 20) using a Bio-Tek 405 plate washer (Bio-Tek Instruments, Winsooki, VT) and blocked with 200 μl/well of Pierce Superblock blocking buffer (Pierce, Rockford, IL) overnight (ON) at 4 °C. The plates were stored at 4 °C until ready for use.

The plates were washed five times with PBST prior to use. 50 μl/well of freshly prepared albumin reference standard (Bethyl, Inc., Montgomery, TX), clinical samples, or quality control samples diluted in PBST were added to the plates and incubated for 1 h at RT. The plates were washed five times with PBST and incubated with 100 μl/well of 10 ng/mL HRP-labeled goat polyclonal anti-albumin (Bethyl, Montgomery, TX) at RT for 1 h. The plates were washed five times with PBST and incubated with 100 μl/well of TMB peroxidase substrate solution (R&D Systems, Minneapolis, MN) at RT for 30 min. Finally, 50 μl/well of 1 M H_2SO_4 was added to stop the reaction and the optical density at 450 nm was measured using a Molecular Devices Spectromax plate reader (Molecular Devices, Sunnyvale, CA). The concentration of albumin in the clinical samples was determined from a 4-parameter logistic standard curve generated from the albumin reference standards.

2.3.2. Elastase ELISA

The levels of human elastase were determined by a double sandwich enzyme-linked immunosorbent assay. Briefly, Nunc ImmunoPlate Maxi Sorp microtiter plates were incubated with 60 μl/well of 2 μg/mL mouse monoclonal anti-human elastase antibodies (Cell Science, Canton, MA) in PBS at RT overnight. The plates were washed three times with PBST, blocked 2 h at RT with 200 μl/well of Pierce Superblock blocking buffer, and stored in blocking buffer at 4 °C until ready for use.

The plates were washed five times with PBST and incubated for 1 h at RT with 50 μl/well of elastase reference standards (Cell Science, Canton, MA), clinical samples, or quality control samples. The plates were then washed five times, incubated with 60 μl/well of 1.5 μg/mL biotinylated mouse monoclonal anti-human elastase antibodies (Cell Science, Canton, MA) for 1 h at RT, and washed five times with PBST. Next, the plate was incubated for 20 min at RT with 60 μl/well of HRP–streptavidin conjugate (R&D Systems, Minneapolis, MN) diluted according to the manufacturer's instruction, washed five times, and incubated with 60 μl/well of TMB substrate for 30 min at RT. The reaction was stopped by addition of 50 μl/well of 1 M H_2SO_4 stop solution and the optical density at 450 nm was determined. The concentration of elastase in the clinical samples

was determined from a 4-parameter logistic standard curve generated from the elastase reference standards.

2.3.3. IL-6, IL-8, lactoferrin, and myeloperoxidase and nerve growth factor assays

IL-6 and IL-8 were measured using Quantikine ELISA Kits from R&D Systems (Minneapolis, MN). Lactoferrin was measured using Oxis Research Bioxytech Lactoferrin EIA assay (Portland, OR). Myeloperoxidase (MPO) was measured using an ELISA kit from Assay Designs Inc. (Ann Arbor, MI). Nerve growth factor (NGF) was measured using the Luminex *x*MAP®-based NGF kit from Millipore (St. Charles, MO).

Each assay was performed according to the manufacturer's instructions. The concentration of IL-6, IL-8, lactoferrin, MPO and NGF in the clinical samples was determined from a 4-parameter logistic standard curve generated from the reference standards.

Assay quality control samples were prepared by spiking a pool of nasal lavage samples with the recombinant protein standards. At least three quality control samples were used for each assay with biomarker concentrations falling in the low, middle, and high regions of the calibration curves, respectively.

2.4. Statistical analysis

The lower limit of quantitation (LLQ) for each assay was calculated, which is defined as the lowest measurable concentration of reference standard in which the coefficient of variation (CV) of the replicates was <30% and the measured value was within 30% of expected concentration (accuracy). We used the JMP 7.0.1 statistical software package (SAS Institute Inc., Cary, NC) to perform all subsequent statistical analysis on the biomarker data. The data were log-transformed to reduce skewness. The effects of pre-washing on biomarker levels in the lavage samples were determined via a paired *t*-test and the Wilcoxon's signed rank test. The day-to-day correlation of biomarker concentrations was evaluated with Pearson's product–moment correlation. For the analysis of morning-to-afternoon and day-to-day changes, a three-way analysis of variance (ANOVA) model was applied. The factors in the model were subject (as a random factor), study day, time point and study day by time point interaction. A p-value of <0.05 was considered statistically significant for all the analyses unless otherwise stated.

3. Results

3.1. Technical performances of biomarker assays

We selected a panel of biomarkers which would provide an indication of changes that occur in the nasal epithelium, mucosa and olfactory regions. The assay characteristics and the biomarker physiological significance are listed in Table 1. These assays were assessed for their technical range, accuracy and precision before they were used in this study. Table 1 provides the calculated lower limit of quantitation (LLQ) and upper limit of quantitation (ULQ) for each assay as defined by the lowest or highest reference standard concentration in which the deviation from nominal values and the coefficient of variation (CV) between replicates were within 30%. The data in Table 1 show that the albumin, elastase, lactoferrin, and myeloperoxidase assays all had LLQ values in the single ng/mL range, and IL-6, IL-8, and NGF assays had low pg/mL ranges. The measured ULQs for these assays ranged from 0.65 to 1000 ng/mL. We assessed the reproducibility of these assays using a minimum of four replicates of each quality control sample on each plate (intra-assay) and repeating the assay at least three times on different days (inter-assay). At least three quality control samples with biomarker concentrations falling in the low, middle and high regions of the calibration curves were used to control the inter-assay variability. The results in Table 1 show that each of these assays had good reproducibility (less than 25% CV). Fig. 1 shows representative 4-parameter-based curves generated from the reference standards for each biomarker assay used in this study. Based on the data in Table 1 and Fig. 1, we determined that each of these assays would provide useful and reliable data to complete the biomarker analysis.

3.2. Measurement of biomarkers in clinical samples

Once the technical performance of these assays was established, we wanted to establish baseline levels of each biomarker in our clinical samples. Nasal lavage fluids collected from assumed normal healthy volunteers as described in Section 2, were analyzed for each biomarker. Each of the lavage samples was analyzed at a range of sample dilutions to ensure that the analytes were within the technical range of the assays. We determined that samples should be diluted 1/2 or 1/4 for elastase, IL-6, IL-8, NGF and MPO, while the analysis of albumin and lactoferrin required at least a 1/100 dilution. Fig. 2 shows the measured concentrations of each biomarker. Box–whisker diagrams generated with JMP 7.0.1 were used to graphically summarize the distribution of the results and identify concentration range for each biomarker tested. More than 98% of the samples contained measurable concentrations of the albumin, elastase, lactoferrin and MPO. The concentration ranges of albumin and lactoferrin were 1–50 μg/mL (albumin) and 0.5–50 μg/mL (lactoferrin). The measured concentration ranges of the other biomarkers were 10–150 ng/mL for elastase, 0.2–200 ng/mL for MPO, and 60–1000 pg/mL for IL-8. However, IL-6 could only be quantified in 19% of the samples, since most fell below the lower limit of quantitation for the assay (4.8 pg/mL). We also found that only 28% of samples had quantifiable levels of NGF (above LLQ of 5 pg/mL). These findings are similar to published studies showing low levels of IL-6 and NGF in nasal lavage from healthy volunteers [6,25]. The mean concentration of IL-6 in healthy

Table 1
Biomarker assay performance characteristics.

Biomarkers	Physiological significance	LLQ[a]	ULQ[b]	% Mean inter-run CV[c]	Sample dilution
Albumin	Vascular permeability	4.0 ng/mL	1000 ng/mL	9.7	1/125
Elastase	Neutrophil activation	2.0 ng/mL	225 ng/mL	8.7	1/4
IL-6	Pro-inflammatory cytokine	4.8 pg/mL	3000 pg/mL	22.6	1/2
IL-8	Neutrophil chemoattractant	8.0 pg/mL	1000 pg/mL	12.7	1/2
NGF	Neurochemical changes	5.0 pg/mL	650 pg/mL	24.2	1/2
Lactoferrin	Glandular stimulation	1.5 ng/mL	100 ng/mL	15.3	1/100–1/6000
Myeloperoxidase	Neutrophil activity	0.1 ng/mL	25 ng/mL	22.8	1/4

[a] Lower limit of quantitation (LLQ) was determined as the lowest standard concentrations where the deviation from nominal values and the replicate imprecision are within 30%.

[b] Upper limit of quantitation (ULQ) was determined as the highest standard concentrations where the deviation from nominal values and the replicate imprecision are within 30%.

[c] % Mean inter-run CV was calculated using three quality control samples with value at low, middle, or high regions of the calibration curve for each biomarker.

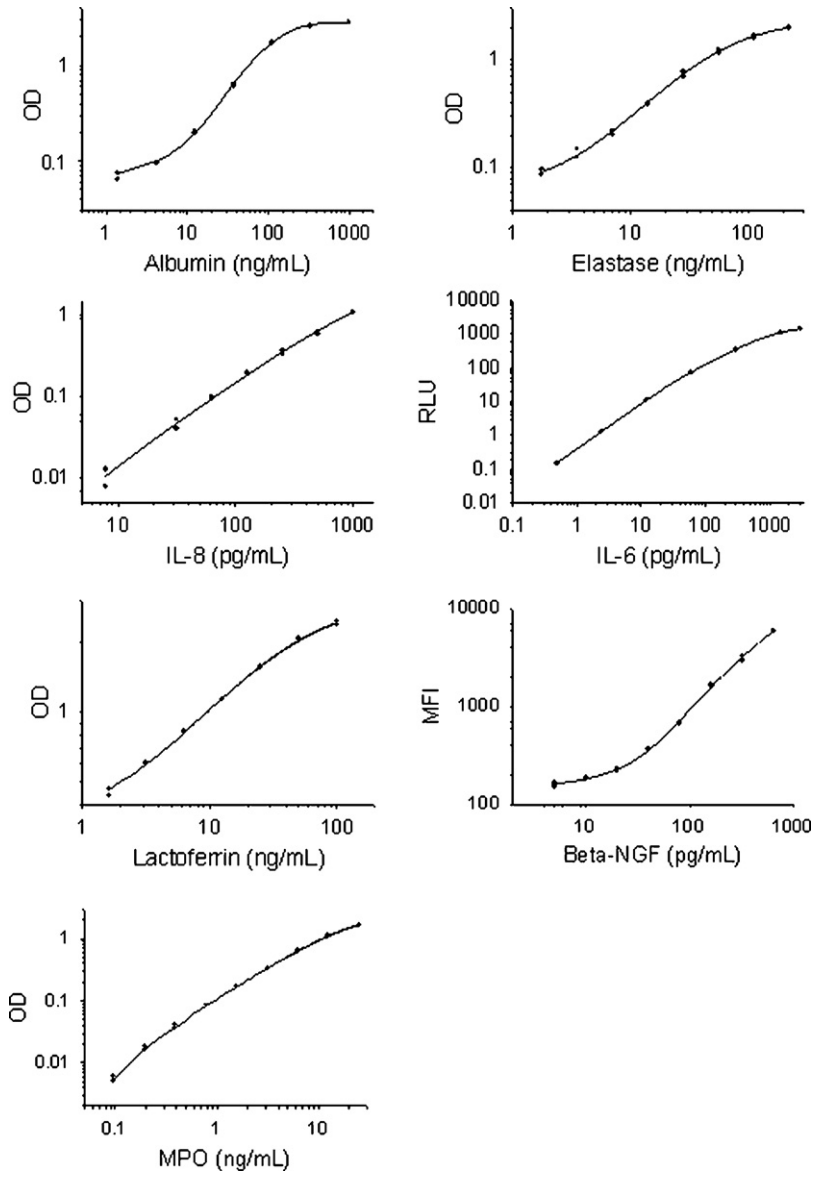

Fig. 1. Biomarker standard curves. Standard curves for each assay determined from the measured optical density (O.D.), median fluorescent intensity (MFI), or relative light unit (RLU) of each standard concentration plotted against the nominal concentrations of each biomarker and fitted with a 4-parameter model using SigmaPlot 8.0 software (Chicago, IL). Duplicated measurements are shown for each standard concentration.

volunteers was approximately 5 pg/mL as reported by Bachert et al. [25], and a range of 3–40 pg/mL of NGF concentrations was reported in the study by Millqvist et al. [6]. However, increases in IL-6 or NGF concentrations are often associated with disease or drug-induced alteration in the nasal epithelium, mucosa, and olfactory regions, thus these assays could still prove useful in analysis of these type samples. Based on the small percentage of measurable IL-6 and NGF in these healthy lavage samples, we did not perform further statistical analysis on these data.

3.3. Influence of pre-washing on biomarker measurement in nasal lavage

A pre-wash of the nasal cavity was included in this study to help establish a consistent starting point for the lavage samples and to improve the reproducibility of the sample collections. A number of previous studies utilized a pre-wash step [35,36], whereas others did not [3,5,28,33]. Based on these published findings, it is not clear if the inclusion of a pre-wash step influenced the measurement of the biomarker levels. Thus, we analyzed the differences between pre-wash and lavage samples collected at each sampling time point to assess the effect of a pre-wash on the biomarker levels in the nasal lavage. The samples from the pre-washes were collected and processed in the same procedure as that used for nasal lavage to ensure consistency in the study. We implemented a 10-min delay between the pre-wash and collection of the nasal lavage. The data had a non-normal distribution, thus we utilized both parametric (paired *t*-test) and nonparametric (Wilcoxon sign-rank test) tests for the analysis. We also log-transformed the data to reduce skewness. The pre-wash and lavage samples from the same subject at each time point were treated as a matched pair in the analysis to evaluate the difference. Table 2 shows the mean difference of the pre-wash lavage samples and the corresponding lavage samples for each biomarker and its associated *p*-values of the paired *t*- and Wilcoxon sign-rank tests. The *p*-values shown in Table 2 demonstrate a significant difference in the levels of elastase between the pre-wash sample and the corresponding lavage sample taken 10 min later (by either test). However, the results show

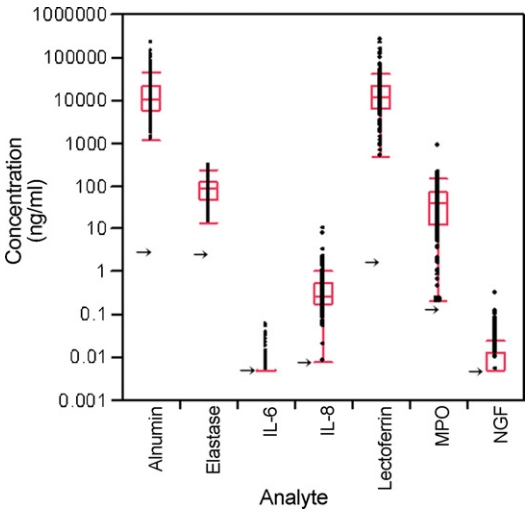

Fig. 2. Measured biomarker concentrations in clinical nasal lavage fluids. The measured biomarker concentrations (ng/mL) from all samples tested for each biomarker were displayed. The results which are below lower limit of quantitation (LLQ) (identified by arrows) are represented by the LLQ of the assay. The box and whisker diagrams summarize the distribution of data for each biomarker. The ends of the box are the 25th and 75th quartiles. The line across the middle of the box identifies the median concentration. The differences between 25th and 75th quartiles are interquartile range (IQR). The whisker lines extend to the outer-most data that falls within 1.5× IQR lower than the 25th quartile or 1.5× IQR higher than the 75th quartile. The two lines across the ends of whisker lines identify the concentration ranges.

Table 2
Matched pairs analysis to evaluate difference between pre-wash and lavage after the pre-washing.

Biomarker	Paired *t* test		Wilcoxon sign-rank test
	Mean difference [a] (lower 95%, upper 95%)	*p*-Value	*p*-Value
Albumin	−0.051 (−0.109, 0.007)	0.082	0.123
Elastase	−0.040 (−0.077, −0.004)	0.032	0.047
IL-8	0.041 (−0.032, 0.113)	0.272	0.113
Lactoferrin	0.002 (−0.076, 0.079)	0.958	0.535
MPO	−0.050 (−0.140, 0.041)	0.280	0.065

[a] The biomarker concentrations (ng/mL) were log-transformed prior to the analysis. The difference here is the difference between log-transformed concentrations for each time point.

that there were no significant differences between the pre-wash and lavage samples for albumin, IL-8, lactoferrin and MPO measurements. These findings show that pre-washing may reduce the levels of elastase but not the other biomarkers in the corresponding lavage sample taken 10 min later.

3.4. Influence of pre-washing on measurement consistency

We next evaluated if adding a pre-wash step improved the consistency of the biomarker measurement on consecutive days. Therefore, if pre-washing improved the consistency, the levels of each biomarker should have a higher correlation between the same clock times on two consecutive days. We used Pearson's product–moment correlation analysis tool to assess the correlation. The analysis showed that there was a significant correlation of albumin measurements on consecutive days in both the morning ($r = 0.641$, $p = 0.0002$) and the afternoon ($r = 0.721$, $p < 0.0001$) lavage samples when there was a pre-wash step performed. We performed the same analysis on the pre-wash lavage samples, which represented samples that did not have a pre-wash step. Interestingly, there was no significant day-to-day correlation of albumin measurements in the morning samples ($r = 0.053$, $p = 0.788$). However, there was a significant, but relatively low level of correlation with the afternoon samples ($r = 0.429$, $p = 0.023$). This suggests that pre-washing the nasal cavity can improve the consistency of albumin measurements on consecutive days. We also looked for a similar finding with the other biomarkers, but found no significant correlation with any of the other biomarkers.

3.5. Morning-to-afternoon changes

Previous studies showed that the levels of albumin, fibrinogen and secretory IgA changed at different times during a 24-h period, in accordance with a diurnal cycle [29,30,37]. It was important for us to investigate if our panel of biomarkers was influenced by this cycle, since future clinical studies would involve more than one time point throughout the day. We compared the measured concentration of each biomarker collected in the morning versus an afternoon time point 6 h later. The time interval for nasal lavage samples in the study has been selected to coincide with the 6-h post-dose time point in the future drug study. The 6-h time point post-dose in the drug study is expected to capture the acute drug-related effect.

The design of the planned clinical study incorporated a pre-wash step 10 min prior to the collection of the lavage samples. We mea-

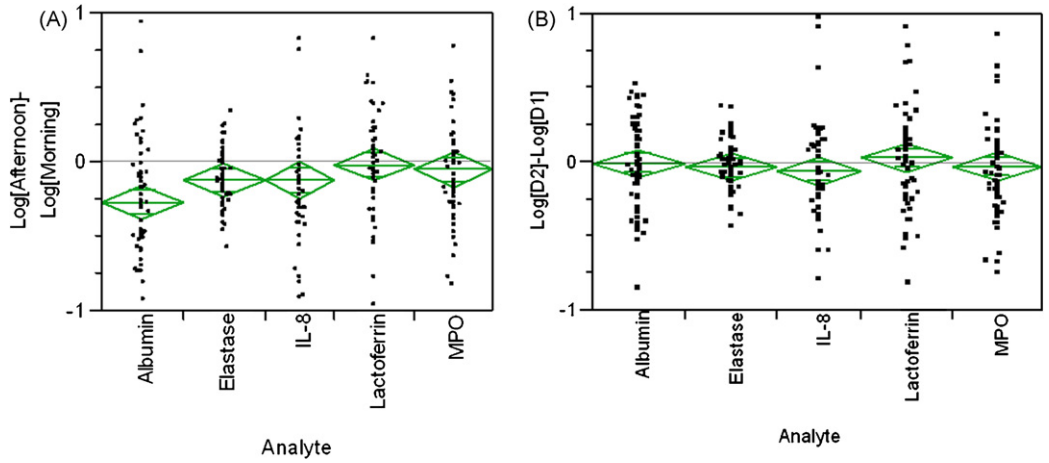

Fig. 3. Morning-to-afternoon and day-to-day variations. The graphs show the difference between the measured levels of each biomarker in the morning vs. the afternoon time points for each subject (A) and in the day 1 vs. day 2 for the matched clock time points for each subject (B). The measured biomarker concentrations (ng/mL) were log-transformed. The results were plotted using JMP 7.0.1 software. The horizontal line in the center of means diamond shows the mean of the difference for each biomarker. The vertical span of each diamond represents the 95% confidence interval (CL).

Table 3
Analysis of variance to evaluate difference between afternoon and morning samples.

Biomarkers	Least squares mean ± std error		
	Morning	Afternoon	*p*-Value
Albumin (μg/mL)	14.7 ± 0.2	8.0 ± 0.2	0.0001
Elastase (ng/mL)	81.8 ± 0.1	62.1 ± 0.1	0.0001
IL-8 (pg/mL)	388.0 ± 0.1	268.3 ± 0.1	0.0046

sured the level of each biomarker in the morning and afternoon lavage samples as described in Section 2. We also log-transformed these data to reduce the observed skewness. Fig. 3A shows the difference between the morning and afternoon biomarker levels from each subject on the same day. Consistent with the finding in previous studies [29,30], the levels of albumin were lower in the afternoon in comparison to the morning. Interestingly, the data show a similar pattern for elastase and IL-8. We performed further analysis using an analysis of variance (ANOVA) model with factors as subject (as a random factor), study day, time point and study day by time point interaction. Table 3 shows the least squares mean concentrations of these three biomarkers at the morning and afternoon sampling time points and the associated *p*-values of the analysis. The results confirmed that all the three biomarkers were at significantly lower concentration in the afternoon in comparison to the morning time point ($p < 0.05$). Using either statistical model, we found no significant changes in concentrations between the morning and afternoon time points for the other biomarkers. These findings suggest that the levels of albumin, elastase, and IL-8 changed at different time points within a day, implying that these biomarkers are influenced by diurnal cycles.

3.5.1. Day-to-day changes

We compared the measured concentrations of these biomarkers at matched clock times on different days. Fig. 3B shows the difference between matched clock times on consecutive days for each subject. The analysis showed that there were no significant mean differences from zero between the two consecutive days at the 95% confidence level. The same ANOVA used in assessing morning-to-afternoon effects above was also applied to analyze the day-to-day changes. The *p*-values from the ANOVA for the effect of study day were >0.05 for all the biomarkers analyzed. Based on these findings, we report no significant differences with any of the biomarkers as measured at the same time on consecutive days.

4. Discussion

In this study, we assessed a panel of clinical biomarkers in assumed-healthy volunteers that could be used to monitor changes in the nasal epithelium, mucosa, and olfactory nerve regions. Effects of assay performance, pre-washing the nasal cavity, and sample collection timing on biomarker baseline values were investigated. We found that the biomarker assays for albumin, elastase, IL-8, lactoferrin, and myeloperoxidase provided sufficient sensitivity, precision and technical ranges for use in clinical samples. However, the assays used to measure IL-6 and NGF were found only to be suitable for analysis of clinical samples for significant increases. We found that the levels of albumin, elastase, and IL-8 had concentrations that varied between morning and afternoon within the same day. The data also demonstrate that pre-washing the nasal cavity prior to collecting nasal lavage caused a significant decrease in elastase levels and improved the consistency of albumin measurements on consecutive days.

Among the seven biomarkers included in this panel, albumin and lactoferrin were the most abundant (1–50 μg/mL), followed by elastase (10–150 ng/mL) and MPO (0.2–200 ng/mL). IL-8 concentrations ranged from 60 to 1000 pg/mL, whereas, IL-6 and NGF concentrations were less than 30 pg/mL in most samples. The biomarker concentrations reported in this study are comparable with the results previously published in numerous studies using immunoassays [5,6,13,24,29,34,35,38].

Interestingly, the levels of some biomarkers fluctuate throughout the day as we found for albumin, IL-8, and elastase. Evidence of circadian modifications in concentrations of biomarkers such as sIgA, albumin and fibrinogen in nasal lavage has been reported previously [28,29,37,39]. Passali and Bellussi [30] reported that there is a circadian pattern in the production of secretory immunoglobins, with concentrations which reach maximal values at 4:00 am. Greiff et al. [29] showed that the levels of albumin and fibrinogen obtained at morning time (4:00 am) were significantly greater than the levels at the afternoon time (16:00). The albumin levels at 8:00 and 12:00 were also higher than that at 16:00, although the difference was not statistically significant. Our results on changes in albumin levels in nasal lavage between morning and afternoon (Fig. 3A) were consistent with the previous finding of the diurnal effects. In addition, we demonstrated similar morning-to-afternoon changes in elastase and IL-8 concentrations in nasal lavage. The diurnal differences in nasal lavage proteins could be related to many factors such as nasal cycle, variation of local production or plasma biomarker levels, and nasal clearance regulated by mucociliary transport. Passali et al. [28] reported that the patency degree of nasal fossa changes at interval of 30 min to 4 h. It was suggested that the periodic congestion and decongestion of the nasal venous sinuses associated with the nasal cycle may contribute to plasma exudation and nasal fluid formation [40]. Greiff et al. hypothesized that the recumbent position during night may lead to affect nasal cycle and increased airway microvascular pooling of blood, and thus, results in increased plasma exudes in the morning hours [29]. Passali et al. [28] reported that sIgA concentration in nasal lavage was related to mucociliary transport, indicating the role of nasal clearance on sIgA concentrations. In our study, with a pre-wash step prior to the lavage sample collection, the nasal clearance effect was minimized. The morning-to-afternoon changes on albumin, elastase and IL-8 concentrations were mainly contributed by the rate of plasma exudation or local production. At the current state of knowledge, the detailed mechanisms of the within day changes is still speculative. It is interesting that we did not observe any significant changes in lactoferrin and MPO concentrations between the morning and afternoon sampling. As these biomarkers in this panel were produced from different cells, it is not surprising that the mechanisms regulating their secretion are different. Unlike albumin, elastase and IL-8 which were at least partially from plasma extrudes or nasal epithelia, lactoferrin was secreted by glandular cells that cover the surface of mucosa [14]. Thus, the nasal cycles that affect plasma extrudation may not affect lactoferrin secretion. Another possibility of no significant morning to afternoon effect is that the sample size in this study may not be sufficient to determine any significant morning-to-afternoon changes on lactoferrin and MPO.

Different factors such as humidity, temperature, sneezing, or length of time between samplings can influence clearance of mucosal surface materials and thus affect nasal biomarker levels [28,41,42]. A pre-wash step was expected to reduce any pre-existing secretions, extraneous matter and accumulated debris. Consequently, the results in lavage samples after the pre-wash were expected to reflect mostly the newly secreted biomarker levels. Therefore, the variations caused by any uncontrollable effects on nasal clearance should be minimized. A number of previous studies utilized a pre-wash step [35,36], whereas others did not [3,5,28,33]. It was not clear whether pre-wash steps had any effects on the nasal biomarker measurements. In this study, we compared biomarker concentrations from pre-wash and lavage samples after pre-washing. Our results showed that the difference in the measured biomarker concentrations between the pre-wash and lavage

samples was significant for elastase (Table 2). Furthermore, the pre-wash increased the correlation of albumin measurements between the matched clock times on two consecutive days. The decrease in the biomarker level after the pre-wash likely resulted from removal of the biomarker in the pre-existing secretion. The increase in day-to-day correlation indicated that the pre-wash increased reproducibility in measurement of albumin, presumably by reducing the variability related to nasal clearance. The extent of the effect of a pre-wash is biomarker dependent as each biomarker has its own rate and mechanism of secretion and clearance. In this study, we did not observe any significant effect of a pre-wash on IL-8, lactoferrin or MPO levels in nasal lavage. Interestingly, a previous study showed that some biomarker concentrations including lactoferrin and IL-8 dropped to ~60% in nasal fluid 30 min after the initial sampling [13]. The difference between studies remains to be understood. Many factors including different sampling procedures and subject populations could have affected study outcomes. It is also possible that this study may not have enough statistical power to detect any significant effect of a pre-wash on IL-8, lactoferrin or MPO levels due to large inter-subject variability. However, the results of the significant effects of a pre-wash on albumin and elastase measurement indicate that a pre-wash can influence biomarker levels in nasal lavage. A pre-wash is a useful step to remove the pre-existing secretion and accumulated debris which are potential sources of variability influenced by uncontrollable nasal clearance.

In addition to the factors of diurnal cycles and nasal clearance, sampling methods could also contribute to the data variability. Unlike the collection of serum or plasma samples, the nasal secretion sampling methods have not been standardized. A number of techniques including spontaneous secretion (nose blowing, suction, microsuction), absorption with filtered paper or foam sampler, nasal washing (nasal lavage and nasal spray blow) have been compared previously [13,23]. Spontaneous secretions, which yielded insufficient amount in many cases, were not regarded as useful for biomarker determination in healthy subjects. Absorption methods are based on capillary suction and thus remove the serous phase of biphasic nasal mucus. Absorption with filter paper often yielded dry specimens and was thus considered as a less suitable method [13]. Riechelmann et al. [13] and Klimek and Rasp [23] both reported that absorption using a foam sampler was superior to obtain specimen for measuring analytes with low concentration in nasal secretions. Nasal lavage and nasal spray blow methods use isotonic sodium chloride solution to wash mucosa and the solution is recovered. The volume of solution in nasal spray blow can be kept considerably smaller to minimize sample dilution. The main concern of using lavage techniques is the unknown dilution factor of the secretions by the washing buffer. Accidental swallowing of saline buffer by subjects during sample collection could add unpredictable variations in the final biomarker measurements. Consistent with previous reports [5,13], considerable variability in the biomarker levels among subjects was observed in this study. The mean inter-subject CV at each sampling time point ranged between 54% and 126%. To assess drug-induced nasal effects with such high variability between subjects, large sample size is needed in drug trials and data analysis should focus on changes within subject. The unpredictable dilution factor of the lavage may be one of the reasons for the large variability. However, lavage techniques allow collection of not only nasal secretions but also cells shed from nasal mucosal surface. The nasal lavage technique has thus been most widely used to assess both cellular and biochemical changes caused by disease, pollution, or drug treatment [3,5,24,43]. In our study, a nasal lavage method was used so that both nasal secretions and circulating cells can be collected for analysis.

Sample matrix effects are also potential sources of assay variability. In our study, we found that IL-6 and IL-8 assays performed better on ELISA platforms as opposed to Luminex-based assays due to the sample viscosity problem. The nasal lavage samples exhibited various levels of viscosity, even though 2.5 mL of saline buffer was used per nostril for the sample collection. Thorough washing between assay steps is critical to minimize any matrix effects and non-specific binding. Our findings suggest that plate-based assays, such as ELISA, are better for lavage samples than bead-based assays. One reason for this is that bead-based assays often utilize filter plates to facilitate the washings which are prone to filter clogging and debris trapping, and promote bead aggregation during the assay process. Sample pretreatment steps such as filtration, dilution, sonication, etc. should be carefully evaluated to reduce these problems without compromising analysis sensitivity and biomarker integrities.

Understanding the variation in baseline values is essential for determining the power of a study in assessing therapeutic or clinical effects. Establishing a consistent starting point for the lavage samples, such as including a pre-wash of the nasal cavity can improve the reproducibility of the sample collections. In clinical studies, drug effects were often assessed by collecting samples at multiple time points. As diurnal fluctuations are common in many nasal lavage biomarkers, careful design with placebo controls for diurnal effects is necessary to exclude confounding diurnal effects. In studies with a single point per day, it is important to compare samples collected at matched time points. The evaluation on intra- and inter-subject variation, circadian rhythm, and effects of sampling and assay methods in this study has provided important information in determining optimal subject number and sampling schedule for the desirable end-points of a clinical study.

Acknowledgements

The authors would like to thank Holly Palme, Oi Wong, and Flora Berisha for their advice and support, and Dr. Faud Baroody for support in study design and sample collection.

References

[1] C.L. Graff, G.M. Pollack, J. Pharm. Sci. 94 (2005) 1187–1195.
[2] L. Illum, Eur. J. Pharm. Sci. 11 (2000) 1–18.
[3] R. Walinder, D. Norback, B. Wessen, P. Venge, Arch. Environ. Health 56 (2001) 30–36.
[4] M. Pupek, W. Mikulewicz, J. Mielnik, B. Batycka, I. Katnik-Prastowska, Arch. Immunol. Ther. Exp. (Warsz) 51 (2003) 259–265.
[5] L. Nikasinovic-Fournier, J. Just, N. Seta, F. Callais, F. Sahraoui, A. Grimfeld, I. Momas, J. Lab. Clin. Med. 139 (2002) 173–180.
[6] E. Millqvist, E. Ternesten-Hasseus, A. Stahl, M. Bende, Environ. Health Perspect. 113 (2005) 849–852.
[7] H. Riechelmann, T. Deutschle, A. Rozsasi, T. Keck, D. Polzehl, H. Burner, Clin. Exp. Allergy 35 (2005) 1186–1191.
[8] J. Mochca-Morales, Arch. Med. Res. 31 (2000) 409–414.
[9] H.C. Kaulbach, M.V. White, Y. Igarashi, B.K. Hahn, M.A. Kaliner, J. Allergy Clin. Immunol. 92 (1993) 457–465.
[10] X. Xu, D. Zhang, H. Zhang, P.J. Wolters, N.P. Killeen, B.M. Sullivan, R.M. Locksley, C.A. Lowell, G.H. Caughey, J. Exp. Med. 203 (2006) 2907–2917.
[11] F.T. Allocco, V. Votypka, M. deTineo, R.M. Naclerio, F.M. Baroody, Ann. Allergy Asthma Immunol. 89 (2002) 578–584.
[12] J.A. Nadel, Chest 117 (2000) 386S–389S.
[13] H. Riechelmann, T. Deutschle, E. Friemel, H.J. Gross, M. Bachem, Eur. Respir. J. 21 (2003) 600–605.
[14] D. Legrand, E. Elass, M. Carpentier, J. Mazurier, Cell. Mol. Life Sci. 62 (2005) 2549–2559.
[15] X. Wu, A.C. Myers, A.C. Goldstone, A. Togias, A.M. Sanico, J. Allergy Clin. Immunol. 118 (2006) 428–433.
[16] T. Miwa, T. Moriizumi, I. Horikawa, N. Uramoto, T. Ishimaru, T. Nishimura, M. Furukawa, Microsc. Res. Technol. 58 (2002) 197–203.
[17] E.R. Wilfong, R.D. Dey, Am. J. Respir. Cell. Mol. Biol. 30 (2004) 793–800.
[18] J.B. Watelet, C. Bachert, C. Claeys, P. Van Cauwenberge, Allergy 59 (2004) 54–60.
[19] M.F. Kramer, T.R. Jordan, C. Klemens, E. Hilgert, J.M. Hempel, E. Pfrogner, G. Rasp, Am. J. Otolaryngol. 27 (2006) 190–199.
[20] M.F. Kramer, G. Burow, E. Pfrogner, G. Rasp, Clin. Exp. Allergy 34 (2004) 1086–1092.
[21] H. Riechelmann, G. Rettinger, S. Lautebach, S. Schmittinger, T. Deutschle, J. Occup. Environ. Med. 46 (2004) 316–322.
[22] R. Alam, T.C. Sim, K. Hilsmeier, J.A. Grant, J. Immunol. Methods 155 (1992) 25–29.
[23] L. Klimek, G. Rasp, Clin. Exp. Allergy 29 (1999) 367–374.

[24] H.S. Koren, G.E. Hatch, D.E. Graham, Toxicology 60 (1990) 15–25.
[25] C. Bachert, M.J. van Kempen, K. Hopken, G. Holtappels, M. Wagenmann, Eur. Arch. Otorhinolaryngol. 258 (2001) 406–412.
[26] T. Heikkinen, M. Shenoy, R.M. Goldblum, T. Chonmaitree, Acta Paediatr. 88 (1999) 150–153.
[27] Y.S. Cho, S.Y. Park, C.K. Lee, B. Yoo, H.B. Moon, J. Allergy Clin. Immunol. 112 (2003) 695–701.
[28] D. Passali, L. Bellussi, M. Lauriello, Acta Otolaryngol. 110 (1990) 437–442.
[29] L. Greiff, A. Akerlund, M. Andersson, C. Svensson, U. Alkner, C.G. Persson, Acta Otolaryngol. 116 (1996) 85–90.
[30] D. Passali, L. Bellussi, Acta Otolaryngol. 106 (1988) 281–285.
[31] N. Terada, N. Hamano, W.J. Kim, K. Hirai, T. Nakajima, H. Yamada, H. Kawasaki, T. Yamashita, H. Kishi, T. Nomura, T. Numata, O. Yoshie, A. Konno, Am. J. Respir. Crit. Care Med. 164 (2001) 575–579.
[32] D.A. van Zuijlen, E.A. van de Graaf, E.M. van Bolhuis, C. Versluis, E.F. Knol, S. van der Baan, J. Immunol. Methods 256 (2001) 1–10.
[33] R.J. Salib, L.C. Lau, P.H. Howarth, Clin. Exp. Allergy 35 (2005) 995–1002.
[34] M. Abu-Harb, F. Bell, A. Finn, W.H. Rao, L. Nixon, D. Shale, M.L. Everard, Eur. Respir. J. 14 (1999) 139–143.
[35] S. Repka-Ramirez, K. Naranch, Y.J. Park, D. Clauw, J.N. Baraniuk, Allergy Asthma Proc. 23 (2002) 185–190.
[36] G.D. Raphael, Y. Igarashi, M.V. White, M.A. Kaliner, J. Allergy Clin. Immunol. 88 (1991) 33–42.
[37] N. Mygind, J. Thomsen, Acta Otolaryngol. 82 (1976) 219–221.
[38] U. Westin, E. Lundberg, J.A. Wihl, K. Ohlsson, Allergy 54 (1999) 857–864.
[39] T. Harada, Y. Hamaguchi, Y. Sakakura, Y. Miyoshi, Acta Otolaryngol. 97 (1984) 359–362.
[40] R.B. Eccles, Acta Otorhinolaryngol. Belg. 54 (2000) 281–286.
[41] L. Illum, J. Aerosol Med. 19 (2006) 92–99.
[42] R.J. Soane, A.S. Carney, N.S. Jones, M. Frier, A.C. Perkins, S.S. Davis, L. Illum, Clin. Otolaryngol. Allied Sci. 26 (2001) 9–15.
[43] P.A. Steerenberg, P.H. Fischer, L. van Bree, H. van Loveren, Arch. Toxicol. Suppl. 19 (1997) 207–216.