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Methodology to quantitate Total Nitrate/Nitrite in Human Sputum samples

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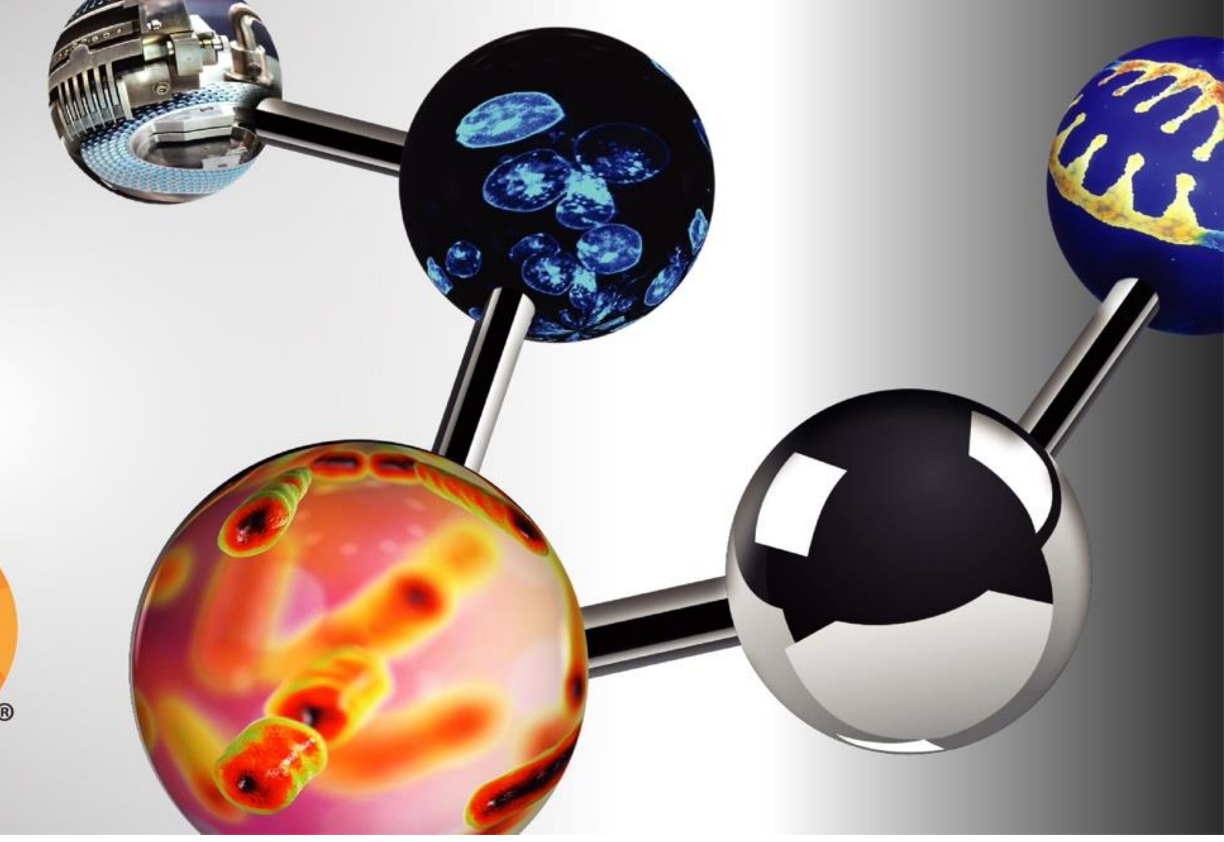

PURPOSE

Nitric oxide (NO) is a free radical gas that has been shown to play a role in a variety of lung processes and is important in the pathophysiology of cystic fibrosis patients. Cystic fibrosis (CF) is characterized by chronic airway infection and inflammation, which accounts for most morbidity and deaths. Exhaled nitric oxide (NO), elevated inmost inflammatory lung diseases, is decreased in CF, suggesting decreased formation, increased metabolism or loss of NO. Nitric oxide (NO) can be detected in exhaled gas in human subjects, however this NO measurement can be challenging and cumbersome as it is relatively unstable. In patients with cystic fibrosis there is increased arginase activity in sputum. This elevated arginase activity can limit the availability of L-arginine for nitric oxide (NO) synthesis, as the arginase catalyzes the hydrolysis of L-arginine to form L-ornithine and urea. NO is produced by nitric oxide synthase (NOS) and is rapidly metabolized to nitrite and nitrate (NO_2^-/NO_3^-). Here we describe a methodology (Somru Biosciences) approach to measure total nitrate and nitrite in human sputum.

OBJECTIVES

1. To formulate a processing buffer to treat highly viscous sputum samples before using it in total nitrate/nitrite colorimetric assay.
2. To validate the total nitrate/nitrite colorimetric assay for human sputum matrix.

METHODS

Human Sputum Processing

Human sputum is composed of white blood cells, cellular debris, dead tissue, serous fluid and mucus. In addition to diluting the viscous matrix, a reducing mucolytic agent must be added to dissolve the mucin present. Human sputum processing buffer (pH 6.0) contains sodium phosphate, sodium chloride and dithiothreitol (DTT). Following treatment with sputum processing buffer, samples are centrifuged at high speed (13,000 g) for 30 minutes to remove any cell debris.

Assay Outline

To the processed human sputum sample, nitrate reductase cofactor and nitrate reductase enzyme are added. Following an incubation with the nitrate reductase cofactor and enzyme, Greiss reagent 1 and 2 are added. Greiss reagent reacts with nitrite to form an azo dye which is read at 540 nm. The assay range is 15 µM – 500 µM. An endogenous quality control sample (QC Endo), comprised of pooled human sputum samples, was used in all assays to monitor inter-day and intra-day assay variability and sample stability.

RESULTS

Table 1. Basal level of Total Nitrate/Nitrite in Human Sputum

Lot#	Observed Concentration (µM)
1	88.8
2	35.8
3	QMV
4	59.1
5	719
6	42.6
7	89.9
8	27.9
9	43.9
10	53.3

QMV = Questionable Multiple Values

Table 2. Short-Term Stability of Total Nitrate/Nitrite in Human Sputum at Ambient Temperature Under White Light Conditions

Run	STS QC Endo 133 µM
28	128
	125
	127
	128
	127
	127
Mean	127
% CV	0.9
% Theoretical	95.5
n	6

Table 3. Stability of Total Nitrate/Nitrite in Human Sputum Following Freeze (-80℃)-Thaw (Ambient Temperature) Cycles Under White Light Conditions

Run	FT QC Endo 133 µM
28	132
	142
	139
	135
	140
	133
Mean	137
% CV	3.0
% Theoretical	103.0
n	6

Table 4. Long-Term Stability (258 days) of Total Nitrate/Nitrite in Human Sputum at -80℃

Run	QC Endo 133 µM
36	151
	150
	152
	151
	152
	149
Mean	151
% CV	0.8
% Theoretical	113.5
n	6

Table 5. Within-Run and Between-Run Accuracy and Precision for Total Nitrate/Nitrite in Human Sputum

Run	LLOQ QC 15.0 µM	QC A 40.0 µM	QC B 100 µM	QC Endo 133 µM	QC C 375 µM	ULOQ QC 500 µM
24	15.7	39.6	98.2	121	396	572
	15.2	38.5	97.6	123	396	498
	14.6	37.8	87.5	125	372	553
Within-Run Mean	15.2	38.6	94.4	123	388	541
Within-Run SD	0.551	0.907	6.01	2.00	13.9	38.4
Within-Run % CV	3.6	2.3	6.4	1.6	3.6	7.1
Within-Run % Bias	1.3	-3.5	-5.6	-7.5	3.5	8.2
n	3	3	3	3	3	3
31	15.0	40.5	100	142	380	505
	14.5	37.4	96.6	141	373	489
	16.5	35.1	90.6	125	348	474
Within-Run Mean	15.3	37.7	95.7	136	367	489
Within-Run SD	1.04	2.71	4.76	9.54	16.8	15.5
Within-Run % CV	6.8	7.2	5.0	7.0	4.6	3.2
Within-Run % Bias	2.0	-5.8	-4.3	2.3	-2.1	-2.2
n	3	3	3	3	3	3
32	15.0	35.7	93.5	123	369	476
	14.5	34.6	90.5	120	355	472
	14.4	34.3	90.2	115	350	462
Within-Run Mean	14.6	34.9	91.4	119	358	470
Within-Run SD	0.321	0.737	1.82	4.04	9.85	7.21
Within-Run % CV	2.2	2.1	2.0	3.4	2.8	1.5
Within-Run % Bias	-2.7	-12.8	-8.6	-10.5	-4.5	-6.0
n	3	3	3	3	3	3
33	12.3	38.3	91.1	139	331	554
	12.1	37.8	88.6	138	314	456
	~10.8	43.1	88.7	143	304	420
Within-Run Mean	11.7	39.7	89.5	140	316	477
Within-Run SD	0.814	2.93	1.42	2.65	13.7	69.3
Within-Run % CV	7.0	7.4	1.6	1.9	4.3	14.5
Within-Run % Bias	-22.0	-0.8	-10.5	5.3	-15.7	-4.6
n	3	3	3	3	3	3
34	14.2	36.2	92.7	134	355	476
	14.3	35.1	93.2	139	358	474
	14.0	35.2	91.4	133	358	467
Within-Run Mean	14.2	35.5	92.4	135	357	472
Within-Run SD	0.153	0.608	0.929	3.21	1.73	4.73
Within-Run % CV	1.1	1.7	1.0	2.4	0.5	1.0
Within-Run % Bias	-5.3	-11.3	-7.6	1.5	-4.8	-5.6
n	3	3	3	3	3	3
35	14.4	38.7	102	145	365	483
	13.6	37.8	96.5	141	360	474
	12.9	41.3	94.3	142	338	477
Within-Run Mean	13.6	39.3	97.6	143	354	478
Within-Run SD	0.751	1.82	3.97	2.08	14.4	4.58
Within-Run % CV	5.5	4.6	4.1	1.5	4.1	1.0
Within-Run % Bias	-9.3	-1.8	-2.4	7.5	-5.6	-4.4
n	3	3	3	3	3	3
Between-Run Mean	14.1	37.6	93.5	133	357	488
Between-Run SD	1.37	2.46	4.16	9.69	24.4	37.6
Between-Run % CV	9.7	6.5	4.4	7.3	6.8	7.7
Between-Run % Bias	-6.0	-6.0	-6.5	0.0	-4.8	-2.4
Between-Run % Total Error	15.7	12.5	10.9	7.3	11.6	10.1
n	18	18	18	18	18	18

~ = Greater than 25% theoretical

CONCLUSION

Human sputum samples are directly measured using a spectrophotometer, and any cell debris or other particle could interfere with the optical reading. Treating the sputum sample with sputum processing buffer containing a mucolytic agent and spinning down the sample at high speed yields a cleaner sample for direct use in the colorimetric assay.

For validating the method to quantitate total nitrate/nitrite in human sputum samples, matrix effect, inter-run and intra-run accuracy and precision and stability testing were carried out. Basal level of total nitrate/nitrite was tested in sputum from 9 unique healthy control human subjects. Data are reported from these 9 lots (Table 1). Results showed highly variable total nitrate/nitrite measurements between subjects, ranging from 25-720 µM. To assess the inter-run and intra-run variability of the assay, screened control human sputum lots were pooled together to make an Endogenous Quality Control sample (QC Endo).

The within run and between run % CV of the QC Endo across 9 different runs was 7.3% indicating that the sputum sample processing and assay quantitation are highly precise (Table 5). QC Endo sample in polypropylene tubes were subjected to freeze (-80℃)-thaw (ambient temperature) cycles under the white light conditions and were assessed against a freshly prepared calibration curve. Stability was demonstrated for up to 6 freeze-thaw cycles (Table 3). QC Endo replicates were maintained in polypropylene tubes at ambient temperature under white light conditions for 17 hours and were assessed against a freshly prepared calibration curve to assess short-term (bench-top) stability (Table 2). Stability of QC Endo was demonstrated for up to 258 days when frozen at 80℃ (Table 4).

This colorimetric method can be used to quantitate total nitrate/nitrite in the human sputum samples. This method is highly reproducible and can be used as an easier alternative for nitric oxide measurement in cystic fibrosis patients leading to improved therapeutic treatments and evaluations in this diseased population.

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