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Diagnosis of Gulf War Illness Using Laser-Induced Spectra Acquired from Blood Samples

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Abstract

Gulf War illness (GWI) is a chronic illness with no known validated biomarkers that affects the lives of hundreds of thousands of people. As a result, there is an urgent need for the development of an untargeted and unbiased method to distinguish GWI patients from non-GWI patients. We report on the application of laser-induced breakdown spectroscopy (LIBS) to distinguish blood plasma samples from a group of subjects with GWI and from subjects with chronic low back pain as controls. We initially obtained LIBS data from blood plasma samples of four GWI patients and four non-GWI patients. We used an analytical method based on taking the difference between a mean LIBS spectrum obtained with those of GWI patients from the mean LIBS spectrum of those of the control group, to generate a "difference" spectrum for our classification model. This model was cross-validated using different numbers of differential LIBS emission peaks. A subset of 17 of the 82 atomic and ionic transitions that provided 70% of correct diagnosis was selected test in a blinded fashion using 10 additional samples and was found to yield 90% classification accuracy, 100% sensitivity, and 83.3% specificity. Of the 17 atomic and ionic transitions, eight could be assigned unambiguously to species of Na, K, and Fe.

Keywords

Laser-induced breakdown spectroscopy, Gulf War illness, chemometrics, spectral analysis, classification algorithms, blood plasma, chronic low back pain, chronic multisymptom illness, diagnostic biomarker

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Introduction

Gulf War illness (GWI) is a chronic illness with multiple symptoms spanning several domains in individual patients.[1,2] First defined by the Centers for Disease Control and Prevention (CDC) after the 1990–1991 Gulf War,[3] established medical diagnoses, laboratory tests, and hypothesis-driven research have failed to explain its multifaceted symptomatology.[1,2,4] The etiology of GWI is still unknown and hypotheses involving exposures to vaccines,[5] medications, pesticides, chemical munitions, inhalation of depleted uranium dust and smoke from burn pits, and burning oil fields have all been investigated.[6] GWI is reflected in a multifaceted syndrome with varied presentation in individual patients comprising physical symptoms (fatigue,[7] joint and muscle pain[8]), gastrointestinal disorders,[9] cognitive symptoms,[10] co-morbid syndromes (chronic fatigue syndrome, fibromyalgia, irritable bowel syndrome), and other clinical aspects such as depression and anxiety. Impairment due to GWI can include both cognitive and emotional/behavioral symptoms.

According to the Research Advisory Committee on Gulf War Veterans' Illnesses,[2] at least a quarter of the nearly

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700 000 U.S. veterans who served in the 1990–1991 Gulf War are affected by GWI.[11–13] The health effects associated with GWI have significant impact on quality of life. There is no effective treatment and patients do not appear to recover with time. This is a complex condition that is not well understood, and its management may require individualized health care plans.[1]

The broad spectrum of GWI significantly complicates not only clinical assessment but also the likelihood of effective treatment–intervention efficacy depends upon clear diagnosis, patient follow up, and cooperation in the treatment plan. An objective and widely deployable tool to confirm GWI diagnosis could also improve disease monitoring and treatment outcomes. Currently, there are no established biomarkers or other lab tests for diagnosis of GWI or for prediction of the success rate of treatment interventions in patients with GWI symptoms, primarily due to our incomplete understanding of the disease etiology. Several previous studies have searched for GWI diagnostic biomarkers with limited success so far in terms of broader validation of the findings and applicability into clinical practice.[14–19] The complexity of GWI symptoms suggests implication of multiple pathways with concomitant dysfunction. Together, these aspects of GWI suggest that a global, rather than targeted, approach in diagnostic biomarkers is warranted. Concomitant markers of multiple pathways inhere in intact human-derived biological specimens.

In this work, we report on the application of laser-induced breakdown spectroscopy (LIBS) to comprehensively survey blood plasma from GWI patients and to identify characteristics that distinguish them from non-GWI patients who might share similar symptoms. LIBS is an analytical technique that relies on the generation and spectral analysis of atomic, ionic, and molecular lines emitted by the laser-induced plasma (LIP) plume induced by the interaction of pulsed laser with a sample.[20] This work is based on the fact that LIBS can provide a uniquely comprehensive, elemental-level assessment, which will reveal the presence of key, concomitant components in biologic specimens, necessary to correlate with the range of symptoms experienced in GWI. As we recently reviewed,[21] biomedical applications of LIBS include imaging, guided laser surgery, pathogen identification, medical diagnosis and the first demonstrations of LIBS "liquid biopsy" for diagnosing asymptomatic cancers, either with tagging approaches[22,23] or with tag-free untargeted methods.[24–27] The latter are usually coupled with machine learning that has been gaining increasing attention in the LIBS community and in spectroscopy at large.[28–34]

One of the main advantages of LIBS over other spectroscopic methods is that it can be used to interrogate tissues without preparation protocols that select for or inadvertently alter sample constituents or disrupt constituent interactions, which may be important in the pathobiology of GWI. This approach does not hypothesize the importance of a single pathway but rather seeks to provide an unbiased survey and assessment of the spectroscopic signatures of all potential biological markers present, and their interactions, in intact tissue.

Experimental

We analyzed two sets of samples: blood plasma samples from a group of subjects with GWI (GWI pos) and from subjects with chronic low back pain (cLBP) as controls (GWI neg). Patients with cLBP were used as a comparison group (disease control), as they also suffered from a chronic illness and shared some symptoms with GWI but lacked the GWI-associated exposures during deployment which are believed to play a causative role in GWI. Below we describe how each set of samples was collected.

Blood Sample Collection

The blood samples of the GWI group were drawn from subjects enrolled to a Randomized Controlled Trial testing the effectiveness of acupuncture.[35] Volunteers were invited in for screening into the study if they had (i) been deployed to the "Gulf Theater of operations", (ii) at least two of the following symptoms from the three CDC clusters of symptoms which lasted for more than six months, including fatigue for 24 h or more after exertion, depression, anxiety, irritability, difficulty in thinking or concentration, problems finding words, sleep disturbances, and muscle or joint pain.[3] Candidates for enrollment were recruited from the New England area using advertisements in various media and mailings to military personnel from the Federal Manpower Database. The study was approved by the New England School of Acupuncture Institutional Review Board (IRB) (NEIRB #09-204).

The blood samples from the cLBP group were drawn from enrolled patients for a pilot clinical trial "Structural Integration for Chronic Low Back Pain".[36] The study, including the protocol for obtaining, processing, storing, and analyzing blood samples (including consent for future research activities), was approved by the Spaulding Rehabilitation Hospital Institutional Review Board (# 2010p000014). Candidates for enrollment were recruited in the Boston area by self-referral in response to posters placed at Spaulding Outpatient Rehabilitation clinics, presentations to their clinical staff, and emails broadcast to individuals who had registered their interest in clinical trials for low back pain in a recruitment database maintained by Partners Healthcare. Enrollment criteria included men and women, aged 18–65 with low back pain of at least six months duration not attributed to radiculopathy, infection, neoplasm, fracture, or inflammatory rheumatic process. The severity of pain was self-rated on average over the

preceding six months, and only patients with pain score ≥ 3 on an 11-point ordinal scale were included.

Both studies were conducted approximately during the same period (2010–2013) and the exact same protocol was used for sample collection, processing, and storage in both groups. Briefly, blood was drawn by venipuncture in a heparinized tube. Plasma was separated by centrifugation within 30 min of blood collection and stored for up to four weeks at –20 °C followed by long-term storage at –80 °C. All samples were previously thawed once on ice for making aliquots.

For the present study, nine samples from the GWI group (GWI pos) and nine samples from the cLBP group (GWI neg) were analyzed.

LIBS Experimental Setup

The LIBS experimental setup and method used for this work are described in a previous publication from our group.[37] Essentially, they consist of a focusing 7 ns neodymium-doped yttrium aluminum garnet (Nd:YAG) laser (Surelite II, Continuum) pulses operating at 1064 nm on samples using an air-spaced doublet lens with focal length of 30 mm. The samples to be analyzed were loaded onto a three-dimensional computer-controlled translation stage located within a chamber (SciTrace, AtomTrace). The LIP emission was collected at an angle of 45° with respect to the laser beam by means of a 50 μm core-diameter optical fiber, which was coupled to an Echelle spectrograph (Andor Technology, ME 5000) and a thermoelectrically cooled iStar intensified charge-coupled device camera (Andor Technology, DH734-18 F-03). The time parameters used for this work were: 1 μs gate delay, 5 μs gate width. The focused laser spot diameter was about 100 μm, the repetition rate was 0.5 Hz, and the laser energy was 130 ± 2 mJ. All measurements were carried out in air at atmospheric pressure. We deposited 5 μL of each individual blood plasma specimen on the unpolished side of pure Si wafers, previously rinsed in 2-propanol, and dried the samples for 10 min with a tungsten infrared lamp. To make up for possible inhomogeneity in the liquid distribution on the substrate, we acquired 96 single-shot spectra for each sample and ensured that each spectrum came from a fresh spot on the surface of the dried plasma drop by displacing the sample with the translation stage. Averages of the 96 single-shot spectra were used for each sample, after removing those with total emission intensity lying outside the interval mean $\pm$ 1 standard deviation.

Results and Discussion

The data analysis approach we adopted for this work is a modified version of the difference spectrum method that we developed in Gaudiuso et al.[37] We started by generating a classification model by using eight samples of known status (four GWI pos, four GWI neg). We averaged the LIBS spectra of the GWI pos samples, normalized over the total emission intensity, and did the same for the controls, to generate two mean spectra, one for each class. We then subtracted the mean GWI neg spectrum from the mean GWI pos one and obtained the difference spectrum shown in Fig. 1. This was used to identify the atomic and ionic transitions to include in the classification test, by means of a two-step feature selection procedure.

The first step was to eliminate the possibility of spectral interference from the substrate (pure silicon wafer), by including in the analysis only the emission peaks that either were completely absent in the spectra of clean silicon or that had intensity lower than 50% of the intensity in the samples' average spectra. This left us with 82 transitions (from about the 200 peaks visible in the spectral range 200–900 nm) that we used to validate our model.

For this, we employed a leave-one-out cross-validation approach, i.e., we used seven of the eight known samples as training set, built a model difference spectrum, and tested it with the eighth, left-out sample. We then swapped the training and testing subsets, until we built eight different models and used each to obtain a GWI pos or GWI neg diagnosis for each left-out sample.

As described in Gaudiuso et al.,[37] this was done by comparing the polarity of each transition in the test difference spectrum against those in the model. The polarities were determined by simply subtracting the mean normalized GWI neg spectrum from the analogous GWI pos one. For some transitions, the polarity could not be immediately established with this simple approach, because the difference peaks were asymmetric, most likely due to a slight wavelength shift in the source spectra. Such spectrum-to-spectrum wavelength shift, which corresponds to about one to two pixels, is most likely due to electronic noise. In such cases, we determined the intensity of the given peaks in the mean normalized GWI neg spectrum and in the analogous GWI pos by Lorentzian fitting, and we used the difference between the resulting numerical values to determine the polarity of the transition in the difference spectrum. Transitions having the same polarity as the model received a GWI pos label, while those with opposite polarity received a GWI neg label. The diagnosis for each sample was then obtained through a majority vote, i.e., based on the number of GWI pos or GWI neg labels. While we did not observe it in this work, it is possible that samples receive an equal number of GWI pos/GWI neg labels. In such cases, no diagnosis would be possible, and the status of the samples would remain undetermined.

The second step of our feature selection procedure was to identify the transitions that contribute the most to the classification and rank them based on the percentage of correct labels assigned by each transition to the samples. To establish the number of spectral features providing optimal classification, we repeated the test using various

subsets of the 82 features, each corresponding to different percentages of correct diagnoses. To do so, we checked the labels assigned by each transition to each sample, and we tallied the number of correct labels to determine the corresponding percentage of correct diagnoses. We then ranked the transitions based on this percentage, and ran separate tests using different thresholds, which results are reported in Table I. In this table, for example, threshold >70% indicates that the specific test was run using only the transitions that provided more than 70% correct labels.

The results shown in Table I are expressed in terms of the classification metrics typically used to predict true and false positives and negatives.[37]

When using all the selected transitions, two of the control samples are misclassified as GWI pos (false positives) but using a threshold of at least 70% correct diagnoses ($N = 17$) brought the number of misclassified samples to only one. Since changing the threshold to 80% correct diagnoses ($N = 3$) did not improve the results, we selected 70% as the optimal threshold for the blind test, so to minimize the risk of overfitting the cross-validation data set.

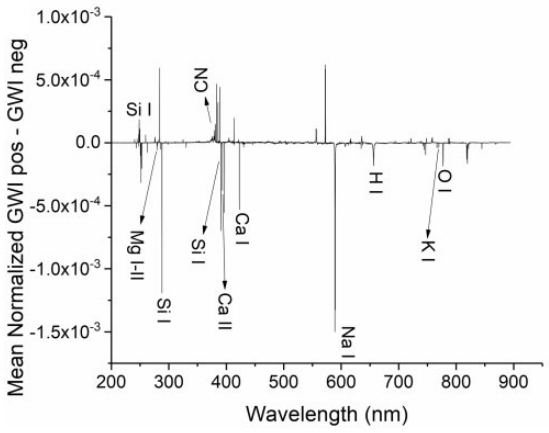

Figure 1. Difference spectrum obtained by subtracting the mean normalized GWI neg spectrum (obtained by averaging together all the LIBS spectra of the four known GWI neg samples) from the mean normalized GWI pos spectrum (obtained by averaging together all the LIBS spectra of the four known GWI pos samples).

These 17 top transitions are listed in Table II. Due to the fact that some of these transitions are not listed in the existing atomic spectra databases,[38,39] several could not be assigned with certainty and were therefore left blank. While this can hinder establishing a possible causality link between elemental dyshomeostasis and GWI physiopathology, it does not affect the scope of the current work, i.e., investigating the feasibility of an atomic spectroscopy technique to distinguish between two groups of samples that are characterized by the presence/absence of GWI. Moreover, as Table II shows, some of the elements contributing the most to the classification accuracy can be identified as alkaline metals (Na, K) and Fe.

Table II shows that different transitions from the same emitter, Na(I), have a different contribution to the classification accuracy. Two aspects could contribute to this. The first is the experimental uncertainty associated to Lorentzian fitting. Different transitions can be affected by a different fitting error (e.g., associated to Lorentzian deconvolution of adjacent peaks), which can result in a slightly different contribution to the classification accuracy. The second is that, while they belong to the same emitter, these two groups of lines (589–590 nm, 818 nm) have different energies. In particular, the 589 nm Na(I) lines are more likely to be affected by self-absorption than the 818 nm ones. This can affect their contribution to classification accuracy in two ways, i.e., (i) by altering the line profile and thus increasing the fitting error and the polarity of the difference peaks and (ii) by altering the proportionality between the spectral intensity and the population of the excited level. Moreover, we note that, while the percentages appear significantly different (87.5% with Na(I) 818.33–818.49 nm and 75% with Na 589.00–589.56 nm), in absolute numbers the actual difference is minimal (respectively, seven/eight and six/eight correct diagnoses.) We expect that with larger training sample sets, these differences would even out.

The blind test was carried out with 10 additional samples, using the top transitions reported in Table II. The difference spectra for the unknown samples were obtained by subtracting the normalized mean spectrum of the four known

Table I. Results of cross-validation test with different subsets of transitions in the LIBS difference spectra.

	Results with different subsets of transitions[a]			
Classification metrics (training set)	$N = 82$	$N = 32$	$N = 17$	$N = 3$
Classification accuracy (%)	75	75	87.5	87.5
Sensitivity (%)	100	100	100	100
Specificity (%)	50	50	75	75

[a]All transitions indicates results that were obtained using all the spectral features that were visible in the sample spectra, but absent in the substrate ($N = 82$). The remaining columns in the table indicate three different thresholds used to select the most diagnostic features, i.e., results obtained using only the transitions providing at least 60% ($N = 32$), 70% ($N = 17$), and 80% ($N = 3$) of correct diagnoses.

Table II. Spectral assignation of the $N = 17$ top transitions, ranked based on the percentage of correct diagnoses provided by each.

Wavelength (nm)	Emitter	% Correct diagnoses (cross-validation)
606.97	–	87.5
818.33	Na(I)	87.5
819.48	Na(I)	87.5
276.43	–	75.0
275.63	Fe(I)	75.0
556.25	–	75.0
281.21	–	75.0
758.73	–	75.0
324.91	–	75.0
330.27	Na(I)	75.0
556.80	–	75.0
769.90	K(I)	75.0
589.00	Na(I)	75.0
380.98	–	75.0
379.80	–	75.0
244.77	Fe(I)	75.0
589.59	Na(I)	75.0

Note: Only those with more than 70% correct diagnoses are reported in this table, as this threshold was chosen to carry out the blind test.

Table III. Results of blind test.

Classification metrics (blind test)	Results with $N = 17$ top transitions (threshold > 70%)[a]
Classification accuracy (%)	90.0
Sensitivity (%)	100
Specificity (%)	83.3

[a]Threshold > 70% indicates that the results were obtained using only the $N=17$ spectral features that provided at least 70% correct diagnoses in the cross-validation test (reported in Table II).

GWI neg specimens from the normalized spectra of each unknown sample, and the resulting polarities compared to those of the model difference spectrum comprised of all eight known specimens. The results of the blind tests are reported in Table III. As previously seen for the cross-validation set, also in this group of samples there was only one misclassification, and it was a false positive.

Conclusion

This work is the first example of the application of an optical spectroscopy technique, LIBS, to the diagnosis of GWI in Veterans. No known biomarkers have so far been validated for GWI, and as a result there is an urgent need for the development of an untargeted and unbiased method to distinguish GWI-positive patients. We adopted a liquid biopsy approach, a minimally invasive procedure based on analyzing microliter droplets of blood plasma specimens from two groups, those with and without GWI, after depositing and drying the specimens on solid substrates. For this work, we used the difference spectrum method, a home-developed multivariate analysis approach. This method is based on generating a model difference spectrum with known samples and comparing the transitions' polarities with those of the unknown samples' difference spectra to obtain a diagnosis. In this work, we used eight known samples (four GWI pos, four GWI neg) to cross-validate our method, identify the most diagnostic transitions, and set a threshold for the optimal number of spectral features to be used for the blind test. In the cross-validation results, only one sample was not correctly identified, and it was a false positive for GWI. This may be explained by the fact that, while GWI is twice as prevalent in deployed veterans, it has also been described in 15% of non-deployed veterans.[13]

To further test the validity of our approach, we performed a blind test using 10 additional samples, whose status was unknown to the researchers performing the LIBS measurements and analysis. The results of the blind tests yielded a classification accuracy 90.0%, sensitivity 100%, specificity 83.3%. While these results may not be considered conclusive, due to the small scale of this first study, they nonetheless demonstrate that LIBS shows a clear potential for minimally invasive GWI diagnosis. Additional investigations are currently underway, using a larger number of GWI patients and controls (disease controls and healthy individuals) to further validate the diagnostic accuracy of our LIBS-based test. We are also in the process of evaluating spectral changes in GWI samples collected over time and in response to acupuncture treatment.[35]

Declaration of Conflicting Interests

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Disclaimer

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense or the Department of Veterans Affairs.

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