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Improved Quantification of CSF Bilirubin in the Presence of Hemoglobin using Least Squares Curve-Fitting

Joshua Butler, Blaine Booher, Peggy S. Bowman, Joseph F. Clark, and
Fred R. Beyette, Jr. *Member, IEEE*

***Abstract*—Subarachnoid hemorrhage (SAH) is a dangerous neurological event with a very short time window for early diagnosis. Clinical diagnoses performed in a lab seek to quantify bilirubin in cerebrospinal fluid (CSF) as a biomarker for SAHs; however laboratory assays suffer from lengthy protocols, interference from hemoglobin, and the availability of expertise. Substantial improvements in the determination of bilirubin concentration in the presence of hemoglobin in CSF are demonstrated in this work. Concentration estimates within 15% for bilirubin in the range of 0.2 to 1.6 mg/dl were determined for CSF samples containing fresh hemoglobin concentrations ranging from 0.05 to 0.25 g/dl. To demonstrate extensibility of the system with respect to more complete mock SAH samples, sample sets with one additional species of both hemoglobin and bilirubin, methemoglobin and alpha-bilirubin, respectively, were tested and yielded results within 25% of actual values, as measured by standard chemical assays of preparations prior to mixing.**

I. Introduction

ACCURATE and precise quantification of bilirubin, a blood component normally present at low concentrations in human cerebrospinal fluid (CSF), can provide evidence of the life-threatening neurological condition, subarachnoid hemorrhage (SAH). The often rapid progression of neurological events following the onset of an SAH may require aggressive treatments such as emergency brain surgery in order to avoid irreversible brain damage, coma, or death. Because many patients experiencing SAH report only "thunderclap" or migraine-like headaches, a symptom shared with many medical conditions, symptomatic diagnosis without quantitative analysis is highly unreliable [1].

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J. Butler is a graduate student in the Electrical and Computer Engineering Department at the University of Cincinnati, Cincinnati, OH 45221 (phone: 513-633-4572; e-mail: butlerj9@email.uc.edu).

B. Booher is an employee with Xanthostat Diagnostics, Cincinnati, OH 45236 (email: frikker@gmail.com).

P. S. Bowman is an employee with the Electrical and Computer Engineering Department at the University of Cincinnati, Cincinnati, OH 45221 (email: Peggy.Bowman@uc.edu).

J. F. Clark is with the Neurology Department at the University of Cincinnati, Cincinnati OH 45221 (email: clarkjf@ucmail.uc.edu).

F. R. Beyette, Jr. is with the Electrical and Computer Engineering Department at the University of Cincinnati, Cincinnati, OH 45221 (e-mail: beyettfr@ucmail.uc.edu).

II. Background

A. Clinical Need for Point-of-Care Bilirubin Quantification

The primary tool for diagnosis of SAH when hemorrhaging is suspect is the computed topography (CT) scan. However CT scans can result in false negatives, so it is standard clinical practice to co-administer a CT scan of the brain with a bilirubin assay of spinal fluid obtained from a lumbar puncture. The assay requires two hours to complete, and every minute that an SAH is allowed to progress untreated diminishes the patient's prognosis by limiting opportunities for less aggressive treatment options. For this reason, a reliable, robust point-of-care device is needed to address the important clinical need for immediate SAH diagnosis [1].

B. Interference from Hemoglobin

When CSF is obtained from a spinal tap, a doctor may inspect the sample against a white background such as a lab coat sleeve to determine if the sample appears yellow and thus warrants further tests such as quantification of CSF bilirubin or a CT. Although simple and usually reliable, visual examination for bilirubin in a CSF sample is often impaired by hemoglobin or its decomposition products, present from either a hemorrhage or a bloody spinal tap making visual examination unreliable. Additionally, chemical and spectrophotometric analysis using assays of bilirubin is also impaired when hemoglobin is present using conventional quantification methods [2].

C. Previous Work

To date, the collaborators on this project have been involved with the development of two previous generations of the device platform reported here for the quantification of bilirubin in CSF samples that also contain moderate concentrations of oxyhemoglobin. The initial effort by Bhadri et.al validated the basic proof-of-concept device [3]. It was developed as a working prototype; however, it was not capable of reliable quantification of real CSF samples. The device consisted of a laptop computer and an Ocean Optics USB-2000 spectrophotometer connected to a laptop via a USB 2.0 port. More recently Beyette et al. reported on the migration of the original proof-of-concept implementation into a stand-alone point-of-care device with onboard computing [4].

The second version of the device (called the Bilibox by the development team) features more robust functionality and

software modularity. For this version, three duplicate prototype systems have been assembled and tested. They use a 600MHz Overo board (Gumstix Inc.) running a Linux operating system and utilizing a partial least-squares regression (PLSR) signal analysis algorithm. Computing modules written in C are managed by Python scripts. The user interface is displayed on a touch screen that also provides readouts of estimated concentrations and error codes [5]. The Bilibox is expected to be submitted for FDA approval in 2011. In this work, the second generation device performance is used as the benchmark for evaluating the alternative computation module written for this purpose.

D. Objective of This Work

The previous effort has demonstrated a clear ability to quantify bilirubin in CSF with the confounding presence of hemoglobin also in the CSF sample. However, the previous work treats the hemoglobin as a single constituent molecule. This simplification does not account for the reality that the hemoglobin molecule takes on slightly different optical characteristics based on the atomic species bonded to the hemoglobin molecule. More specifically, it is well known that various forms of hemoglobin (meth-, oxy-, deoxy-, etc.) contribute slight variations in the absorbance spectrum. The primary goal of this work is to establish a new signal processing methodology that enables the independent and simultaneous quantification of these various hemoglobin constituents. It is believed that the ability to independently quantify the major forms of hemoglobin will have two primary advantages over the previously reported approaches. First, more accurate quantification of the various hemoglobin forms will lead to an improved accuracy and dynamic operating range for bilirubin quantification. Second, by providing accurate quantification of the individual hemoglobin forms, the point-of-care device for analysis of CSF will add additional clinical data that may have a substantial impact on patient diagnosis and subsequent medical care in cases of suspected SAH.

III. Experimental Set-up and Methods

A. Sample Preparation

Bloody CSF samples are prepared using fresh hemoglobin from human blood, drawn within a week previous to testing. Red blood cells are first separated from the plasma using centrifugation and rinsed with 0.9% NaCl prior to lysing to remove plasma-sourced protein and bilirubin. Bilirubin is sourced from a bilirubin calibrator (Wako Diagnostics, Inc.) that consists of 40% solubilized and unconjugated and 60% conjugated bilirubin, lyophilized to allow rehydration to a precise concentration.

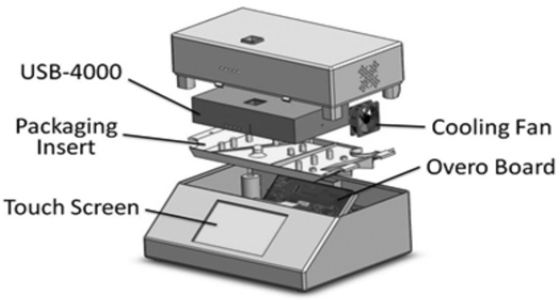

Figure 1 - Bilibox Prototype SLA Packaging and Electronics

B. Device Hardware

The hardware configuration used for data collection consists of a USB-4000 compact spectrometer and UV-VIS light source (Ocean Optics). Identical to the cuvette-loaded device previously described by Beyette [4], with the exception of an alternative software computation module for calculating estimates, the system is capable of capturing and analyzing absorbance spectra in the wavelength range of 350 to 700 nm. The point-of-care system hardware is shown in Figure 1.

C. Chemical Analysis

Multiple assays were used to provide a quantitative tool for development of the device and validation of the prototype performance. The hemoglobin assay uses Drabkin's reagent in a 96-well plate and is measured using the absorbance of the reaction product, cyanomethemoglobin, at 540 nm. This method is noted by Han et al. [5]. A commercially available fresh hemoglobin calibrator from Sigma-Aldrich was used to produce a first-order linear trend line over the concentration range of interest for each assay. Bilirubin was quantified with the standard diazo assay at 600 nm [6] on a 96-well plate using a bilirubin calibrator from Wako, Inc. Calibration of the assay was performed similarly to the hemoglobin, instead using a bilirubin calibration standard. All samples were prepared in duplicate, assayed in triplicate, and measured spectrophotometrically in duplicate. Data presented are averages of multiple measurements for identical preparations.

D. Formation of Hemoglobin Species

For the preparation of methemoglobin from oxyhemoglobin, potassium cyanide was titrated into a dilute oxyhemoglobin solution until the 540 nm peak characteristic of methemoglobin stopped increasing with further incremental additions and the two secondary oxyhemoglobin absorbance peaks between 500nm and 600nm were no longer present. This was taken to be 100% methemoglobin. The residual potassium cyanide could potentially introduce error by continuing to convert oxyhemoglobin after mixing, although no such error was readily apparent from the data or observations.

E. Software

The primary computation software package and development environment used for development was Matlab with the Optimization and Signal Processing toolboxes. Final implementation for the curve-fitting algorithm will be in C and Python using substantially equivalent methods to those used in Matlab.

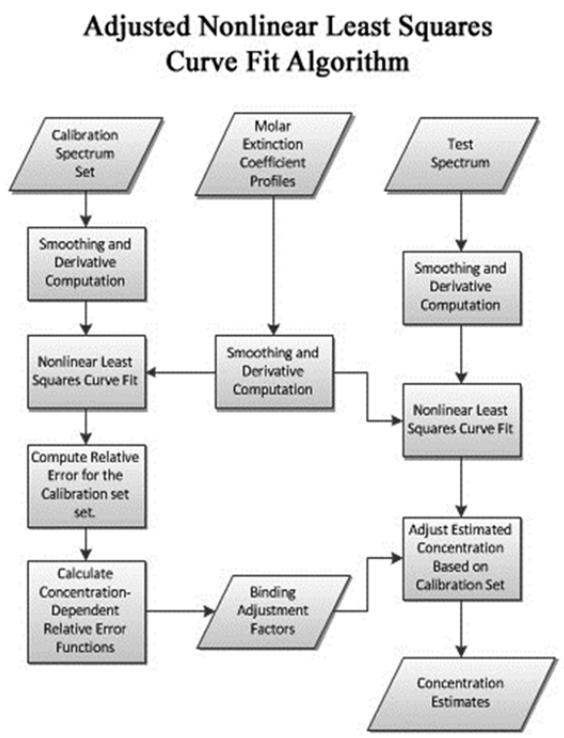

Figure 2 - Spectral Analysis Algorithm Flowchart

IV. Spectral Analysis

A. Algorithm Overview

The absorbance data collected from the spectrometer are analyzed as indicated by the flow chart in Figure 2. An absorbance spectrum of a CSF sample is captured and analyzed with curve-fitting according to the additive model. Concentration-dependent adjustment functions that were previously computed offline are used to adjust the estimates provided by the fit.

B. Spectrum Preparation

Spectra are collected in the form of vectors, with each entry corresponding to wavelengths between 460 nm to 615 nm. Smoothing and baseline shifting is performed in order to remove noise and sample variations not attributable to the species of interest.

Reference library curves, consisting of pure-species absorbance spectra of all species of interest, were collected, smoothed using cubic splines, and normalized to obtain the molar extinction coefficient (MEC) profiles. These spectra generally correspond to literature MEC curves, but are somewhat unique to the spectrometer and pre processing steps used. To avoid baseline shifts due to varying levels of unanalyzed CSF proteins, derivative curves are computed and fit using the two-point slope equation using forward differencing, rather than fitting using the absorption curves.

C. Model Development

The mathematical model used for analysis is fundamentally the sum of the relationship described by Beer-Lambert's Law for all species present, i. e.

$$A_i = \sum \epsilon_i l c_j \qquad (1)$$

where A_i is the total absorbance at wavelength i, c_j is the concentration of species j, ε_i is the molar extinction coefficient at wavelength i, and l is the path length.

When computing single-species molar extinction coefficients where A_i, c, and l are known, ε_i can be computed for all wavelengths. This is essentially the normalized pure-spectrum absorbance, unique to the species but mostly independent of the spectrometer.

D. Non-Linear Least Squares Fit

A non-linear curve fitting algorithm was used to provide estimates by fitting molar extinction coefficient profiles to a sample spectrum by iteratively adjusting coefficients for each species according to the provided model to get a composite curve [7]. The iterative adjustment continues until the sum of the square of the differences between the sample spectrum and the composite curve computed at each point is minimized below a given tolerance for the entire wavelength range of interest. In other words, the solution found for the Equation (2) [8],

$$\begin{aligned} &\min_x \|F(x, xdata) - Absorbance\|_2^2 \\ &= \min_x (\sum_i F(x, xdata_i) - Absorbance_i)^2 \end{aligned} \qquad (2)$$

where $F(x,xdata)$ is the wavelength-dependent function for absorbance described by the chosen model equation.

E. Calibration Adjustment

To correct for binding of hemoglobin to bilirubin, a calibration set of samples with known concentration is analyzed with the least squares algorithm using the model in Equation (1) and the estimates compared with the known values. Concentration dependent functions that relate relative error to the sample space are computed for each species. After initial analysis of a sample, the error functions are computed and negated from the initial estimates to produce the adjusted concentrations that are reported.

V. Results

A. Comparison with Predicate Device

Performance was evaluated on the basis of relative error between estimates provided by the algorithms and concentrations obtained from standard chemical assays. Relative error was calculated for each of 42 samples and values averaged for all samples of the same bilirubin concentration, excluding those below 0.25 mg/dL bilirubin.

Figure 3 shows a comparison of the algorithm proposed here versus the algorithm used in the Bilibox (i.e. the second generation prototype). The plot shows average relative error in the quantification of bilirubin. For the lower end of the evaluated concentration range, the new algorithm performs better than the PLSR algorithm currently in use in the Bilibox. This is significant as this is the concentration range that is most likely to enable earlier diagnosis for questionable cases. It is also worth noting that at the high

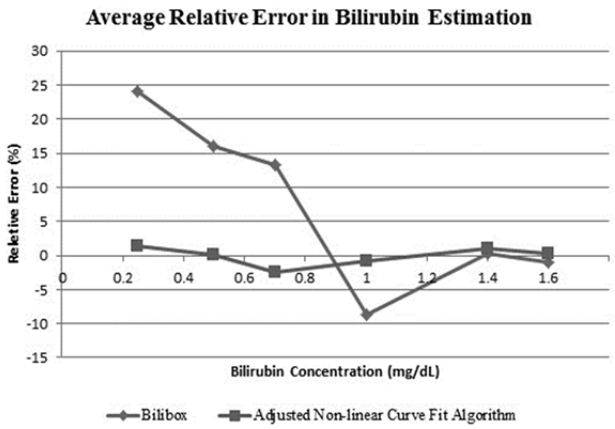

Figure 3 - Comparison of average error in bilirubin estimations for measurements with hemoglobin present in the range of 0 to 0.25 g/dL for the Bilibox and the nonlinear curve fit algorithm.

end of the concentration range, the proposed algorithm has performance comparable to the PLSR algorithm.

B. Performance with Additional Species

To further investigate the utility of the system, two additional species, unconjugated bilirubin, also called alpha-bilirubin (Frontier Scientific), and methemoglobin, prepared from oxyhemoglobin from fresh human blood were tested in combinatorial mixtures with the Wako bilirubin and untreated oxyhemoglobin. A calibration set of 81 (or 3^4) samples was used. The system was then validated with 225 samples (or 3^2x5^2) samples.

The results are presented in Figure 4. Bilirubin concentrations tested were in the range of 0 to 1.6 mg/dL, and hemoglobin concentrations ranged from 0 to 0.25g/dL. For comparison, the horizontal axis designates the concentration as a percentage of that species' highest concentration in the sample set. As expected, the relative error generally decreases with increasing concentration, however because of the large number of samples needed to calibrate the system for many species, resolution of the concentrations used for the calibration set is sacrificed in order to maintain a manageable sample set size using standard manual laboratory methods. All estimations were within 25% of their actual values for the samples tested, with error averages for all samples of the same concentration below 20%.

VI. CONCLUSIONS

A least squares curve-fitting approach, along with derivative spectra analysis, has been shown to improve both the accuracy and the extensibility of absorbance spectrophotometric analysis of real CSF samples with added blood components of known concentrations. The reliability of the concentrations estimated by the algorithm is further strengthened in several areas by preprocessing steps such as smoothing and derivative curve fitting. One explanation for the improved performance over the PLSR algorithm is the model equation is isolated from the training or calibration set, reducing the impact on the entire system of noise in the data. the curve-fitting approach allows for separate specification of the model equation used for analysis, isolating the model from extraneous term Applications of this approach extend beyond the current quantification of bilirubin to include a wide variety of other species absorbent in the UV-visible range in CSF and potentially other media.

Further development is necessary to capture all concentration-dependent binding and other interactions for all species commonly present in hemorrhagic CSF. It may be necessary to extend the wavelength range in order to reliably estimate several similar hemoglobin species in a single sample. Also of concern is the large number of samples used to calibrate a many-species mixture that may more accurately describe clinical test samples. High throughput calibration and automated sample mixing processes would reduce development cost and time. Statistical analysis of many clinical samples with a variety of CSF protein and blood species that have arisen from natural biological events is necessary before any claims about clinical accuracy can be made.

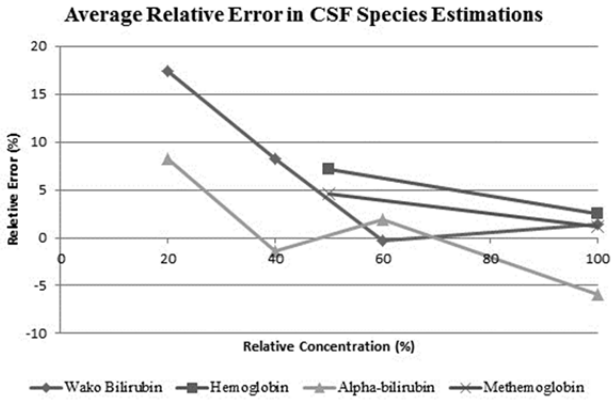

Figure 4 - Estimation of Species in CSF using the Adjusted Least Squares Curve Fit Algorithm.

REFERENCES

[1] Zivin J. Hemorrhagic cerebrovascular disease. In: Goldman L, Ausiello D, eds. *Cecil Medicine*. 23rd ed., ch. 432, 2007.

[2] J. A. van der Woerd-de Lange, et al., "Studies on the interference by haemoglobin in the determination of bilirubin," *J Clin Chem Clin Biochem*, vol. 21, pp. 437-43, Jul 1983.

[3] P. R. Bhadri, et al., "Spectrophotometric quantification of bilirubin in hemorrhagic spinal fluid using an innovative algorithm," *Med Chem*, vol. 3, pp. 21-7, Jan 2007.

[4] F. R. Beyette, Jr., et al., " Device for quantification of bilirubin in cerebral spinal fluid," *IEEE Trans. Biomed. Eng.*, 2010.

[5] V. Han, et al., "A comparative study of common techniques used to measure haemolysis in stored red cell concentrates," *Vox Sanguinis*, vol. 98, pp. 116-123, Feb 2010.

[6] P. J. Lindsberg and L. Uotila, "The diagnostics of subarachnoid hemorrhage and warning leaks by spectrophotometric analysis of the cerebrospinal fluid," *Duodecim*, vol. 125, pp. 2677-85, 2009.

[7] Coleman, T.F. and Y. Li, "An interior, trust region approach for nonlinear minimization subject to bounds," *SIAM Journal on Optimization*, Vol. 6, pp. 418-445, 1996.

[8] Dennis, J. E. Jr., "Nonlinear Least-Squares," *State of the Art in Numerical Analysis*, ed. D. Jacobs, Academic Press, pp. 269-312, 1977.