Measurement of Physiologic Glucose Levels Using Raman Spectroscopy in a Rabbit Aqueous Humor Model

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ABSTRACT

We have elicited a reliable glucose signature in mammalian physiological ranges using near infrared Raman laser excitation at 785 nm and multivariate analysis. In a recent series of experiments we measured glucose levels in an artificial aqueous humor in the range from 0.5 to 13X normal values. Data were obtained in 100 μL samples to mimic the volume constraints imposed by the human and rabbit anterior chamber of the eye. Feature extraction and data analysis were accomplished using linear multivariate analysis techniques (partial least squares fit). The spectra of the artificial aqueous humor closely approximate spectra acquired from rabbit aqueous humor.

Keywords: *Raman, spectroscopy, glucose, multivariate analysis, diabetes mellitus, rabbit, aqueous humor, eye.*

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Introduction and Purpose:

Non-invasive measurement of blood glucose by any method including optical spectroscopy techniques has remained an elusive target for at least two decades. Blood, tissue, and most excreted fluids contain numerous substances which confound glucose spectral signatures. On the other hand, Aqueous humor (AH) filling the anterior chamber of the eye (between the lens and cornea) contains relatively few molecules capable of interfering with the spectroscopic detection of glucose. These are primarily lactate, ascorbate, and urea [1]. This fact, and its optically accessible location behind the cornea make AH an obvious choice as a site on which to attempt non–invasive analysis of glucose.

A further advantage of aqueous humor is that its glucose concentration appears linearly related to plasma glucose concentration in animal studies. Furthermore, the rate constant for transport of glucose into the AH from the plasma is not affected by diabetes [2]. Lactate and urea levels in AH are also felt to vary with blood levels, while ascorbate is concentrated in the AH by active transport mechanisms.

The potential for non–invasive measurement of blood glucose using Raman spectroscopy on AH has been suggested before [3]. Previous work has demonstrated that the principle AH metabolites, including glucose, can be distinguished in water solutions containing mixed metabolites [4]. In addition, techniques have been described which could increase laser Raman sensitivity so that these metabolites could be measured at laser intensities which can be used safely in the eye [5,6]. Reliable measurement, however, of these metabolites at physiologic levels with Raman spectroscopy has yet to be described.

The purpose of this study is to demonstrate that the Raman spectra of a solution of mixed metabolites approximates that of aqueous humor, and that physiologic concentrations of glucose in such a solution of mixed metabolites can be measured with Raman spectroscopy.

Technical Background: Raman spectroscopy offers the possibility of remotely obtaining a measurement of glucose *in vivo* because, in contrast to infrared spectroscopy, its spectral signature is not obscured by water. In addition, Raman spectral bands are considerably narrower than those produced in classical infrared spectral experiments and Raman excitation in the near infrared region (700–1300 nm) encounters minimal fluorescence in aqueous media.

In a photon activating event, the majority of the photons incident on a target molecule scatter with unchanged frequency. A small proportion of light scatters with a shift in photon energy. This Raman shift occurs when photon energy transfers to (or from) the molecule during an inelastic collision. The vibrational spectra produced as a result of Raman scattering reveals the state of the atomic nuclei and chemical bonding within a molecule, as well as the interactions between the molecule and its local chemical environment.

Attempts to employ Raman techniques to directly measure glucose concentration in serum, plasma, and whole blood have met with encouraging success *in vitro* [7,8]. However, efforts to utilize these (and other) techniques *in vivo* for transcutaneous measurement of whole blood glucose levels have met with considerable difficulty. This is partly because whole blood and most tissue are highly absorptive, containing many floure scent and Raman active confounders.

Aqueous humor (AH), on the other hand, is relatively non–absorptive, and contains few Raman–active molecules. The four dominant, Raman–active molecules in AH are (concentrations are for rabbit AH) glucose (97 mg/dl), lactate (84 mg/dl), urea (36 mg/dl), and ascorbate (16 mg/dl) [1]. There is also a small amount of protein (26 mg/dl) that may produce fluorescence activity in sufficient strength to adversely affect Raman signal to noise ratios. Raman spectra from aqueous humor specimens of rabbits and humans, as well as spectra obtained through fresh excised rabbit corneas, have demonstrated detectable peaks of activity attributed to glucose, lactate, urea, amino acids, and proteins [9].

Methods: For our work, we have chosen a Raman excitation wavelength in the near infrared region to diminish extraneous biological fluorescence and minimize tissue damage. The price for these advantages is that Raman scattering efficiency decreases inversely with wavelength to the fourth power. We used a 250mW external cavity stabilized laser diode emitting at 785 nm and a Kaiser Optical Systems f/1.8 holographic imaging spectrograph with holographic filter and HoloPlex transmission grating. The holographic probe head was mounted on an Olympus BX60 microscope with 10X objective. Data were collected using a Princeton Instruments camera with an EEV back illuminated, NIR optimized 1024x256 CCD array operated at -80°C.

An artificial aqueous humor was designed to provide random fluctuations in concentration for the four major AH metabolites across a range of concentrations from 0.5X to 13X normal values for rabbit (Table 1). Metabolite levels in this range can be seen in hypoglycemia and diabetes (glucose), renal failure (urea), and myocardial infraction (lactate). The analytes were dissolved in pH buffered physiological (0.9%) saline. Variation in the other three

analytes can dramatically alter glucose estimation. To develop a tool for estimating AH glucose levels we obtained spectra from the 20 randomly generated mixtures depicted in Table 1. Concentrations of the four principal constituents of the aqueous humor (AH) were randomly mixed in physiological buffered saline. Individual component levels range from 0.5X to 13X levels expected in rabbit AH. Mixtures are listed in order of increasing glucose concentration. Correlation coefficients across the 6 possible combinations ranged from –0.37 (ascorbate | urea) to 0.44 (lactate | glucose). Two aliquots were taken from each mixture and analyzed separately to produce a total of 40 samples and to permit estimation of test/re–test accuracy.

Table I: Metabolite Concentration in Artificial Aqueous Test Mixtures (mg/dl)

Mixture No.	Glucose	Lactate	Ascorbate	Urea	Mixture No.	Glucose	Lactate	Ascorbate	Urea
1	50	80	1000	50	11	400	1100	1300	100
2	60	700	600	120	12	500	1300	500	170
3	80	60	1200	400	13	600	900	300	1200
4	100	100	400	250	14	700	50	140	1000
5	120	120	50	140	15	800	1200	100	800
6	140	250	800	500	16	900	300	60	200
7	170	140	80	60	17	1000	400	250	700
8	200	170	200	900	18	1100	1000	1100	80
9	250	800	170	600	19	1200	1000	900	80
10	300	500	120	1100	20	1300	600	700	1300

Samples were placed in quartz cuvettes designed to limit sample volume to 100 µL and to permit direct access to the test solution without traversing quartz walls or coverslips. Data acquisition and multivariate analysis were accomplished using Holograms and Grams, commercial software packages provided by Princeton Instruments and Galactic Industries Corporation, respectively. The integration time for each spectra was 100 seconds with an average power delivered to sample of 100 mW. Since we are interested in minimal exposure times for future *in vivo* measurements, only a single spectra was collected for each sample. However, each mixture of concentrations was duplicated and two independent measurements performed on each aliquot.

A cross–correlational analysis of the spectra identified 38 wavenumbers or spectral bins correlating significantly with glucose concentration. (Table 2) This made it possible to implement a partial least squares algorithm for data reduction and calibration using fewer test measurements than data samples. This is particularly important when attempting to build a robust spectral prediction algorithm capable of identifying the effect of multi–metabolite concentration variable on spectral signatures [10].

Table II: Wavenumbers Selected for PLS Analysis

213	846	1539	2718
426	906	1850	2751
442	1010	1900	2778
513	1053	1950	2827
535	1059	2000	2871
628	1119	2050	2904
644	1255	2100	2980
671	1332	2150	3362
693	1359	2200	
742	1457	2250	

For our multivariate analysis algorithm, we have chosen the partial least squares (PLS) technique, a multivariate analysis and spectral decomposition algorithm that, unlike principal component analysis, uses concentration information to calculate the eigenvectors. In our design, test and training samples were put into a "round–robin" or autocorrelation training mode to iteratively employ all but one of the sample set in the minimization and eigenvector extraction process. Hence, the system trains on all but one of the samples, estimates the glucose level in that sample, then rotates the test sample back into the general pool and repeats the cycle until all samples have served as an unknown test subject. In this manner, the system is masked for the concentration of analytes in each unknown sample.

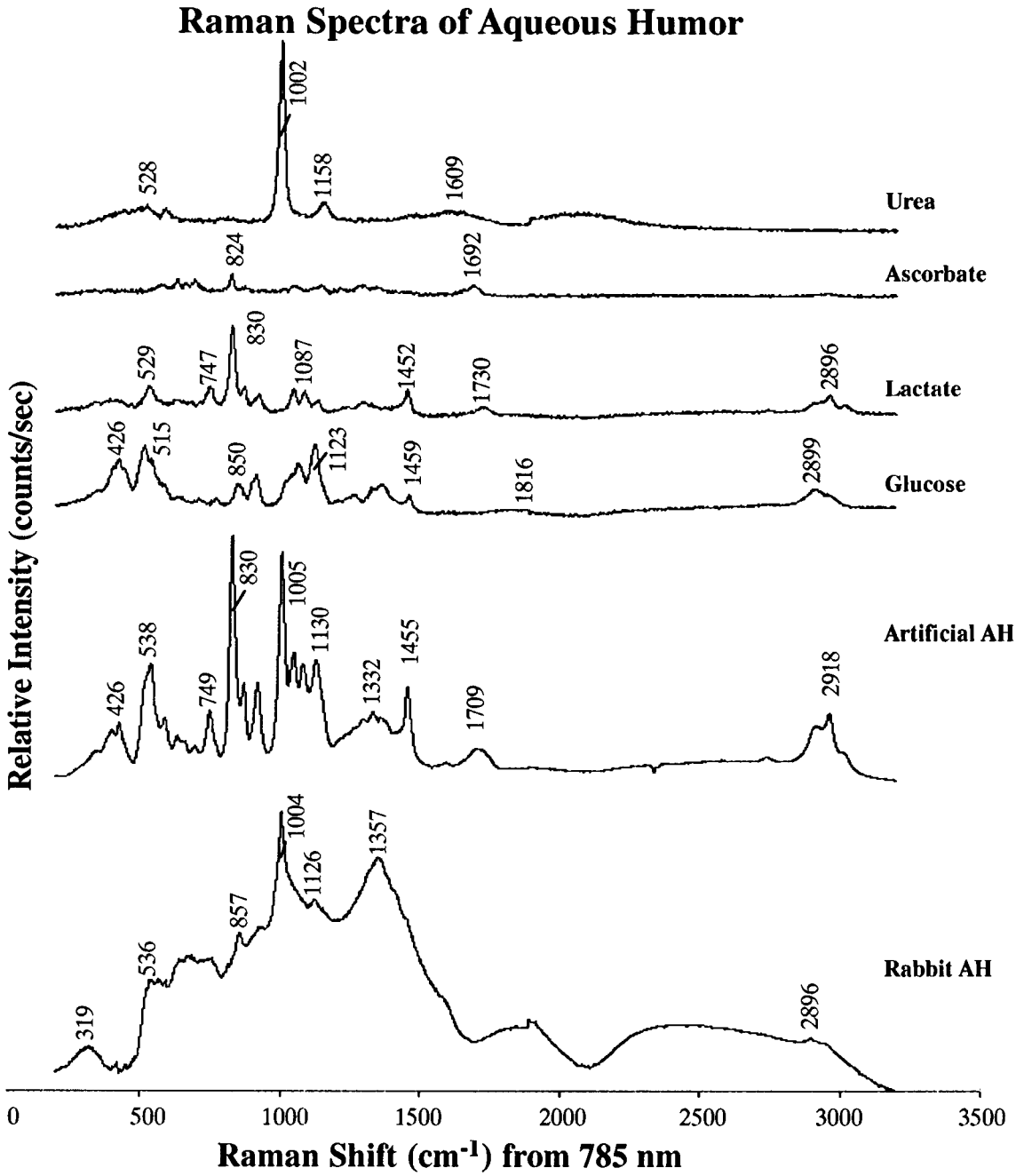
Rabbit AH was obtained from three animals within five minutes of sacrifice by other investigators. These animals had experienced experimental myocardial infarction 48 hours prior to euthanasia. They were sacrificed with a rapid exsanguination technique. Rabbit AH samples were kept on ice until glucose levels could be measured and Raman spectroscopy performed. Glucose concentration in rabbit AH samples was measured with a commercial glucometer (Glucometer Elite, Bayer) and confirmed against concentration standards.

Results and Discussion: The individual Raman signatures for the four principal metabolites found in AH (glucose, lactate, ascorbate, and urea) are quite distinct (first four spectra of Figure 1). When the four analytes are mixed at concentrations approximating the normal levels, the composite signature shows marked

similarity to that of the rabbit AH (last two spectra of Figure 1). The rabbit AH spectra also contains evidence of broadband fluorescence and elevated lactate activity (secondary to the myocardial infarction). Nevertheless, we can still detect common peaks attributable to lactate, urea, and glucose. The similarity between spectra of artificial AH and rabbit AH is quite striking.

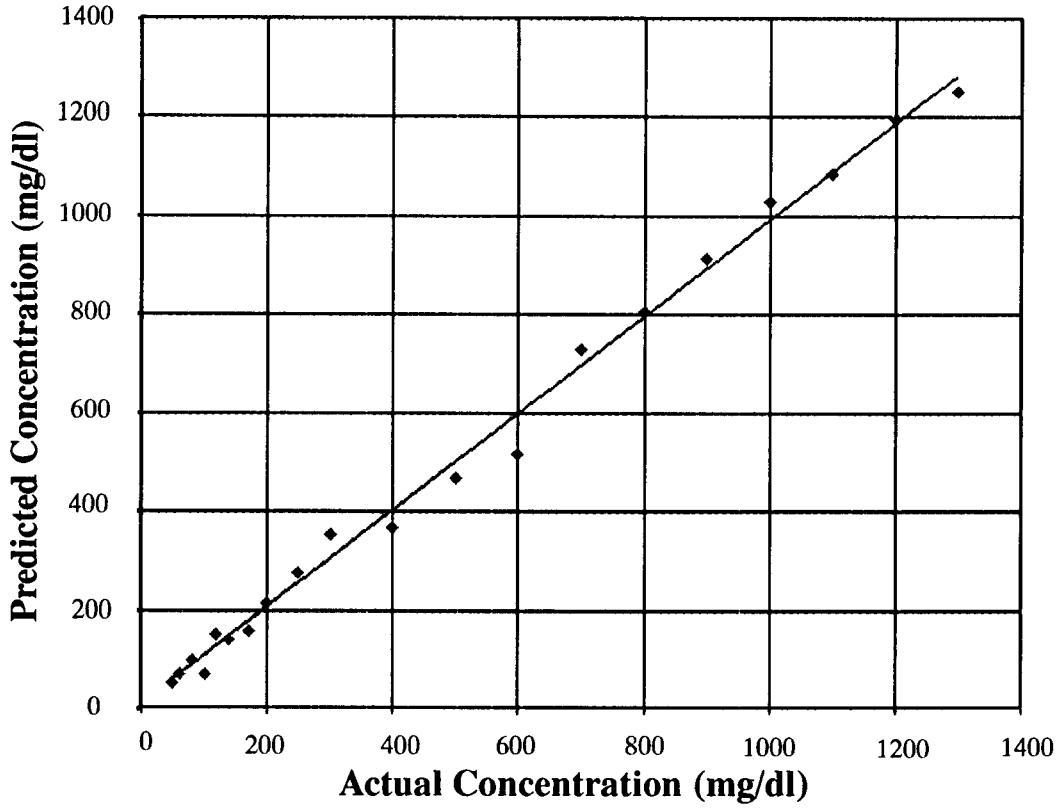
In our experiments with artificial AH, the unknown glucose concentration was extracted with no preprocessing. The glucose concentration was estimated from the artificial AH mixtures using the raw spectra while the concentrations of the various metabolites was randomly varied over a broad range of clinical conditions.

Following our determination of the spectral regions producing significant correlations to glucose concentration, we ran a series of experiments employing both the full 3200 wavenumbers acquired and the restricted minimal set. There was no diminution in algorithm accuracy using the 38 selected bins instead of the entire spectra. The partial least squares algorithm



▼ *Fig 1* The top four spectra are of pure solutions of the major AH metabolites in physiologic saline at 100X the normal rabbit AH concentration (urea 3600 mg/dl; ascorbate 1600 mg/dl; lactate 8400 mg/dl; glucose 9700mg/dl). The acquisition time for these spectra was 1 second. The fifth spectra is from a mixture of the major AH metabolites at normal rabbit AH concentrations. The bottom spectra is from rabbit AH. The two bottom spectra were acquired over 100 seconds.

Glucose Measurement Across the Physiological Range in Artificial Aqueous Humor Using Raman Spectroscopy and Multivariate Analysis



▼ *Fig 2* Multivariate analysis provides a quantitative assay for glucose concentrations across the hypoglycemic (50 mg/dl) to severe diabetic 1300 mg/dl) range. Mixtures included random concentrations of other metabolites from 0.5X to 13X normal rabbit AH concentrations. The composition of each of these mixtures is listed in Table 1. Each mixture was measured twice and the average predicted concentration is reported.

learned quickly and proved capable of predicting glucose concentrations of unknown samples across a wide range of clinically significant metabolic states.

Figure 2 demonstrates the excellent predictive ability of the current system, with the PLS algorithm producing correlation coefficients of 0.99 for both predicted vs actual glucose concentrations and for test/re-test accuracy.

The rabbit AH signature (last spectra of Figure 1) is complicated by both the expected elevation of lactate secondary to severe myocardial infarction, and the drugs introduced as part of the experimental procedure. Potential Raman-active molecules include aspirin, ketamine, xylazine, pentobarbital, and heparin. We are currently investigating the Raman response of these and several other potentially confounding substances. Nevertheless, expected optical activity appears at a variety of sites including 1004, 1126, and 2896 cm^{-1}.

The actual rabbit AH glucose levels were measured to be higher than normal (332 ± 19 mg/dl in the left eyes; 328 ± 37 mg/dl in the right eyes). Raman spectral estimation of glucose in the rabbit AH against the standards in artificial AH produced even higher readings of 561 ± 62 mg/dl (left eyes) and 602 ± 46 mg/dl (right eyes). This may be due to the broadband fluorescence (possibly due to protein) apparent between 800 and 1500 cm^{-1} and between 2300 and 2800 cm^{-1}. This fluorescence artificially elevates glucose estimation if such activity is not present in the training set.

Significantly, the elevated glucose level in rabbit AH was expected in response to xylazine. Xylazine is commonly utilized in conjunction with Ketamine as an anesthetic in veterinary surgical procedures. It appears to interfere with the release of insulin by the pancreas. This results in elevated blood glucose levels for two to six hours [11].

We find these initial results most encouraging and propose that Raman spectroscopy of aqueous humor in the near infrared combined with multifactor analysis techniques constitutes a new technology capable of estimating levels of blood glucose and other metabolites non-invasively.

We are currently proceeding to investigate the next three important factors in the development of this technology:

1.) determining the minimum laser power and data acquisition time required for *in vivo* application;

2.) elucidating the temporal correlation between glucose levels in AH and steady state blood concentrations;

3.) deconvolving and identifying the broadband fluorescence apparent in rabbit AH.