Identification of hepatitis C in human blood serum by near-infrared Raman spectroscopy

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Abstract. Hepatitis C has become one of the higher health problems around the world. Near-infrared Raman spectroscopy had been used to spectrally differentiate among health human blood serum from the one with hepatitis C contamination *in vitro*. In this study a Raman spectrometer with 80 mW, 830 nm excitation, liquid-nitrogen cooled CCD and imaging spectrograph were used to collect Raman scattering from 24 blood samples (14 healthy and 10 diseased) with collection time of 120 s. It has been used an algorithm based on the Principal Components Analysis (PCA) for main spectral features identification and Mahalanobis distance for blood spectrum classification depending on the serology. It was observed that the highest spectral differences between the two types of human blood serum were found in 1002, 1169, 1262 and 1348 cm^{-1} Raman bands. The spectral analysis using multivariate statistics presented good results when compared to classical diagnosis for viral hepatitis C, showing that Raman spectroscopy can classify human blood serum spectrum in one of the two categories by identifying biochemical alterations that occur in the presence of viral infections.

Keywords: Raman spectroscopy, hepatitis C, laser diagnosis, PCA, blood serum, Mahalanobis distance

1. Introduction

Facing the transmissible endemic–epidemic illnesses remains a big challenge and a great problem for public health. Among them, the viral hepatitis, whose epidemiological behavior has undergone great changes worldwide, must be particularly considered. The viral hepatitis is an illness caused by different etiologic agents of universal distribution, having in common the hepatotropism. The major importance given to hepatitis regards not only the great amount of infected people, but also the complications in their chronic and acute forms [1–3]. The viruses are responsible for hepatitis engender in a wide variety of clinical cases, from non-symptomatic porter or chronic or acute hepatitis, to cirrhosis and hepatocellular carcinoma [1,4,5].

Before the rigorous selection of blood donators in 1991, viral hepatitis C (VHC) was the biggest cause of post-transfusion hepatitis. In present days the VHC is one of the highest causes of chronic hepatic illness worldwide, and the most frequent indication of hepatic transplant. The chronic VHC is a disease of variable evolution, generally slow and progressive evolution. Today, there must be at least 170 millions of people porters of VHC, part of them may develop further complications such as cirrhosis and hepatocarcinoma. Furthermore, the infection by VHC has been implied on the beginning of some

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non-hepatic illnesses [1,6]. In the last few years, VHC has become one of the highest global problem in public health [7].

The diagnosis of hepatitis is classically developed by serological analysis. The serological trial that is elaborated in order to obtain the hepatitis C diagnosis, usually used since the 1990s, is an immunoenzymatic test (ELISA) made to detect specific antibodies against the virus of this disease (anti-VHC). In spite of ELISA to be a useful method to diagnosis of chronic hepatitis, this test commonly presents negative results in the first months after contamination. This fact precludes the etiological diagnosis in the initial phases of the acute hepatitis caused by viruses, generating false negative results in donors of contaminated blood. On the other hand, the possibility of false positive results remains in blood donors or any group of patients that present small probability of contamination for VHC [8–10].

The molecular biology techniques used to direct detection of RNA associated to VHC are more complexes, not widely available, and furthermore, present high costs. However, this biological procedure has been widely considered a necessary tool to the confirmation of the respective diagnosis. For instance, Polymerase Chain Reaction (PCR) is one of the most used techniques of molecular biology. Nevertheless, due to the difficulties to obtain enough specificity and precision, PCR has presented significant limitations [11]. Other technique employed in this kind of approach is called branched DNA (bDNA), which presents higher reproductivity and lower sensitivity when compared with PCR [12]. Thus, in the present days the diagnosis of hepatitis C involves complex processes that demonstrate significant percentual mistakes. Moreover, the results are not obtained immediately, taking several days in some cases.

Optical techniques such as Raman spectroscopy and correlated vibrational techniques have been used at diagnosis of a variety of human illnesses, analyzing biological tissues and body fluids [13]. The development of reliable Raman spectrometers began since the 1990's and has shown its potential for quantitative and qualitative investigations in biological samples [14–20]. Various human tissues have been exploited and characterized by Raman spectroscopy. Cancer diagnosis through Raman spectroscopy has received special consideration, as tumors in different neoplastic tissues have already been characterized [21–26]. Urine and blood are also subjects of studies, aiming the detection of metabolites *in vitro* [27–29]. Viral infections such as HIV and others have been successfully diagnosed with complementary techniques in the near-infrared combined with multivariate statistical analysis as PLS, PCA and PCRA [30,31]. However, there is no study with Raman technique used to identify blood serum alterations in porters of hepatitis C.

The process of Raman scattering can be viewed as an inelastic phenomenon in which the scattered photon is shifted in frequency from the incident photon as it either gains energy from or loses energy to a particular vibrational mode of the molecule. Thus, Raman spectroscopy can be used to access the molecular constitution of a specific sample and then classify it according to differences observed in the spectra [32,33].

Principal Components Analysis (PCA), a multivariate statistical tool, has been applied with success in spectral analysis of biological samples, detecting spectral alterations that occur after changes in the morphology and physiology of biological fluids and tissues. Within a set of spectral data, there are usually many different variations that make up a particular spectrum. Background, differences in constituents, instrument variations, sample handling, and others affect the appearance of the final spectrum. Yet with several changes occurring at the same time, there are only few independent variables accounting for all spectral differences. Normally, the largest variation in the spectral data is due to differences in the constitution of the samples, a few variables. One can achieve simplification of information redundancy or reduction of variables by using PCA due to the fact that PCA extracts the relevant information from the original data and generates a new set of variables, called principal components (PC) and scores (S). The

PC are related to the most important variation of all spectra; first PC account for most of the variation of the data, the last ones carry only noise [34].

PCA has been used for statistical analysis and classification tool in a variety of human diseases, such as near-infrared fluorescence spectroscopy of Alzheimer disease [35], near-infrared Raman spectroscopy of cervical precancer [36], FT-Raman spectroscopy of atherosclerosis in human carotid artery [37], Raman spectroscopy for discrimination of normal and malignant mucosal tissues of the colon [21], parathyroid tissue pathology [38], human breast cancer and skin [39,40].

The Mahalanobis distance is a very useful way to find the similarity of a set of values from one group of samples compared to another group for discriminant analysis. The Mahalanobis distance has advantages over Euclidean distance because it takes not only the relative distance from the sample to the mean of the group but also takes into account the covariance matrix of the data and can be used to classify the sample into well-defined classes [41]. Mahalanobis has been applied to classify Raman spectra of atherosclerosis with high sensitivity and specificity [37].

The objective of the present work is to verify if near-infrared Raman spectroscopy, compared to classical diagnostics analysis for viral hepatitis C as a gold standard, is capable of identifying the hepatitis C human blood serum from the health human blood serum, using an algorithm based on the results of PCA applied over all spectra for main spectral features identification and Mahalanobis distance for blood sample classification compared to standard serology.

2. Material and methods

2.1. Human blood serum samples

A total of 14 healthy blood serum and 10 hepatitis C blood serum samples were obtained from the ATHEL clinic (Atendimento Hemoterápico Laboratorial, Campos do Jordão, SP, Brazil) taken from 24 subjects aged from 30 to 40 years, both sexes, that underwent blood analysis. After extracting, blood serum samples were stored in glass tubes and refrigerated (4°C) prior to use. At the moment of Raman spectral analysis the samples were brought to room temperature and placed in quartz cuvette in a controlled temperature room (18°C). This protocol was approved by the Ethics Committee of the University of Vale do Paraíba under no. H273/CEP/2007.

2.2. Raman spectroscopy

Near-infrared Raman spectra were obtained using a dispersive spectrometer described elsewhere [42]. Briefly, the excitation is composed by a wavelength stabilized semiconductor laser at 830 nm operating wavelength and 80 mW excitation power. The laser hits the sample in a 90° geometry and the scattered light is collected by a set of lenses ($f = 100$ mm) which couples the light onto the imaging spectrograph (Chromex, model 2501S, 600 lines/mm grating) that disperses and directs to a liquid-nitrogen, deep depletion CCD camera. Spectra were obtained with integrating time of 120 s under a spectrometer resolution of about 8 cm^{-1} . The blood sample holder was a quartz cuvette with 10 mm optical pathway and 1 mm thickness window. The number total of spectra for each group was 17 for the normal serum and 12 for the hepatitis C group.

All serum samples were conventionally diagnosed by a immunoenzimatic analysis (chemiluminescence) at the ATHEL. Samples were then classified into two categories: (1) healthy and (2) hepatitis C.

2.3. Raman spectral analysis – algorithm based on PCA

All spectra were recorded in binary format and converted to ASCII format for postprocessing. In order to develop an algorithm for sample classification based upon the chemiluminescence assay, the Raman spectra were analyzed through PCA technique. The PC scores and S principal components spectral vectors were calculated from all 29 serum spectra using a PCA routine written in Matlab[®] software (The Mathworks, Inc., PCA) with the NIPALS-CA algorithm [34]. Since S is the weight, or the importance of each PC to configure the original spectrum, they can be used to build an algorithm to correlate the chemiluminescence analysis findings to the Raman features.

In order to separate serum samples in two groups: normal and hepatitis C, a discriminant analysis based on Mahalanobis distance was done. The Mahalanobis distance has the advantage over the Euclidean distance because it uses the covariance of the data. Mahalanobis distance calculations and the discriminant analysis were done using Matlab Statistics toolbox.

3. Results

In the present study, 24 samples were analyzed by the chemiluminescence diagnosis. Among them, 14 were classified as healthy serum and 10 presented biochemical alterations, which constitutes a characteristic behavior of hepatitis C, which are typical such disease, were not very pronounced when compared with the pathological manifestations associated to hepatitis A and B.

Raman spectra of *in vitro* human blood serum are shown in Fig. 1. A Raman spectrum shows vibrational bands from biomolecules and could reveal the composition of the blood serum. Figure 1(a) shows the mean spectrum of a healthy blood serum and Fig. 1(b) shows the mean spectrum of hepatitis C blood serum. The most important bands have been assigned to proteins and lipids present in the blood serum. Figure 1(a) shows weak bands when compared to Fig. 1(b), because blood samples analyzed in this work were not submitted to any previous treatment, what explain the absence of some bands in healthy serum.

Fig. 1. Raman spectra of *in vitro* human blood serum: (a) healthy; (b) hepatitis C blood serum.

The Raman band at 1640 cm^{-1} in Fig. 1 is attributed to asymmetric bending of CH_3 which is assigned to lipids presents in both, healthy and diseased serum. The predominant bands observed at 1170 , 1257 and 1344 cm^{-1} in Fig. 1(b) are due to CO-O-C asymmetric stretching and CH_2 wagging band progression, respectively, presented in lipids and phospholipids [42]. These phospholipids are associated to enzymes that are activated by the destruction process of hepatocytes in hepatitis. [43–47]. In consequence of this destruction process, alterations can occur in the concentration of the various enzymes, proteins, lipids and phospholipids that are found in blood. The biochemical characterization of the concentrations of these biological molecules, such as enzymes, constitutes in important markers of the hepatic function [43,44], and can be used to identify the disease. The most significant differences among normal and hepatitis C spectra are observed in the region of the 1002 cm^{-1} and through the bands at 1170 , 1257 and 1344 cm^{-1} . The bands that occur at 1639 and 1061 cm^{-1} are due in part to quartz cuvette Raman scattering, which was used to hold the blood serum samples.

Principal components analysis was performed using all spectral data in order to identify spectral features that could differentiate the type of serum. Each S spectral vector has a single axis in space and one is perpendicular to the other. When each observation (spectrum) is projected in each S , new variables come up, the PC scores (orthonormals in respect to each other). Since the scores represent the intensity of each principal component vector to reconstruct the original spectrum, they can be used in discriminant analysis as they do not carry redundant information.

The results of PCA calculation show that the first four PC spectra represent about 95% of all spectral variations. PC1 spectrum represents 90% of such variation and the remaining three ones contribute with about 5%. By analyzing Fig. 2 it was observed that the highest spectral differences between the two types of human blood serum can be found in the PC2, PC3 and PC4 spectra (i.e., peaks in 1002 , 1169 , 1262 and 1348 cm^{-1}), showing the presence of relevant peaks in the same positions as the blood serum spectrum and therefore they were used to develop the diagnostic algorithm. PC1 spectrum (Fig. 2)

Fig. 2. Spectra of the first four PC vectors calculated using 29 Raman spectra. Labeled features represent main peaks of each PC that can be related to Raman bands found in hepatitis C blood serum.

Fig. 3. Scatter plot of the first and third PC scores calculated using 29 Raman spectra of human blood serum. Black line represents Mahalanobis based classification surface.

Table 1

Results from traditional hepatitis C diagnosis based on chemoluminescence analysis compared to Raman analysis algorithm and correspondent sensitivity/specificity values

Traditional diagnosis	PCA analysis				
	HS	CS	Total	Sensitivity (%)	Specificity (%)
Healthy	15	2	17	—	88
Hepatitis C	1	11	12	92	—

HS – Health serum; CS – Hepatitis C serum.

reproduces the average spectrum and carry most important spectral information and therefore could be used for diagnosis.

Figure 3 shows the scatter plot of PC1 versus PC3 evidencing differences in scores among the two types of blood serum. A discriminant line based on mean Mahalanobis distance among both groups was drawn aiming for the best separation of two categories. A total of 15 out of 17 normal blood serum were classified as HS (healthy serum) and a total of 11 out of 12 hepatitis C blood serum were classified as CS (hepatitis C serum). Algorithm's sensitivity and specificity indices were calculated for each one of the blood categories. These results are summarized in Table 1.

4. Discussion and conclusion

This work has proposed a method for *in vitro* analysis of hepatitis C in human blood serum using near-infrared Raman spectroscopy collected with dispersive imaging spectrograph and CCD camera from serum samples placed in quartz cuvette with spectral analysis performed by PCA and discriminant by Mahalanobis distance. The PCA scores bring details about which spectral information is relevant for fluid differentiation and Mahalanobis distance showed to be efficient in discriminating among the two types of human serum, healthy and hepatitis C blood serum. The calculated sensitivity for the healthy serum was about 88% and the specificity for the hepatitis C blood serum was about 92%.

Triglycerides, phospholipids and cholesteryl esters constitute the classes of lipid that occur in human blood serum. However, the carbonyl peaks are heavily overlapped, being too close in order to allow

a clear distinction. Moreover, the concentrations of these components are usually in the range 0.03–0.3% in healthy human blood, which is significantly low to allow a sensitive detection. In this way, it is necessary a preliminary separation of blood samples to make the analysis with low sensibility in order to detect these bands [42]. The blood samples analyzed in this work were not submitted to any previous treatment, what explain the absence of some bands in healthy serum as presented in those spectra.

Previous results have indicated that near-infrared Raman spectroscopy could be an efficient technique to detect IgM antibodies against *Toxoplasma Gondii* in serum samples of domestic cats [48]. Analysis of lactic acid of the *in vitro* human serum by Raman spectroscopy has shown that this tool is an interesting technique in order to study clear biofluids [27]. Measurements of concentration of blood serum and urine samples, such as glucose, albumin, cholesterol, triglycerides, creatinine and others, have been reached also with success [29].

In the present study has been shown that Raman spectroscopy is able to classify the two categories of human blood serum identifying biochemical alterations that occur in the presence of viral infections. Raman spectrum not only discriminates between healthy and pathological serum with molecular specificity, but also provides an exclusive diagnostic assignment for each category. These results suggest that Raman spectroscopy may be turned into a promising diagnostic technique in therapeutics.

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