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Measurement of Amphotericin B concentration by Resonant Raman Spectroscopy – a novel technique that may be useful for non-invasive monitoring

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We wished to determine whether Resonant Raman Spectroscopy (RRS) could be used to measure Amphotericin B (AmB) at therapeutic and subtherapeutic concentrations in a model system mimicking the anterior chamber of the eye. The goal was to develop a technique for non-invasive measurement of AmB levels in the aqueous humor (AH) of the eye. A krypton-ion laser source (406.7 nm) was used for excitation and Resonant Raman Spectra were captured with a confocal system in an anterior chamber (AC) model. These spectra were used to develop a correlation curve for prediction of AmB levels. Subsequently, one rabbit was evaluated with this system after 5 days of intravenous AmB administration (1 mg/kg/day) and AmB concentrations measured by RRS were compared to those measured by high-performance liquid chromatography (HPLC). AmB exhibited a unique spectral peak at 1557 cm^{-1}. Integrated area of this peak linearly correlated with AmB concentration in our model AC. When integrated peak area from multiple *in vivo* measurements in one animal at steady-state was plotted on this correlation curve, we were able to predict AmB levels. These closely approximated those measured by HPLC. These measurements were not significantly affected by photobleaching or depth profile at acquisition. RRS at 406.7 nm is a method that may be useful for non-invasive monitoring of intraocular AmB levels. This instrument can help physicians decide when repeat, invasive delivery of this drug is warranted based on measurement of actual drug levels in the AH. Also, there is the potential to measure the ocular concentrations of other pharmaceutical agents with similar instruments.

Keywords Resonant Raman Spectroscopy (RRS), Amphotericin B (AmB)

Introduction

Non-invasive measurement of drug levels has been difficult to achieve for a number of reasons. Most antimicrobial agents accumulate in tissues at concentrations too low to be detected with most spectroscopic techniques. In addition, most tissues contain numerous optically active and fluorescing molecules effectively obscuring spectral signatures. Aqueous humor (AH), on the other hand, contains relatively few optically active molecules capable of interfering with detection of drugs. The AH concentration of glucose, which is actively transported, correlates closely with that seen in blood [1–3]. However, drugs have variable ocular

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penetration resulting in ocular levels that may be similar to or, substantially different than, blood levels after systemic administration. In addition, newer drug delivery methods may influence the AH levels and the ability to easily measure these levels may be useful in the management of intra-ocular infection.

Resonant Raman Spectroscopy is a laser vibrational spectroscopy technique capable of eliciting highly specific chemical signatures of the constituents of AH. Previously published data from our laboratory have documented that combining Raman Spectroscopy (at 785 nm) with partial least squares analysis makes possible the quantification of glucose concentrations in the physiologic range in aqueous solution [1].

Operating at 406.7 nm, the laser deposits minimal power on the cornea or AH. Amphotericin B (AmB) is a large molecule and few studies have evaluated its eye penetration. Similar results have been obtained for rabbit and human eyes. AmB achieves eye levels that are 7–40% of the plasma levels after i.v. administration when inflammation is present [4,5]. In the absence of inflammation, ocular levels are not measurable after a single i.v. dose of AmB [4]. Therefore, patients with fungal endophthalmitis require direct injection of AmB into the eye. If non-invasive monitoring were available it might help determine when to re-inject AmB, thereby avoiding unnecessary procedures. It might also allow determination of effective intraocular levels with intravenous administration alone. In addition, levels consistently below the minimum inhibitory concentration (MIC) for the infecting organism might predict those patients who may need adjunctive or alternative therapy.

The purpose of this study was to investigate the possibility of non-invasively measuring AmB levels in the AH of the eye. One previous study has demonstrated the ability to visualize AmB Raman spectra *in vitro* and *in vivo* at therapeutic concentrations [6]. We used a model anterior chamber to determine if AmB at pharmacologic concentrations can be measured with Resonant Raman Spectroscopy (RRS) *in vitro*. Subsequently, we collected spectral information from a rabbit that had received AmB intravenously for 5 days to see if the spectral peaks we observed in our anterior chamber (AC) model were visible *in vivo*.

Methods

A model anterior chamber was used to measure known concentrations of AmB in aqueous solution. This consisted of a poly(methyl methacrylate) (PMMA) contact lens and an underlying quartz slide. This model anterior chamber held $\sim$50µl and had a depth of 1.6mm from the slide to the superficial surface of the contact lens. This distance represents about half of that from the lens to the corneal surface in humans.

Ten aqueous solutions of AmB were prepared ranging in concentration from 0.039–10 µg/ml. This large range encompasses the range of AmB concentrations expected in AH and plasma in rabbits at steady state after administration of 1 mg/kg/day of AmB [7]. In order to keep the AmB in solution, desoxycholate was maintained at a concentration of 40 mmol/l which is approximately 8 times the critical micelle concentration [8]. Previous work in our laboratory reveals no desoxycholate Raman peaks that interfere with those of AmB (data not shown).

All Raman spectra were collected with a Kaiser Optical Systems (Ann Arbor, MI) Raman microscope. Light at 406.7 nm from a krypton-ion laser was filtered by a bandpass filter and injected into the excitation optical fiber connected to the Raman microscope. We chose 406.7 nm because this wavelength matched an absorption band of AmB that led to resonance enhancement of the Raman signal. In addition, the wavelength of Raman scattering was weakly absorbed by the AmB sample. The optical fiber was single mode for 450 nm light, but supported higher order modes at 406.7 nm. A 20x, 0.4 N.A. objective lens was used to focus laser light onto the sample 1 mm below the surface of the contact lens in the model anterior chamber [1]. The laser power was measured and adjusted prior to each Raman measurement to provide 0.6 mW at the sample. Integration times were 20 s.

The collection optical fiber connecting the microscope to the Raman spectrograph had a 50 µm diameter metallized aperture vapor deposited on the end connected to the microscope. This aperture served as a confocal aperture, limiting the depth-of-field of the microscope [9]. The depth-of-field for the Raman microscope was measured by recording the Raman intensity from a thin (20 micrometers thick) nylon film as the film was translated through the focus normal to the optic axis. The resulting Raman intensity profile was approximately Lorentzian with a full-width-at-half-maximum of 90 micrometers. The thickness of the film had a negligible effect, adding approximately 2 micrometers to the experimentally observed profile.

Animal preparation

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Childrens Hospital Los Angeles. At the end of the experiment, the animal was euthanasized with an overdose of anesthetic without ever regaining consciousness.

A New Zealand red rabbit (3.9 kg) was housed for several days in the vivarium with food and water provided *ad libitum*. A heparinized, silastic central venous catheter (CVC) was placed in the external jugular vein. This was performed under general anesthesia with ketamine (35 mg/kg) and xylazine (7 mg/kg) administered by intramuscular (i.m.) injection followed by inhaled isoflurane, as previously described by Walsh *et al*. [9]. AmB deoxycholate was reconstituted with sterile water to 5 mg/ml and then further diluted with 5% dextrose solution to 1 mg/ml prior to infusion. The rabbit received 1 mg/kg of the final dilution by steady i.v. bolus over 5 min, daily for 5 consecutive days through the CVC. This dose represents the FDA approved dosage for treatment of invasive fungal infections in humans.

Two hours after the last AmB dose, the rabbit was anesthetized with ketamine (35 mg/kg) and xylazine (7 mg/kg) administered by i.m. injection. When the animal was fully anesthetized, the microscope was focused on the corneal surface along the visual axis and a Raman spectrum was obtained. Subsequently, Raman spectra were collected from the AH along the visual axis at depths of 0.5 mm, 1 mm and 1.5 mm below the corneal surface. Immediately thereafter, AH was withdrawn through the cornea with a 1 ml syringe attached to a 25 gauge needle for high-performance liquid chromatography (HPLC) confirmation of AH AmB levels. The animal was then euthanized with i.v. pentobarbital sodium. Immediately after euthanasia, blood was withdrawn into a heparinized syringe by cardiac puncture for plasma AmB level determination.

In the Raman spectra of Am B there is a characteristic peak near 1557 cm^{-1}. This band was integrated using an iterative curve fitting utility (Curvefit) in GRAMS/32 Spectral Notebase (Galactic Industries Corp, Salem, NH). Curve fitting often provides more precise band area determinations in the presence of significant baseline noise and curvature [11]. The curve fitting utility modeled the Raman band profile as a mixture of Guassian and Lorentzian bandshapes. The Gaussian character was introduced by the Raman instrument since its resolution was about half the width of the amphotericin Raman band. The same iterative curve fit starting conditions were used for all Raman spectra. The results of the curve fitting were exported to Excel for further analysis.

AmB concentrations in plasma and AH were determined after methanol extraction by modification of an internally validated reversed-phase high-performance liquid chromatographic method [12]. The mobile phase (flow rate 1.6 ml/min) was composed of methanol (500ml)-acetonitrile (350 ml) and 0.0025 M Na-EDTA buffer (200 ml). All components were manufactured by Fisher Scientific (Fair Lawn, N.J.). The injection volume was 75 µl for plasma samples and 100 µl for aqueous. AmB was detected by UV absorbance (Spectroflow 783 Programmable Absorbance Detector, Kratos Analytical) at 382 nm using a C_{18} analytical column (Waters, Milford, Mass.) with a Guard C_{18} in-line precolumn filter (Perkin-Elmer, Norwalk, Conn.), and Shimadzu CR501 Chromatopac integrator. Quantification was based on the peak height-concentration response of the external calibration standard. Nine-point standard curve, prepared in drug-free plasma (0–5.0µg/ml, extraction 1:2 v/v sample/methanol), and eight-point standard curve (0–1.0 µg/ml, extraction 1:1 v/v sample/methanol) was prepared in Hanks (90%) with addition 10% of plasma, were linear with $R^2 > 0.990$. Coefficient of variation was less than 15% for both matrixes.

Results

AmB exhibited unique spectral peaks at 1152 and 1557 cm^{-1} (Fig. 1). The most prominent of these peaks (1557 cm^{-1}) was evaluated in our anterior chamber model in order to examine the relationship between various peak parameters and AmB concentration. We observed a linear correlation ($r^2 = 0.9954$) between AmB concentration and integrated peak area (Fig. 2) in the range of AmB concentrations studied. This curve was used to predict concentration in the rabbit eye after daily administration of i.v. AmB for 5 days. The 1557 cm^{-1} peak was resolvable from the background in the spectra of the rabbit anterior chamber (Fig. 3). This peak was very consistent in size and shape despite the background 'noise' as these overlaid curves in Fig. 3 demonstrate.

Integrated peak areas from 7 rabbit eye measurements were calculated as previously described. The

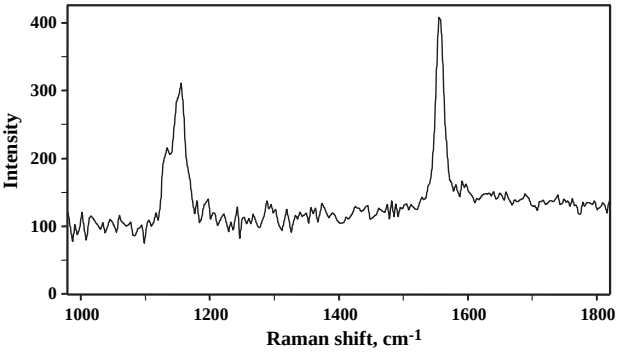

Fig. 1 Resonant Raman Spectrum of 0.625 µg/ml amphotericin B in 40 mmol/l desoxycholate.

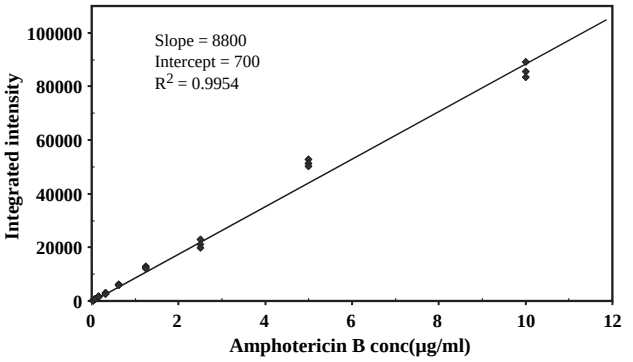

Fig. 2 Integrated area of 1557 cm^{-1} Raman peak vs. amphotericin B concentration in artificial anterior chamber.

mean ($\pm$SE) integrated area of 2220.8 ± 654 was plotted on the calibration curve in Figure 2 and predicted an AmB concentration of 0.173 μg/ml. Subsequent measurement of the AmB concentration in AH by HPLC yielded a comparable value of 0.18 μg/ml. Both of these are an order of magnitude less than the HPLC determined plasma value (1.109 μg/ml). These 7 *in vivo* spectra were obtained at depths ranging from 0.5 mm to 1.5 mm below the corneal surface. The peak area was similar regardless of the depth at which the confocal spectra were acquired (Fig. 3).

Collection of 19 consecutive spectra from a 10 μg/ml AmB solution in our anterior chamber model over 27 min revealed no evidence of photobleaching. In addition, collection of 6 spectra from another 10 μg/ml AmB solution at depths ranging from 0.2 mm to 1.6 mm below the contact lens surface, revealed no evidence of self-absorption (data not shown).

Discussion

This is the first study to demonstrate the ability of Raman spectroscopy to accurately measure AmB levels at therapeutic levels in an *in vitro* model that simulates the anterior chamber (Fig. 4). In addition, data from one animal suggest that this may be a useful tool for non-invasively measuring ocular levels of AmB *in vivo*. The ability of this instrument to measure AmB in the AH after i.v. administration shows that Raman Spectroscopy is very sensitive and capable of detecting levels well below those expected after direct ocular injection. A previous study demonstrated the ability to use Raman spectroscopy to measure AmB in a cuvette and to visualize Raman peaks in the anterior chamber however, the authors did not attempt to predict concentration in the latter [6].

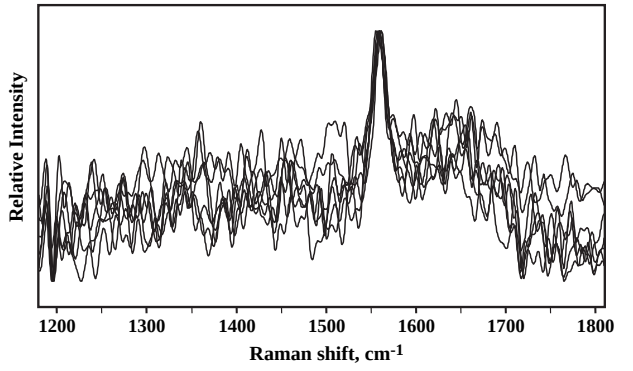

Fig. 3 Overlaid *in vivo* Raman spectra from rabbit anterior chamber at depths 0.5–1.5 mm below corneal surface. The AmB peak at 1557 cm^{-1} is consistent in all spectra.

The lack of significant self-absorption *in vitro* suggests that this should be a practical tool for measuring AmB concentration in clinical situations since the depth of acquisition of Raman spectra is not critical for accuracy. This observation was further substantiated by the data we obtained from one animal in which the Raman spectra were consistently reproducible when the confocal Raman system was focused at a variety of depths. In addition, since photobleaching does not appear to be a concern, even after more than 25 min of exposure, the only limiting factor for integration times would be from light toxicity. Since toxicity should be minimal at the studied wavelength and intensity, this would allow us to increase integration time and further reduce our signal-to-noise ratios if necessary to improve instrument performance.

This instrument may become important in the management of fungal endophthalmitis. Using this technology could help physicians decide when repeat dosing is necessary based on measured ocular drug levels. A non-invasive way to measure these levels in 'real-time' would avoid the possibility of unnecessary invasive injection of this difficult to deliver, toxic agent without the risk of the AmB concentration dropping below the MIC of the infecting organism.

New lipid associated preparations of AmB are currently in widespread clinical usage. These have less toxicity than AmB desoxycholate and may have better efficacy for certain types of difficult to treat fungal infections as well as better penetration into various tissue compartments [13,14]. One of these compounds, liposomal AmB, may achieve ocular levels above the MIC for certain fungi [13]. A non-invasive drug level monitoring tool such as this one may help us understand these phenomena and predict those patients likely to be successfully treated with systemic therapy. If levels above the fungal MIC in that compartment can be shown to correlate with cure using this non-invasive tool, the physician can predict early in the course of treatment the likelihood of success. If adequate levels

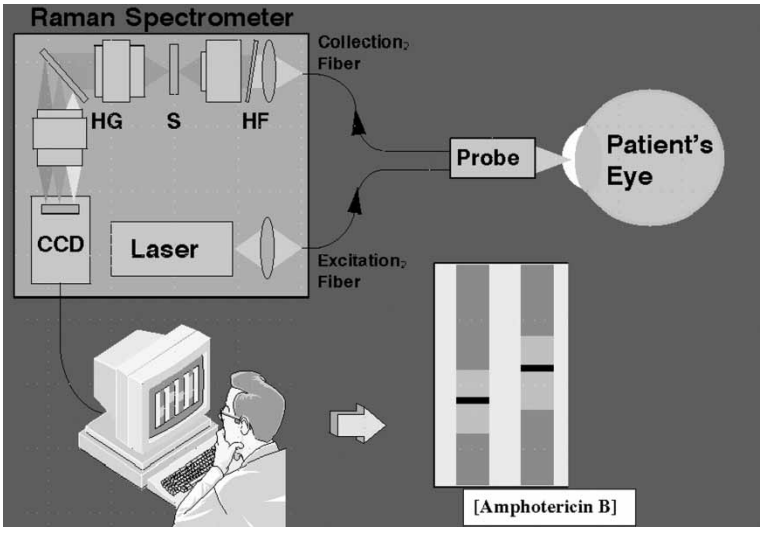

Fig. 4 Schematic diagram of Raman instrument used in this study.

are not achieved, alternative therapy can be prescribed early in the course of treatment, which should improve long-term success. We have not yet demonstrated the ability of this system to measure AmB in these lipid preparations however, Raman spectroscopy has been used to characterize AmB in these preparations and the spectral features remain visible [15]. Clearly, this needs to be confirmed in our *in vitro* AC model and in subsequent animal experiments. Currently, it is unknown whether the AmB remains lipid associated once it enters the eye. Nevertheless, it is important to know that it could be accurately measured whether free drug or lipid-associated drug is present.

Future studies with this instrument should be focused on evaluation *in vivo* including correlation with levels measured by HPLC in addition to correlation of tissue compartment levels with cure rates from fungal infection in those compartments. These studies will need to be performed in a variety of clinical scenarios including those with a significant inflammatory response in order to assure that fluorescence from protein and other substances that may enter the eye do not interfere with the Raman spectra. If Raman spectroscopy is successful in monitoring AmB levels in those models, other compounds with similar pharmacodynamic issues should be studied since it may be useful for other drugs currently in clinical use. This technology has the potential to revolutionize intra-ocular drug management.

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References

1 Lambert JL, Morookian JM, Sirk SJ, Borchert MS. Measurement of aqueous glucose in a model anterior chamber using Raman spectroscopy. *J Raman Spectrosc* 2002; **33**: 524.
2 Pohjola S. The glucose content of the aqueous humor in man. *Acta Ophthalmol* 1966; **88**(Suppl.): 1–80.
3 Schrader WF, Grajewski RS, Meuer P, Kiefer W. The glucose content of the aqueous humor compared with capillary blood in man. *Investigat Ophthalmol Vis Sci* 2000; **41**(Suppl.): S77.
4 Fisher JF, Taylor AT, Clark J, *et al*. Penetration of amphotericin B into the Human Eye. *J Infect Dis* 1983; **147**: 164.
5 Green WR, Bennett JE, Goos RD. Ocular penetration of amphotericin B. *Arch Ophthal* 1965; **73**: 769–775.
6 Hosseini K, Jongsma FH, Hendrikse F, Motamedi M. Non-invasive monitoring of commonly used intraocular drugs against endophthalmitis by Raman spectroscopy. *Lasers Surg Med* 2003; **32**: 265–270.
7 Walsh TJ, Jackson AJ, Lee JW, *et al*. Dose-dependent pharmacokinetics of amphotericin B lipid complex in rabbits. *Antimicrob Agents Chemother* 2000; **44**: 2068–2076.
8 Norman A. The beginning solubilization of 20-methylcholanthrene in aqueous solutions of conjugated and unconjugated bile acid salts. *Acta Chem Scand* 1960; **14**: 1295–1299.
9 Slater JB, US Patent #5,917,971, June 29,1999.
10 Walsh TJ, Bachar J, Pizzo PA. Chronic silastic central venous catheterization for induction, maintenance, and support of persistent granulocytopenia in rabbits. *Lab Anim Med* 1988; **38**: 467–471.
11 Pelletier MJ. Quantitative analysis using Raman spectrometry. *Appl Spectrosc* 2003; **57**: 20A–42A.
12 Brassinne C, Laduron C, Coune A, *et al*. High-performance liquid chromatographic determination of amphotericin B in human serum. *J Chromatogr* 1987; **419**: 401–407.
13 Goldblum D, Rohrer K, Frueh BE, *et al*. Ocular distribution of intravenously administered lipid formulations of amphotericin b in a rabbit model. *Antimicrob Agents Chemother* 2002; **46**: 3719–3723.

14 Groll AH, Giri N, Petraitis V, *et al*. Comparative efficacy and distribution of lipid formulations of amphotericin B in experimental *Candida albicans* infection of the central nervous system. *J Infect Dis* 2000; **182**: 274–282.

15 Bunow MR, Levin IW. Vibrational Raman spectra of lipid systems containing amphotericin B. *Biochim Biophys Acta* 1977; **464**: 202–216.