Analytical Methods

Identification of substandard and counterfeit antimalarial pharmaceuticals chloroquine, doxycycline, and primaquine using surface-enhanced Raman scattering

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ARTICLE

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Counterfeit antimalarial pharmaceuticals are a worldwide problem with negative public health implications. Here, we develop a surface-enhanced Raman scattering (SERS) protocol to recognize substandard and falsified antimalarial drugs present in commercially available tablets. After recording SERS spectra for pure chloroquine, primaquine, and doxycycline, SERS is used to measure these drugs formulated as active pharmaceutical ingredients (APIs) in the presence of common pharmaceutical caplet excipients. To demonstrate viability of our approach, a red team study was also performed where low-quality and counterfeit formulations of all three drugs presented as unknowns were identified. These data in conjunction with promising results from a portable Raman spectrometer suggest that SERS is a viable technique for on-site analysis of drug quality.

Introduction

Low quality and falsified medications are a global problem with dire humanitarian consequences.[1-7] Counterfeit antimalarial pharmaceuticals, specifically, are an especially important aspect of this crisis.[2] The World Health Organization (WHO) reported more than 200 million cases of malaria resulting in 445,000 deaths across 91 countries in 2016, where up to 200,000 deaths could have been avoided by identifying and avoiding substandard antimalarials.[1,3] Estimates suggest one third of the available antimalarial pharmaceutical treatments in sub-Saharan Africa are of low quality.[4] Low quality pharmaceuticals are those in which the amount of active pharmaceutical ingredient (API) present is not the amount recommended for effective treatment, often contradicting the label claim.[5,6] These deficits come from a variety of sources such as deliberate counterfeiting, lack of supply-chain regulation, and degradation of APIs in poor storage conditions.[2,5] Low quality or falsified drugs greatly hinder our ability to treat malaria as low dosage quantities of API foster drug resistance among carriers of the disease and medications lacking APIs entirely are clearly ineffective, increasing incidents of illness, mistrust in available healthcare, and fatalities.[4,5] Chloroquine, doxycycline, and primaquine are a few of the antimalarial pharmaceuticals included by the WHO in its list of essential medicines indicating their important role in malaria treatment and are therefore ideal candidates for quality studies.[1]

A variety of chromatographic and mass spectrometric techniques are routinely used for testing drug quality and are therefore preferred methods of analysis.[3] However, analytical methods such as these are not feasible for pharmaceutical analysis in developing countries and low-resource settings where malaria is prevalent as they commonly lack the supplies and skilled analysts to accommodate such endeavors.[6] Other schemes relying on optical spectroscopies are relatively inexpensive and can be portable, showing promise in addressing the shortcomings of other analytical techniques. Of optical methods such as UV/vis absorption and Raman spectroscopy, Raman is well suited for pharmaceutical identification over other techniques because it is non-destructive and yields fingerprint vibrational spectra unique to each analyte molecule.[3,8] Raman scattering, however, is a very weak phenomenon with only approximately one photon Raman scattered out of 10 million scattered photons.[9] An additional disadvantage of Raman scattering is the high degree of fluorescence exhibited by pharmaceuticals the near-UV and visible region which regularly interferes with Raman analysis.[7] Placing analyte molecules on the surface of a plasmonic metal has two benefits: it enhances the signal compensating for weak Raman intensity by amplifying scattering by a factor of 10^6 to 10^9 and also quenches problematic interferences due to fluorescence.[7-10] Surface-enhanced Raman spectroscopy (SERS) is therefore a promising tool in pharmaceutical analysis due to the technique's high sensitivity and capacity for detecting analytes at femtomolar concentrations as well as its ability to offer quantitative results.[11]

Experimental

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Silver nitrate, sodium citrate tribasic, sodium bromide, chloroquine phosphate, doxycycline hyclate, primaquine phosphate, and 4-mercaptobenzoic acid (4-MBA) were purchased from Sigma-Aldrich and used without further purification. Dosage forms of chloroquine (chloroquine phosphate, 250 mg) and of doxycycline (doxycycline hyclate, 100 mg, lot 150806, HPLC assay: 107%) used in the blinded field samples were manufactured by CBF Pharmacopeia and Unidoxy (SPIC) China respectively. Additional dosage form doxycycline was obtained in Kenya. Silver colloidal nanoparticles were prepared by the Lee and Meisel method:[12] a 2.29mM silver nitrate solution was prepared using ultrapure water and allowed to boil. 118.7 mg sodium citrate was dissolved into the silver nitrate solution and boiled for an additional 25 minutes resulting in a yellow-gray silver colloid suspension. The final suspension was diluted to 1 L. This process yielded generally spherical nanoparticles approximately 70nm in diameter, which was verified using UV/vis absorption, dynamic light scattering, and transmission electron microscopy (Figure S1).

For each sample, 50uL of pharmaceutical stock solutions at varying dilutions were added to a 5 mL aliquot of the silver nanoparticle suspension. Stock solution concentrations of each pharmaceutical were optimized to give a SERS signal falling within the linear response range. Samples made at a concentration of analyte above the concentration sufficient saturate the substrate surface do not produce spectra whose peak intensities scale linearly with concentration, and thus do not produce quantitative results. To avoid this, linear response limits were found and stock solutions for each pharmaceutical were made accordingly, ranging from approximately 0.1mM to 1mM for chloroquine, 0.05mM to 10mM for primaquine, and 1mM to 10mM for doxycycline. After thorough mixing, 50uL of a 10uM 4-MBA solution were added to each sample used for correlation or peak-area ratio analysis to serve as an internal standard. 1 mL of 1M sodium bromide solution was used to aggregate silver nanoparticles for a final bromide concentration of 0.16M and final concentrations of 1uM to 10uM for chloroquine, 0.5uM to 100uM for primaquine, and 10uM to 100uM for doxycycline in analytical samples. Nanoparticle aggregates were allowed to settle to the bottom of the sample vial before analysis.

SERS samples for evaluating unknown samples were prepared in a similar fashion. The red team independent lab prepared unknown samples in two ways: by mixing pure API with common excipients such as starch and by crushing actual unit dose caplets and cutting them with said excipients. Unknowns were first crushed and placed in water before being sonicated and centrifuged to dissolve active ingredients and remove insoluble excipients. The supernatant solution was used immediately as a pharmaceutical stock solution. A reference concentration of 0.48mM was used when analyzing unknown chloroquine samples, 0.5mM for primaquine, and 5mM for doxycycline. Detailed information on formulation of red team samples can be found in supporting information (Table S1).

Raman and SERS spectra were obtained using a custom-built Raman spectrometer composed of a 633nm HeNe laser (Thorlabs) aligned into the back of an inverted microscope (Nikon) and focused onto the sample using an objective lens (20X, NA=0.5). Backscattering was collected through the same objective, passed through a Rayleigh rejection filter (Semrock), and directed into a liquid nitrogen-cooled spectrometer (Princeton Instruments, Acton Research, f = 0.3m, 1200 grooves per mm). Each spectrum was acquired for 30s using Winspec software. Laser powers, measured at the objective, were 7mW for the acquisition of Raman spectra and 50μW for SERS spectra. For each sample concentration, triplicate scans of three or more samples were taken and analyzed.

Portable SERS spectra were obtained using a CBEx instrument (Snowy Range Instruments) powered by a 785nm laser at 50mW power. 2 mL of the above-described SERS samples were transferred to glass vials compatible with the CBEx instrument for analysis. The instrument acquired spectra using orbital raster scanning with a 0.2 s acquisition time.

Results and Discussion

Raman and SERS spectra for pure chloroquine and doxycycline without internal standard are displayed in Figure 1. Peaks that appear in the Raman spectra of both drugs generally correspond to those in the equivalent SERS spectra, demonstrating that the Raman signal of each drug was effectively enhanced. The SERS spectrum for chloroquine is consistent with past reports showing strong bands at 1373cm^{-1} corresponding to the ν(C-C) stretching mode and the δ(C-H) bending mode of the quinoline as well as the ring deformations of quinoline observed at 757cm^{-1}.[13,14] Similarly, the SERS spectrum for doxycycline agrees with previous research,[15] as the most intense bands centered at 1279cm^{-1} and 1333cm^{-1} are pronounced. Primaquine was omitted here because the Raman

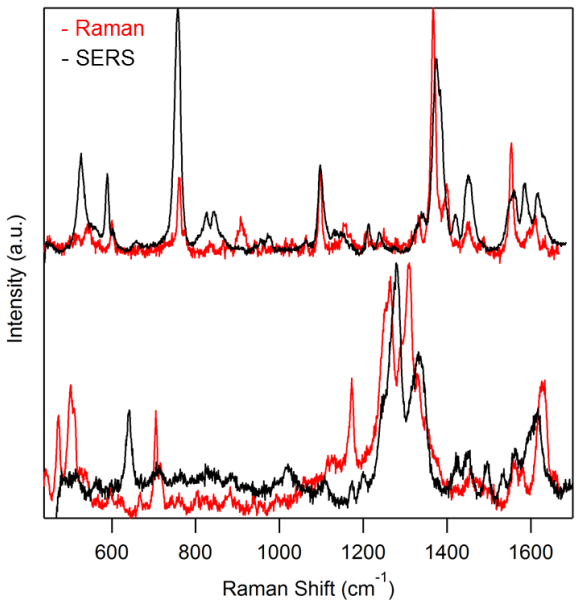

Figure 1: Raman (red) and SERS (black) of chloroquine (top) and doxycycline (bottom) obtained in the absence of internal standard on a benchtop Raman system using a 633nm HeNe laser. While there are differences between the SERS and Raman spectra arising from the sensitivity of SERS to the adsorbate geometry, the overall similarity between spectra indicates successful enhancement of analyte Raman signal and illustrates the unique SERS signal for each analyte.

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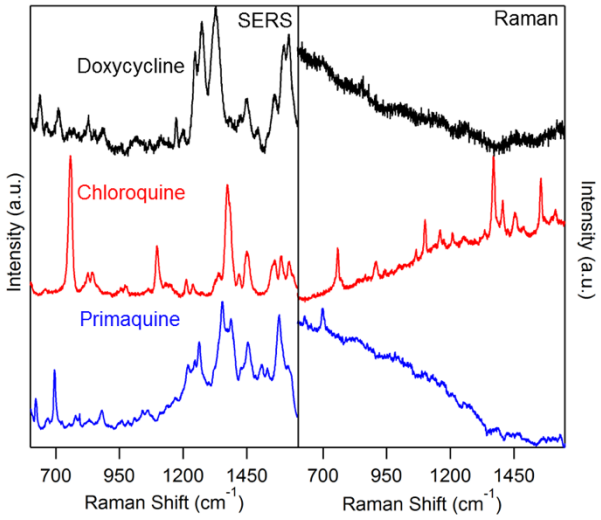

Figure 2: SERS spectra (left) and Raman spectra (right) of dosage form doxycycline, chloroquine, and primaquine demonstrating the viability of spectroscopic analysis of antimalarial pharmaceuticals in a common excipient matrix. Raman spectra obtained from dosage-form API formulations exhibit fluorescence interferences which are clearly absent from the SERS spectra.

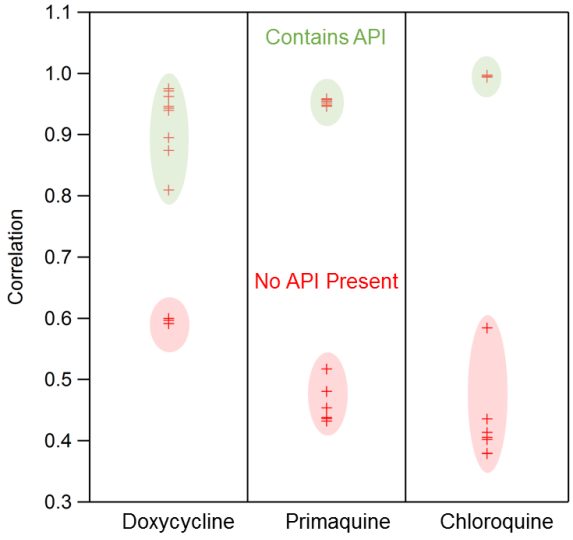

Figure 4: Correlation values for samples analyzed during the red team study of doxycycline, primaquine, and chloroquine. For each compound, the samples with and without API are easily distinguished using their correlation to the reference compounds.

spectrum of the pure pharmaceutical displayed strong fluorescence in agreement with literature.[16] Additional band assignments are reported in supporting information (Table S2). Generally, differences between SERS and Raman spectra arise because vibrational modes of analytes on a plasmonic metal surface are preferentially enhanced when they align with the local electric field.[17,18]

Figure 2 shows the SERS and Raman spectra of all three drugs presented as APIs in dosage-form caplets with excipients. Clearly, SERS benefits over Raman as the process quenches interferences that are attributed to the excipient matrix. Further, our results (Figure 3, top) demonstrate that packing materials typically found in unit dose forms of antimalarial pharmaceuticals do not interfere with the SERS signal of the active ingredient. The spectra of drugs in the presence of excipients were invariant when compared to SERS obtained from pure compounds.

To verify the efficacy of our method, a red team study was performed where an independent lab prepared dosage form samples of unknown API concentrations for identification (Table

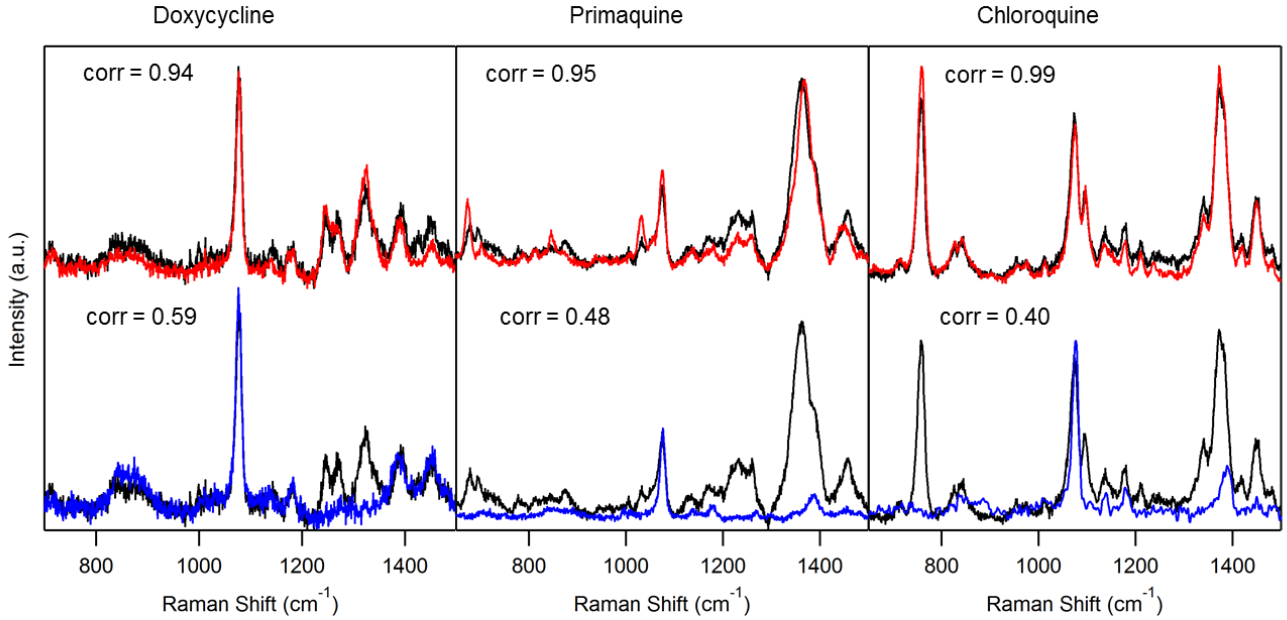

Figure 3: Results of the red team study demonstrating the effectiveness of SERS analysis for antimalarial pharmaceuticals. For each compound, reference spectra of API with internal standard (black) were compared to samples of each pharmaceutical presented as an unknown quantity of API in the presence of excipients and internal standard. Samples containing label-claim levels of API were identified by a high correlation value and deemed good samples (red). Samples with little or no API present are identified with a low correlation value (blue) were deemed low-quality. Correlations of the reference and unknown spectra were evaluated in the 1100-1600cm^{-1} region to avoid overlap with internal standard peaks.

S1). The method described here was able to successfully identify the pharmaceutical content of all of the unknowns provided in agreement with what was prepared by the independent lab. Unknown samples containing no API were identified by determining the correlation coefficient of the reference and unknown spectra (Matlab, Figure S2). Correlations were assessed in the 1100-1600cm^{-1} region of each spectrum to avoid overlap with signal from the 4-MBA internal standard. Selected results of this study are given as a comparison of the SERS spectra of reference samples of each pure pharmaceutical to the SERS spectra of the unknown caplets (Figure 3). Correlations between the unknown spectra and the reference samples for each drug are shown in Figure 4 which displays a clear separation between correlation values for unknowns that contained API and those that were composed entirely of excipients. Correlation coefficient threshold values of 0.8 for each drug were established to compare future correlations of reference and unknown samples. Unknown samples found through correlation to have coefficients above the 0.8 threshold can be said to contain API (Table S3).

To further elucidate the quality of the unknown samples, the SERS spectra were recorded in the presence of 4-MBA as an internal reference and evaluated through peak-area ratio analysis. Peak-area ratio quantification with an internal standard has previously shown to account for fluctuations in SERS signal while retaining high sensitivity.[19,20,21] 4-MBA was selected as the internal standard as it is a well-characterized and commonly used SERS-active molecule,[22,23] and its peaks are easily distinguishable from those derived from pharmaceuticals in a SERS spectrum containing both species. The SERS spectrum for 4-MBA can be found in supporting information (Figure S3). The 4-MBA bands at 1076 cm^{-1} and 1585 cm^{-1} corresponding to the ν(C-C) stretching mode were used as reference bands.[22,23] The 1076 cm^{-1} peak was used as reference for primaquine and doxycycline analysis and the band at 1585 cm^{-1} was used for chloroquine. Among samples determined to contain API from the correlation analysis (Figure 4), a peak-area ratio analysis was performed where the area of the 4-MBA band was divided by the area of a prominent API band (765 cm^{-1} chloroquine, 1333 cm^{-1} doxycycline, 1364 cm^{-1} primaquine). An acceptable range for confirming legitimate API content was defined as the mean ratio value plus or minus one standard deviation of the nine reference spectra for each drug. Using these criteria, we were able to identify low quality caplets of both doxycycline and chloroquine, 48% and 50% of the label claim respectively, and differentiate them from caplets containing the full reported API content in the previously mentioned red team studies (Table S3). No caplets containing lower concentrations of API were presented as unknowns in the red team study of primaquine.

Figure 5 displays representative SERS spectra of chloroquine acquired on the custom-built Raman system and portable, handheld Raman spectrometer. The portable spectrometer shows promise as the results are comparable to the bench-top laser configuration. While the signal-to-noise ratios are similar between the two, the higher resolution of the benchtop instrument yields a more detailed spectrum than one attained on the portable device. In general, however, the good agreement between the two spectra suggests a promising outlook for application in field-based pharmaceutical assays.

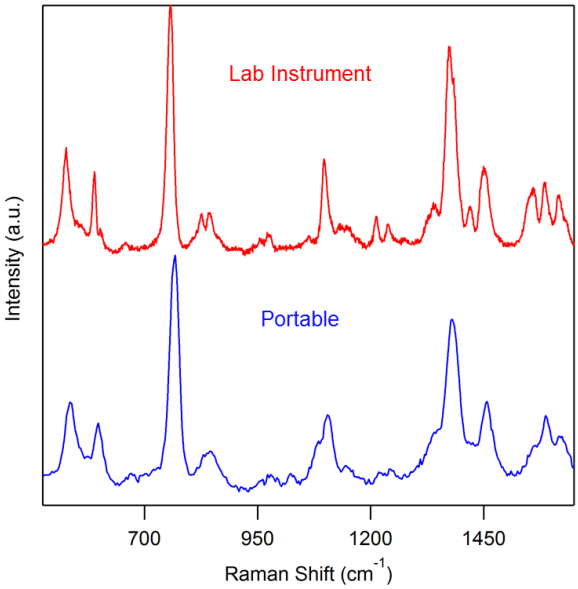

Figure 5: Comparison of representative SERS spectra of chloroquine acquired using a custom-built bench top instrument (red) and a handheld portable Raman device (blue). The similarity of the spectra obtained on the portable instrument and the bench top instrument illustrates the potential for portable SERS analysis of antimalarial pharmaceuticals.

Conclusions

We demonstrate that SERS is a useful technique for evaluating substandard and falsified drugs by successfully obtaining Raman and SERS spectra for doxycycline, chloroquine, and primaquine as well as assessing the quality of dosage-form samples of all three pharmaceuticals. We further show that pure pharmaceutical and pharmaceutical as an API in dosage-form medications yield comparable signal, meaning that common excipients do not affect SERS analysis. Our method has great potential for in-field pharmaceutical analysis proven by the comparable spectra obtained using our custom-built Raman spectrometer and a portable Raman spectrometer.

Conflicts of interest

There are no conflicts to declare.

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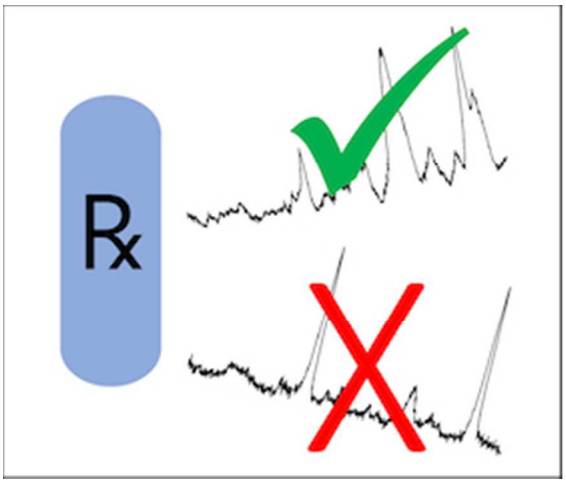

A method for determining the quality of substandard and falsified antimalarial pharmaceuticals using SERS spectroscopy.