LABEL-FREE QUANTIFICATION OF EMTRICITABINE IN HUMAN PLASMA BY SURFACE-ENHANCED RAMAN SPECTROSCOPY (SERS)

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Abstract

Surface-enhanced Raman spectroscopy (SERS) offers promising capabilities for sensitive detection and quantification of pharmaceuticals in complex biological matrices. This study presents a novel approach for SERS-based reproducible, label-free quantification of the antiretroviral drug emtricitabine (FTC) in human plasma, achieving a limit of quantification (LOQ) of 78 ng/mL. The methodology consists of using silver colloidal nanoparticles (Ag CNPs) and silver nitrate ($AgNO_3$) for plasma pretreatment, evaporating the samples onto an aluminum well plate, and collecting spatially resolved SERS measurements using a custom-built Raman scanning system. Calibration was performed using three analytical methods: the total population method, the quality index (Qi) sample method (selective sampling via a figure-of-merit), and the cumulative distribution function (CDF) method (modeling Qi distributions through CDFs). Among these, the Qi sample method demonstrated superior linearity (R^2=0.99) and optimal signal-to-noise ratios (S/N) near the LOQ, while the CDF method provided enhanced analytical sensitivity at higher concentrations. This study not only advances the analytical rigor of SERS-based quantitative assays but also establishes a practical framework for integrating SERS into clinical monitoring of medication adherence, specifically for FTC-based HIV treatment and prevention.

Introduction

Medication adherence can be defined as the extent to which patients consistently follow prescribed medication regimens, which significantly impacts therapeutic outcomes for human immunodeficiency virus (HIV) prevention and treatment. Antiretroviral therapy (ART) and pre-exposure prophylaxis (PrEP) require strict adherence for optimal efficacy, as poor adherence can lead to reduced therapeutic benefits, the emergence of drug-resistant viral strains, and increased healthcare costs.[1, 2] While traditional laboratory-based methods (i.e., liquid chromatography-tandem mass spectrometry (LC-MS/MS)) used to monitor adherence are highly sensitive and accurate, they often require sophisticated instrumentation, considerable expense, and extensive expertise.[3] Consequently, alternative analytical methods for adherence monitoring suitable for widespread implementation, particularly in resource-limited settings, remain highly desirable.

Surface-enhanced Raman spectroscopy (SERS) presents a compelling solution due to its high sensitivity, rapid analysis capability, and suitability for integration into portable analytical platforms. Raman spectroscopy is an optical technique that detects molecular vibrations by measuring inelastically scattered photons from laser-irradiated samples. Each molecule produces a unique vibrational "fingerprint," allowing for specific identification and quantification. However, conventional Raman spectroscopy suffers from inherently weak signal intensities, limiting its practicality for quantitative analysis. SERS overcomes this limitation by adsorbing molecules onto metallic nanostructures, typically gold or silver nanoparticles, to significantly amplify the Raman scattering signals. This amplification occurs primarily through localized surface plasmon resonance (LSPR), where incident photons induce collective oscillations of conduction electrons on the metallic nanoparticle surfaces, producing highly concentrated electromagnetic fields around the nanoparticle surface.[4]

Enhancement factors achieved by SERS can reach up to 10^{10}, greatly improving the ability to detect trace-level analytes by increasing signal intensity while preserving molecular specificity.[4]

Prior research published by our group established the utility of SERS for detecting antiretroviral drugs, specifically demonstrating quantitative analysis of tenofovir and FTC, which are both key components of first-line HIV prevention and treatment regimens. Previous studies achieved detection limits down to 25 ng/mL for tenofovir and 40 ng/mL for FTC in aqueous solutions.[5-8] However, extending these analytical capabilities to complex biological matrices, such as human plasma, remains challenging due to significant signal interference from endogenous biomolecules, background variability, and quantitative irreproducibility.

The current study addresses these critical challenges by developing a robust SERS-based assay capable of reproducibly detecting and quantifying FTC directly in human plasma samples at therapeutically relevant concentrations. This work introduces an innovative plasma sample pretreatment protocol using silver nitrate ($AgNO_3$) and silver colloidal nanoparticles (Ag CNPs) to effectively reduce interference and enhance analyte detection. Spatially resolved SERS measurements collected using a custom-built Raman scanning system facilitate detailed statistical analyses, further improving quantitative accuracy and precision. Three analytical methods, total population averaging (total population method), selective spectral sampling (Qi sample method), and CDF modeling (CDF method), are compared to determine the most reliable strategy for FTC quantification. The results presented in this paper establish a foundation for translating SERS-based methodologies from laboratory proof-of-concept toward practical clinical applications, particularly in medication adherence monitoring for FTC-based HIV treatment and prevention programs.

Experimental

Preparation of Silver Colloidal Nanoparticles

Silver colloidal nanoparticles (Ag CNPs) were synthesized following a previously described protocol.[9] Briefly, silver nitrate ($AgNO_3$) was reduced by hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) in an aqueous alkaline medium at room temperature, yielding nanoparticles averaging approximately 70 nm in diameter.

Plasma Sample Pretreatment

Commercial human plasma (BioIVT, K_2 EDTA anticoagulant) was thawed and spiked with aqueous FTC solutions to achieve concentrations ranging from 78 to 5000 ng/mL. Samples were initially filtered using size-exclusion centrifuge filters (3 kDa molecular weight cutoff) to remove high molecular weight components. To eliminate chloride ions, filtered plasma was treated with 1 M $AgNO_3$, forming a white AgCl precipitate removed by centrifugation. The supernatant underwent two subsequent centrifugation treatments with Ag CNPs: an initial treatment to remove residual silver ions, followed by a final treatment step for analyte adsorption. The prepared samples were then deposited (5 technical replicates of each concentration, each a 20 µL aliquot) onto machined aluminum well plates and dried at room temperature prior to SERS analysis.

SERS Spectral Acquisition

Spatially resolved SERS spectra were collected using a custom-built Raman scanning system equipped with a 785 nm laser (Wasatch Photonics Raman spectrometer, 15 mW laser power, and 800 ms integration time). Each well on the aluminum well plate was scanned systematically in a raster pattern, acquiring a total of 1806 spectra per well.

Analytical Methods and Data Processing

Calibration curves of three separately FTC-spiked plasma sample sets (prepared as described in section *Plasma Sample Pretreatment*) were generated using three

analytical approaches for quantitative analysis of FTC.

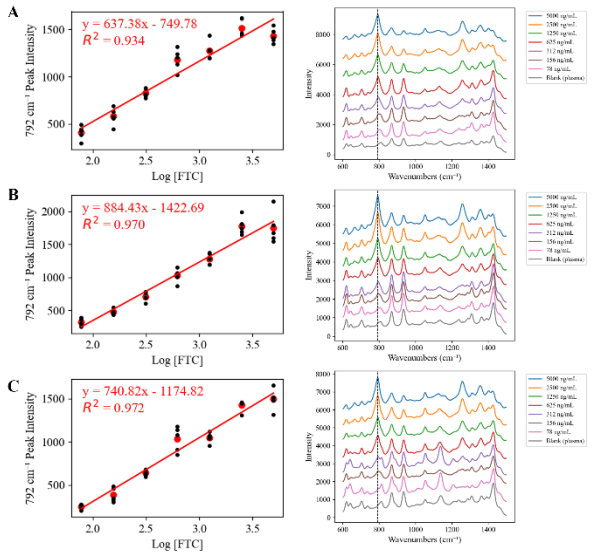

Fig. 1. (A – C) Calibration curves prepared using the total population method for three replicate experiments with accompanying SERS spectra. Each spectrum shown is an average of all collected spectra from all replicates for each concentration with a vertical line shown at 792 cm^{-1}. Each black data point represents the difference in SERS intensities at 792 cm^{-1} and 723 cm^{-1} for each technical replicate. Linear regression lines were calculated using the average of all concentration replicates (red data points). The regression line, equation, and correlation coefficient for each replicate experiment are shown in red. Spectra were offset for clarity.

Total Population Method. For the total population method, all acquired spectra (n = 1806 per sample) were averaged for each FTC concentration. The calibration response was calculated as the difference in SERS intensity between the FTC-specific peak, corresponding to a ring breathing mode[10], at 792 cm^{-1} and the baseline at 723 cm^{-1}. A linear trendline was calculated from the average response of all replicates (Fig. 1). This traditional approach served as a benchmark by utilizing the entirety of available spectral data without discrimination based on signal quality.

Qi Sample Method. In the Qi sample method, spectra were selectively sampled based on a calculated figure-of-merit termed the quality index (Qi), which quantifies the signal-to-noise (S/N) ratio specifically for the FTC peak at 792 cm^{-1} relative to baseline intensities at 723 cm^{-1} (see Eq. 1). The Qi calculation involves summing the SERS intensities (I_j) across ±n wavenumber points around the peak of interest p (792 cm^{-1}) and around two baseline regions b_1 and b_2 (both set at 723 cm^{-1}). The summed baseline intensities are subtracted from the summed peak intensities to produce background-corrected peak values. These corrected values for each peak t are then multiplied together and raised to the power of 1/t (in the current study, t = 1), resulting in the final Qi value (Eq. 1). Any negative Qi values are set to zero.

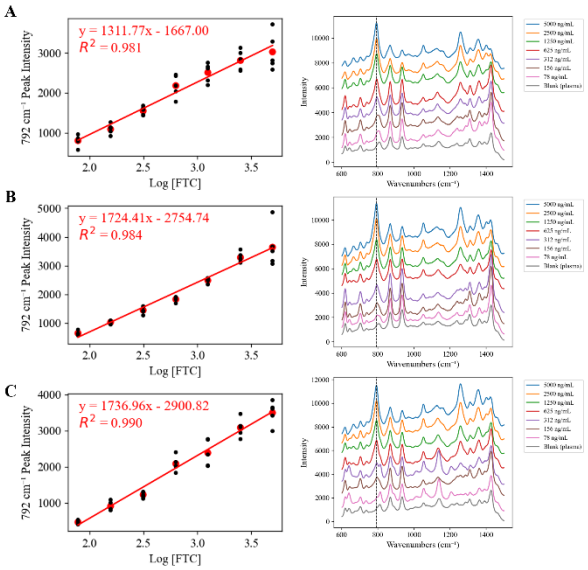

Fig. 2. (A – C) Calibration curves prepared using the Qi sample method for three replicate experiments with accompanying SERS spectra. Each spectrum shown is an average of the top 20 spectra from each replicate corresponding to the highest 792 cm^{-1} Qi (total of 100 spectra for each concentration) with a vertical line shown at 792 cm^{-1}. Each black data point represents the difference in SERS intensities at 792 cm^{-1} and 723 cm^{-1} for each technical replicate. Linear regression lines were calculated using the average of all concentration replicates (red data points). The regression line, equation, and correlation coefficient for each replicate experiment are shown in red. Spectra were offset for clarity.

$$Qi = \left(\prod_{k=1}^{k=t} \left\{ \frac{1}{2n+1} \left[\left(\sum_{j=p-n}^{p+n} I_j - \sum_{j=b_1-n}^{b_1+n} I_j \right) \times \left(\sum_{j=p-n}^{p+n} I_j - \sum_{j=b_2-n}^{b_2+n} I_j \right) \right] \right\}_k \right)^{1/t} \quad (1)$$

$$\{Qi < 0 \stackrel{\text{def}}{=} Qi = 0\}$$

For each replicate (one aluminum well), only spectra corresponding to the highest 20 Qi values were selected and averaged. The intensity at 723 cm^{-1} was then subtracted from the intensity at 792 cm^{-1} of each averaged spectrum and plotted as function of concentration (Fig. 2). A linear regression analysis was then conduced on the average of all of these differences for each concentration.

CDF Method The CDF method modeled the entire distribution of nonzero Qi values (see Eq. 1). For each replicate (one aluminum well), all nonzero Qi values were sorted in ascending order and assigned incremental index values starting from one. The cumulative probability for each Qi value was then calculated by dividing its index value by the highest index number, and these probabilities were plotted against the logarithm of the corresponding Qi values to form replicate CDFs. To generate a model CDF for each FTC concentration, Qi values from all replicates for each concentration were combined into a single array, and the sorting, indexing, and probability calculation process was repeated. Thus, replicate CDFs represent the Qi distributions within individual sample wells, while model CDFs represent the overall Qi distribution at each concentration.[5]

Calibration curves using the CDF method were generated from the model CDFs by calculating cumulative errors (Σ ΔQCDF) for each concentration (Eq. 2). Specifically, the 0.6 - 0.9 probability range of each model CDF was fitted using a fourth-order polynomial, from which 500 evenly spaced points were extracted across this truncated probability range. Cumulative errors were then computed as pointwise differences between each CDF and summed across all probability points.

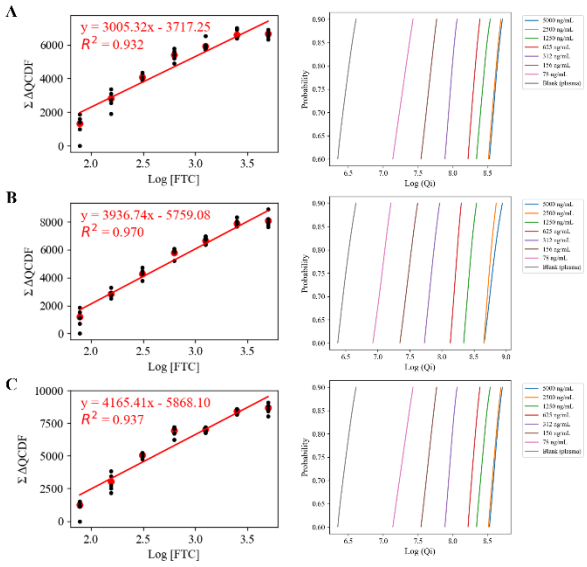

Fig. 3. (A-C) Calibration curves prepared using the CDF method for three replicate experiments with accompanying model CDFs of each FTC concentration. The CDFs were constructed based on the Qi calculated from the 792 cm^{-1} spectral region. A 4^{th} order polynomial was fitted to each CDF in the probability range of 0.6 – 0.9. The Σ ΔQCDF was calculated for each concentration (see Eq. 2) and plotted as a function of the logarithm of FTC concentration. The Σ ΔQCDF values of the model CDFs (red data points) were used for linear regression. Black data points represent the Σ ΔQCDF of concentration replicates. The regression line, equation, and correlation coefficient for each replicate experiment are shown in red.

Table 1. Slope and R^2 values from calibration curves obtained from the three methods of quantification. All slope and R^2 values in this table are an average of the slope and R^2 values of each of the three replicate experiments (see Figs. 1 - 3). The cells corresponding to the highest R^2 and slope are highlighted in yellow.

	Quantitative method		
	Total population	Qi sample	CDF
Slope	754.21	1591.05	3702.47
R^2	0.97	0.99	0.95

These differences for both the replicate and model CDFs were then plotted as a function of FTC concentration with a linear calibration curve being calculated from the model CDF responses (Fig. 3).

$$\Sigma\,\Delta QCDF = \sum_{x=n}^{x=n+7} [CDF(n)-CDF(x)] \qquad (2)$$

Results and Discussion

Calibration curves were established using the three analytical methods described in section *Analytical Methods and Data Processing*: the total population method (Fig. 1), the Qi sample method (Fig. 2), and the CDF method (Fig. 3). Each method was evaluated based on linearity (R^2), analytical sensitivity (slope), reproducibility (relative standard deviation, RSD), and signal-to-noise ratio (S/N) (see Tables 1-3).

Table 2. RSD values of each concentration for the three methods of quantification. For each method, the quantitative information of the three replicate experiments (see Figs. 1 - 3) were averaged prior to RSD calculations. The RSD values were calculated by dividing the average response of all concentration replicates by the standard deviation. The cells corresponding to the lowest RSD of each concentration are highlighted in yellow.

	Quantitative method		
Concentration (ng/mL)	Total population	Qi sample	CDF
5000	0.09	0.15	0.05
2500	0.06	0.08	0.03
1250	0.07	0.09	0.04
625	0.11	0.09	0.06
312	0.07	0.07	0.06
156	0.15	0.10	0.17
78	0.16	0.13	0.56

The total population method yielded acceptable linearity ($R^2 = 0.97$) but had limited analytical sensitivity (slope = 754.21) due to averaging both strong and weak analyte spectra across entire surfaces (Table 1). In contrast, the Qi sample method provided significantly improved linearity ($R^2 = 0.99$) and analytical sensitivity (slope = 1591.05), particularly at concentrations near the LOQ (78 ng/mL). At the LOQ, the Qi sample method demonstrated an S/N of 7.4 and RSD of 0.13, surpassing the total population method's S/N of 6.2 and RSD of 0.16 (Tables 2 and 3).

The CDF method achieved the highest analytical sensitivity overall (slope = 3702.47) but showed decreased linearity ($R^2 = 0.95$) and substantially reduced performance at the LOQ, with a S/N of 1.8 and high variability (RSD = 0.56). However, at higher FTC concentrations (312–5000 ng/mL), the CDF method consistently demonstrated superior precision (lowest RSD values, ranging from 0.03 to 0.06) and higher S/N ratios (16.6–31.5) relative to the other methods (Tables 2 and 3).

Table 3. S/N values of each concentration for the three methods of quantification. For each method, the quantitative information of the three replicate experiments (see Figs. 1 - 3) were averaged prior to S/N calculations. The S/N values were calculated from the reciprocal of the concentration RSD values shown in Table 2. The cells corresponding to the highest S/N of each concentration are highlighted in yellow.

	Quantitative method		
Concentration (ng/mL)	Total population	Qi sample	CDF
5000	10.7	6.8	20.8
2500	16.2	13.2	31.5
1250	14.9	10.7	25.5
625	9.1	10.6	16.6
312	14.5	13.9	17.4
156	6.7	9.7	5.7
78	6.2	7.4	1.8

Spectral Interference and Sample Complexity. Despite sufficient quantification, residual spectral interference arising from the inherent complexity of the plasma matrix was evident. Notably, spectral overlap between the primary FTC peak at 792 cm^{-1} (assigned to a ring breathing vibration) and an endogenous

glutathione peak at approximately 807 cm^{-1} could be observed in some spectra, as shown in Fig. 4.[11] Such overlap complicates accurate quantification at lower FTC concentrations, highlighting the necessity of spectral deconvolution and multivariate statistical approaches. Additionally, multiple low molecular weight plasma components exhibited SERS enhancement, further increasing spectral complexity. Therefore, ongoing method refinement, including advanced data processing, is essential to enhance analyte specificity within a complex plasma matrix.

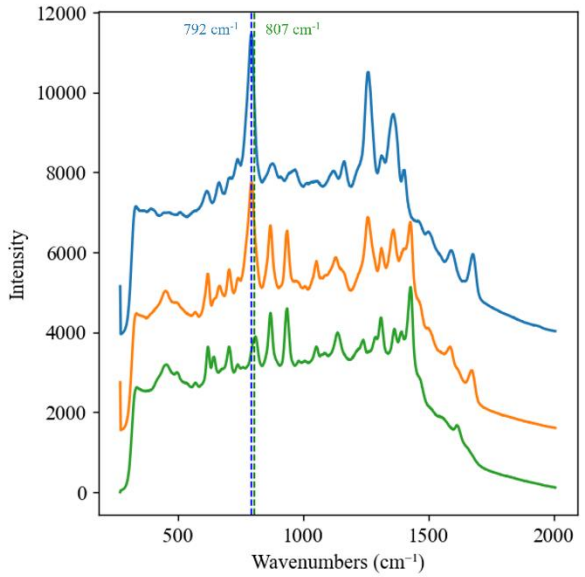

Fig. 4. SERS spectra of aqueous 1250 ng/mL FTC (blue), plasma containing 1250 ng/mL of FTC (orange), and plasma containing no FTC (green). Vertical lines highlighting the 792 cm^{-1} peak from the aqueous and plasma FTC spectra and the 807 cm^{-1} peak from the blank plasma spectrum are shown in blue and green, respectively.

Reproducibility of Plasma Pretreatment Protocol. The plasma pretreatment protocol yielded consistent results across experimental replicates, effectively isolating FTC from the complex plasma matrix and minimizing background signals. Treatment steps involving size-exclusion filtration, $AgNO_3$ addition for chloride ion removal, and subsequent interactions with Ag CNPs reliably produced samples suitable for reproducible SERS analysis. Nevertheless, potential redox reactions involving residual silver ions and endogenous plasma reducing agents (e.g., ascorbic acid and glutathione) could introduce variability in SERS signal intensities. Consequently, careful sample preparation and advanced statistical treatment of spatially resolved spectral data remain crucial to ensure the reproducibility and reliability of SERS-based quantitative assays in biological samples.

Conclusion

This study successfully demonstrates the capability of surface-enhanced Raman spectroscopy (SERS) to quantify the antiretroviral drug emtricitabine (FTC) in human plasma with high sensitivity and reproducibility. A novel plasma pretreatment protocol employing silver colloidal nanoparticles (Ag CNPs) combined with silver nitrate ($AgNO_3$), coupled with spatially resolved SERS measurements using a custom-built Raman scanning system, enabled reliable and label-free detection and quantification of FTC at therapeutically relevant concentrations (LOQ = 78 ng/mL). Three distinct analytical approaches, the total population method, the Qi sample method, and the CDF method, were compared, with the Qi sample method demonstrating superior linearity ($R^2 = 0.99$) and an optimal signal-to-noise ratio at the LOQ (S/N = 7.4). The CDF method provided enhanced analytical sensitivity at higher concentrations, highlighting the importance of statistical treatment of SERS data. Integrating this SERS-based methodology into clinical practice holds promise for resource-limited regions lacking access to specialized, costly instrumentation such as LC-MS/MS. This advancement presents a practical, affordable solution to enhance global HIV medication adherence monitoring, ultimately contributing to improved healthcare outcomes worldwide.

Acknowledgements

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