In Vivo Cytochrome P450 Drug Metabolizing Enzyme Characterization Using Surface Enhanced Raman Spectroscopy

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ABSTRACT

The development of a rapid, inexpensive, and accurate *in vivo* phenotyping methodology for characterizing drug-metabolizing phenotypes with reference to the cytochrome P450 (CYP450) enzymes would be very beneficial. In terms of application, in the wake of the human genome project, considerable interest is focused on the development of new drugs whose uses will be tailored to specific genetic polymorphisms, and on the individualization of dosing regimens that are also tailored to meet individual patient needs depending upon genotype. In this investigation, chemical probes for CYP450 enzymes were characterized and identified with Raman spectroscopy. Furthermore, gold-based metal colloid clusters were utilized to generate surface enhanced Raman spectra for each of the chemical probes. Results will be presented demonstrating the ability of SERS to identify minute quantities of these probes on the order needed for *in vivo* application.

1. Introduction

Cytochrome P450 enzymes are responsible for metabolism of many commonly used medications. Significant efforts have been put forth to find accurate and minimally invasive methods of describing in-vivo CYP enzymes activities. The optimal method of describing real-time enzyme activity is phenotyping, where metabolism of a carefully selected probe compound is used to estimate the activity of one or more of the enzymes involved in its metabolism. Phenotyping provides the most clinically relevant information because it is a reflection of the combined effects of genetic, environmental, and endogenous factors on CYP activity. It terms of application, phenotyping drug metabolizing activity could be routinely incorporated into the tailoring of drug dosages to meet patient-specific needs and development of new drugs depending on genotype. Phenotypes can be assessed by both *in vitro* and *in vivo* approaches, both using substrate drugs (i.e. probes) that are known to be metabolized by the specific enzyme being evaluated. Concentrations of an unchanged probe drug and its metabolites are measured in an incubation medium *in vitro* or in a biological matrix such as plasma, urine, or saliva *in vivo*. The major forms of CYPs involved in the metabolism of drugs in humans include CYP3A4, CYP2C9 and CYP2D6. CYP2E1, CYP1A2 and CYP2C19 are also involved but to a lesser extent[1]. Several potential phenotyping probes have been proposed for most CYP enzymes[1, 2], such as chlorzoxazone for CYP2E1, warfarin and phenytoin for CYP2C9, and ethosuximide for CYP3A4[3].

High performance liquid chromatography (HPLC) and fluorescence polarization immunoassay (FPIA) are the current methods used for *in vivo* and *in vitro* phenotyping[4, 5]. However, both are time-consuming and require complicated sample preparation. To overcome these limitations, we investigated the potential of using surface-enhanced Raman spectroscopy (SERS) to both identify and quantify these probes *in vivo*. The approach is based on the trace analytical capabilities of the SERS effect together with its high structural selectivity and the opportunity to measure Raman spectra from extremely small volumes. SERS has demonstrated extreme sensitivity allowing for the detection of single molecules[6, 7]. Thus, there is considerable potential to develop rapid, inexpensive and accurate *in vivo* phenotyping strategies for characterizing drug-metabolizing phenotypes with reference to the CYP450 enzymes in plasma as well as oral-based fluids with SERS.

In 1974 it was discovered that unexpectedly high Raman signals from pyridine which was in close proximity to a roughened silver electrode[8]. This phenomenon has been extensively studied since then and it is now established that the effect occurs when molecules adsorb to metal surfaces with roughness on the order of tens of nanometers. The mechanism is generally explained as the electromagnetic and chemical effects on the adsorption system. Many different types of substrates have been developed to produce surfaces or solutions containing metals with optimum roughness or diameter on the order of tens nanometers. These substrates include roughened electrodes[9-12], metal

colloids[13-16], metal island films[17], and metal-coated substrates such as silver-coated alumina[18]. Among these substrates, metal colloids are used most frequently to produce SERS-active media in solutions.

Metal colloids, in general, show strong SERS enhancement and are easily prepared and manipulated. Many studies have been carried out to characterize the formation and morphology of these colloids[19-22]. To generate a greater SERS signal, the colloids must first be aggregated by the analyte itself or by the addition of inorganic salts such as sodium chloride or acid solutions as aggregation agents[22-24]. More recently, other methods using poly-L-lysine to aggregate the colloid have been suggested[24]. Fractal clusters are formed where the electric field intensity is very high in the interstices of the aggregates, which is the prerequisite for a strong SERS effect. However, aggregated metal colloids tend to coagulate. This makes them unstable and difficult to use, resulting in poor reproducibility of SERS signals and incapability of quantitative measurements. To extend the application of SERS technology to various experiment situations, research efforts have been made to find new and simple procedures to prepare SERS active media with high sensitivity and stability. Silver-doped sol-gels were developed to give reproducible, quantitative SERS measurements[25]. A simple drying method[26] was investigated to prepare controllably aggregated silver particle films that are supported directly on a glass slide. Although there are standard procedures of preparing gold colloids[14], commercial gold colloids could also be used to generate SERS signals[27]. To eliminate the preparation and sorting steps required for the use of gold colloids, we exploited the use of some commercially available gold colloids for SERS in relation to sensing various chemical probe drugs that can be used to characterize cytochrome P450 enzymes *in vivo*.

2. Experiments

All chemicals employed in these experiments were research grade (Sigma Chemical, St. Louis, Mo). For the SERS measurements, individual solutions containing 10^{-2}—10^{-5} M analyte were prepared in triply distilled water or methanol (99%). The method of preparing the sample followed the technique used to form silver particle films, in which 10μl of a given concentration of analyte solution was added to 100μl of 0.01% 20nm metal colloid solution (in our case gold). To promote the aggregation, 5μl 0.1mol/L NaCl solution was introduced to the mixture as preaggregation agent. For analysis, a 5μl drop of the mixture was deposited on a glass microscope cover slide. The resulting drop of gold colloid and adsorbate was allowed to dry in air naturally to form a gold particle film.

SERS spectra were obtained with a confocal microscopy Raman spectrometer (Jobin Yvon, Edison, NJ) with a Peltier cooled CCD detector. The spectrometer used for the Raman measurements consisted of a stigmatic 300mm focal length monochromator with a 600 grooves/mm holographic grating. Slits were typically set at 0.5mm. The spectral resolution of the Raman spectrometer was $3cm^{-1}$. A 632.8nm He-Ne laser was used to excite spectra with 180° geometry. The laser power at the sample position is approximately 3 mW. The laser was focused on the desired cluster by means of an Olympus microscope equipped with ×50 and ×100 objectives. Optical images of the gold particle films were captured by the CCD camera. All data acquisition and processing were accomplished with LabSpec™ software (Jobin Yvon, Edison, NJ).

3. Results and Discussion

3.1 Chlorzoxazone

Chlorzoxazone (CZX) has been used for many years as a potent long-acting central nervous relaxant indicated for relief of painful musculoskeletal conditions[28]. Currently, it is the only available in-vivo cytochrome P450 2E1 (CYP2E1) phenotyping probe[2]. The drug is largely, though not extensively metabolized in the liver via 6'-hydroxylation by CYP2E1[29]. It has been demonstrated that a single measurement of plasma CZX at 6 hour post-dose can be used to accurately estimate CZX clearance after a single 500mg oral dose[30]. The expected CZX plasma concentration is 10mg/L. Figure 1 compares the normal and surface-enhanced Raman spectra of CZX . The Raman spectra of pure CZX powder is shown in Fig 1a. Since CZX is insoluble in water, we dissolved it in methanol at 10^{-3}M and 10^{-4}M. The final concentrations of CZX in the SERS sample were 8.7×10^{-5}M and 8.7×10^{-6}M respectively. Fig. 1b shows the SER spectra of CZX with a 20s integration time after baseline correction and filtration at the concentration of 8.7×10^{-5}M (14.7mg/L). Fig. 1c is the spectrum with 30s exposure time for the concentration of 8.7×10^{-6}M (1.47mg/L) well below the expected plasma concentration.

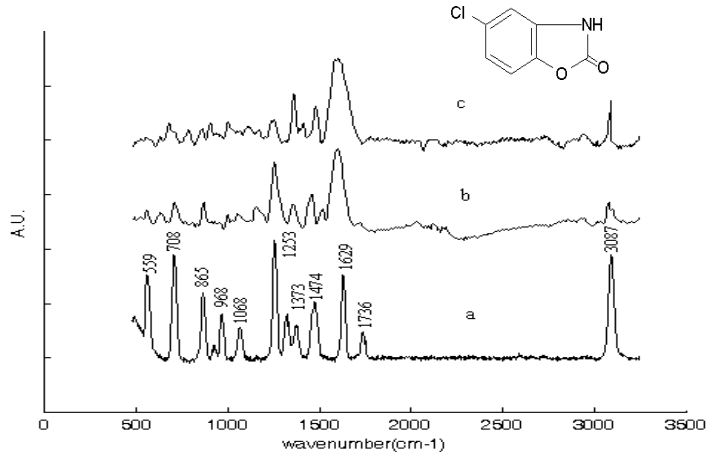

Figure 1. a) Raman spectrum of CZX powder, b) and c) SER spectra of CZX, 7×10^{-5}M (14.7mg/L) and 8.7×10^{-6}M (1.47mg/L) respectively

3.2 Ethosuximide

Ethosuximide which has been widely used for treatment of absence seizures, is the most effective drug among the succinimide antiepileptic agents[31]. Under usual conditions, long-term therapy with ethosuximide is necessary. A plasma concentration of 40-100mg/L is required for satisfactory control of absence seizures in most patients. During long-term therapy, the average plasma concentration is about 2mg/L out of 1mg/kg per daily dose. Ethosuximide can be used as a single sample probe of CYP3A4 activity in humans[3]. Figure 2 shows the Raman and SER spectra of ethosuximide, respectively. The final ethosuximide concentration is 8.7×10^{-5}M (12.3mg/L) using methanol as solvent. The SER spectrum was obtained with 25 seconds exposure time. Since the methanol was used as the solvent, it introduced extra peaks to the SERS, such as the characteristic band of methanol at 1603 cm^{-1} in Fig 2b.

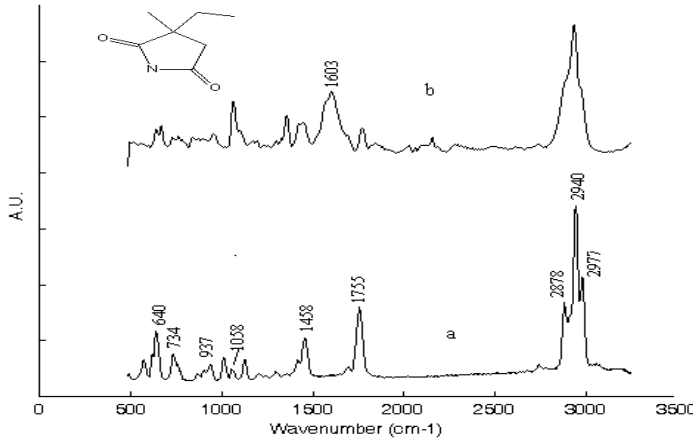

Figure 2. a) Raman and b) SER spectra of ethosuximide solution at the concentration of 8.7×10^{-5}M (12.3mg/L)

3.3 Warfarin

Warfarin is an anticoagulant prescribed for the treatment of thrombosis. Although warfarin is a racemic compound, with (R)- and (S)-forms, it is (S)-warfarin that is mainly responsible for the anticoagulant effect[5]. Approximately 80-85% of (S)-warfarin elimination is through 6- or 7-hydroxylation via CYP2C9, while several different CYP enzymes mediate the metabolism of (R)- warfarin[2]. Several researchers have considered warfarin as a phenotyping and polymorphisms probe for CYP2C9[5, 32]. The expected warfarin plasma concentration at usual doses would be about 1mg/L. The raw Raman spectrum of warfarin is shown in Figure 3a, which was taken using pure warfarin (98%)

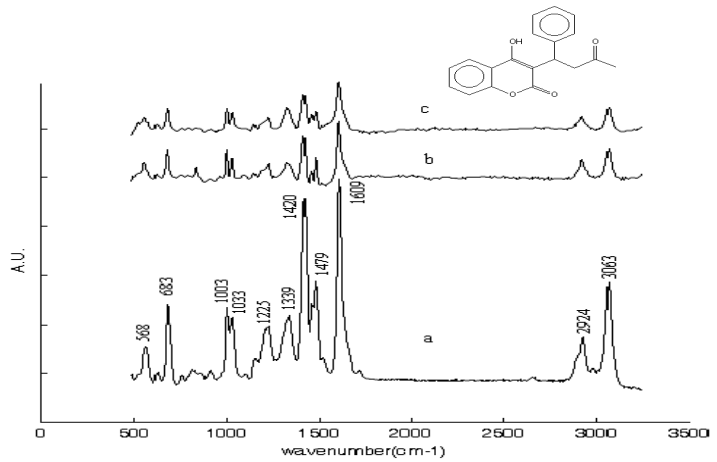

Figure 3. a) Raman spectrum of warfarin powder, b) and c) SER spectra of warfarin solution, 8.7×10^{-5}M (26.8mg/L) and 8.7×10^{-6}M (2.68mg/L)

powder with 10 seconds exposure time. Warfarin powder was dissolved in triple distilled water at the concentration of 10^{-3}M and 10^{-4}M. So the final warfarin concentration in the SERS sample were 8.7×10^{-5}M (26.8mg/L) and 8.7×10^{-6}M (2.68mg/L) respectively. Figure 3b are 3c are the SER spectra of warfarin at this two concentrations. Both were collected with 30 seconds integration time and processed with background deduction.

3.4 Phenytoin

Phenytoin is a well-known antiepileptic agent used to control tonic-chronic seizures and partial seizures. Its anti-convulsant activity is due to an effect of phenytoin on sodium and calcium transport across the membrane, thereby limiting the repetitive firing of action potentials during an epileptic period. At low plasma concentrations, phenytoin is processed by hepatic CYP2C9, but at therapeutic concentrations (10-20mg/L) CYP2C19 may also participate in the oxidation of phenytoin[33]. However, phenytoin elicits subtherapeutic plasma concentrations (about 5mg/L) with a single 4.5mg/kg oral dose, which in turn can be fit to a linear model. Therefore, phenytoin is used as a probe for CYP2C9 activity[2]. 5×10^{-4}M and 5×10^{-5}M phenytoin solutions were made by dissolving pure phenytoin (99%) in methanol. The final phenytoin concentration in the SER sample are 4.35×10^{-5}M (10.966mg/L) and 4.35×10^{-6}M (1.0966mg/L). Figure 4a is the standard Raman spectrum taken with phenytoin powder as a comparison. SER spectra of phenytoin solution are shown in Figure 4b and 4c, with 25 seconds and 30s integration time respectively.

When compared with normal Raman spectra, the characteristic bands of these probes are easily identified and are

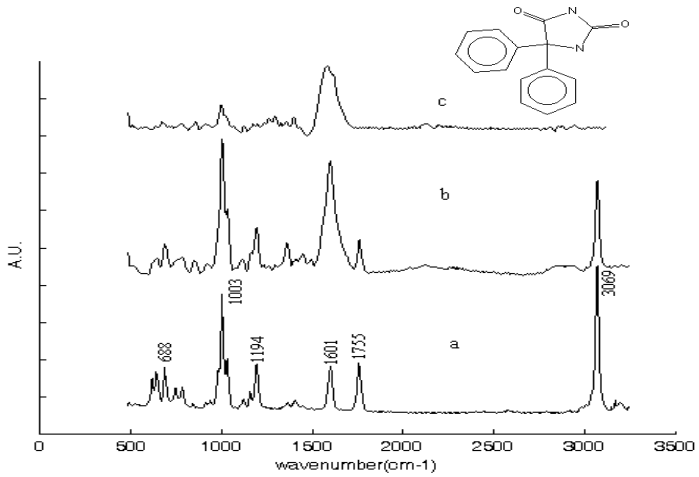

Figure 4. a) Raman spectrum of phenytoin powder, b) and c) SER spectra of phenytoin solution at 4.35×10^{-5}M (10.96mg/L) and 4.35×10^{-6}M (1.096mg/L)

consistent with those observed them. It should be noted, however, that the variation in the relative intensities is due to differences in the collection parameters and the use of non-uniform colloid structures. The standard Raman spectra could not be observed at the respective concentrations other than in the powdered form.

4. Conclusions

Based on the above discussion, we could identity those CYP450 probes with SERS with their own unique vibration peaks. We have also demonstrated that SERS could detect CYP450 probes at the level of the plasma concentration with normal oral dose. The sample is easily prepared and the spectra could be collected in a few seconds, therefore, SERS has the potential to provide a rapid, inexpensive, and accurate method for characterizing drug-metabolizing phenotypes and with the application therapeutic drug monitoring (TDM). Moreover, due to the microlitre sample volumes coupled with high sensitivity, SERS also has the potential to be a non-invasive diagnostic method if such fluids as saliva are utilized. However, due to the quality and non-uniformity of our aggregates, we couldn't quantitate the analyte concentrations at this stage. In future work, improvement and better control in the process of aggregation and colloid development with specific interactions and enhancements of Raman signatures to target molecules of interest for *in vivo* testing will allow for better identification and quantification for each of the respective probes.

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