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Short communication

Identification of lamivudine conformers by Raman scattering measurements and quantum chemical calculations

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Abstract

Characterization of nucleoside and non-nucleoside human immunodeficiency virus (HIV) reverse transcriptase inhibitors conformers, NRTIs and NNRTIs, respectively, is fundamental for an improved treatment of infected individuals. Three conformers in lamivudine I powder are quickly identified in this work by assignment of some Raman peaks to their vibrational frequencies, as obtained by first principles quantum chemical calculations. The method is proposed as a practical procedure for non-destructive identification, analysis, and process monitoring of NRTIs and NNRTIs conformers.

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1. Introduction

In the treatment of individuals infected with human immunodeficiency virus (HIV), the key case of antiretroviral therapy failing is the emergence of drug-resistance mutations. Conformational flexibility and adaptability are important in the design of nucleoside and non-nucleoside HIV reverse transcriptase inhibitors, NRTIs and NNRTIs, respectively [1]. They have a lot of sites forming inter and intramolecular hydrogen bonds, presenting conformations strongly dependent on the solvent characteristics and experimental conditions. Accurate knowledge of the conformational properties of NRTIs and NNRTIs would be important for the interpretation of drug-target interactions since adaptation to the binding pocket changes due to HIV mutations involves a combination of conformational adjustments, and rotational and translational shifts of the inhibitor within the binding pocket. Improvement of antiretroviral therapy for the human immunodeficiency virus is related to conformational adjustment of drugs to the binding pocket. Consequently, it is important to have information on NRTIs and NNRTIs conformers, and to develop practical procedures for their non-destructive identification, analysis, and process monitoring.

The conformation analysis of several anti-HIV nucleoside analogues shows that the preferred sugar-ring orientation is based on C3′-exo conformations [2,3]. Fisher et al. [4] proposed 144 potentially bioactive conformations (due to relevant torsional angles φ_1, φ_2, φ_3) for the important compound 3′-azido-2′,3′-dideoxythymidne (AZT), the first clinically successful drug for acquired immunodeficiency syndrome (AIDS) as well for AIDS-related diseases. The structural flexibility of 9-CI TIBO was investigated using high level of calculations, ab initio, and density functional theory (DFT), demonstrating the existence of eight pronounced local minima within an energy difference of less than 10 kJ/mol [5]. Finally, a molecular modeling study of the mechanism of tenofovir against 3TC- and AZT-resistance mutant HIV-1 reverse transcriptase [6] has indicated

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clearly that the conformational flexibility of acyclic natures of TFV provides energetically indistinguishable multiple conformations, which do not experience the cross-resistance conferred by mutants RTs.

The focus of this work is on lamivudine (3TC), 2(1H)-pyrimidinone,4-amino-1-((2*R*, 5*S*)-2(hydroxylmethyl)1,3-oxathiolan-5-yl), one of the almost 40 compounds that have been officially approved for clinical use, having activity spectrum against HIV (types 1 and 2) and hepatitis B virus (HBV). It is indicated in combination with other anti-HIV agents such as zidovudine and abacavir [7], acting as a chain terminator, following intracellular phosphorylation to 3TC 5′-triposphate, and, after removal of the diphosphate group, incorporation of 3TC 5′-monophosphate at the 3′-end of the viral DNA chain.

2. Lamivudine conformers

Lamivudine crystals are found in two polymorphic modifications: form I ($C_8H_{11}N_3O_3S{\cdot}0.2H_2O$) has an orthorhombic asymmetric unit ($P2_12_12_1$) containing five non-equivalent molecules (with some disorder) and a bonded water associated to one of them, with lattice parameters $a = 10.427$ Å, $b = 14.327$ Å, $c = 34.851$ Å [8]; form II ($C_8H_{11}N_3O_3S$) has a highly symmetrical tetragonal ($P4_32_12$) crystal lattice with one molecule in the unit cell with lattice parameters $a = b = 8.7490$ Å, $c = 26.523$ Å [8]. Solution-state infrared (IR) and neutron magnetic resonance (NMR) spectra of the two forms are identical, while the only difference (a peak at 3545 cm^{-1}due to a single molecule of bonded water) in the solid-state IR spectra of the two forms do not, however, adequately reflect the fundamental differences in symmetry between them [8]. Whatsoever, it is not an easy task to identify the existence of lamivudine conformers in samples with IR and NMR standard techniques.

An exploratory conformational study of the molecule lamivudine in vacuum was performed by Fidanza et al. [9] using an ab initio method at the HF/3-21G level. The existence of four low-energy conformers was pointed out, as probably the most favorable structures for biological activity [9]. The choice of the conformers was based on the stabilization by intramolecular hydrogen bonding interactions of the C–H···O and O–H···O types between the rings. The conformers, labeled A, B, C, and D in Fidanza's et al. work [9], were classified as *anti* (A, B) or *syn* (C, D), according with the relative orientation of the two rings around the C–N bond (optimized structural parameters for such conformers are listed in the mentioned report). *Anti* conformations A and B contain the pyrimidine ring and the sugar analogue residue nearly perpendicular to each other [9]. In the first one the C–H···O is a hydrogen bond between the C_6–H aromatic bond and the oxygen atom in the hydroxymethyl group. In the other it is the $C_{5'}$–H···O(=C_2) interaction involving the $C_{5'}$–H at the modified sugar and the carbonyl oxygen of the thymine. On the other hand, *syn* conformations C and D exhibit C–H···O and O–H···O hydrogen bonding that involves the C=O group of thymine [9]. The C conformer shows hydrogen bonding interactions between the C=O of the pyrimidine ring and two hydrogen atoms, and the D conformer shows three hydrogen bonding interactions involving the oxygen atom of the thymine carbonyl group. Arissawa et al. [10] have calculated the vibrational frequencies and have investigated the effects of solvents, Mulliken, and natural bond orbital charge distribution, as well as HB effects in nucleoside analogs with anti-HIV activity such as AZT, d4T, ddI, 3TC, and ddC. They tried to correlate very low and very high anti-HIV activity with charges, vibrational stretching frequencies, interatomic distances, and the effect of solvents. Yekeler [11] have studied conformational properties and the energy barriers between the *anti* and the *syn* configurations of 3TC, ddC, AZT and d4T using the density functional theory and taking into account the solute-solvent interaction via the self-consistent reaction field. However, no comparison with measurements was performed in the above mentioned works.

The purpose of this short communication is to show that Raman spectroscopy can be used for the detection of lamivudine conformers. This is accomplished through assignment of some Raman peaks to the vibrational modes of lamivudine conformers A, B, C, and D as obtained by quantum chemical calculations. Raman analysis of composites that show pharmacological activity is a promising new tool for their characterization, since it offers some advantages, such as the identification of raw materials, quantitative determination of active substances in different formulations, and polymorphic screening support. Vankeirsbilck et al. [12] pointed out potential applications of Raman spectroscopy in pharmaceutical analysis, weighting the advantages and disadvantages of this technique when applied to drug molecules. It is known that Raman, rather than infrared, is more useful to identify different crystalline polymorphic forms and molecular conformers, while near-infrared is better suited to detect similar carbohydrate species and varying hydration states. Raman spectroscopy moved out of the shadow of IR spectroscopy, providing real-time, multi-component chemical analysis for process monitoring and control for industrial liquid and solid process applications. Several pharmaceutical forms have already been studied by Raman spectroscopy [12]. The ability to theoretically predict physical properties in order to differentiate and/or explain molecular activity (i.e. different conformers and/or slightly different structures for molecules) is receiving attention from industry due to the cost reduction obtained with this strategy by avoiding the unnecessary use of expensive reagents and techniques.

The basic structure of the lamivudine molecule is depicted in the left side of Fig. 1. The atomic numbering follows the convention, with the hexagonal ring labeled with arabic numbers and the primed numbers labeling atoms out of this ring. The hexagonal ring comprises 12 atoms, the pentagonal ring is formed with 9 atoms and the $OHCH_2$-radical has 5 atoms, summing up a total of 26 atoms in the molecule. Lamivudine is a flexible molecule, which means that it can adopt a variety of dynamically-interconverting conformations, as shown in the right side of Fig. 1 for the A, B, C and D most relevant conformers according to the work of Fidanza et al. [9] Moreover, Harris et al. [8] have shown that form I of lamivudine crystals can host five different conformers in the unit cell, leading us to infer that the Raman spectra of lamivudine crystals may have contributions from different molecular geometries. As suitable conformations that may explain the Raman spec-

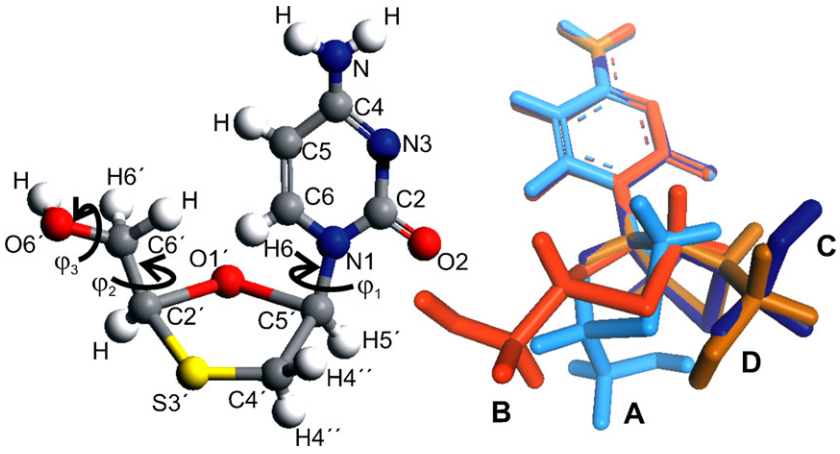

Fig. 1. *Left*: the structure of the lamivudine molecule showing the torsional angles φ_1, φ_2, φ_3. *Right*: superimposed spatial profiles of the lamivudine conformers A, B, C, and D.

trum, we selected four minimum energy conformers which are very similar to those described by Fidanza et al. [9].

3. Raman scattering and vibrational modes assignment

Lamivudine is a white crystalline solid with melting point in the range of 160–162 °C and solubility of 70 mg/ml in water at 20 °C [15]. Its *p*Ka is equal to 4.3 and it is sufficiently stable in visible light and at room temperature in the solid state and water solution. There are two polymorphs of lamivudine in the crystalline phase, one partially hydrated (form I, orthorhombic, $P2_12_12_1$) and one anhydrous (form II, tetragonal, $P4_32_12$) [8]. X-ray measurements suggested a surprisingly large unit cell ($a = 10.427(2)$ Å, $b = 14.327(3)$ Å, $c = 34.851(7)$ Å) for the lamivudine form I, empirical formula $C_8H_{11}N_3O_3S{\cdot}0.2H_2O$, with ∼0.2 Å inter-molecular hydrogen bonds. This is consistent with five independent molecules being accommodated within the asymmetric unit, each showing a different conformation [8]. On the other hand, the lamivudine form II, empirical formula $C_8H_{11}N_3O_3S$, shows only one molecule in the crystallographic asymmetric unit ($a = b = 8.7490(12)$ Å, $c = 26.523(5)$ Å), with ∼2.5 Å inter-molecular hydrogen bonds [8]. Lamivudine can be obtained as acicular crystals (form I, 0.2 hydrate) from water or methanol and as bipyramidal crystals (form II, non-solvated) from many nonaqueous solvents. Form II is thermodynamically favored in the solid state (higher melting point and greater density than form I) at ambient relative humidities. Solution calorimetry data indicated that form I is favored (less soluble) in all solvents studied on the basis of enthalpy alone. In higher alcohols and other organic solvents, form I has a larger entropy of solution than form II, which compensates for the enthalpic factors and results in physical stability for form II in these systems [16]. The infrared spectra of both lamivudine forms are remarkably similar, but the hydrated form I reveals a peak at 3545 cm^{-1} which is due to a single molecule of bonded water associated with one of the five molecules in the asymmetric unit.

The Raman spectrum of lamivudine form I powder was measured with a LAB-RAM single spectrometer (Jobin-Yvon) equipped with a Charged Coupled Device (CCD) detection apparatus. As exciting radiation the 647.0 nm line from a He–Ne laser was employed. The laser beam was focused by an OLYMPUS microscope with a 100× objective. All measurements were performed in the back-scattering geometry and at room temperature. The spectral resolution imposed by the equipment was better than 2 cm^{-1}. In order to analyze the measured Raman spectrum, we performed first principles quantum chemical calculations for all the four lamivudine conformers A, B, C, and D. This calculation used the Gaussian 03 software [13] within the density functional formalism with the Becke's hybrid functional (DFT/B3LYP). A 6-31+G(d) basis set was adopted and geometry optimization was carried out for all selected conformations. The equilibrium configuration was considered, as the structure for which the rms force acting on all atoms was smaller than 0.3 mHartree/Å. An energy threshold of 10^{-6} Hartree was adopted for the quadratically self-consistent field (SCF) convergence, and after energy minimization the energies obtained for the lamivudine conformers A, B, C, and D are listed Table 1. All molecules were simulated using the vacuum as dielectric environment. After molecular geometry optimization was achieved

Table 1
Angles φ_1, φ_2, φ_3 (in °) and calculated energies of the stable lamivudine conformers A, B, C, and D obtained after geometry optimization

	A	B	C	D
φ_1 [C6–N1–C5′–O1′]	17.73	0.67	−109.88	−109.64
φ_2 [O1′–C2′–C6′–O6′]	64.55	−69.00	−65.59	57.32
φ_3 [C2′–C6′–O6′–H]	61.28	−77.42	−60.25	47.60
Energy (a.u., Hartree)	−1099.58069	−1099.57336	−1099.57716	−1099.57918

through the search of a minimum for the total energy, the molecular polarizability tensor was calculated from the response theory. Table 1 lists the angles φ_1, φ_2, φ_3 of the lamivudine conformers A, B, C, and D obtained after geometry optimization, which agree very well with those obtained by Fidanza et al. [9]. The gradients for both the mean polarizability and anisotropy were calculated allowing to obtain the Raman differential cross section for each normal mode of vibration [14]. In this way, the Raman spectra for all the four lamivudine conformers A, B, C, and D were calculated. As an extra convergence criterion, the calculated normal modes of vibration were analyzed in order to ensure that all frequencies were positive. When a negative eigenvalue was found, this minimum would represent a saddle point and the corresponding molecular geometry was not suitable, even though the energy cutoff criterion was achieved. Finally, in order to allow theoretical comparison with the experimental results, we have scaled the computed frequencies by a factor of 0.9614 according to the work of Scott and Radom [17]. It is important to remark that a drawback of our work is that we have not simulated the Raman spectrum considering the orthorhombic cell with five independent lamivudine molecules accommodated within the asymmetric unit, which is a formidable computational task due to the number of atoms involved. However, the X-ray data of Harris et al. [8] shows that the lamivudine molecules are quiet far apart in the orthorhombic crystal, with inter-molecular hydrogen bond pairs with bond lenghts in the 1.950–2.296 Å range. This suggest that our approach is a good approximation.

The Raman peaks of lamivudine I powder in the [260–660] cm^{-1} and [960–1360] cm^{-1} ranges assigned to the calculated vibrational frequencies of the lamivudine conformers are depicted in Figs. 2 and 3, respectively. Analysis of these data together with eigenvectors determination, allowed us to select a region in the spectra where the B and D lamivudine conformers give distinct contributions. This is the region between 360 and 660 cm^{-1}, that will be detailed next. Within the aforementioned region, the peak observed at 276 cm^{-1} is probably due to the rocking C6′–H2 + wagging NH_2 + wagging

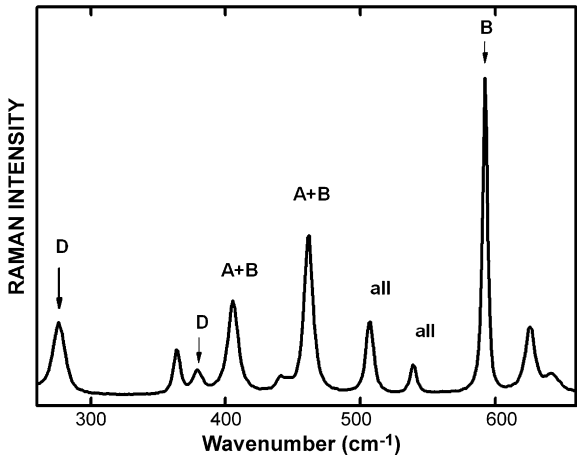

Fig. 2. The Raman spectrum of the lamivudine powder in the [260–660] cm^{-1} frequency range. The arrows indicate first-principles calculated vibrational frequencies of selected lamivudine conformers.

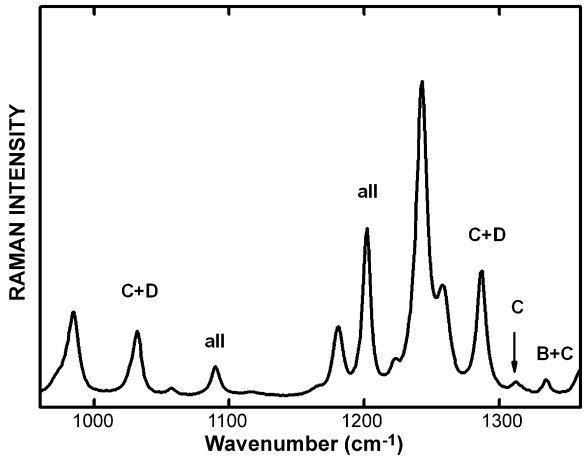

Fig. 3. The Raman spectrum of the lamivudine powder in the [960–1360] cm^{-1} frequency range. The arrows indicate first-principles calculated vibrational frequencies of selected lamivudine conformers.

N1–C5′O1′ of the lamivudine D conformer. The lamivudne D conformer can be identified by the peak at 380 cm^{-1} as well, which is assigned to NH_2 twisting + C2′–C6′–O6′ bending + N1–C6–C5 wagging + C4′H2 rocking. The strong peak at 592 cm^{-1} can be identified with the lamivudine B conformer asymmetric stretching C2′–S3′–C4′ + bending C2′–O1′–C5′, C5–C4–N3, and C6–N1–C2 + rocking NH_2. There are other bands that can be reasonably well attributed as isolated conformations, as will be shown next. In the region comprised between 960 and 1360 cm^{-1}, we can assign the peak at 1310 cm^{-1} as being an isolated contribution from the C conformation (although its intensity being small) related to C5′–C4′ stretching + H–C2′–O1′ bending and H–C6′–O6′ bending.

The remaining Raman lines cannot be attributed to a single conformation, safely, due to the overlap of bands closely positioned. Moreover, it is possible to define the superposition from two conformers modes appearing as single bands in the spectrum. In this category, the band at 406 cm^{-1} may be due to lamivudine conformer A (bending along C2–C5 + twisting NH_2 + C6′H2 and C2–N1–C6 rocking vibrations) and lamivudine conformer B (twisting NH_2 + torsion in ϕ_3 + wagging N1–C6–C5). Moreover the peak at 462 cm^{-1} can also be attributed to the contribution from both lamivudine conformers A (torsion in ϕ_3 + bending S3′–C2′–O1′ + rocking C4′H2) and B (torsion in ϕ_3 + rocking C4′H2 and C6′H2 + bending C2′–S3′–C4′ + wagging C5–N1–C5′). Likewise, the band at 1031 cm^{-1} can be assigned as a contribution from both lamivudine conformers C (asymmetric stretching O1′–C5′–C4′ and O6′–C6′–C2′ + bending N1–C6–C5 + rocking NH_2) and D (symmetric stretching O1′–C5′–N 1 + stretching C5′–C4′ + rocking C6′H2 + twisting C4′H2 + bending H–O6′–C6′, C6–N1–C2 and C5–C4–N3). Furthermore, the peak at 1287 cm^{-1} can also be assigned as contributions from both C (stretching N1–C6 + bending C1–C5′–H 5′ + wagging C4′H2 + stretching C2–N3 and C4–NH_2) and D (twisting C6′H2 + wagging C4′H2 + stretching N1–C6 + bending C2–N1–C5′ and H–C2′–C6′ + rocking NH_2) lamivu-

dine conformations. Finally, within the 960–1360 cm^{-1} spectra range, we can assign the small peak at 1334 cm^{-1} as being related to the double contribution from B (bending C4–NH_2, C2–N3–C4 and C2′–C6′–H + symmetric stretching N1–C5–C6 + rocking C6′ and C5′) and C (stretching C5′–C4′ and O1′–C2′ + bending H–C6′–O6′) lamivudine conformations. The complete Raman spectra of lamivudine I in the frequency range 100–3600 cm^{-1}, as well as the modes assignment will be presented elsewhere [18].

4. Concluding remarks

In conclusion, the comparison of the first-principles calculated vibrational frequencies of the lamivudine conformers A, B, C, and D with the Raman spectrum of the lamivudine I powder allows to identify the existence of the conformers A, B, C, and D in the sample: the conformer D can be identified by the peaks at 276 cm^{-1} and at 380 cm^{-1}; the conformer B can be identified by the peak at 592 cm^{-1}; the conformer C can be identified by the peak at 1310 cm^{-1}; finally, the conformer A could not be identified by an isolated peak using this procedure. A similar scheme, e.g. comparison of Raman scattering measurements of actual samples with quantum chemical calculated vibrational modes of conformers, can also be used for conformers identification in samples of others NRTIs and NNRTIs. In this sense, Raman scattering can be very useful for non-destructive identification, analysis, and process monitoring of NRTIs and NNRTIs conformers. This identification can lead to a practical monitoring process of the existence of conformers in the development of drugs for the treatment of HIV infected individuals.

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