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Drug-drug interaction: the case of flubendazole and doxycycline hyclate investigated by Raman spectroscopy

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This paper expands the applicability of Raman spectroscopy in veterinary field, aiming to search for spectroscopic signature of the drug-drug interaction between Rombendazole and Doxycycline hyclate, commercially available for veterinary use and prescribed in poultry with simultaneous nematodes and respiratory infections. The failure of treatment in chickens with symptoms of respiratory infections and parasitic worms, who were given the combination of Rombendazole and doxycycline hyclate triggered and motivated this study, to (1) identify the active ingredients in commercially formulated Rombendazole tablets; and (2) to investigate the Raman spectroscopy signature of the mixed, kneading product of the two drugs in solid phase, consistent with the simultaneous administration, in search for their direct molecular interaction as a possible cause of vet treatment failure. Raman spectroscopy enabled the identification of flubendazole as the genuine active ingredient of commercial Rombendazole and found changes in Raman signature of the active ingredient in spectra of solid mixtures, due to the possible interaction of flubendazole through the N atom from benzimidazole ring with the doxycycline. The high degree of overlap between the Raman spectra of the two compounds raised additional difficulties along with the co-existence of excipients, however, spectral evidence of interaction between the two solid drugs are reported.

Keywords: Rombendazole, Doxycycline hyclate, Veterinary drugs, Raman Spectroscopy,

1 Introduction

In the widening field of applied Raman spectroscopy, pharmaceuticals are strongly represented, since Raman technology development assisted by advanced computational methods allowed to accurately characterize, identify active ingredients and excipients. Moreover, Raman spectroscopy enables to quantify, screen counterfeits drugs, detect polymorphs, assist new (nano) formulations, monitor the production lines, identify new drugs from natural resources or drugs residue in living organisms or environment and many others. To cite such rich publication field, a search on "Raman+ pharmaceutical" in Science Direct (as of October 29, 2021) covering the last 20 years (2000-2020) showed a total number of publications of 17,881, out of which 2,307 are review articles, 11,698 research papers, followed by chapters and books. For obvious reasons, such citation space is better represented by a graphic display (Fig 1), constructed from the retrieved data from Science Direct. Refining the results for "antibiotic" only, it revealed 2,659 entries. The graphs in Fig 1 suggested an exponential increase in scientific production relying on Raman spectroscopy of pharmaceuticals in the past two decades, where antibiotics keep the exponential trend, where doxycycline hyclate, considered in this study, is poorly represented (Fig 1 a). A similar increasing tendency is observed when trying to search for keywords Raman+drug-drug interaction (Fig 1 b).

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Doxycycline belongs to the medication class of tetracycline antibiotics, being commercially available as water soluble yellow powders for use in veterinary medicine, particularly in poultry and intended for administration via drinking water or as solid form, for better control of the dose. Flubendazole is an anthelmintic largely used against internal parasites and worms. Aiming to investigate drug-drug interaction in solid form, doxycycline hyclate and flubendazole are absent from the literature data displayed in the Fig 1, although doxycycline is refereed in 123 publications while anthelmintic in 127.

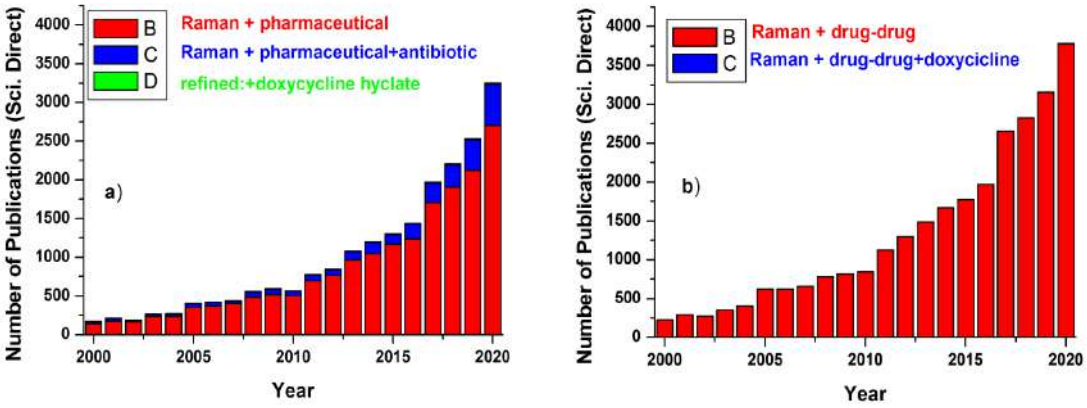

Fig 1. Graphic display of the number of publications involving Raman spectroscopy and pharmaceuticals in the past two decades as revealed by the search in Science Direct: (a) keywords Raman+pharmaceutical, out of them refined "antibiotics" and further "doxycycline hyclate, as showed in the legend color codes; (b) similar search regarding the drug-drug interaction and Raman, with refined "doxycycline". The overall output suggested an exponential increase in scientific publications number, however, the compounds taken in this study, flubendazole and doxycycline hyclate are less represented.

Nevertheless, it appears that a complete vibrational Raman characterization of two widely used drugs, flubendazole and doxycycline hyclate is not available, although a screening tool based on a portable instrument has been developed for counterfeit drugs supposed to comprise doxycycline and a Raman spectrum of such tablet has been released [1]. Raman mapping was also used in investigation of the doxycycline encapsulated in hydroxyl-propyl-beta-cyclodxetrine [2]. The flubendazole crystal structure has been recently investigated [3] and its interaction with maleic acid resulted in a co-crystal with improved solubility and salt character resulted from maleic acid-flubendazole proton transfer [3]. Flubendazole interaction with Pd(II) ions was also reported [4] and the involvement of the nitrogen of the benzimidazole ring was evidenced using the theoretical DFT/B3LYP method for geometry optimization, molecular electrostatic potential maps and natural bond orbital analysis.

Further search criteria regarding Raman spectroscopy for drug-drug interaction revealed a similar trend (Fig 1 b), but again, very limited spectroscopic information is available regarding doxycycline hyclate. Even so, the two drugs of interest mentioned in this paper are poorly represented regarding their complete vibrational characterization.

Here, we aim to apply the Raman spectroscopy technique in search for spectroscopic signature of any drug-drug interaction between Rombendazole and Doxycycline hyclate, which are widely used drugs in veterinary cure and prescribed in poultry and animals with simultaneous symptoms of nematodes and respiratory infections. The failure of treatment in chickens with symptoms of respiratory infections and parasitic worms, when given in combination of Rombendazole and doxycycline hyclate triggered and motivated this study to (1) identify the active ingredient in commercially formulated Rombendazole tablets, and (2) to investigate the Raman spectroscopy signature of the mixed, kneading products resulted from

simultaneous administration of the two drugs, in search for their direct molecular interaction, as a possible cause of treatment failure.

To correctly identify the active ingredient in the commercially available Rombendazole tablets, with active ingredient flubendazole ($C_{16}H_{12}FN_3O_3$, SpectraBase Compound ID 1t48QnTSUk), we used the reference spectra from the SpectraBase (https://spectrabase.com/spectrum/IspwaTQq3c4)[John Wiley & Sons, Inc. SpectraBase;https://spectrabase.com/search?q=flubendazole (accessed 10/29/2021)[5].

In Rombendazole tablets the prospect indicates flubendazole as active ingredient. One single database comprises a Raman and ATR-FT-IR spectrum of this compound, and we used it for reference purpose in Raman screening of the commercial tablets of Rombendazole and Doxyfarm product and subsequently, their physical mixtures.

Rombendazole-F is a popular Romanian veterinary drug formulated for oral administration comprising flubendazole 10 mg / 120 mg tablet. It is widely recommended for birds (hen, turkey, pheasant, quail, quail, peacock, goose, and duck) dogs and cats due to its broad- anthelmintics spectrum. In Europe, frequent brand names of the drug are Flubenol, Flubenvet, Flumoxal, Flimabent, Biovermin, Helminex, Solubenol, Flubengal, Flumoxal, Vermicat [6,7].

When suspicion of avian bacterial infection co-exists, Rombendazole is associated with doxycycline hyclate. This antibiotic is known to bind with Ca, Mg or other minerals [7,8] whichever is present in animal feeding products, thus preventing the treated bird/animal body from full drug bioavailability. Water administration route is also poorly controllable, as the water intake is random and unlikely effective, as the expected dose intake in suffering birds or animals is unlikely to be normal. Rare cases, if any, reported healing effectiveness, particularly in domestic birds which have been treated simultaneously with flubendazole and doxycycline after signs of illness associated with parasitic worms and bacterial infections. In such situations, veterinarians recommend administration to control bird parasites and bacterial infection by prescribing flubendazole 1 tablet/ kg bird (animal), two consecutive days, and repeated three weeks later. When doxyclicline is associated in the treatment, its administration is achieved via drink water, although the solubility is low [9], in food or via oral methods suitable for veterinary practices. The doxycycline formulation for veterinarian use is broaden, comprising four clorhydrate (Doxivit 100, DOXY H 10%, Doxiciclina 10%, Doxicol 60%) and 17 doxycycline hyclate products. However, the commercially available Doxifarm 50%, is not included in the regulation from 2010 because of its later (2013) release. Doxifarm 50% is administered in drinking water or in liquid feed. The real situations are unlikely to be strictly controlled, regarding individual water intake and the degree of illness incidence which can vary from one individual to another. The alternative recommendation to disperse the powder doxycycline hyclate with powdered Rombendazol in meal is also inefficient, due to the induced appetite loss in ill birds or animals [10].

Here, we firstly describe the Raman characteristics of the commercially available products using their solid form and further, their three different mixtures, relevant for the usual veterinary prescriptions ratios in poultry administration per kg body weight, aiming to search spectroscopic signatures of the molecular identity change and/or interaction in binary solid products.

2 Experimental

Samples preparation and characterization

The commercially available drugs appeared as white tablets for Rombendazole and yellow powder for doxycycline, respectively. The physical mixtures of the drugs, Rombendazole and Doxycycline hyclate were prepared in powder state, using an agate mortar with pestle.

Flubendazole ($C_{16}H_{12}FN_3O_3$, methyl [5-(4-fluorobenzoyl)-1H-benzimidazol-2yl] carbamate) has been acquired as commercial veterinary product named Rombendazol-F (code 29337) from Romvac,

Romania, as tablets with 10 mg active ingredient per 120 mg tablet. Excipients included starch, talc, magnesium stearate and lactose.

Doxifarm 50% (500 mg/g) doxycycline hyclate (DH) powder (Chemifarma S.P. A. Italy) has been used as formulated, in fine, yellow powder form. Excipients are not commercially indicated.

The kneading products obtained as physical mixtures of these two drugs were prepared in three different ratios, denoted P2, P3 and P4 and comprising (P2) one tablet of Rombendazole to 0.33 mg doxycycline hyclate (P2 molar mixture 1:1); (P3): two tablets of Rombendazole : 0,33mg of doxycycline hyclate (molar mixture 2:1), and P4: 1 tablet of Rombendazole : 0,66 mg of doxycycline hyclate (molar mixture 1:2).

Equipment

Raman spectra were recorded using a Renishaw InVia Reflex Raman spectrometer with a Leica microscope with various objectives available (5×, 20 ×, 50 ×, 100 ×). A laser diode operating at 785 nm line has been employed for Raman excitation. Data acquisitions conditions have been controlled from the Wire 3.4 software, where the exposure time, number or acquisitions and laser power could be set, depending on the sample scattering properties. Different types of measurement and mixtures were realized. Firstly, multiple single point Raman spectra have been acquired from several Rombendazole tablets and subsequently from the powdered forms. Doxycycline hyclate were measured 3 times each for 3 different positions of each sample. Acquisition time was 1 second and the power of laser was set to minimal 1% (3 mW) to avoid sample damage (burning, photodecomposition) under high laser energy exposure. The spectral data were analyzed using Origin 6.0 software.

3 Results and Discussion

Raman spectra of Rombendazole-F tablets and Doxycycline hyclate powder

Molecular structures of the studied drugs are showed in the Fig 2 along with the micrographs taken from the Rombendazole tablet surface (a) and Doxyfarm (b). The micrographs of the physical mixture of powders in the 1:1, 1:2 and 2:1 molar ratio of active ingredients are showed in the bottom (Fig 1c, d, e), revealing yellow spotted areas with high doxycycline concentrations, which indicated the uneven distribution of antibiotic in the physical mixture at microscopic level.

Typical raw vibrational Raman spectra collected under short (1s) or long (10s) exposures in extended mode from multiple Rombendazole tablets are shown in Fig 3.

For correct identification of the active ingredient Raman bands, we first used the reference database of Raman spectra of pharmaceutical excipient [11,12] to establish their contribution to the overall Raman signature of the tablets. Subsequently, prominent Raman bands assigned to the active ingredient structure, including molecular rings characteristic to fused benzene and imidazole (defining benzimidazole), carbamate ester, *p*-fluorobenzoyl group and aromatic ketone are observed [13-16]. The Raman bands of excipients like magnesium stearate, talc, starch and lactose showed certain interference with the main ingredient signal, as revealed by multiple points Raman scan of the tablet. The strongest bands of excipients were discernable in several tablet spectra. These bands appeared fluctuant or absent in other spectra collected from distinct points, suggesting the high tablets inhomogeneity.

Talc shows prominent bands [11] at 361(s), 466(w), 550(m), 675(vs), 1050(w), 1229(m), 1260(m), 1385(w), 1412(w) cm^{-1}, which were randomly observed in the tablet spectra. Magnesium stearate shows Raman bands at 888(w), 945(w), 1061(s), 1102(w), 1128(s), 1294(vs), 1437(s), 1458(m). Starch (white) exhibits Raman bands at 302(w), 359(w), 409(w), 439(w), 477(vs), 576(w), 867(w), 940(m), 1050(w) cm^{-1}.

Lactose Raman bands are located at 256(w), 353(vs), 374(s), 395(m), 474(m), 551(w), 629(w), 847(m), 873(m), 912(w), 950(w), 1015(w), 1037(w), 1049(w), 1083(m), 1117(w), 1139(w), 1258(w), 1323(w), 1344(w), 1377(w) cm^{-1}. .

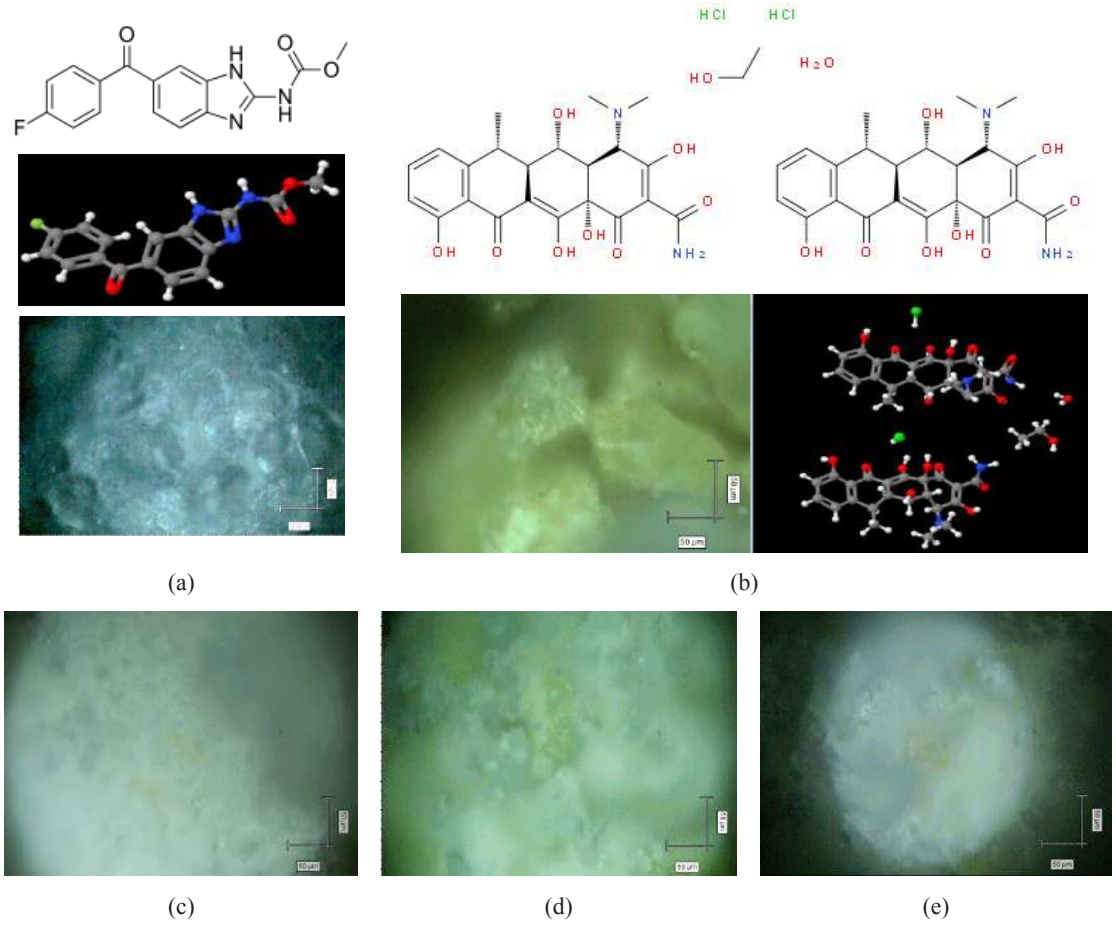

Fig 2. Molecular structure of the active ingredients of the considered drugs, (a) flubendazole; (b) doxycycline hyclate; Both 2D and 3D structures are given. The micrograph of the Rombendazole tablet and Doxifarm powder are also shown. Bottom row: micrographs of the powdered drugs mixtures of P2 (c), P3 (d) and P4 (e), yellow spots highlighting doxycycline microparticles.

The excipients bands have been randomly identified in the micro-Raman spectra collected from tablet surface. The tablet surface under 20× microscope objective appeared as bright spotted micro-grain structure which introduced additional Raman background and required higher numerical aperture objective (50× or 100×) for higher quality Raman spectra acquisition. The main bands of starch (S), lactose (L), magnesium stearate (MS) and talc (T) are indicated in the spectra taken in raw form from the Fig 2. It appeared that the excipients clearly dominate the Raman spectra in the low wavenumber range (100-800 cm^{-1}), thus, this spectral region was considered less relevant relative to the scope of the study. Further, comparing the observed Raman bands of active ingredient with those of flubendazole from SpectraBase (Compound ID1t48QnTSUk, (https://spectrabase.com/search?q=flubendazole), an excellent match of the bands has been observed, indicatig that the commercial tablet is genuine.

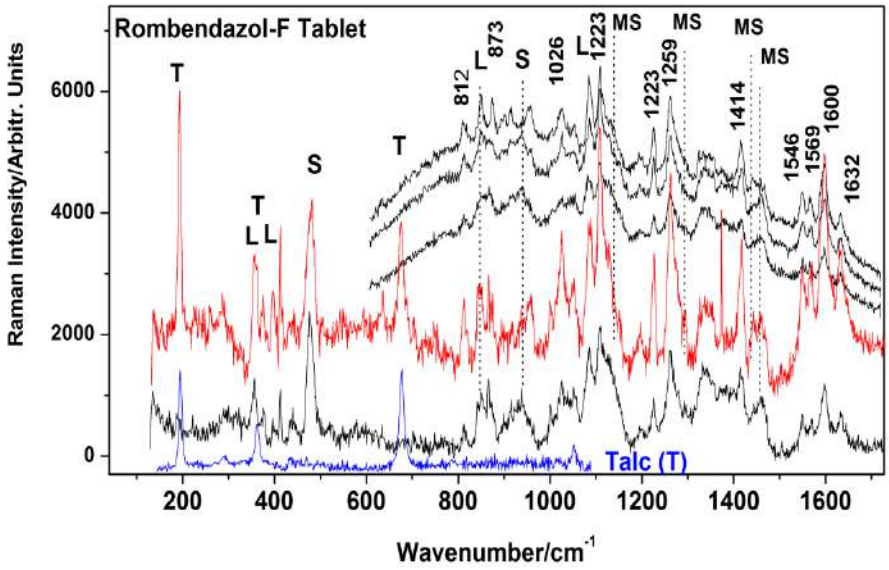

Fig 3. Typical raw micro-Raman spectra collected from different points of Rombendazole-F tablets with short acquisition time (1s, upper spectra, shorter spectral range) or extended acquisition mode (10 s, lower spectra). The spectrum of talc (denoted T) is given in bottom (blue line). Magnesium stearate (MS), starch (S), lactose (L) and Talc (T) bands are indicated. The active ingredient main Raman bands are plotted. Excitation wavelength: 785 nm.

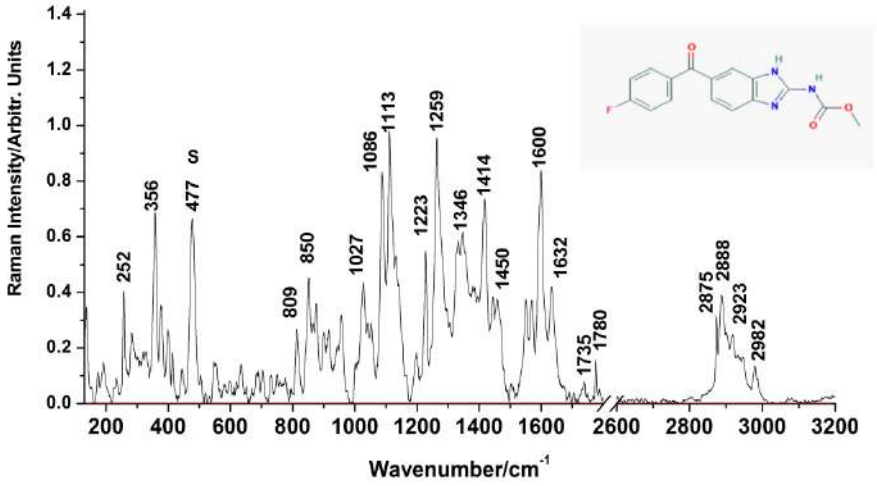

Fig 4. Background subtracted normalized and smoothed spectrum of powdered Rombendazole tablet. Note the decreased intensity or absence of several sharp peaks from excipients in fine powdered tablets. S indicates starch excipient peak. Excitation: 785 nm.

Raman spectroscopy of the powdered tablet of Rombendazole showed quite different feature, as highlighted in the Fig 4. The spurious bands of point spectra collection on tablet are diminished or absent in the fine powder spectrum. Thus, this spectrum is further used for comparative analysis of the spectra collected from mixed drugs in powdered form.

Raman spectra of doxycycline hyclate (Fig 5) showed uniformity in powder structure as revealed by the excellent reproducibility of the spectra collected from one micro-grain to another. The recorded signal was consistent with previous reports of the doxycycline standards [13-15]. The dominant Raman bands are located at 702(s), 844(w), 1176(vs), 1269(s), 1624(vs) cm^{-1} as revealed by the reference Raman spectre [16].

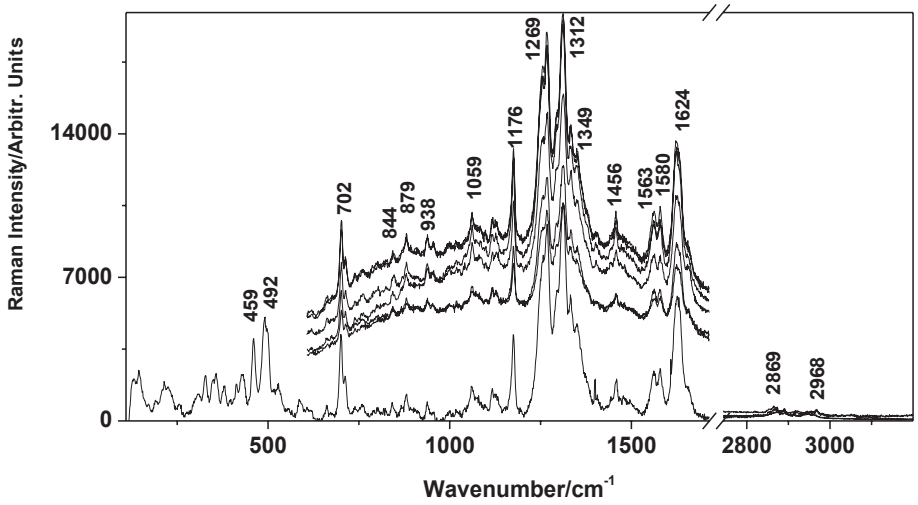

Fig 5. Raman spectra collected from the commercial Doxifarm 50% yellow powder. Excitation: 785 nm, 300 mW. Note the homogeneity of the powder material deduced from reproducible point scans, and shown for the short range single point Raman acquisitions (upper spectra, short range). Bottom spectrum: extended mode acquisition (10 s) covering the whole spectral range (100-3200 cm^{-1}). Excitation: 785 nm.

Analysis of vibrational Raman spectra of drug-drug solid mixtures

Raman spectra of the powdered products in three ratios are shown in the Fig 6. The spectra have been collected from fine powders pressed in holder and expose to Raman excitation. The laser line employed was 785 nm, to avoid excessive fluorescence typical for both compounds.

In the spectra of mixtures the Rombendazole signal is dominant. However, visual observation of possible changes in bands positions and relative intensities changes in spectra of mixtures is difficult. Therefore, we applied the calculation of spectral differences to investigate any spectral evidence of the changes of molecular identity in mixtures.

Figure 7 shows the calculated spectral differences between the P3 and DH. The difference spectrum (P3-DH) is expected to resemble with the spectrum of flubendazole, particularly in the 1100-1800 cm^{-1} range, where excipient bands are less represented. This situation would correspond for the lack of any interaction between the two drugs. Slight changes in flubendazole signature expected from the calculated difference may indicate molecular identity change in mixture, as a result of the interactions with DH functional groups. Lack of molecular interactions would result in identical signature of the Flubendazole with that of the P3-DH spectrum. The difference spectrum clearly showed subtle changes, and a particular band at 1780 cm^{-1}, as highlighted in circle in the Fig 7, where the difference spectrum is displayed in detail in the 700-1800 cm^{-1} range in comparison with the Rombendazole powder spectrum. The characteristic Raman bands at 1273, 1202, 1157, 1135 and 1114 cm^{-1} in benzimidazole are assigned to C-H in-plane bending vibrations [18]. In flubendazole, the benzimidazole moiety is likely to behave as previously described in interaction studies [3,4] and to exhibit two metabolites, the reduced and hydrolyzed forms [19], both being important for oral administration and improved bioavailability.

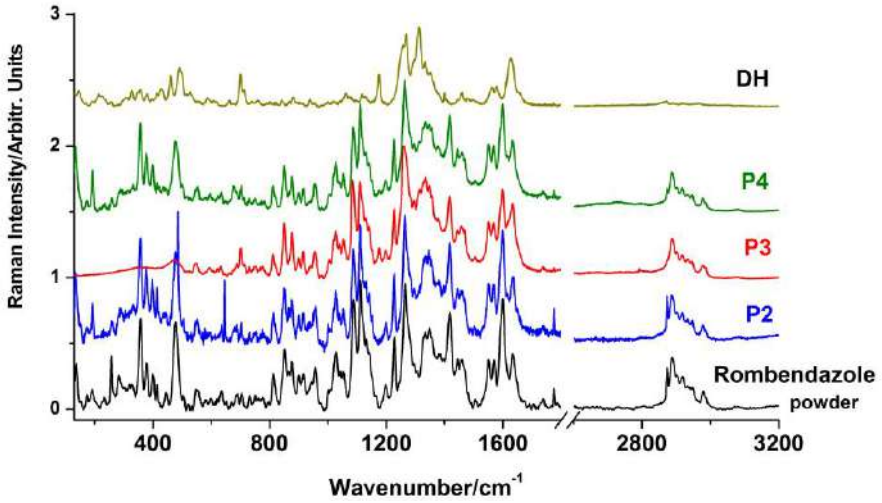

Fig 6. Micro-Raman spectra of the solid mixtures P2 (2:1) P3 (1:2) and P4 (1:1) ratios in comparison with the spectra of doxycycline hyclate (DH) and Rombendazole, as indicated on each spectrum. Spectra were background subtracted, normalized to unit and stacked for clarity.

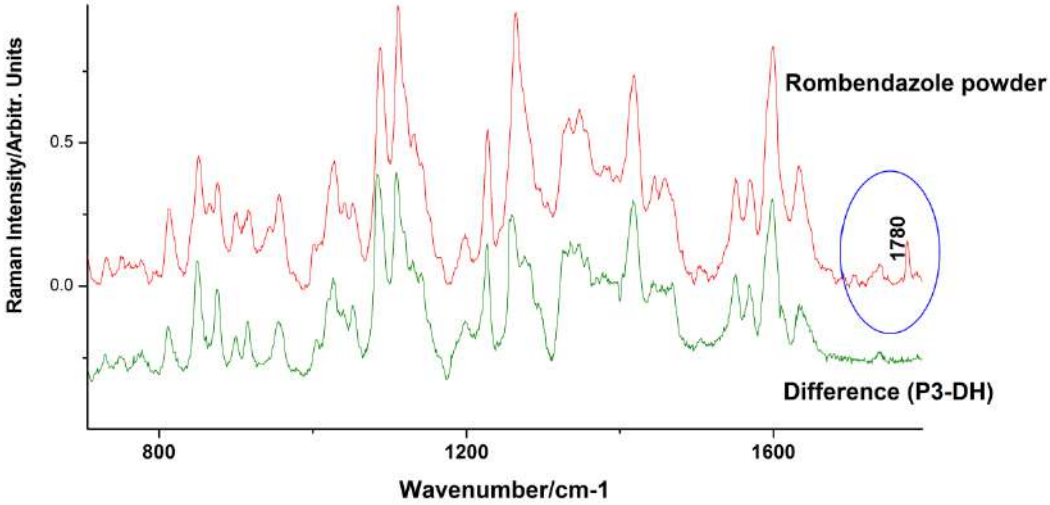

Fig 7. The calculated difference spectrum (P3-DH) from normalized, background subtracted spectra of mixture P3 and the comparison with the Raman spectrum of Rombendazole. The spectral ranges with clear evidence of differences from Rombendazole is highlighted in circle.

The subtle changes are also noted in the 1250-1400 cm^{-1} range, where multiple bands contribution from the benzimidazole ring exhibited prominent Raman characteristics. Above 1600 cm^{-1}, the typical signature of C=O and N-H bending modes are present. These regions are indeed those which presented typical changes in the difference spectrum calculated as P3-DH spectrum from the Fig 7. The observed new band at 1780 cm^{-1} and the changes in the prominent bands profile at 1280 and 1176 cm^{-1}, where C-N and C-C stretching from the benzimidazole ring moiety are involved, may indicate the interaction of the benzimidazole moiety with the DH. Taking into account the high degree of overlap in the Raman spectra of the active ingredients, the similarity in many functional groups and the multiple possibilities of interactions, further

dedicated Raman study of the pure compounds both theoretically and experimentally would be necessary to clarify these aspects. However, this preliminary study of the commercial products mixture provides strong support to assume their interaction and thus, may explain their lack of veterinary medication effectiveness when administrated together.

4 Conclusions

This work evaluated the Raman spectroscopy ability to evidence the drug-drug interaction in the case of two veterinary drugs largely used in bacterial infections and deworming.

We investigated the vibrational Raman properties of Rombendazole, a Romanian formulation of Flubendazole active ingredient, and doxycycline hyclate and found a high degree of overlap in their Raman signature, which raised difficulties in establishing detailed bands contributions of each ingredient in solid mixtures. The vibrational modes observed in Raman spectra of the commercial products are dominated by the excipients in the 100-800 cm^{-1} range. Raman spectra of the commercial drugs have been recorded and interpreted. The Rombendazole tablets were found genuine, highly inhomogeneous, with spectroscopic evidence of excipients (lactose, Mg stearate, talk, starch). The presence of excipients in the study of the interaction between the active ingredients in drug-drug mixtures makes the spectroscopic study difficult, but, based on the correct identification of their bands, relevant conclusions can be drawn. Although this study suggests interaction between Doxycycline and Rombendazole, additional studies in solution would certainly bring consolidated information, particularly for the pH conditions compatible to the veterinary administration.

The results are promising in evidencing the molecular interactions between the drugs and consequently, their change in properties and lack of therapeutic efficiency when administered together. This leads to more careful prescriptions and dosage, when drugs are simultaneously administered.

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