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Raman spectral signatures for the differentiation of benzodiazepine drugs

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The identification of benzodiazepine drugs is important in the Forensics field because they are used in drug-facilitated crimes. Raman spectroscopy has been proven as a non-invasive, fast and reliable technique highly promising for the analysis of drug products. Up to date, attention has been paid on the active ingredient, but the spectral drug product signature has rarely been used in spite of having potential valuable information. In this work, confocal Raman microscopy was used to obtain the spectral signature of the most widely used benzodiazepine products. Firstly, the study aimed at determining an appropriate Raman mapping spectra to obtain each benzodiazepine signature with low sampling error. Then, PCA scores and loadings showed that the variability, measured on the variance, among batches of the same benzodiazepine drug was similar to the variability of the spectral signature of the same tablet (or capsule content) and the same batch, mainly attributed to the heterogeneity of such drug product. Interestingly, differentiation among doses of the same active ingredient (AI), benzodiazepine drugs with different AI manufactured by the same pharmaceutical company, and drugs with the same AI but manufactured by different companies were demonstrated. It is remarkable that for low doses, the active ingredient is almost absent of the spectral signature, but the differentiation is mainly achieved by the excipients. As consequence, spectral signature obtained by confocal Raman microscopy can be used for discriminating among these benzodiazepine drugs without requiring a clearly identifiable band related to the active ingredient in the corresponding Raman spectra.

Introduction

Raman spectroscopy is a non-contact, non-destructive technique able to provide information about the identity of a sample requiring minimal or no sample preparation. This technique is highly selective and provides spectral fingerprints characteristic of compounds, for samples in different states (solid, liquid and gas) in a fast and easy way.[1] For those reasons, Raman spectroscopy has shown an important potential for the analysis of drug products.[2-5]

Up to day, few articles are referred in the literature about the use of this spectroscopic technique for the analysis of benzodiazepine drugs. The characterization of benzodiazepines by Fourier Transform Infrared (FTIR) spectroscopy and Fourier Transform Raman spectroscopy (FTRaman)[4, 6-9] or Surface Enhance Raman Spectroscopy (SERS)[10] has been performed. Raman spectroscopy has also been used for the identification of polymorphism of different drugs[11, 12] and for determining the distribution of benzodiazepine in the pharmaceutical product.[4] However, most of these works focus in assigning the spectral bands to the different molecular vibrations of the chemical groups of the active ingredients (benzodiazepines), and do not study the drug product as such. Nevertheless, pharmaceutical forms contain both active ingredients (AI) and excipient(s), which are added to aid the formulation. In the last years, a tendency on the use of the Raman signature is being observed. Thus, Li and col. classified according to the manufacture and quantified Azithromycin tablets by Raman spectroscopy and chemometric based on the active ingredient and main excipients.[13] De Veij and col. used the complete Raman spectrum as signature for the identification of counterfeit Viagra tablets since those contain less or other inactive (excipients) compounds than the genuine tablets.[14] By using chemometric on the complete Raman spectrum, the distinction between them was automatized. The same researchers published a reference database of Raman spectra of most common pharmaceutical excipients for the interpretation of Raman spectra.[15] Recently, Roggo et al.[16] also studied drugs (including two benzodiazepines) by Raman spectroscopy, obtaining the complete spectrum of each sample (AI and excipients), and applied mathematical approaches (chemometrics) for the identification of unknown

pharmaceutical tables. However, they only focused on bands corresponding to the active ingredients.

This recent approach, based on the consideration of the drug product as a whole (active ingredient and excipients), lacks in the sampling method for micro-Raman analysis of solid dosage forms because tablets can exhibit very different degrees of micro-heterogeneity, i.e., domains of high/low drug contents. However, most of the above mentioned works used a confocal Raman microscope with different objective lens (x5 [14, 15], x20 [4], x50 [2] or facula of 50 x 1000 μm of the laser beam [13]). Neither of them mentioned any particular strategy to perform those measurements except Bell and col. [2] that recorded an average spectrum from 64 points (8 x 8 grid with a 200 μm spacing) on the tablet surface. Those authors compared with the high optical efficiency of a macro-Raman (sampling method of 8x8 grid with 0.5 mm spacing) and concluded than this is consistent with achieving lower sampling error than a micro-Raman for solid dosage forms seized MDMA-caffeine-lactose tablets and keeping overhead times at an acceptable level.[2] In addition, mapping spectra were collected in Alprazolam tablets under the consideration of domain sizes of the AI in the tablet, in a spatial resolution of 25 x 27 μm.[4] However, this approach only took into account the spatial distribution of the AI. As consequence, this work will pay attention to the sampling method required to obtain a Raman spectral signature able to discriminate among benzodiazepine drugs.

Benzodiazepine drugs possess sedative and tranquilizing properties, with muscle relaxant effects, hypnotic and amnesic properties. Their mixture with alcoholic drinks potentiates these effects and increases the vulnerability of the consumer, who suffers loss of memory, not remembering what happened during that time. This fact has triggered an increasing number of forensic cases where these drugs are involved in crimes like assaults, robberies and, especially, sexual assaults. Consequently, the increasing use of these widely prescribed drugs in drug-facilitated crimes is gaining current forensic interest. [17, 18] The present paper focuses on the development of a methodology to differentiate several benzodiazepine drugs considering their commercial product, which includes the active ingredient and the excipients. First part of the work deeps in the sampling methodology. Then, because each pharmaceutical company, and even each dose have a characteristic composition that should help to differentiate them in a forensic case, this work studies if the Raman spectral signature differs among: (i) batches of the same AI (i.e., benzodiazepine); (ii) doses of the same AI; (iii) drugs with different AI manufactured by the same pharmaceutical company; and (iv) drugs with the same AI but manufactured by different pharmaceutical companies.

Experimental Section

Chemicals and Samples

The different benzodiazepine drugs in pharmaceutical products studied in this work were selected considering those more widely prescribed by medical doctors. Table 1 shows each commercial pharmaceutical product studied in this paper, and its AI (benzodiazepine) with the chemical supplier of the standard AI, pharmaceutical company, doses, AI percentage in the product (as %, m/m), nomenclature and number of different studied batches. Commercial drugs were acquired from their respective pharmaceutical companies after filling an official psychotropic document provided by the Agencia Española del Medicamento y Productos Sanitarios (AEMPS).[19] Standard AIs were kindly supplied by the General Commissary of the Spanish Scientific Police. Table 2 shows the excipients contained in the different benzodiazepine drugs studied, except those that are part of the coating of the tablets or capsules since they were not considered in the Raman exploration. All the excipients analysed were obtained from Sigma-Aldrich (St. Louis, MO, USA). Some excipients such as amaranth, blue V, indigo and tartrazine are used as dyes at very low concentration. They are included in the formulation with the main aim of distinguishing by naked-eye among different AIs or doses of the same AI.

Non-coated tablets were analysed on their external surface. Coated tablets (i.e. Tranxilium®) were fragmented in two parts and the cross-section surface was measured. Capsules (i.e. Bromazepam or Dipotassium Clorazepate) were opened and their powder content was analysed by Raman spectroscopy. All samples were analysed after placing them properly on microscope slides (Fig. 1, left).

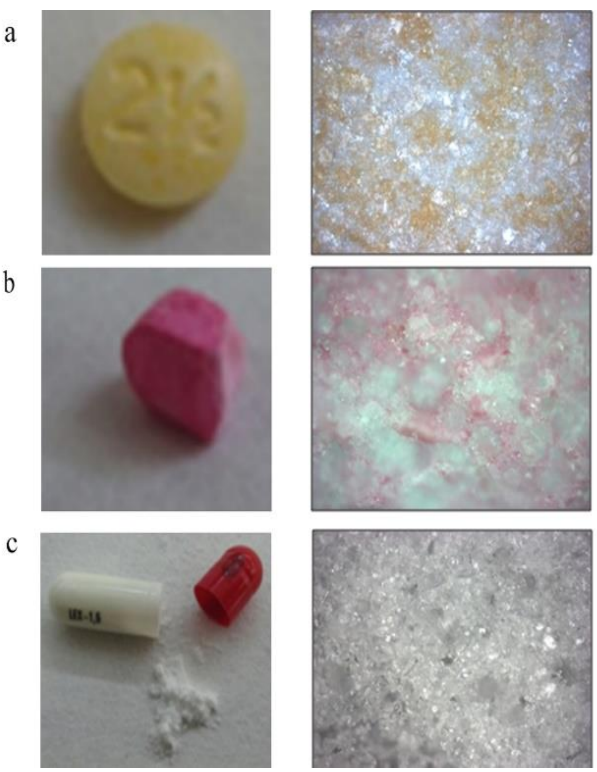

Fig. 1 Macroscopic (left) and microscopic (right) visual images (objective magnification 10x) of the tablets surface for: a) tablet (Kern Pharma Diazepam tablets, 2.5 mg); b) the cross-section of a coated tablet (Tranxilium® coated tablets, 50 mg); and c) a capsule content (Roche Lexatin® capsules, 3 mg).

Table 1 Commercial benzodiazepines studied in this work.

Commercial benzodiazepine-based drug product	Active ingredient (AI) (Standard AI vendor)	Pharmaceutical company	Dose (mg)	AI (%, m/m)	Sign	Number of measured batches
Alprazolam tablets	Alprazolam (Liponed)	Cinfa	**1**	0.74	ALP Ci 1	1
		Kern Pharma	**0.25**	0.21	ALP KP 0.25	1
			0.5	0.41	ALP KP 0.5	1
			1	0.84	ALP KP 1	5
			2	0.83	ALP KP 2	1
		Normon	**0.25**	0.21	ALP N 0.25	1
			0.5	0.41	ALP N 0.5	1
			1	0.81	ALP N 1	5
			2	0.83	ALP N 2	1
		Ratiopharm	**0.25**	0.21	ALP RP 0.25	2
			0.5	0.42	ALP RP 0.5	1
			1	0.84	ALP RP 1	2
Bromazepam capsules	Bromazepam (Roche)	Kern Pharma	**1.5**	0.92	BMZ KP 1.5	5
			3	1.9	BMZ KP 3	1
		Normon	**1.5**	1.25	BMZ N 1.5	6
			3	2.05	BMZ N 3	1
			6	3.90	BMZ N 6	1
Lexatin® cápsules	Bromazepam (Roche)	Roche	**1.5**	1.06	LEX Ro 1.5	7
			3	2.09	LEX Ro 3	1
			6	3.80	LEX Ro 6	1
Dipotassium Clorazepate capsules	Dipotassium Clorazepate (Sigma)	Normon	**5**	4.91	CLZ N 5	2
			10	9.76	CLZ N 10	2
			15	14.10	CLZ N 15	1
Tranxilium® coated tablets	Dipotassium Clorazepate (Sigma)	Sanofi-Aventis	**50**	32.90	TRA Sa 50	1
Diazepam tablets	Diazepam (Roche)	Kern Pharma	**2.5**	3.19	DZP KP 2.5	4
			5	6.23	DZP KP 5	5
			10	12.64	DZP KP 10	4
			25	31.80	DZP KP 25	5
		Normon	**5**	4.67	DZP N 5	2
Lorazepam tablets	Lorazepam	Kern Pharma	**1**	1.0	LZP KP 1	5
		Pensa	**5**	4.83	LZP Pe 5	1
		Normon	**1**	1.06	LZP N 1	5
Lormetazepam tablets	Lormetazepam	Cinfa	**2**	1.77	LMZ Ci 2	1
		Kern Pharma	**1**	0.91	LMZ KP 1	5
			2	1.75	LMZ KP 2	1
		Normon	**1**	0.82	LMZ N 1	1
			2	0.85	LMZ N 2	1
Noctamid® tablets		Bayer	**1**	0.81	NOC Ba 1	3
			2	1.75	NOC Ba 2	2

Table 2 Excipients contained in the different commercial benzodiazepine-based drug products studied. The numbers in the boxes indicate those nominal doses containing that excipient. Abbreviations used: Alprazolam (ALP); Bromazepam (BMZ); Dipotassium Clorazepate (CLZ); Diazepam (DZP); Lorazepam (LZP); Lormetazepam (LMZ); Noctamid (NOC); Traxilium® (TRA); Cinfa (Ci); Kern Pharma (KP); Normon (N); Ratiopharm (RP); Roche (Ro); Sanofi-Aventis (Sa); Pensa (Pe); Bayer (Ba)

Commercial Drugs / Excipients	ALP				BMZ		CLZ	DZP		LEX	LZP			LMP			NOC	TRA
	Ci	KP	N	RP	KP	N	N	KP	N	Ro	KP	N	Pe	Ci	KP	N	Ba	Sa
Cellulose microcristaline																		
Corn starch																		
Lactose																		
Magnesium sterearate								2.5 5 25										
Talc								2.5 5 25										
Aluminium oxide																		
Amaranth (E-123)		1		1														
Blue V (E-131)		1		1														
Carboxymethyl sodium starch																		
Croscarmellose								2.5 5 25										
Colloidal silica								2.5 5 25										
Dicalcium phosphate anhydrous																		
Docusate sodium																		
Erythrosine aluminic (E127)								25										
Indigo (E-132)	1		1															
Mannitol																		
Potassium carbonate																		
Povidone K-25																		
Povidone K-30																		
Pregelatinized starch																		
uinoleine (E-104)		0.5		0.5														
Sodium benzoate																		
Sodium lauril sulfate (SDS)																		
Sodium starch glycolate																		
Sunset yellow FCF (E-110)		0.5	0.5	0.5														
Tartrazine (E-102)								2.5										
Titanium dioxide																		

Instrumentation

A Thermo Scientific DXR Raman microscope (Waltham, MA, USA) with a 780 nm excitation wavelength was used for the Raman spectral mapping of the different samples studied. The mappings were carried out automatically; a 4 x 4 grid on an area corresponding to 400 × 400 μm was defined on the optical image provided by the instrument video camera. Step sizes between two sequential measurements were set to 133 x 133 μm, which resulted in 16-recorded Raman spectra corresponding to the 16 different mapping points. The Raman spectral signature of the drug product was defined as the averaged spectrum of the 16 spectra from every mapping. The wavenumber range extended in the region of interest, from 100 to 1800 cm^{-1}. The microscope was set to 10x magnification for the different samples analysed. 10 spectra with duration of 10 s were recorded for all samples in each mapping point. The laser power on sample was set to 14 mW. All the spectra were automatically corrected for avoiding fluorescence and background effects. Both phenomena were filtered out by means of a 5^{th}-order polynomial baseline and dark current subtraction, respectively. Those correction methods were set directly on the Thermo Scientific OMNIC for dispersive Raman 8.3.103 software.

Data treatment

Thermo Scientific Omnic software was used to calculate the average and variance spectra of the normalized Raman spectra. We have considered a signal to noise ratio in spectra above 10 prior to normalization to avoid to confuse real bands with amplified noise and achieve an appropriate low sampling error. Relative Standard Deviations (RSD) were calculated dividing the intensities of the mean ($\bar{x}$) by the standard deviation (*s*) of the spectral data on the most intense band. Raman spectra were exported to Origin 8.6 (OriginLab Corporation, USA) and to Excel 2010 (Microsoft, USA) in order to organize the different samples data according to their AI, batches, doses or pharmaceutical company. Multivariate analysis and data pre-processing were performed using The Unscrambler X 10.1 (CAMO Software AS, Oslo, Norway).[20] All the spectra were subjected to an offset and baseline correction, normalization by means of SNV (Standard Normal Variate) method, and smoothing with the Savitzky-Golay Transformation (2nd-order polynomial with a 15 points window). All those pre-processing techniques are implemented in The Unscrambler.

Principal Component Analysis (PCA)

PCA model interpretation was carried out by plotting the scores (representing samples) and loadings (representing variables) in order to explain the differences observed among the sample classes (groups of samples organised row-wise). On the other hand, the variables (organised column-wise) comprised every data point collected as Raman shift. The comparison between scores and loadings plots allows to screen and at a glance establish relationships among the sample classes and variables. For PCA, a built-in Non-Linear Iterative Partial Least Squares (NIPALS) algorithm with full validation was used. [21] PCA was performed using different ranges in the 100-1800 cm^{-1} region of the pre-processed spectra. Such ranges were selected according to the highest explicability of the model.

Results and Discussion

Raman spectral signature of a drug product

Firstly, the Raman conditions for obtaining spectral signatures with good signal to noise ratio in short analysis time were established (14 mW, 10x magnification, pinhole aperture diameter of 25 μm, 10 s × 10 acquisitions). From the two laser wavelengths available in our Raman system, 780 nm was selected because at 532 nm most studied drug products exhibited intense fluorescence, which overwhelmed the Raman spectra. That magnification resulted in a laser spot of 2.1 μm. We preferred the largest spot size as possible to obtain information of a wider area rather than a high spatially resolved spectra that could be obtained with higher magnification.

Secondly, under these instrumental conditions and due to the micro-heterogeneity of the benzodiazepine samples studied, the sample measurement settings necessary to obtain a sample Raman signature independent of the analyst were studied. As example, Fig. 1 shows the macroscopic and microscopic images of three different pharmaceutical formulations: one tablet, one coated tablet and one capsule. The microscopic image (Fig. 1, right) clearly shows different colourful domains probably due to a heterogeneous distribution of components. Therefore, different approaches were studied for defining the area, number and spatial distribution of points on tablet surface (or powder content in case of capsules) where spectra should be collected in order to obtain the Raman spectral signature of each pharmaceutical product. Different arbitrary areas of different extension (the largest was 2.5% of the tablet size, approximately), located in different positions and covering the entire tablet surface, provided similar results. In the arbitrary selected areas, different randomly distributed points (12, 16, 28 and 29 points) were measured, and the average (arithmetic mean of the Raman intensity values for each data point) and variance (standard deviation of the Raman intensity values for each data point) spectra of each sample were calculated. Raman spectra had the same Raman shift in all bands, and variability was only observed in the bands intensity. RSD values obtained on the most intense band of the variance spectra were lower than 15 %. Based on this acceptable variance, 12 points were considered enough to obtain an average spectrum representative of the pharmaceutical product with acceptable sampling error. A larger number of points are expected to result in lower sampling error of Raman signatures. However, an increase of measured points also increases the time required for analysis. In addition, the read noise, which is normally insignificant in the most CCD detectors, may become important when a larger number of small signals are read from different points on the same tablets. [2] For this reason, the smallest number of points that results in a low sampling error was selected, in order to perform a faster mapping analysis.

Once decided the minimum number of spectra to be averaged, the spatial distribution of the measured points on the surface of the pharmaceutical product was investigated to diminish the measurement subjectivity. Thus, instead of a random selection of measured points on the tablet (or capsule content), a 4 x 4 Raman spectral mapping grid (mesh) of every sample was recorder, which rendered 16 spectra for each mesh. In turn, every spectra mesh was averaged for calculating a Raman spectral signature of each drug product. Therefore,

different information was obtained: An average spectrum (Raman spectral signature of the pharmaceutical product) from 16 spectra objectively recorded, and the spatial distribution of the main components. As example, Fig. 2 illustrates the distribution of two major components (Diazepam and Lactose) in a 10 mg Diazepam tablet from Kern Pharma. The 16 different positions on the sample over an area of 400 × 400 μm (step sizes of 133 x 133 μm) resulted in 16 spectra. That grid area represented approximately the 5 % of the full tablet size.

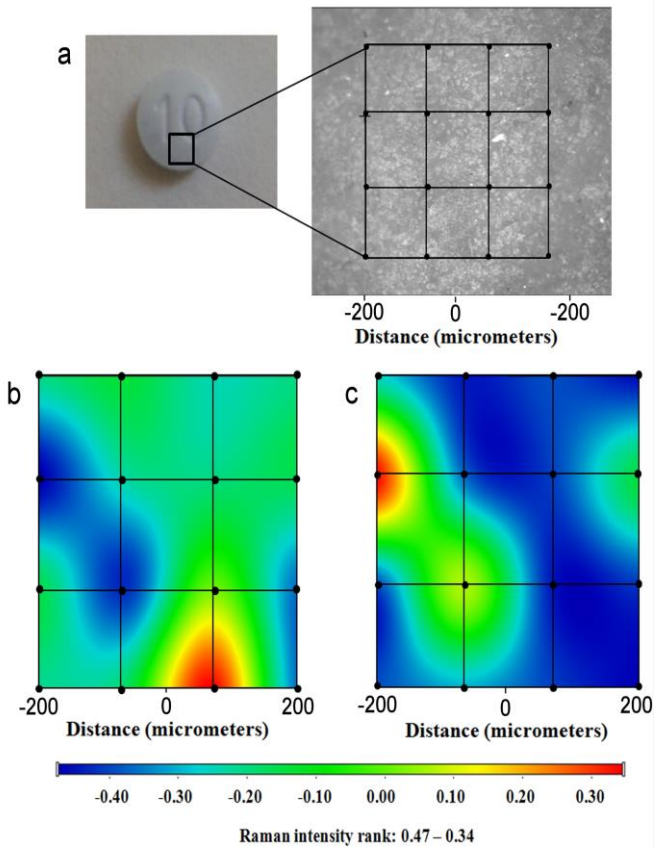

Fig. 2 Chemical maps of Diazepam (Kern Pharma, 10mg) created on an intensity based colour scale where red and blue colours represent high and low intensities, respectively. a) Chemical maps based on the intensity of the spectral band at 1592 cm^{-1} (Diazepam AI); b) chemical maps based on the intensity of the spectral band at 1085 cm^{-1} (Lactose). Raman conditions: laser at 780 nm, 14 mW, 10x magnification, 25 μm pinhole aperture, 10 acquisitions of 10 s each. Raman spectral mapping: 400 × 400 μm mesh-area; step sizes 133 × 133 μm, which corresponds to a 16 spectra record.

The overall spectral intensity of a certain band of these spectra allowed obtaining two chemical contour maps, one for Diazepam and another for Lactose, which is the major excipientof this drug product. These contour maps are made on an intensity based colour scale, where red represents the highest intensity and blue represents lowest intensity. The intense band of the Diazepam's AI at 1592 cm^{-1} is characteristic for Diazepam,[9] thus it was selected to show its distribution on the tablet. In the case of Lactose, its characteristic band at 1085 cm^{-1} was selected.[22]

Fig. 3 displays the Raman spectral signature of the different pharmaceutical products studied in this work (Table 1) compared to the spectra of their AI standards and pure excipients. Fig. 3a marks out the most intense bands in the collected spectra for the common excipients (starch, microcrystalline cellulose, lactose, talc and magnesium stearate) in the different pharmaceutical products (Table 2). Significant excipients bands appear mainly in the 200-1500cm^{-1} region. Fig. 3b illustrates the Raman spectra of the different AIs and their main bands. It is remarkable that the 1500-1700 cm^{-1} region exhibits the characteristic benzodiazepine AI bands. Fig. 3c shows the spectral signatures of the drug products and their most intense bands from the excipients and AIs. However, his work focus on the use of the Raman spectral signature of the whole composition of the drug product. This comprises all the spectral bands belonging to the AI plus the excipients. Such approach tests if these drug products can be distinguished regardless of the batches, doses or pharmaceutical companies that manufactured them.

Variability among different batches of the same drug product.

Raman spectral signatures of the different selected benzodiazepine AIs were analysed to study the variability of several batches of the same AI in pharmaceutical products. Every selected benzodiazepine had at least 4 distinctive batches (Table 1). RSD values, obtained considering the averaged and variance spectra ratio on the most intense band of the variance spectra, were ranged from 9 % (6 batches of Bromazepam Normon, 1.5 mg) to 19 % (5 batches of Bromazepam Kern Pharma, 1.5 mg). As example, Fig. 4 shows the averaged and variance spectra for 5 different batches of Bromazepam Kern Pharma (Fig. 4a) compared with the averaged and variance spectra of the 16 spectra obtained for the Bromazepam Kern Pharma's spectral signature gathered from a single tablet (Fig. 4b). The RSD values are similar, thus there was not variability among batches of the same benzodiazepine AI in the drug products.

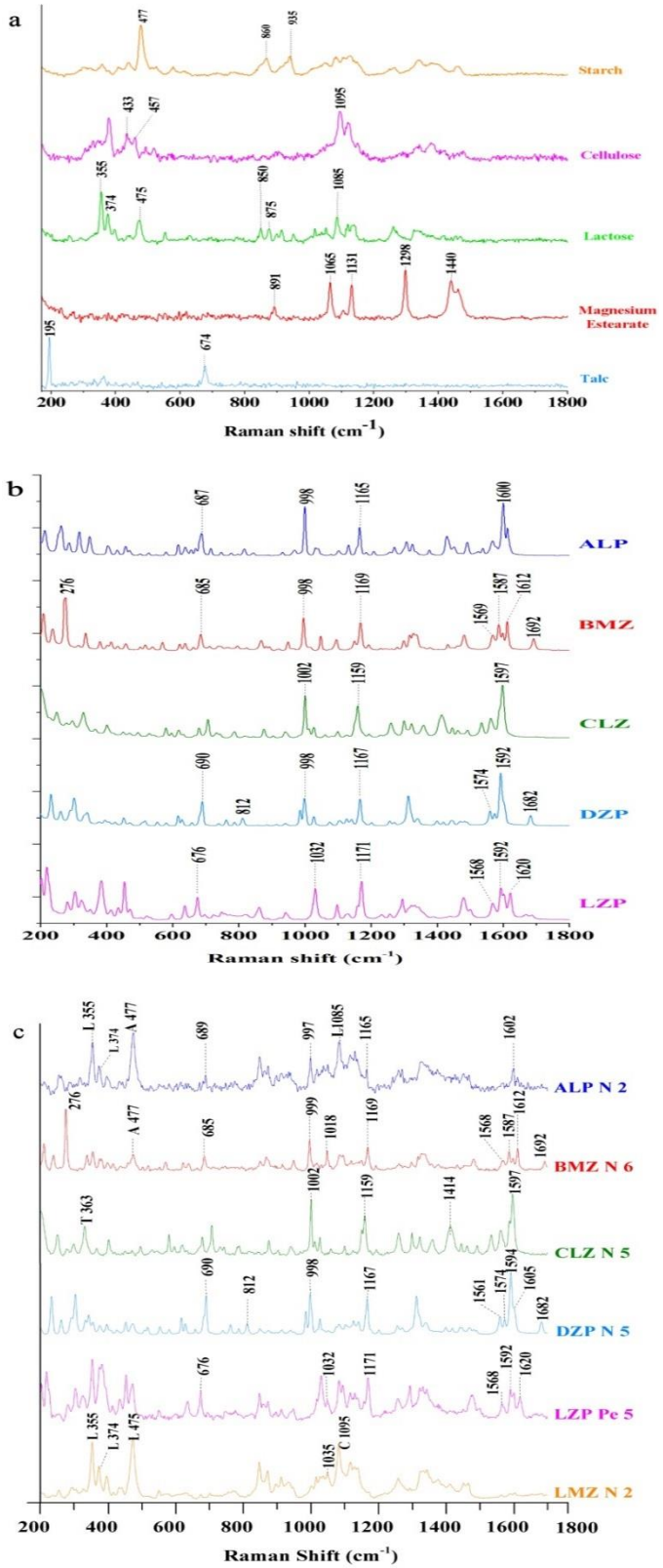

Fig. 3 Raman spectrum of main excipients (a), AI standards (b) and Raman spectral signature (c) of the different commercial drug products studied. Characteristic bands of excipients and AIs are labelled for clarity. Lorazepam and Lormetazepam AIs' bands have been assigned according to Ref 23. Raman conditions as in Fig. 2.

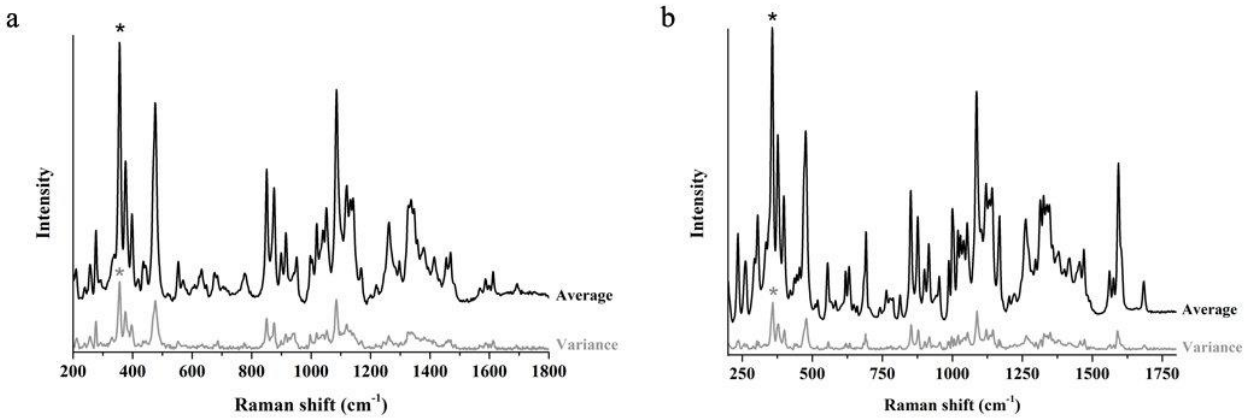

Fig. 4 Average (black line) and variance (grey line) Raman spectra collected for: a) five different batches of Bromazepam Kern Pharma 1.5 mg; b) a same table of Bromazepam Kern Pharma 1.5 mg. (*), Calculation of RSD value on the most intense band of the variance spectra. Raman conditions as in Fig. 2.

Discrimination among different doses of the same benzodiazepine active ingredient in drug products.

Raman spectral signature was also used to investigate the variability of the same benzodiazepine AI at different doses. For this study, benzodiazepines presenting more than 3 different doses were considered (Table 1). Note that the main change in these samples is the concentration of the AI in the drug product. As example, Fig. 5 shows the Raman spectral signature of Diazepam Kern Pharma at different doses (2.5, 5, 10 and 25 mg).

As expected, AI's bands (1550-1620 cm^{-1} region) increased with increasing doses (see inset at up right side, Fig. 5). Complementary to this effect, the Lactose band (1085 cm^{-1}) decreased with increasing AI's doses (see inset at up left side, Fig. 5). Afterwards, PCA of the Raman spectral signatures of all available batches for the same benzodiazepine dose were performed. Scores and loadings plots revealed that a clear differentiation of high doses was possible. However, that was not the case for low doses.

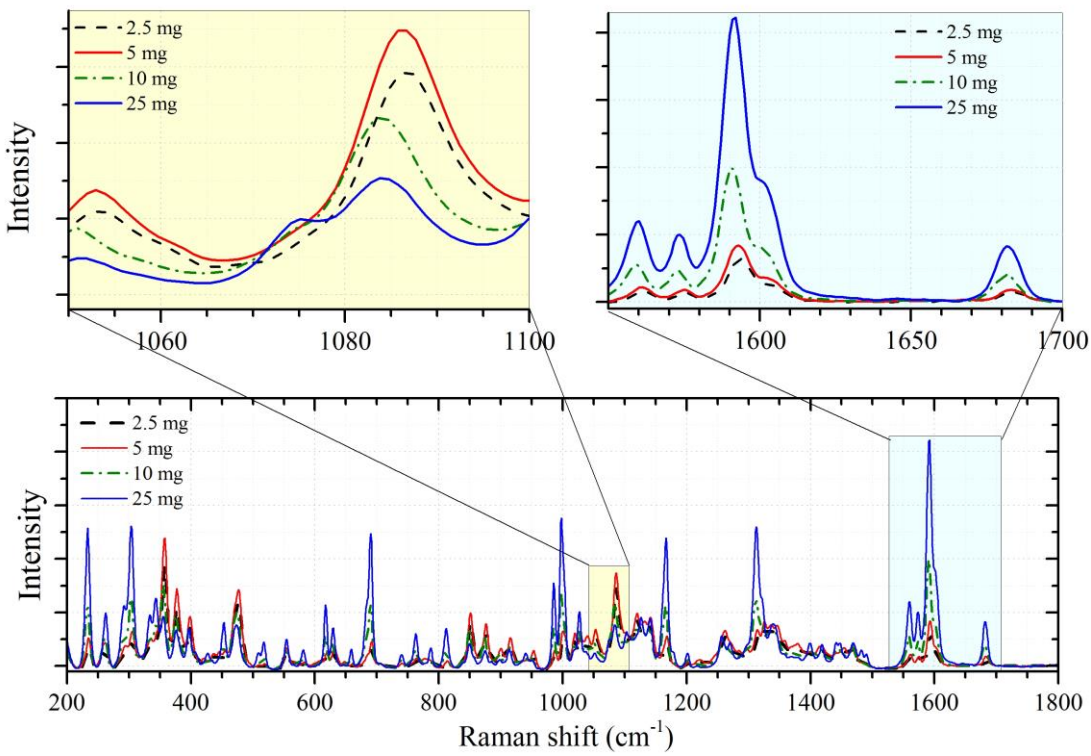

Fig. 5 Raman signature of four different doses of Kern Pharma Diazepam. Up left inset: Magnification of Raman bands assigned to Lactose. Up right inset: Magnification of Raman bands assigned to the Diazepam's AI. Raman conditions as in Fig. 2.

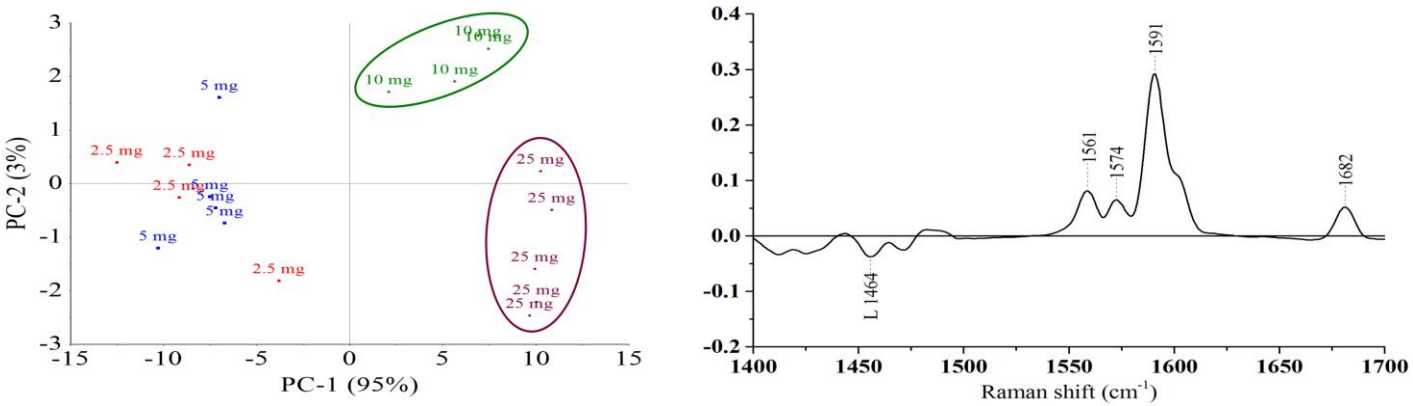

Fig. 6 PCA Scores (PC-1 vs PC-2, 98 % of model explicability) and loadings (for PC-1) for four different doses of Kern Pharma Diazepam in the 1400 to 1700 cm^{-1} spectral range. Raman conditions as in Fig. 2.

Fig. 6 illustrates the scores and loading for 4 different doses (2.5, 5, 10 and 25 mg) of Diazepam Kern Pharma taking into account the 1400-1700 cm^{-1} region where the highest model explicability (95 % for PC-1 and 3 % for PC-2) was obtained. The Scores plot does not allow a clear differentiation among low AI content (2.5-5 mg), but the separation is noticeable for 10 and 25 mg doses. The scores and loadings on a particular PC have the same sign. The larger the values of the scores and loadings the stronger their relation. Considering the fact that the highest AI doses (10 and 25 mg) are placed in the positive axis of PC-1 (X axis), the positive bands in the loadings plot for PC-1 correspond to 1561, 1574 and 1591 cm^{-1} bands. These bands are attributed to the AI, which is at high concentration in the highest doses. This result indicates that differentiation of high doses is possible. However, for low doses, the differentiation is not easy due to a major contribution of the excipients, which are usually the same for the same AI in drug products. Moreover, other important reason for this poor differentiation could also be a non-adequate sampling. This comes from the selection criteria: the smallest number of mapping points for constructing an average spectrum representative of the pharmaceutical product. The number of mapping points is meant to permit a fast mapping analysis with an acceptable variance (see the discussion in the section of Raman spectral signature of a drug product).**Discrimination among drug products with different benzodiazepine active ingredient manufactured by the same pharmaceutical company.**

Raman spectral signatures were also used to test if drug products from the same pharmaceutical company but different AI could be easily differentiated. For this purpose, different AIs from Normon (Table 1) but with equivalent doses (1 mg for Alprazolam and Lorazepam, 1.5 mg for Bromazepam, and 5 mg for Dipotassium Clorazepate and Diazepam) were considered. The study of all drug products at the same dose was not possible because they were commercialized at different doses depending on how intense is its therapeutic effect. Fig. 7 shows the 3D scores plot for the Raman spectral signatures considering the 130-1700 cm^{-1} region (PC-1, PC-2, and PC-3 with 74, 14, and 6 % explicability, respectively). That spectral range exhibits the characteristic bands of both excipients and benzodiazepine AIs. Different groups were observed for each AI. This result is remarkable considering that at low doses the AI bands of the spectral signature of the tablets (capsule content) are minor or are not detected compared to the excipient bands. Consequently, bands corresponding mainly to the excipients allowed discriminating among the different drug products. Taking into account the loadings (Fig. S1 given in supplementary data), only Diazepam was discriminated by its AI (band at 1592 cm^{-1}). On the contrary, most common excipients allowed discriminating among all drug products. Accordingly, talc bands (192 and 674 cm^{-1}) discriminate Dipotassium Clorazepate and Diazepam from the other drugs; starch band (477 cm^{-1}) distinguishes Alprazolam and Diazepam; and microcrystalline cellulose bands (348, 381 and 1097 cm^{-1}) differentiate Lorazepam.

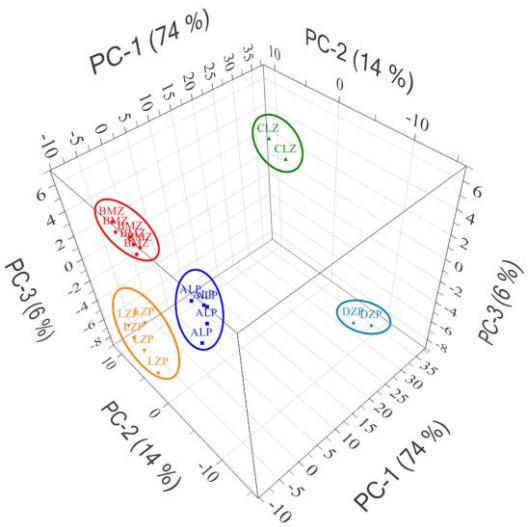

Fig. 7 3D PCA scores plot for pre-processed Raman spectra of low doses of different benzodiazepine AIs provided by the same pharmaceutical company (Normon). Abbreviations as in Table 1. The different AIs are represented by different symbols: ■, ALP; ●, BMZ; ▲, CLZ; ♦, DZP; ▼, LZP.
Discrimination among same benzodiazepine active ingredient manufactured by different pharmaceutical companies.

Finally, Raman spectral signatures were used to discriminate drug products with the same benzodiazepine AI from different pharmaceutical companies. For this study, commercial drug products of the same dose of AI and produced by different pharmaceutical companies were considered (Table 1). PCA results were represented as scores (Fig. 8) and loading (given as supplementary data as Fig. S2) for Alprazolam 1 mg, Diazepam 5 mg, Lorazepam 1mg, Lormetazepam 1mg, and Bromazepam 1.5 mg. Scores plots shown, in general, a good differentiation among companies. Neither Alprazolam from Kern Pharma and Ratiopharma (Fig. 8a) nor Bromazepam from Roche (Lexatin®) and Kern Pharma (Fig. 8e) were distinguishable from each pharmaceutical manufactures. In the case of Alprazolam, the pharmaceutical products from Kern Pharma and Ratiopharma have the same excipients and, according to their technical notes, both were synthesized by the same company (Lacer SA). In the case of Bromazepam from Roche and Kern Pharma, both pharmaceutical companies report a very similar excipients composition, and the AI is at its lowest dose (1.5 mg; Table 1), being the excipient bands predominant in the Raman spectral signatures. Note that Lormetazepam from Kern Pharma and Bayer (Noctamid®) were clearly separated in two groups (Fig. 8d) although their compositions differ only in the croscarmellose dye, which should be at very low content. According to the loadings plots (given as supplementary data), the drug products were mainly distinguished by the presence or absence of certain excipients bands, being the talc (195 and 675 cm^{-1}), starch (477, 860 and 935 cm^{-1}), lactose (355, 850, 875 and 1085 cm^{-1}) and microcrystalline cellulose (378, 433, 457 and 1095 cm^{-1}) the most characteristics ones.

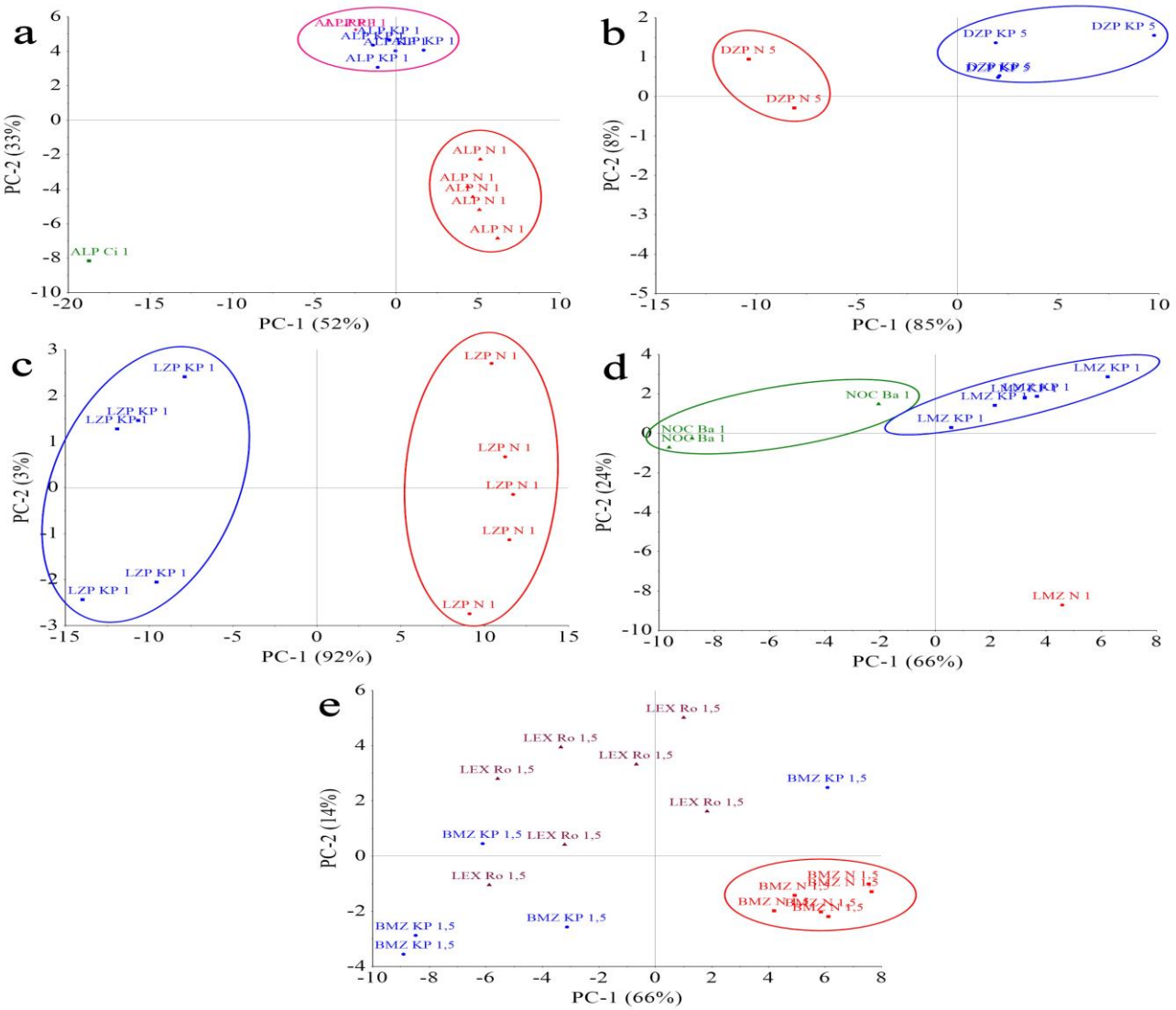

Fig. 8 PCA score plots for pre-processed Raman spectra of the same benzodiazepine AI provided by different pharmaceutical companies labelled as: Cinfa (Ci); Kern Pharma (KP); Normon (N); Ratiopharm (RP); Roche (Ro); Bayer (Ba). Benzodiazepines: a) Alprazolam 1 mg (ALP 1mg); b) Diazepam 5 mg (DZP 5mg); c) Lorazepam 1 mg (LZP 1mg); d) Lormetazepam 1 mg (LMZ 1mg); e) Bromazepam 1.5 mg (BMZ 1.5mg). Complete sign of drug products are given in Table 1.

Conclusions

The differentiation of commercial benzodiazepine-based drug products, which are illicitly used for recreational purposes and involved in drug-facilitate crimes, was demonstrated through their Raman spectral signature. The spectral signature was defined as the averaged spectra of 16 spectra gathered in a total area of 400 x 400 µm on the surface of the tablet or capsule content. Those conditions guarantee a low sampling error. The variability of this spectral signature was similar to that obtained for different batches of the same drug product (RSD < 18 %). In addition, PCA was performed and scores and loadings plots were used to interpret the obtained results. Thus, high doses of the same benzodiazepine AI were differentiated in all the studied benzodiazepine products. For low doses of AI, Raman bands attributed to the AI were negligible or not detectable, being the observed bands mainly attributed to the excipients. Additionally, the discrimination among benzodiazepine-based drug products with different AI, from the same company and comparable doses, was proven even in the most unfavourable conditions with low doses of AI. In the case of drug products containing both the same AI and dose but manufactured by different companies, the discrimination was also confirmed. Only Alprazolam, by Kern Pharma and Ratio Pharma, were not differentiated, but this results is due to both drug products are the same, synthesized by Lacer SA. This last study demonstrate how the differentiation between generic and patented pharmaceutical products is possible. Hence, the drugs spectral signature analysed by PCA is a successful approach for differentiating benzodiazepine-based drugs without the need of benzodiazepine standards

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Electronic Supplementary Information (ESI) available: Fig. S1 contains the PCA Loadings for low doses of the benzodiazepine AIs provided by the same pharmaceutical company (Normon); Fig. S2 contains PCA Loadings (PC-1 or PC-2) for the different pharmaceutical companies and different benzodiazepines. See DOI: 10.1039/b000000x/