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# Analysis of illegally manufactured formulations of tadalafil (Cialis®) by $^1$H NMR, 2D DOSY $^1$H NMR and Raman spectroscopy

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**Abstract**

Counterfeit and/or imitation medicines are becoming a major health problem not only in developing countries but also in wealthier countries. The need of new and easy analytical methods for quality control of drugs is essential. We describe the use of Raman spectroscopy, $^1$H nuclear magnetic resonance (NMR) and 2D diffusion-ordered spectroscopy (DOSY) NMR to analyse genuine Cialis® and seven illegally manufactured formulations of this drug purchased via the internet. Seven out of the eight commercial formulations of tadalafil contain the active ingredient, measured by high performance liquid chromatography (HPLC), within $100 \pm 5\%$ of stated concentration. Vardenafil and homosildenafil instead of tadalafil were found in the Chinese imitation. 2D DOSY NMR spectra clearly showed similarities and differences in the composition of the pharmaceutical formulations of tadalafil, thus giving a precise and global "signature" of the manufacturer. Our data show that the quality of the Cialis imitations manufactured in India and Syria is correct, whereas the Chinese formulation is adulterated with active pharmaceutical ingredients.


*Keywords:* Tadalafil; Vardenafil; Homosildenafil; Raman spectroscopy; $^1$H NMR; 2D DOSY $^1$H NMR; LC–MS; Illegally manufactured drugs

## 1. Introduction

Counterfeit and illegally manufactured drugs become a noticeable problem all over the world. Counterfeit drug detection is often a complex process requiring the use of various chemical analyses. High performance liquid chromatography (HPLC) is considered as the gold standard analytical method in drug analysis of fake drugs [1,2] but in the last few years several methods have been used for quality control, such as refractometry and colorimetry [3], near infrared spectroscopy [4–6], X-ray diffraction [7], mass spectrometry (MS) [8,9] and Raman spectroscopy [10]. It has been previously shown by Witkowski [11] that Raman spectroscopy could be a powerful instrumental technique for the analysis of suspect counterfeit pharmaceutical dosage forms. In previous studies [12,13], we also demonstrated that 2D diffusion-ordered $^1$H nuclear magnetic resonance spectroscopy (2D DOSY $^1$H NMR) is an interesting method for a complete characterisation of pharmaceutical formulations.

Nowadays, drugs for the treatment of erectile dysfunction which function by inhibiting the phosphodiesterase type 5 enzyme (PDE-5), sildenafil citrate (Viagra®), tadalafil (Cialis®) or vardenafil hydrochloride (Levitra®) are among the most counterfeited or imitated. Imitations came generally from Asia (India and China most often) which do not recognise the European and American patent laws so that products manufactured legally in such countries are illegal in Europe, USA and other countries.

In this study we showed an application of (i) Raman spectroscopy and $^1$H NMR to detect fake formulations and (ii) 2D DOSY $^1$H NMR for an acute identification of differences in drug composition. The original Cialis® tablet from Eli Lilly Laboratories and some illegally manufactured formulations of Cialis were analysed. Classical $^1$H NMR and HPLC coupled with tandem MS (LC–MS–MS) were used to achieve this study in the case of a fake Chinese formulation. To the best of our knowledge, this study is the first report dealing with the analysis of imitated pharmaceutical formulations of Cialis.

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Table 1
Cialis commercial formulations analysed in this study

| No. | Formulation name | Color | Batch number | Expiration date | Manufacturer name | Country of manufacturing |
|---|---|---|---|---|---|---|
| 1 | Cialis® 20 mg | Yellow | A170862 | November 2008 | Eli Lilly | Germany |
| 2 | Tadafort 20 mg | Yellow | 06 | May 2007 | Ibn Al Haytham Pharma | Syria |
| 3 | Tadalafil tablets 20 mg | Yellow | 2016 | January 2009 | Guj/Drugs | India |
| 4 | Alegra 20 mg | Blue | 03 | May 2006 | Delta | Syria |
| 5 | Forzest 20 mg | Blue | 9080425 | March 2007 | Ranbaxy | India |
| 6 | Ceebis 20 mg | Yellow | TC-03 | September 2008 | Cooper Pharma | India |
| 7 | Ceebis 20 mg | Red | TC-02 | February 2008 | Cooper Pharma | India |
| 8 | Chinese Cialis | Yellow | Q/CL5: 2004, No. 0487 | 2007 | Baoyi Health Food | China |

## 2. Experimental

### 2.1. Chemicals

Pure tadalafil was obtained from Yick-Vic Laboratories (Hong Kong, China). Excipients were gifts from the Galenic Laboratory of the School of Pharmacy (Paul Sabatier University, Toulouse, France).

### 2.2. Commercial formulations of tadalafil

Eight tadalafil commercial formulations were analysed, one being the brand formulation from Eli Lilly (Cialis®), the others seven being imitation drugs that were purchased over the internet except for those coming from Syria that have been bought in the country. The list of the tablets analysed is given in Table 1.

The composition of the brand Cialis® is as follows—(i) core: lactose monohydrate, sodium croscarmellose, hydroxypropylcellulose, magnesium stearate, microcrystalline cellulose and sodium lauryl sulfate; (ii) film coating: lactose monohydrate, hypromellose, triacetin, titanium dioxide, iron yellow and talc.

All samples, as received, were stored in the dark at ambient temperature and humidity. They were all analysed within expiry dates.

### 2.3. $^1H$ and 2D DOSY $^1H$ NMR

The spectra were recorded in a mixture of $CD_3CN$:$D_2O$ (80:20). The recording and processing conditions have already been described [13].

For $^1H$ NMR, the sole difference was the value of the pulse interval that was 3.64 s for the qualitative analysis of all the samples and was lengthened to 4.64 s for the quantitative analysis of vardenafil and homosildenafil.

For 2D DOSY $^1H$ NMR, the sequence parameters were adapted in order to have the intensity of the $H_{1,1'}$ (5.9 ppm) NMR signal of tadalafil strongly decreased (at least divided by 50) at 95% of the full gradient strength.

### 2.4. Raman measurements

Raman spectra were recorded with a Jobin and Yvon Lambram HR800 equipped with a helium neon laser (excitation line at 632.8 nm). The power of the laser beam at the surface of the sample was about 7 mW. For some samples, beam attenuators were used to avoid destruction of the sample and/or to limit fluorescence emission.

To ensure the homogeneity of the sample (or to take into account its heterogeneity), several measurements were made on each sample. In the case of great heterogeneity, the spectrum presented is the average of all recorded spectra.

### 2.5. LC-DAD apparatus and chromatographic conditions

HPLC was carried out using a Waters 2695 Alliance model with a Waters 2996 diode array detector. The analytical column was a reversed-phase column Luna C18 (100 mm × 3 mm i.d.; 3 μm particle size; Phenomenex, UK). The column temperature was 30 °C. The mobile phase consisted of a mixture (35:65, v/v) of acetonitrile and phosphate buffer (10 mmol $L^{-1}$, pH 3). The flow rate was 0.6 mL $min^{-1}$ and the volume injected 10 μL. A detection wavelength of 225 nm was chosen as it allows the detection of all tadalafil or sildenafil analogues.

For quantitative analysis, a calibration curve was constructed from the analysis of four solutions containing pure tadalafil in a concentration range of 0.01–0.1 mg $mL^{-1}$. Each standard solution was injected in triplicate in the chromatographic system. The linearity ($R^2 > 0.999$) was evaluated by least-squares linear regression analysis.

### 2.6. LC–MS analysis

The HPLC system used consisted of an Agilent 1100 series apparatus. An Applied System QTRAP triple quadrupole mass spectrometer, equipped with a turbo ion spray (TIS) interface, was used for detection. Both were controlled by an Agilent Analyst software (version 1.4). HPLC conditions were as follows. The column temperature was 30 °C. The mobile phase consisted of a mixture (50:50, v/v) of acetonitrile and a buffer solution (ammonium acetate 10 mmol $L^{-1}$, pH 7). The flow rate was 0.6 mL $min^{-1}$ and the volume injected 5 μL.

The mass spectrometer was operated in positive ionisation mode with TIS heater set at 450 °C. Nitrogen served both as auxiliary, collision gas and nebuliser gas. The operating conditions for TIS interface were—(i) in MS mode: mass range 200–550 μm (1 s), step size 0.1 μm; Q1 TIS MS spectra were recorded in profile mode, IS 5000 V, DP 85 V; (ii) in MS–MS mode: precursor mass 489 μm; mass range 10–500 μm (0.35 s); step size 0.15 μm; LC–MS–MS spectra were recorded in profile mode, IS 5000 V, DP 85 V and CE 40 V.

### 2.7. Sample preparations

#### 2.7.1. Raman analysis

All the excipients tested are powder and were analysed directly under the excitation beam. Tablets were analysed without any preparation. For some samples, the coloring of the coating induced strong fluorescence signal. In these cases, a small part of the coating of the tablet was eliminated and the spectrum was then recorded directly on the uncoated tablet.

#### 2.7.2. NMR analysis

The tablet was powdered and dissolved in 5 mL of solvent under magnetic stirring for 15 min, then sonicated for 10 min. The solvent used was a mixture (80:20, v/v) of $CD_3CN$ and $D_2O$. The suspension was then centrifuged (10 min, 3000 rpm) and the supernatant analysed. For quantitative analysis, a tablet of the Chinese formulation was powdered and dissolved in 100 mL of methanol under magnetic stirring for 45 min, then sonicated for 10 min. An aliquot of 2 mL was evaporated to dryness and the residue dissolved in 2 mL of a $2 \times 10^{-3}$ mol $L^{-1}$ solution of maleic acid in MeOH-$d_4$ before the NMR analysis.

#### 2.7.3. Chromatographic analysis

For all chromatographic analyses, the tablets were powdered and dissolved in 100 mL of methanol under magnetic stirring for 2 h, then sonicated for 30 min. This experimental procedure was adapted from the extraction method described by Aboul-Enein and Ali [14]. The solutions were then diluted 10-fold for quantitative analysis by LC-DAD or 100-fold for LC–MS analysis and then filtered through a 0.45-μm filter before injection.

#### 2.7.4. Purification from tablets

Pure vardenafil and homosildenafil were obtained by preparative chromatography from tablets. Five tablets were powdered and resuspended in 100 mL of methanol under magnetic stirring for 1 h, then sonicated for 30 min. The solution, after filtration and concentration to 5 mL by evaporation, was injected into the preparative HPLC apparatus (DeltaPrep Water 4000) equipped with a Hyperprep C18 column (250 mm × 20 mm). The fractions containing vardenafil or homosildenafil were evaporated under vacuum (Speed Vac) and analysed with NMR in a mixture of $CD_3CN$:$D_2O$ (80:20, v/v).

## 3. Results

### 3.1. Raman spectroscopy

The first step of our work was to analyse the original tablets from Eli Lilly Laboratories and the known excipients in order to obtain reference spectra and to identify specific absorption frequencies. We then analysed the seven imitation tablets.

A pharmaceutical dosage form such a tablet is composed of a coating and a core. The tablet coating of brand Cialis® consists of organic components (lactose monohydrate, hypromellose, and triacetin), inorganic components (titanium dioxide and talc) and a coloring component (iron yellow). The tablet core contains the active pharmaceutical ingredient (tadalafil) and various excipients such as fillers (lactose monohydrate and hydroxypropyl cellulose), flow agents (magnesium stearate), wetting agent (sodium lauryl sulfate) and disintegrants (sodium croscarmellose and microcrystalline cellulose).

Table 2
Raman bands detected for aromatic C=C bond vibrations for pure tadalafil and the eight formulations analysed

| Pure tadalafil | Formulations 1–7, mean ± S.D. | Formulation 8 |
|---|---|---|
| Wave number ($cm^{-1}$) | | |
| 3071 | 3072 ± 2 | 3089 |
| 1676 | 1677 ± 3 | 1701 |
| 1641 | 1644 ± 3 | 1621 |
| 1629 | 1630 ± 1 | 1603 |
| 1611 | 1612 ± 3 | 1586 |
| 1594 | 1596 ± 2 | |
| 1575 | 1575 ± 2 | |
| 1567 | 1568 ± 1 | |

All bands were detected for formulations 1–3 and 5. For formulations 4, 6 and 7, two, three and four bands, respectively, were not observed due to the poor resolution of the spectra.

#### 3.1.1. Analysis of pure tadalafil

Tadalafil (Fig. 1) is the sole constituent of the tablet that has aromatic unsaturated cycles giving characteristic responses around 1500–1700 $cm^{-1}$ and 3000–3100 $cm^{-1}$ (Fig. 2A). These bands correspond to unsaturated C=C bond vibrations and to unsaturated or aromatic C–H bond vibrations, respectively. These vibrations offer a tool to detect the presence or absence of tadalafil in the imitated tablets. The main peaks are indicated in Table 2.

#### 3.1.2. Analysis of excipients

The excipients contained in the Eli Lilly Cialis® were listed above. Raman spectra were recorded for all of them. In order to choose markers of the presence of these excipients in illegally manufactured tablets, we selected for each one strong and thin representative bands located in a part of the spectrum with few bands.

All cellulose derivatives (i.e. croscarmellose, cellulose, hydroxypropyl cellulose and microcrystalline cellulose) gave very similar spectra with large bands that are not characteristic of each compound. Talc led to characteristic bands at 363, 468 and 677 $cm^{-1}$, iron yellow at 271 $cm^{-1}$, titanium dioxide at 392, 514 and 636 $cm^{-1}$, triacetin at 895 and 1739 $cm^{-1}$, magnesium stearate at 1295, 1441 and 2728 $cm^{-1}$, lactose at 348, 358 and 475 $cm^{-1}$, and sodium lauryl sulfate at 1087 and 2732 $cm^{-1}$.

#### 3.1.3. Analysis of the genuine formulation of Cialis®

The Raman spectrum of the whole tablet of Eli Lilly Cialis® (formulation 1) is shown in Fig. 2B. The main signals observed are those of titanium dioxide (✶) and talc (⅄) present in the coating of the tablet. Iron yellow and triacetin could not be detected. In order to obtain more information, we eliminated the coating and recorded a new spectrum (Fig. 2C and D). The unsaturated

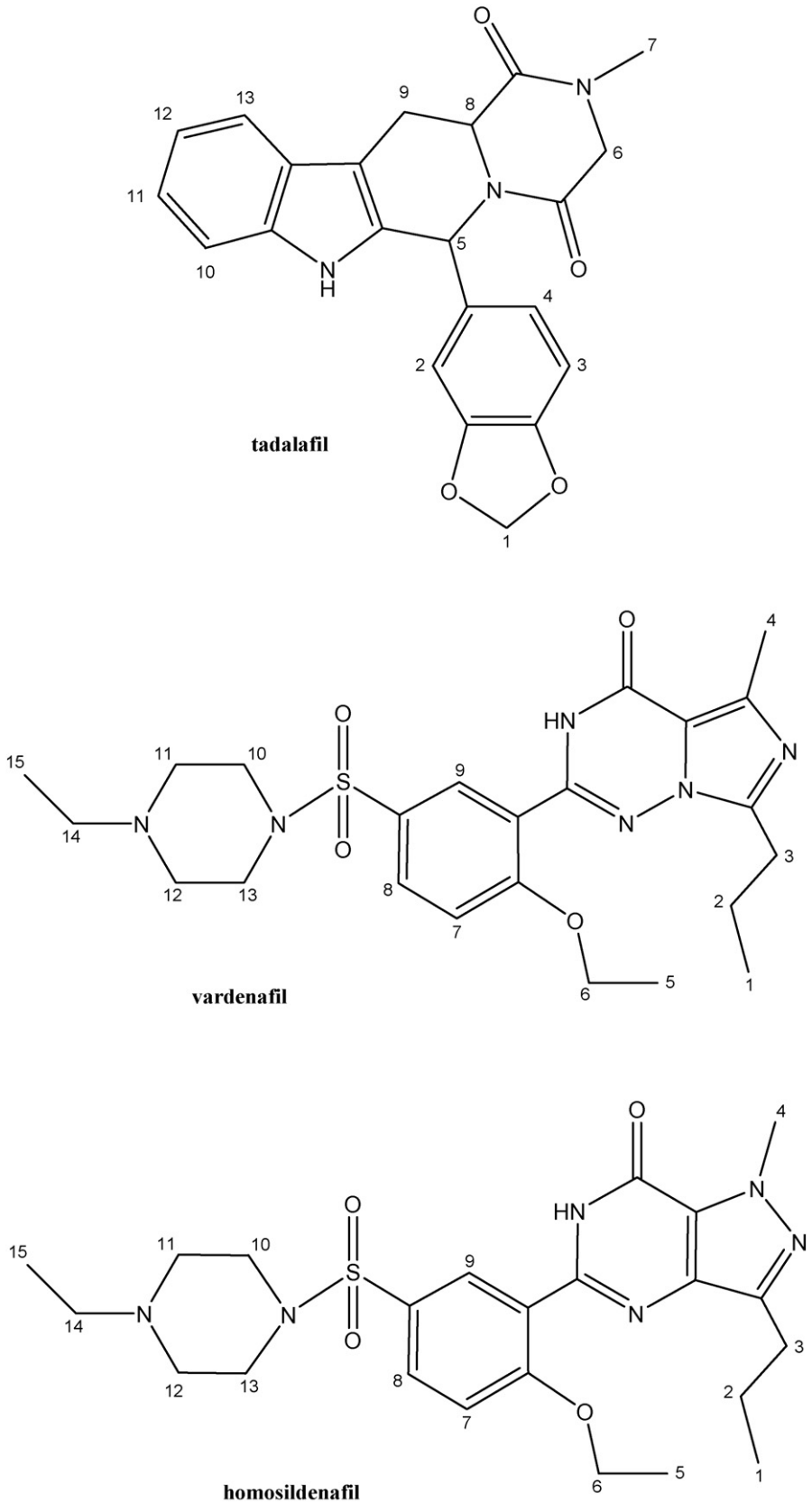


Fig. 1. Structures of tadalafil, vardenafil and homosildenafil.

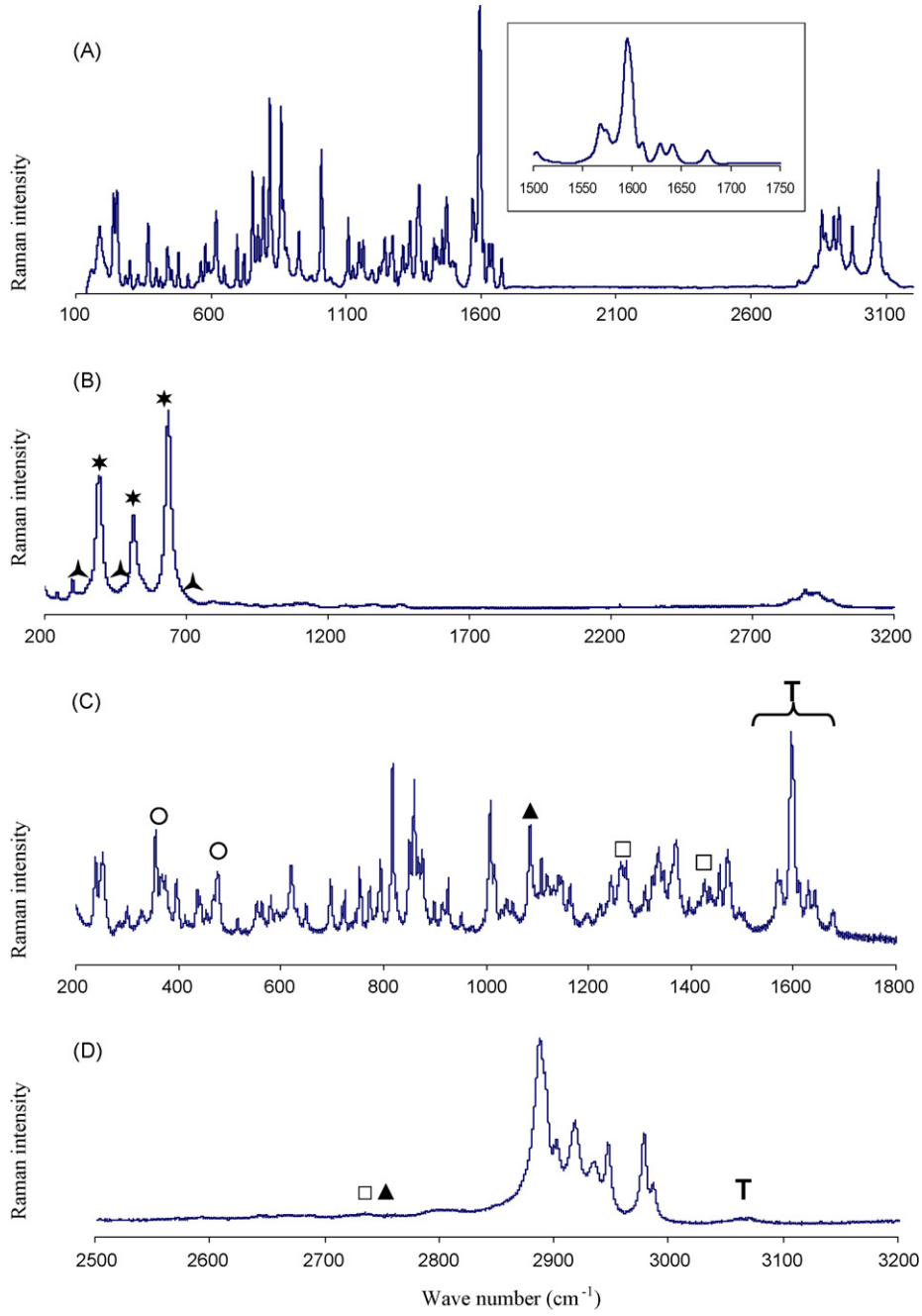


Fig. 2. Raman spectra of pure tadalafil (A) and genuine Eli Lilly Cialis®: whole tablet (B), uncoated tablet from 200 to 1800 $cm^{-1}$ (C), from 2500 to 3200 $cm^{-1}$ (D). ✶ $TiO_2$; ⅄ talc (as shoulders of $TiO_2$ bands); (◯) lactose; (▲) sodium lauryl sulfate; (□) magnesium stearate; (T) tadalafil.

structures of tadalafil (T) appeared clearly between 1568 and 1676 $cm^{-1}$ and around 3070 $cm^{-1}$. Tadalafil gives an intense response to the laser excitation at 632.8 nm, much stronger than that of the other components of the tablets, explaining why its bands are easily distinguished even if it represents only ≈5–10% of the tablet weight. In addition, most of the excipients used in the formulations do not contain aromatic, unsaturated or amide moieties. The peaks between 1550 and 1700 $cm^{-1}$ can thus be attributed to tadalafil by comparing the observed wave numbers with those of the pure substance. On the other hand, the region below 1550 $cm^{-1}$ was not considered for detecting tadalafil in the formulations as it is not specific since numerous bond vibrations (from aliphatic CH, CO and CN groups) give Raman signals.

We also detected the presence of the characteristic bands of magnesium stearate (□), lactose (◯) and sodium lauryl sulfate (▲). We were thus able to detect the active substance and five excipients (titanium dioxide, talc, magnesium stearate, lactose and sodium lauryl sulfate) by this technique.

### *3.1.4. Analysis of imitation formulations*

The Raman spectra were recorded with and without coating. The results on the active ingredient, i.e. tadalafil, are summarized in Table 2. In formulations 2, 3 and 5, tadalafil was clearly

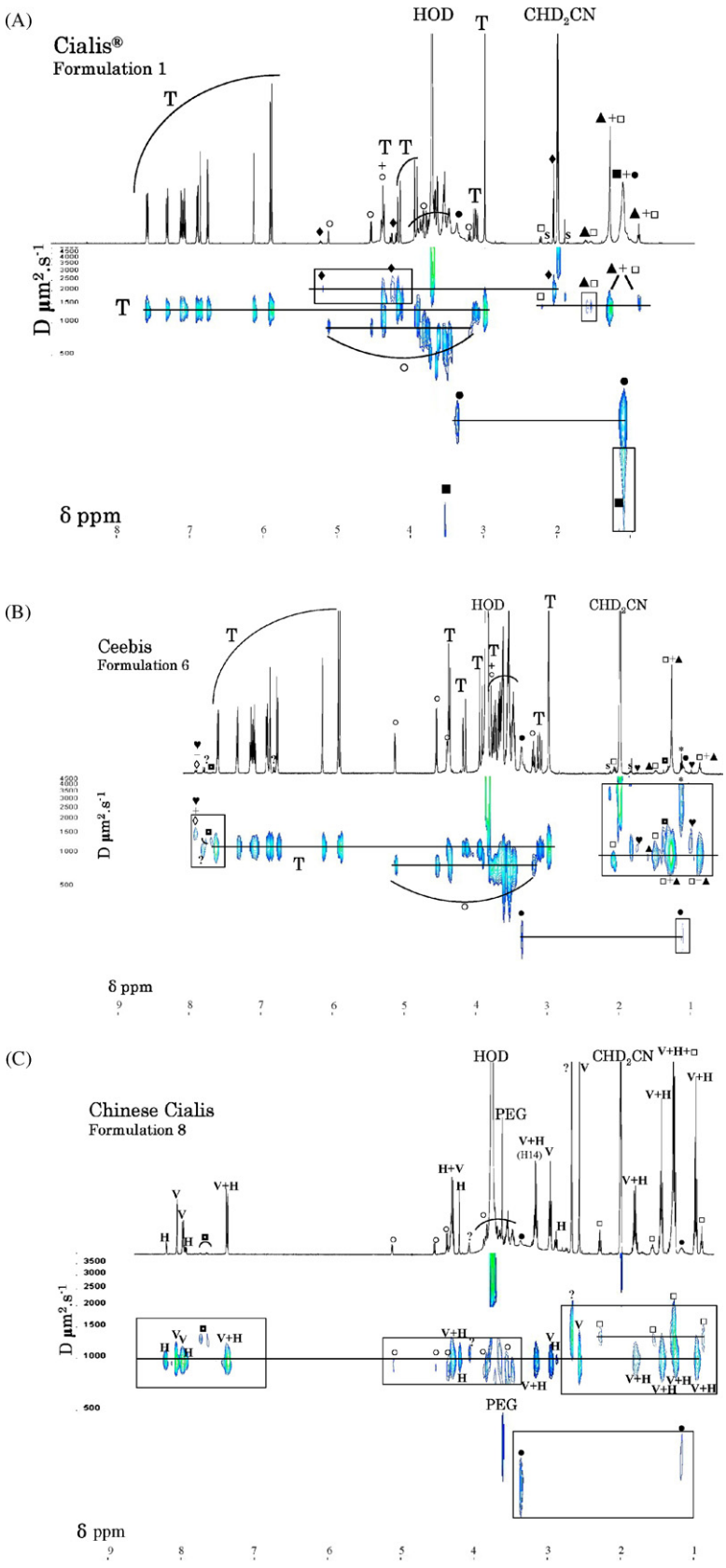


Fig. 3. DOSY NMR spectra in $CD_3CN$:$D_2O$ (80:20) of genuine Eli Lilly Cialis® (A), formulation 6 (B) and formulation 8 (C). (■) Hydroxypropylcellulose; (●) hypromellose; (◯) lactose; (□) magnesium stearate; (▲) sodium lauryl sulfate; (⧫) triacetin; (*) isopropanol; (🞏) diethylphthalate; (♥) propylparaben; (◊) methylparaben; (T) tadalafil; (V) vardenafil; (H) homosildenafil; (PEG) polyethylene glycol; s satellite; ? unknown.

identified from its eight characteristic bands around 1600 and 3000 $cm^{-1}$. For three formulations (4, 6 and 7), the identification was not obvious as some signals of the aromatic region were weak; nevertheless, the remaining signals have the same wave numbers as in pure tadalafil. Concerning the excipients present in the coating, titanium dioxide was unambiguously identified in all formulations; in contrast, talc was never detected. Raman spectroscopy allows the detection of magnesium stearate and sodium lauryl sulfate in four formulations (2–4, 6 and 2, 3, 6, 7, respectively) and lactose in five (formulations 2–4, 6 and 7). For the formulation 8, the only excipient detected was titanium dioxide. Furthermore, the Raman spectrum of 8 was atypical showing the presence of some aromatic moieties but the positions of the signals were not those of tadalafil (Table 2). This fact suggests the presence of other(s) active compound(s) in this formulation which is probably a fake formulation of Cialis containing no tadalafil. In order to confirm this assumption and to identify the active component(s) of this formulation, we then carried out NMR and LC–MS analyses.

### 3.2. Conventional and 2D DOSY $^1$H NMR analysis

All formulations of tadalafil were analysed with 2D DOSY $^1$H NMR. 2D DOSY spectra of genuine Cialis® and formulations 6 and 8 along with their corresponding 1D spectra are presented in Fig. 3. The peaks at 3.68 and 1.99 ppm correspond to the residual signals of water and acetonitrile, respectively. All the peaks of tadalafil are lined up and the value of the self-diffusion coefficient was measured for each peak; an average self-diffusion coefficient was determined for each formulation (Table 3). Several excipients could also be observed. All the formulations contain the lubricant magnesium stearate (□) that leads to four signals located at 0.89 (t), 1.28 (broad s), 1.52 (quin) and 2.13 (t) ppm. Lactose peaks (◯) were identified in all formulations at 3.20 (t), 3.4–3.9 (m), 4.36 (d), 4.54 (d) and 5.12 (d) ppm. Except formulation 2, all the pharmaceutical preparations also contain a cellulose derivative as coating agent, giving three aligned signals at 1.11 (broad s), 3.37 (broad s) and 3.53 (broad s) ppm that is known to be hypromellose (●) (hydroxypropylmethyl cellulose) for the brand formulation 1 but is unknown for the other formulations. Another cellulose derivative with a higher molecular weight leads to broad signals at 1.1 ppm (s) and between 3.3 and 4.0 ppm; as for the first cellulose derivative, it is known to be hydroxypropylcellulose (■) for the brand formulation 1 but is unknown for the other formulations where it is observed (2 and 5). Signals of sodium lauryl sulfate (▲) (0.89 (t), 1.28 (broad s), 1.61 (quin) and 3.90 (t) ppm), a wetting agent, are present in the brand formulation 1 and in formulations 2, 3 and 5–7. The brand formulation 1 and the formulation 5 contain the hydrophilic plasticizer triacetin (♦) (glyceryl triacetate; 5.22 (m), 4.23 (AB d), 2.06 (s) and 2.05 (s) ppm). The antimicrobial preservatives methylparaben (◊) (methyl-4-hydroxybenzoate, 3.83 (s), 7.88 (d) ppm) and propylparaben (♥) (propyl-4-hydroxybenzoate; 0.99 (t), 1.74 (app q), 4.20 (t) and 7.88 (d) ppm) were found in formulations 4, 6 and 7. Diethylphthalate (▭) (1.32 (t), 4.33 (q) and 7.68 (m) ppm), a plasticizer, could be detected in formulations 6–8. Propylene glycol, an anticaking agent, was found in formulations 2–4 (1.07 (d), 3.39 (AB d) and 3.74 (m) ppm). The presence of isopropanol, a coating solvent, is detected only by the signal of its methyl group at 1.1 ppm (d); this excipient was detected in formulations 4, 6 and 7 but its amount was subjected to a great inter-tablet variability even from the same batch. Only three formulations (2, 7 and 8) include polyethylene glycol as a lubricant agent (3.61 ppm (s)). Formulation 2 contains signals of citrate giving a characteristic AB system pattern at 2.53 ppm.

Classical $^1$H NMR was then used to establish the structure of the active pharmaceutical ingredient(s) present in the Chinese fake formulation of Cialis (8) that had an atypical Raman spectrum. Fig. 3C shows the $^1$H NMR spectrum of the Chinese formulation, which is very different from that of the original Cialis® analysed in the same conditions (Fig. 3A). Tadalafil is not present in the Chinese formulation. Indeed, only the signals of excipients were observed in the 2.4–0.8 ppm spectral

Table 3
Self-diffusion coefficients[a] ($\mu m^2\ s^{-1}$) measured in the formulations studied with DOSY $^1$H NMR

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Tadalafil | 1168 ± 48 | 1186 ± 15 | 1219 ± 36 | 1224 ± 23 | 1191 ± 18 | 1133 ± 16 | 1339 ± 60 | – |
| Homosildenafil | – | – | – | – | – | – | – | 984 ± 14 |
| Vardenafil | – | – | – | – | – | – | – | 971 ± 8 |
| Lactose | 816 ± 20 | 831 ± 20 | 858 ± 46 | 869 ± 13 | 797 ± 63 | 796 ± 6 | 917 ± 63 | 860 ± 7 |
| Hypromellose | ∼120 | | ∼228 | ∼185 | ∼153 | 189 | ∼353 | ∼195 |
| Hydroxypropylcellulose | ≤120 | 172 | – | – | ≤150 | – | – | – |
| Triacetin | 1619 ± 62 | | – | – | 1673 | – | – | – |
| Magnesium stearate | 1245 ± 96 | 1218 ± 30 | 1335 ± 26 | 1105 ± 32 | 1300 ± 79 | 947 ± 42 | 1457 ± 47 | 1320 ± 46 |
| Sodium lauryl sulfate | 1233 ± 119 | 1219 | 1282 ± 645 | – | 1288 ± 60 | 952 ± 35 | 1414 ± 35 | – |
| Propylene glycol | – | 2062 ± 91 | nm[b] | 3822 | – | – | – | – |
| Isopropanol | – | | – | 3198 | – | 2701 | 2886 | – |
| Diethylphthalate | – | | – | – | – | 1378 | 1625 | 1868 |
| Citrate | – | 735 | – | – | – | – | – | – |
| Methylparaben | – | | – | 1905 | – | 1670 | 1467 | – |
| Propylparaben | – | | – | 1979 | – | nm[b] | 1326 | – |
| Polyethylene glycol | – | 367 | – | – | – | – | 476 | 315 |

[a] The value of the self-diffusion coefficient was measured for each peak, and an average self-diffusion coefficient was determined for each formulation.
[b] Non-measurable.

Table 4
$^1$H NMR data (500 MHz) of pure tadalafil, vardenafil and homosildenafil dissolved in $CD_3CN$:$D_2O$ (80:20)

| Tadalafil | | | Vardenafil | | | Homosildenafil | | |
|---|---|---|---|---|---|---|---|---|
| δ (ppm) | Number of protons, multiplicity[a] (J, Hz) | Position[b] | δ (ppm) | Number of protons, multiplicity[a] (J, Hz) | Position[b] | δ (ppm) | Number of protons, multiplicity[a] (J, Hz) | Position[b] |
| 7.57 | 1H, d (8.0) | 13 | 8.01 | 1H, d (2.5) | 9 | 8.20 | 1H, d (2.5) | 9 |
| 7.30 | 1H, d (8.0) | 10 | 7.91 | 1H, dd (8.9; 2.5) | 8 | 7.88 | 1H, dd (9.5; 2.5) | 8 |
| 7.11 | 1H, m | 11 | 7.32 | 1H, d (8.9) | 7 | 7.34 | 1H, d (9.5) | 7 |
| 7.07 | 1H, m | 12 | 4.26 | 2H, q (6.9) | 6 | 4.30 | 2H, q (7.0) | 6 |
| 6.89 | 1H, dd (8.1; 2.0) | 4 | | | | 4.19 | 3H, s | 4 |
| 6.85 | 1H, d (2.0) | 2 | 3.01 | 4H, broad s | 10/13 | 3.03 | 4H, broad s | 10/13 |
| 6.75 | 1H, d (8.1) | 3 | 2.91 | 2H, t (7.4) | 3 | 2.87 | 2H, t (7.2) | 3 |
| 6.12 | 1H, s | 5 | 2.53 | 3H, s | 4 | | | |
| 5.87 | 1H, d (1.0) | 1 | 2.51 | 4H, broad s | 11/12 | 2.53 | 4H, broad s | 11/12 |
| 5.90 | 1H, d (1.0) | 1′ | 2.38 | 2H, q (7.2) | 14 | 2.40 | 2H, q (7.0) | 14 |
| 4.36 | 1H, dd (11.5; 3.8) | 8 | 1.77 | 2H, sext (7.4) | 2 | 1.79 | 2H, sext (7.2) | 2 |
| 4.13 | 1H, dd (17.5; 1.2) | 6 | 1.42 | 3H, t (6.9) | 5 | 1.45 | 3H, t (7.0) | 5 |
| 3.91 | 1H, d (17.5) | 6′ | 0.99 | 3H, t (7.2) | 15 | 0.99 | 3H, t (7.0) | 15 |
| 3.63 | 1H, dd (15.8; 3.8) | 9 | 0.95 | 3H, t (7.4) | 1 | 0.97 | 3H, t (7.2) | 1 |
| 3.10 | 1H, ddd (15.8; 11.5; 1.2) | 9′ | | | | | | |
| 2.97 | 3H, s | 7 | | | | | | |

[a] d: doublet; dd: doublet of doublet; ddd: doublet of doublet of doublet; s: singlet; m: multiplet; t: triplet; q: quadruplet; sext: sextuplet.
[b] Fig. 1 shows the numbering of the hydrogen atoms.

region of the brand Cialis® formulation 1, whereas signals of alkyl groups from active(s) were observed in the Chinese formulation 8. Moreover, the intensity of the peaks located in the aromatic region of the spectrum demonstrates that formulation 8 contains a mixture of two active pharmaceutical ingredients.

After chromatographic purification of these two active ingredients, their NMR spectra were recorded and the compounds were thus identified as vardenafil and homosildenafil (Fig. 1). Table 4 reports the NMR spectral data of pure tadalafil, vardenafil and homosildenafil recorded in a mixture of $CD_3CN$:$D_2O$ (80:20).

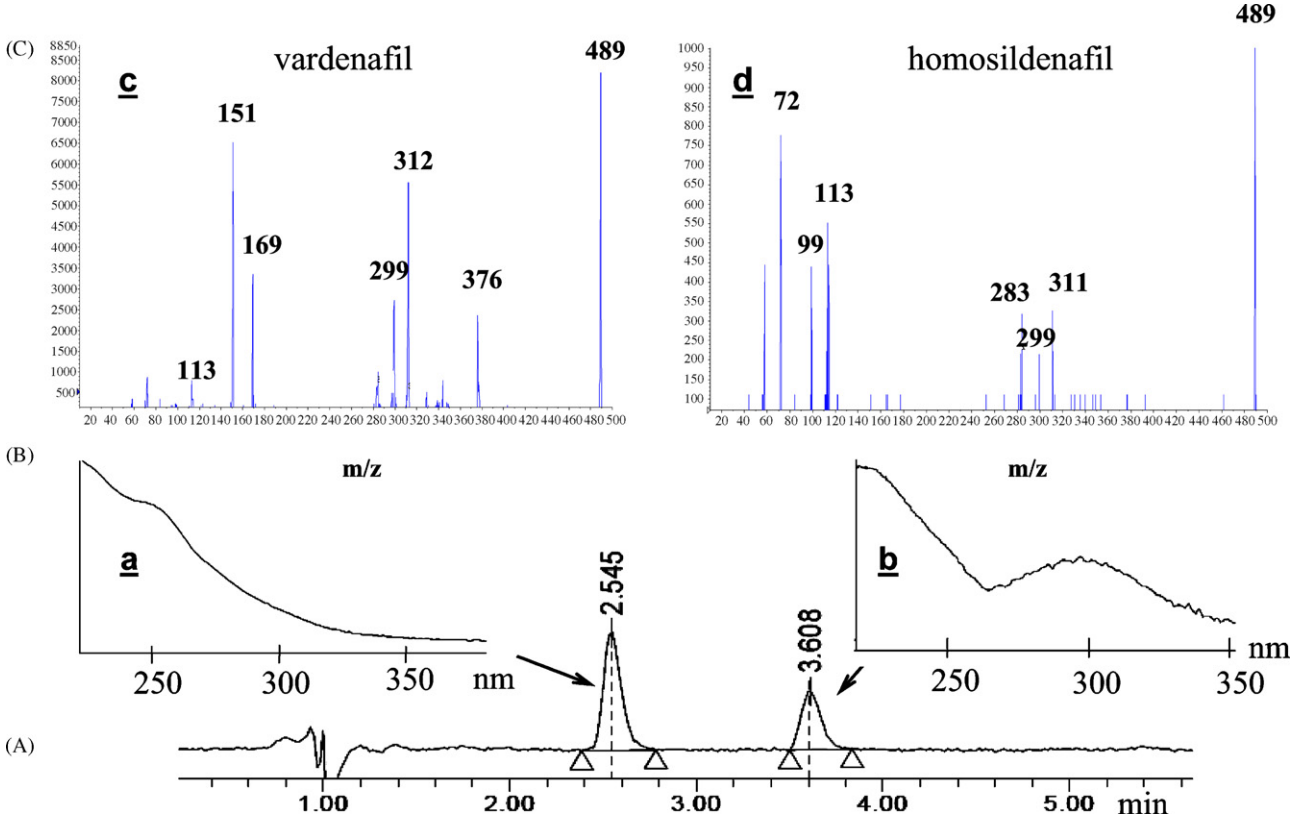


Fig. 4. (A) Chromatogram obtained at λ = 225 nm of a solution of the Chinese formulation 8; (B) corresponding UV spectra of vardenafil (a) and homosildenafil (b); (C) MS–MS fragmentation pattern of parent ion *m/z* 489 for vardenafil (c) and homosildenafil (d).

The $^1$H NMR resonances were assigned by 2D NMR experiments (gCOSY, gHSQC, gHMBC) and comparison to published NMR data on tadalafil, sildenafil and their analogues [15–17].

To confirm the identification of these two active ingredients, we employed LC–MS–MS. Fig. 4A shows the chromatogram ($\lambda = 225$ nm) of a solution of formulation 8 in methanol. Two main peaks were detected, one at 2.55 min and the other at 3.61 min. The first eluted compound presents a UV spectrum typical of vardenafil (Fig. 4Ba), and the UV spectrum of the second compound eluted is typical of sildenafil or its analogues (homosildenafil or hydroxyhomosildenafil) (Fig. 4Bb) [18]. The same value *m*/*z* 489 is measured for the pseudo molecular ion $[M+H]^+$ of these two compounds; however the LC–MS–MS spectra (Fig. 4C) allowed an unambiguous structural elucidation. Indeed, vardenafil produced molecular ion and some fragment ions at the same *m*/*z* as homosildenafil (e.g. ions at 113, 299), but the fragment ions at *m*/*z* 169 and 151 are characteristic product ions from vardenafil [18].

One can notice on the $^1$H NMR spectrum of the Chinese formulation 8 (Fig. 3C) the downfield shift for the $H_{14}$ signals of homosildenafil and vardenafil that have a $\delta$ of 3.15 ppm whereas the $\delta$ of these two hydrogen atoms in the pure compounds after isolation are around 2.4 ppm (Table 4), which is in accordance with literature [16]. A protonation/deprotonation of the nitrogen bearing the ethyl group in the piperazine ring (Fig. 1) could induce this chemical shift variation that is function of the pH of the aqueous phase of the mixture of solvents ($CD_3CN$:$D_2O$) used for the NMR analysis. The amounts of vardenafil and homosildenafil present in formulation 8 were found to be $34.3 \pm 0.2$ mg and $7.2 \pm 0.1$ mg/tablet, respectively.

### 3.3. LC-DAD analysis

The tadalafil content of each formulation, measured by HPLC, is reported in Table 5. Seven out of the eight commercial formulations of tadalafil (1–7) contain the active ingredient within $100 \pm 5\%$ of stated concentration. The relative standard deviations (R.S.D.) are correct as they are <5% for all the tablets except one and even <2% for five formulations. A greater R.S.D. (6.7%) was observed for formulation 4. Formulation 8 does not contain tadalafil.

Table 5
Amount of tadalafil found in eight commercial formulations ($n = 3$)

| Formulations[a] | % | S.D. | R.S.D. (%) |
|---|---|---|---|
| (1) Cialis® | 98.9 | 1.7 | 1.7 |
| (2) Tadafort | 98.9 | 0.4 | 0.4 |
| (3) Tadalafil tablets | 98.3 | 1.4 | 1.4 |
| (4) Alegra | 103.6 | 7.0 | 6.7 |
| (5) Forzest | 99.3 | 0.8 | 0.8 |
| (6) Ceebis yellow | 99.9 | 0.2 | 0.2 |
| (7) Ceebis red | 99.3 | 4.2 | 4.2 |
| (8) Chinese Cialis | 0[b] | – | – |

[a] The stated concentration is 20 mg of tadalafil per tablet.
[b] This formulation contains $34.3 \pm 0.2$ mg of vardenafil and $7.2 \pm 0.1$ mg of homosildenafil per tablet.

## 4. Discussion

In this study, Raman spectroscopy was employed for the screening of imitation or fake pharmaceutical formulations of Cialis. Raman spectra allow the qualitative identification of active pharmaceutical ingredient and some excipients used to manufacture the product. However, the interest of Raman spectroscopy was limited by (i) fluorescence phenomena induced by the coating and (ii) the small volume sampled by the weak spot size of the laser beam (1–5 μm). In our experimental conditions, identification of tadalafil was possible but only partial information was obtained for the identification of excipients. These experimental problems should probably be overcome firstly by using another excitation wavelength (1064 nm) to avoid fluorescence phenomena and secondly by the use of a probe yielding a spot size of around 3 mm for the incident excitation radiation, which should provide better representative sampling.

2D DOSY $^1$H NMR spectra clearly show similarities and differences in the composition of pharmaceutical preparations, thus giving a chemical fingerprint of the formulation and a signature of the manufacturer. This spectral signature includes not only the active pharmaceutical ingredient(s) but also the excipients. The analytical methods that provide most information on excipient composition are solid-state methods such as Raman spectroscopy [10,11,19], near infrared spectroscopy [4,5] or X-ray diffraction [7], whereas chromatography allows an accurate identification of active pharmaceutical ingredients but does not give information on excipients. DOSY NMR is thus a global method that permits to consider the drug preparation as a whole.

2D DOSY NMR relies on differences in translation diffusion as a means to separate components in a solution mixture. The diffusion coefficient generally decreases with increasing molecular weight. The differences in the values of the diffusion coefficients (Table 3) are due to the various compositions of the formulations that resulted in media of different viscosity. In addition to the active tadalafil, several excipients could be observed depending on the formulation studied. In all the formulations, the tablet diluent lactose, the lubricant magnesium stearate and the coating agent hypromellose (except in formulation 2) were detected. Other excipients are function of the formulations studied.

Table 6 shows a comparison on information obtained by various analytical methods on fake drug composition and highlights the interest of DOSY NMR for a global qualitative analysis of pharmaceutical formulations. Limitations for DOSY NMR are not restrictive; all compounds containing protons which are soluble in the NMR solvent are detected. DOSY NMR is thus a powerful analytical method which produces a chemical fingerprint of different types of pharmaceutical samples, and so provides precise information on the samples analysed. Not only it can distinguish between genuine and imitated tablets, but it is also helpful in determining the relationships between different samples and so assists in the investigation of the sources of these drugs. Nevertheless, when a fake formulation is detected, classical analytical methods such as $^1$H NMR or MS are necessary for an unambiguous structural determination of adulterated active pharmaceutical ingredients.

Table 6
Comparison between information on the composition of tablets provided by different analytical methods for fake drug analysis

| | Maurin et al. [7] | Vredenbregt et al. [5] | de Veij et al. [10] | Hall et al. [19] | | This study | |
|---|---|---|---|---|---|---|---|
| Drug | PDE-5 inhibitor | PDE-5 inhibitor | Antimalarial | Antimalarial | | PDE-5 inhibitor | |
| Expected active pharmaceutical ingredient detected | Sildenafil | Sildenafil | Artesunate | Artesunate | | Tadalafil | |
| Analytical method | X-ray diffraction | NIR[a] spectroscopy | Raman | Raman | LC–MS | Raman | DOSY NMR |
| Data analysis | Diffraction pattern | PCA[b] | PCA[b] | | Multivariate clustering | Spectral pattern | Spectral pattern |
| Other active pharmaceutical ingredient detected | | Clomifene citrate, amphetamine, yohimbine, dipyrone, quinine | Paracetamol | Paracetamol | Erythromycins, paracetamol | | Vardenafil, homosildenafil |
| Other pharmaceutical ingredient detected | Microcristalline cellulose, anhydrous calcium hydrogenophosphate | | $TiO_2$, $CaCO_3$, starch, unknown | $CaCO_3$, starch, starch-related excipient | Starch-related excipient | $TiO_2$, talc, lactose, magnesium stearate, sodium lauryl sulfate | Lactose, hypromellose, hydroxypropylcellulose, triacetin, magnesium stearate, sodium lauryl sulfate, propylene glycol, isopropanol, diethylphthalate, citrate, methylparaben, propylparaben, polyethylene glycol |

[a] NIR: near infrared.
[b] PCA: principal component analysis.

The two active ingredients found in the fake Chinese formulation 8 were vardenafil and homosildenafil. As tadalafil, vardenafil is a PDE-5 inhibitor, and indications and contraindications for this medicine are close to those of other PDE-5 inhibitors. The brand formulation of vardenafil (Levitra®) is available in 2.5, 5, 10, and 20 mg doses. The normal starting dose is 10 mg. 34.3 mg of vardenafil were found in the Chinese formulation, which can induce an overdosage in patients. Moreover, in the case of vardenafil, there was no evidence of a dose-dependent improvement in efficacy beyond the 10 mg dose but problems of drug–drug interactions may occur. For example, vardenafil is contraindicated with nitrates as it potentiates their hypotensive effect. Also vardenafil (10 or 20 mg) when concomitantly administered with α-blockers (such as terazosin and tamsulosin) and with nifidipine to healthy volunteers resulted in some subjects experiencing hypotension [20]. Concerning homosildenafil, to the best of our knowledge, toxicological data are not known or not available. Thus, due to possible side-effects or drug–drug interactions, a significant risk is faced by consumers who purchase drugs marketed for erectile dysfunction via the internet.

## 5. Conclusion

The results reported herein demonstrated that 2D DOSY $^1$H NMR, which allows detecting active pharmaceutical ingredients but also most excipients, is a powerful tool for screening of pharmaceutical formulations and checking the quality of drugs. The results obtained in this study from the analysis of eight formulations of tadalafil by complementary analytical methods (Raman spectroscopy, $^1$H NMR, 2D DOSY $^1$H NMR and LC–MS) show that the quality of products manufactured in India and Syria is correct in contrast to a Chinese formulation that is adulterated with active pharmaceutical ingredients.

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