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The usefulness of 2D DOSY and 3D DOSY-COSY ^{1}H NMR for mixture analysis: Application to genuine and fake formulations of sildenafil (Viagra)

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Two-dimensional diffusion ordered spectroscopy (DOSY) ^{1}H NMR is proposed to analyze drugs that are complex mixtures in order to discriminate genuine from fake formulations. The method was applied to the analysis of 17 formulations of sildenafil, one being genuine Viagra and the others illegally manufactured formulations of this drug coming from India, Syria and China. It enabled (i) distinguishing imitations or counterfeit from the authentic formulation, (ii) detecting the presence of sildenafil or adulterants, (iii) gaining information on the formulation process by detection of various excipients, thus giving a precise and global 'signature' of the manufacturer. Even though some samples are slightly overdosed, the quality of products manufactured in India and Syria was better than that of Chinese formulations which were adulterated with vardenafil and homosildenafil. This study also presents a three-dimensional DOSY-COSY ^{1}H NMR experiment that provides both virtual separation and structural information.

Supporting information may be found in the online version of this article.

Keywords: NMR; ^{1}H; 2D DOSY NMR; 3D DOSY-COSY NMR; counterfeit drugs; PDE-5 inhibitors; sildenafil; vardenafil; homosildenafil; excipients

Introduction

Nowadays, all over the world counterfeit and illegally manufactured drugs pose a threat to consumer safety and confidence in the area of drug security. According to the World Health Organization (WHO), a counterfeit medicine is one which is deliberately and fraudulently mislabeled with respect to identity and/or source.[1] This includes products with correct ingredients, wrong ingredients, without active ingredients, with incorrect quantity of active ingredients or with fake packaging.

Since the original marketing authorization of Viagra (sildenafil citrate) in 1998 by Pfizer Laboratories as first treatment for erectile dysfunction which functions by inhibiting the phosphodiesterase type 5 enzyme (PDE-5), this drug is among the most counterfeited or imitated. Imitations come generally from Asia (India and China most often) which do not recognize the European and American patent laws so that products manufactured legally in these such countries are illegal in Europe, USA and other countries. However, people can easily purchase 'Viagra' tablets on the numerous websites that have appeared over the Internet with no indication of their origin.

Differentiation of counterfeit *versus* genuine drugs is more and more difficult as counterfeiters use more sophisticated methods to falsify drugs, and a complex process including complementary analytical techniques is now often required for identification. So far, numerous analytical methods for the determination of sildenafil in pharmaceutical formulations have been proposed including high-performance liquid chromatography (HPLC) with UV[2–6] or mass spectrometry[4,6,7] (MS) detection, capillary gas chromatography,[8] spectrophotometric methods,[9] infrared spectroscopy[10] (IR), polymer membrane sensors,[11] micellar electrokinetic capillary chromatography,[12] thin-layer chromatography[4] (TLC) and high-performance TLC.[13,14] All these methods focus on the active pharmaceutical ingredient sildenafil and do not provide any other information on the pharmaceutical formulation.

Recently, three studies to specifically detect counterfeit Viagra were published. Vredenbregt *et al*.[15] described a near-infrared (NIR) spectroscopy method used to check the homogeneity of a batch, distinguish counterfeits and imitations from authentic Viagra, and screen for the presence of sildenafil citrate in 103 samples with a diversity of appearance, chemical composition and origin. Maurin *et al*.[16] screened seven Viagra samples with a X-ray powder diffraction method which correctly predicts the presence or absence of sildenafil and/or particular excipients. The advantages of these methods are that they are fast and directly applied to intact tablets. However, they are only qualitative and no indication is gained on the excipients. More informative is the very recent study of de Veij *et al*.[17] who analyzed 19 Viagra tablets with Raman spectroscopy and differentiated genuine from counterfeit drugs on the basis of their content of inactive compounds as all

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contained the active ingredient. All these qualitative methods are useful analytical tools for quality assessment of drugs and can be proposed for detecting counterfeits or imitations of Viagra.

This paper describes the application of 2D diffusion ordered spectroscopy ^{1}H nuclear magnetic resonance (2D DOSY ^{1}H NMR)[18] as a method to authenticate commercial products and identify differences in drug composition. It requires sample pretreatment but has the advantages of being global as it gives accurate information on the chemical structures of all organic components. Moreover, the method is nonselective and requires no prior knowledge of the structures of the various components present in the mixture. The original Viagra tablet from Pfizer Laboratories and 16 illegally manufactured formulations of Viagra were used as examples. This study is also the first application of a 3D DOSY-COSY experiment to a pharmaceutical formulation.

Experimental

Chemicals

Pure sildenafil, designated chemically as 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*pyrazolo[4,3-*d*]pyrimidin-5-yl)-4-ethoxyphenyl]sulfonyl]-4-methylpiperazine citrate, was obtained from Molekula Laboratories (La Tour du Pin, France). The excipients were gifts from the Galenic Laboratory of the Faculty of Pharmacy (University of Toulouse, France).

Standards of pure vardenafil and homosildenafil were obtained by preparative chromatography from counterfeit tablets of Cialis, as described in a previous study.[19]

Commercial formulations of sildenafil

Seventeen sildenafil commercial formulations were analyzed, one being the brand formulation from Pfizer (Viagra), the others being counterfeit or imitation drugs. They were purchased over the Internet, except those coming from Syria (formulations **12**–**14**) and the Chinese formulation **17,** which were bought in the country. The different web sites from where the drugs were purchased did not mention the origin of the manufacturers. It happened that 10 came from India and one from China. The list of the tablets analyzed is given in Table 1.

The composition of the brand Viagra is as follows: (i) core: sidenafil, microcrystalline cellulose, sodium hydrogenophosphate, sodium croscarmellose, magnesium stearate; (ii) film coating: hypromellose, titanium dioxide, lactose, triacetine and FD & C Blue #2 aluminum lake.

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

Sample preparations

NMR analysis

The tablet was powdered and dissolved in 5 ml of D_2O under magnetic stirring during 15 min and then sonicated for 10 min. The suspension was then centrifuged (10 min, 3000 rpm) and the supernatant analyzed. The resulting pHs of solutions (uncorrected from isotopic effect) were 4.5 ± 0.2 for formulations **1**–**15** whereas the pH was slightly higher for formulations **16** (pH = 5.8) and **17** (pH = 5.7).

For quantitative analysis, tablets were powdered, dissolved in 100 ml of methanol under magnetic stirring during 45 min and then sonicated for 10 min. An aliquot of 2 ml was evaporated and redissolved in 2 ml of a 2×10^{-3} mol l^{-1} solution of maleic acid in MeOH-d_4 immediately before NMR analysis.

Chromatographic analysis

The tablets dosed at 50 mg were powdered, dissolved in 100 ml of methanol under magnetic stirring during 45 min and sonicated for 10 min. Those dosed at 100 mg were treated in the same conditions except that the final volume was 200 ml. The solutions were then diluted 100-fold and filtered through a 0.45-µm film before injection. Each solution was injected twice in the chromatographic apparatus.

Table 1. Formulations of sildenafil analyzed in this study

	Formulation name	Batch number	Expiry date	Manufacturer's name	Country of manufacture	Color	Shape/ inscription
1	Viagra 50 mg	5160307F	07/2010	Pfizer	France	Blue	Diamond/VGR 50
2	Progra 100 mg	AR3061	06/2006	Cipla	India	Blue	Ovale/
3	Silagra 100 mg	S5-21	09/2007	Cipla	India	Blue	Ovale/
4	Silagra 50 mg	DH5415	08/2008	Cipla	India	Blue	Ovale/
5	Kamagra 100 mg	MLNOAD/219ABN1205E	04/2008	Ajanta Pharma Limited	India	Blue	Diamond/KGR 100
6	Manly 100 mg	DH5094	04/2010	Cooper Pharma	India	Blue	Diamond/100
7	Sildenafil soft tabs	930804	11/2006	Unknown	India	White	Diamond/100
8	Caverta 100 mg	1534370	05/2007	Ranbaxy	India	Red	Diamond/100
9	Penegra 100 mg	ME 1133	12/2006	Zydus Alidac	India	Pink	Diamond/100
10	Sildenafil citrate tablets no 1	880805	05/2007	Unknown	India	Blue	Diamond/100
11	Sildenafil citrate tablets no 2	6002	12/2006	Unknown	India	Blue	Diamond/
12	Extra	51570	08/2007	Alpha	Syria	Blue	Diamond/
13	Viagra 100 mg	5160307F	07/2010	Pfizer	Syria	Blue	Diamond/VGR 100
14	Vega 50 mg	11065	09/2007	Asia Pharmaceutical industries	Syria	Blue	Diamond/50
15	Vega 100 mg	2315	11/2008	Marketed by MTM EXPORTS	Unknown	Blue	Diamond/100
16	Sex power	Unknown	Unknown	Unknown	China	Blue	Diamond/VAG
17	King IX boost	Unknown	03/2009	HONGKONG TIANLONG	China	Blue	Diamond/VAG

^{1}H NMR, 2D DOSY ^{1}H NMR and 3D DOSY-COSY ^{1}H NMR

The ^{1}H NMR experiments were performed on a Bruker AVANCE 500 spectrometer operating at 500.13 MHz equipped with a 5-mm proton cryoprobe at 298 K on 600 µl samples. All chemical shifts (δ) were referred to an internal trimethylsilylpropane sulfonic acid (TMPS) reference. Typical acquisition parameters for the ^{1}H NMR experiments were as follows: acquisition time 1.64 s, spectral width 10 000 Hz, pulse width 3 µs (flip angle $\approx$35°), relaxation delay 2 s and number of scans 128. For quantitative analysis, relaxation delay was lengthened to 3 s. Three tablets from each formulation were analyzed in triplicate.

For 2D DOSY ^{1}H NMR, stimulated echo bipolar gradient pulse experiments[20] were used with a pulse delay of 3 ms after each gradient, a pulse field gradient length of 1 ms and a diffusion delay of 100 ms. Sequence parameters were adapted in order to have the intensity of the H_9 NMR signal of sildenafil strongly decreased (at least 50 times) at 95% of the full gradient strength. Forty experiments were recorded with gradient intensity linearly sampled from 5 to 95%. The gradient system had been calibrated to 46.25 G cm^{-1} at maximum intensity. All data were processed using the Gifa 5.2 software with the inverse Laplace transform method using the maximum entropy (MaxEnt) algorithm.[21]

The processing parameters were 2048 points along the Laplace spectrum diffusion axis and 20 000 MaxEnt iterations. The inverse Laplace transform was computed only on the columns presenting a signal 32 times greater than the noise level of the experiment. DOSY spectra are presented with chemical shifts on the horizontal axis and diffusion coefficients on the vertical axis (expressed in µm^2 s^{-1}).

For 3D DOSY-COSY acquisition, a variant of 3D DQF-COSY iDOSY[22] including water presaturation pulse was recorded on formulation **5**. In the COSY dimension, 8182 × 104 data points were used, corresponding to 0.68 s for acquisition time and 8 ms for the other dimension, with a spectral window of 12 ppm and the transmitter offset frequency located at 4.704 ppm. The sine-shaped gradients for DQF selection, of 4.62 and 9.25 G cm^{-1} strength, were applied for 1 ms. Twenty eight gradient steps were used for the diffusion dimension from 5 to 95%, where 46.25 G cm^{-1} was the maximum gradient intensity. The diffusion time was 60 ms, the gradient pulse length 6.8 ms and the recovery delay 3 ms. Water presaturation was applied during the relaxation delay of 1 s, and 8 scans were used (half-day experiment). The spectrum was processed in COSY dimension with Fourier transform in magnitude mode before applying the inverse Laplace transform method (MaxEnt algorithm) to obtain the DOSY dimension. The processing parameters were 192 points along the diffusion axis and 20 000 MaxEnt iterations. The algorithm was computed only on the columns with a signal-to-noise ratio above 32. Two-dimensional COSY and DOSY experiments were acquired with DQF-COSY iDOSY parameters. The experiments were processed using the NPK software[23] and analyzed with NMRnotebook package.[24]

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 × 3 mm i.d., 3 µm particle size; Phenomenex, UK). The column temperature was 30 °C. The mobile phase consisted of a volumetric mixture (50 : 50) 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. A detection wavelength of 225 nm was chosen, allowing the detection not only of sildenafil, which has another λ_{max} around 295 nm, but also of all sildenafil analogs or other PDE-5 inhibitors.

For quantitative analysis, a calibration curve was constructed from the analysis of four solutions containing pure sildenafil in a concentration range 0.0012 to 0.05 mg ml^{-1}. Each standard solution was injected in triplicate in the chromatographic system. The linearity ($R^2 > 0.998$) was evaluated by least-squares linear regression analysis.

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 interface, was used for detection. Both were controlled by the Agilent Analyst software (version 1.4). HPLC conditions were the same as those described above for LC-DAD analysis.

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

Results

Two-dimensional DOSY ^{1}H NMR analysis of genuine Viagra and imitations

In a first step, the homogeneity of the batch from the genuine formulation of Viagra was checked by analyzing separately three tablets. The three 2D DOSY ^{1}H NMR spectra were identical, thus providing a 'spectral signature' representative of the brand formulation. One of them is illustrated in Fig. 1A. The only constituent of the tablet core that was detected is the active pharmaceutical ingredient sildenafil as magnesium stearate, which is insoluble in water. Other pharmaceutical ingredients are those of the coating, i.e. lactose, hypromellose and triacetine (see below for detailed attributions). In a second step, all the other formulations of sildenafil were analyzed. The spectra of formulations **5** and **7** along with their corresponding 1D spectrum are presented in Fig. 2A and B.

The peak at 4.85 ppm corresponds to the signal of residual HOD in D_2O. All the peaks of sildenafil are lined up, and the chemical shifts of this active compound are reported in Table 2. Sildenafil is present as a citrate salt which gives a characteristic AB system pattern at 2.65 and 2.80 ppm. In addition, several excipients could be observed depending on the formulation. Lactose peaks were identified in 10 formulations (**1–4, 6, 7, 9, 10, 15–17**) giving signals at 3.31 (t, 8.3 Hz), 3.55–3.99 (m), 4.48 (d, 7.8 Hz), 4.70 (d, 8.0 Hz) and 5.25 (d, 3.7 Hz) ppm. All the formulations except **7** and **12** also contain a cellulose derivative, i.e. hypromellose (hydroxypropylmethyl cellulose) as tablet binder, giving three aligned signals at 1.19, 3.45 and 3.62 ppm. Formulations **1–4** include the hydrophilic plasticizer triacetine (glyceryl triacetate: 2.13 (s), 2.15 (s), 4.35 (ABX system; 12.2, 5.9 and 3.8 Hz), 5.33 (m) ppm). Formulation **7**, which is commercialized as a 'soft' formulation, contains various sugars, sweetener and aroma as sucrose (3.49 (t, 9.5 Hz), 3.6 (m), 3.70 (s), 3.80 (t, 9.6 Hz), 3.84–3.94

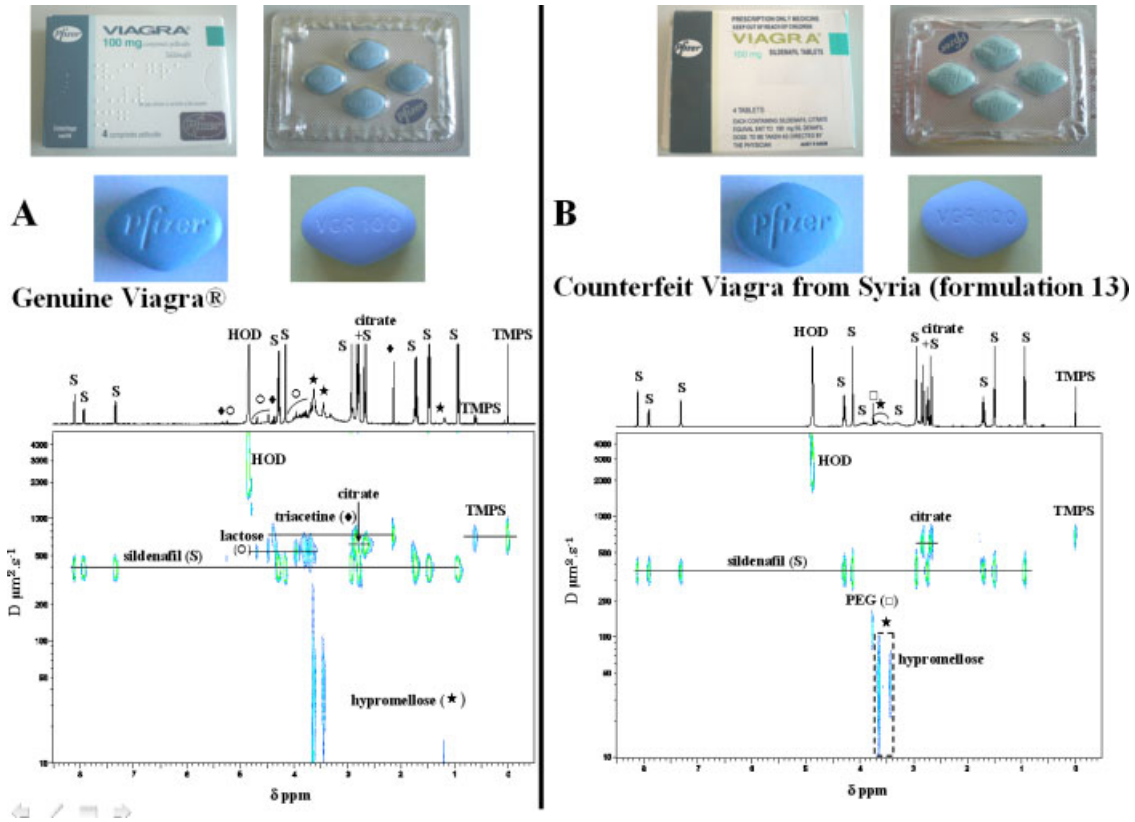

Figure 1. 2D DOSY ^{1}H NMR spectra in D_2O of tablets from (A) genuine Viagra and (B) the Syrian counterfeit formulation **13**. (S) sildenafil; (★) hypromellose; (♦) triacetine; (○) lactose; (□) polyethylene glycol (PEG). TMPS (trimethylsilylpropane sulfonic acid) is the internal reference. Part B: a deeper section of the two signals of hypromellose is shown in a box.

(m), 4.07 (t, 8.6 Hz), 4.23 (d, 8.8 Hz), 5.44 (d, 3.8 Hz) ppm), mannitol (3.68–3.91 (m)), aspartame (2.75 (ddd, 17.5, 8.1 and 5.1 Hz), 3.10 (ddd, 14.0, 9.0 and 5.7 Hz), 3.73 (s), 4.19 (m), 4.75 (m), 7.27–7.41 (m) ppm) and menthol (0.81 (d, 6.9 Hz), 0.91 (m), 0.82–1.13 (m), 1.41 (m), 1.62 (m), 1.67 (m), 1.93 (m), 2.21 (m), 3.33 (m) ppm) for improving the taste of tablets (Fig. 2B). The antimicrobial preservative methylparaben (methyl 4-hydroxybenzoate: 3.85 (s), 6.72 (d, 8.8 Hz), 7.67 (d, 8.8 Hz) ppm) was found in formulations **6** and **11.** The presence of isopropanol, a coating solvent, is generally only detected by the signal of its methyl group at 1.19 ppm; this excipient was detected in formulations **2–5,10,11, 12,14** and **15,** but its amount was subjected to a great inter-tablet variability. Diethylphtalate (1.27 (t, 7.2 Hz), 4.29 (q, 7.2 Hz), 7.64 (m) ppm), a plasticizer, could be detected in formulations **5**,**10** and **14**. Propylene glycol, an anticaking agent, was found only in formulation **15** (1.17 (d, 6.5 Hz), 3.47 (ddd, (11.6, 6.7, 4.2 Hz), 3.85 (m) ppm). Four formulations (**9, 12–14**) contained polyethylene glycol as flow agent, which enhances the effectiveness of the tablet binder, and it gives one signal at 3.76 ppm.

Special attention has been paid to formulation **13**. Indeed, as can be seen in Fig. 1B, the box shows the Pfizer logo, the blister is very similar to the genuine one and the tablets are identical. However, the 2D DOSY ^{1}H NMR spectrum proves that the tablet composition is different. Sample **13** indeed contains sildenafil citrate and hypromellose but lactose and triacetin are absent, whereas polyethylene glycol is found.

The value of the self-diffusion coefficient was measured for each peak, and an average self-diffusion coefficient was determined for each formulation (Table S1, Supporting Information). The diffusion coefficient generally decreases with increasing molecular weight. The differences in the values of the diffusion coefficients are due to the variability in the composition of different tablets, resulting in media of different viscosities.

Analysis of the two Chinese formulations 16 and 17: structural identification of wrong active pharmaceutical ingredients detected

From the conventional and 2D DOSY ^{1}H NMR analyses, it was obvious that formulations **16** and **17** contained different active pharmaceutical ingredients than those of the Viagra brand formulation. The intensities of the peaks located in the aromatic region of the spectrum demonstrated that formulation **16** contains a mixture of three related active components, one being sildenafil, and formulation **17** has a mixture of two (Fig. 3(I)). The ^{1}H NMR spectral data of pure sildenafil, vardenafil and homosildenafil recorded in D_2O are reported in Table 2. The ^{1}H NMR resonances were assigned by 2D NMR experiments (gCOSY, gHSQC, gHMBC) and comparison to published NMR data on sildenafil and their analogs.[25,26] By adding authentic vardenafil and homosildenafil into solutions of formulations **16** and **17** and recording 2D NMR spectra of the mixtures, we could assign each ^{1}H NMR signal of the formulations to vardenafil, homosildenafil or sildenafil as depicted in Fig. 3.

HPLC-DAD chromatograms of formulations **1**–**15** were all identical, with a peak for sildenafil at a retention time of 2.85 min and a characteristic UV spectrum.[6] Formulations **16** and **17** have atypical chromatograms with an additional peak at a retention

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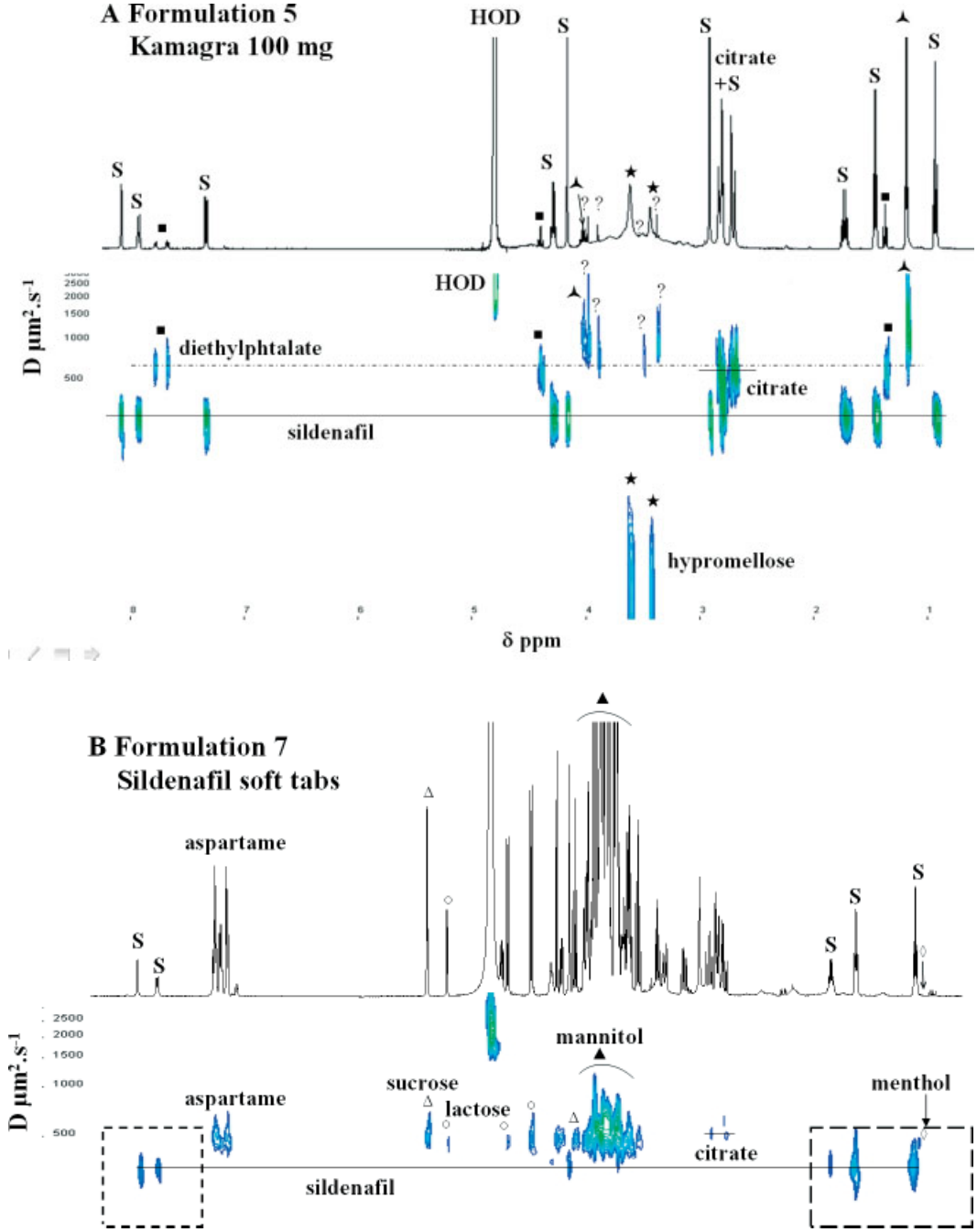


Figure 2. 2D DOSY ^{1}H NMR spectra in D_2O of tablets from the Indian (A) formulation **5** and (B) formulation **7**. (S) sildenafil; (★) hypromellose; (■) diethylphtalate; (⅄) isopropanol; (○) lactose; (△) sucrose; (▲) mannitol; (◊) menthol; (?) unknown. A deeper section of some signals is shown in boxes.

time of 3.85 min. LC-MS and LC-MS-MS were therefore employed to confirm the chemical structures of the active ingredients in both formulations. Figure 4A shows the total ion current chromatogram of a solution of formulation **16** in methanol. Two main peaks were detected, one at 2.7 min with a shoulder at ~2.8 min and the other at 3.85 min. The peak at 2.7 min has a pseudo-molecular ion signal $[M + H]^+$ at *m/z* 489, and those at ~2.8 min and 3.85 min had signals at 475 and 489, respectively (Fig. 4B). From LC-MS-MS spectra, it is obvious that the three peaks correspond to vardenafil (2.7 min), sildenafil (~2.8 min) and homosildenafil (3.85 min). The same *m/z* value was measured for the pseudo-molecular ions of vardenafil and homosildenafil; however, the LC-MS-MS spectra allowed an unambiguous structural elucidation. Indeed, vardenafil produced the 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 of vardenafil.[6,27] The results were similar for formulation **17**, except that no sildenafil was detected. The formulation thus contained only vardenafil and homosildenafil.

3D DOSY-COSY ^{1}H NMR analysis

The main interest of the 3D DOSY-COSY ^{1}H NMR experiment is to extract the COSY spectrum of each component of the mixture

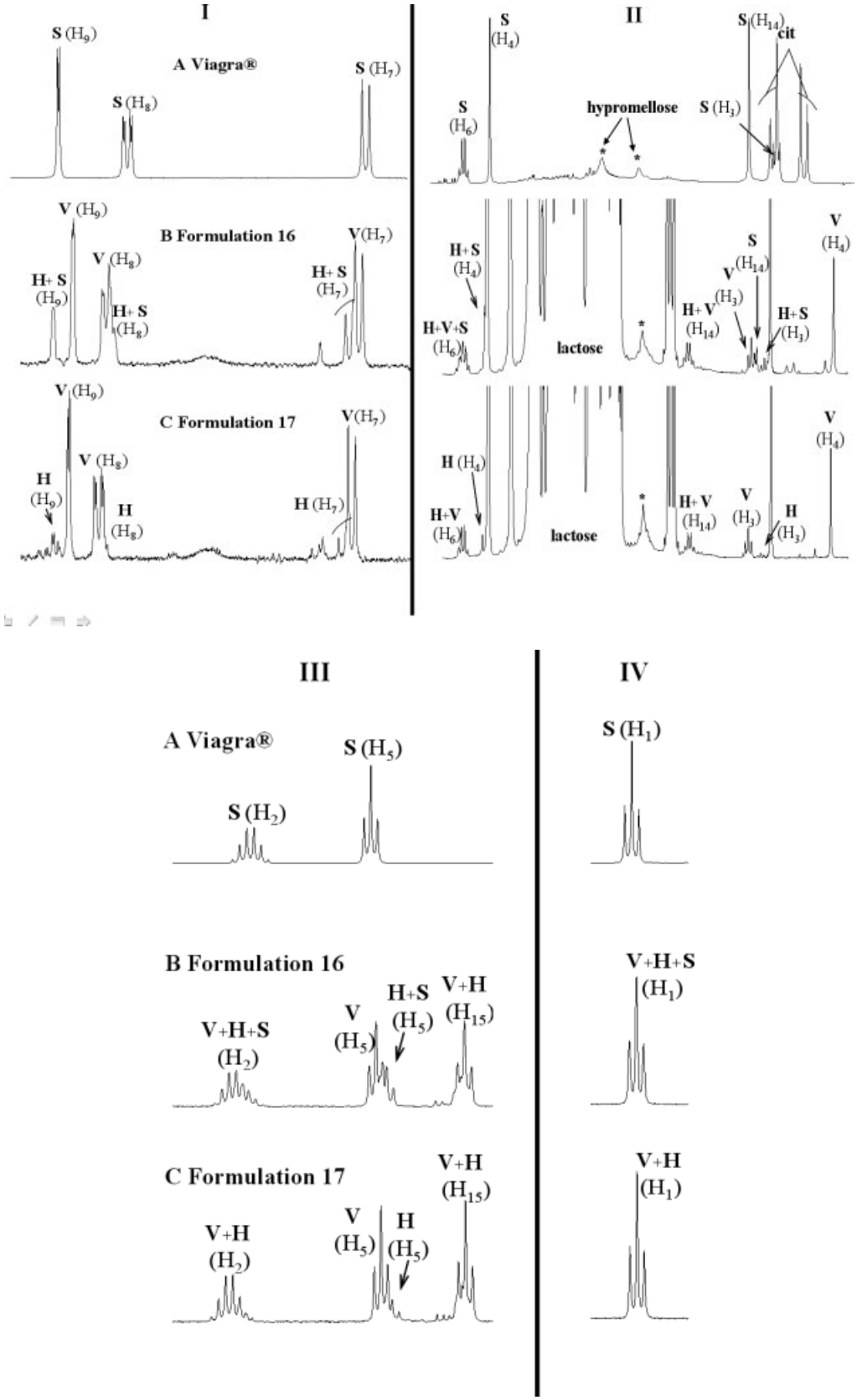
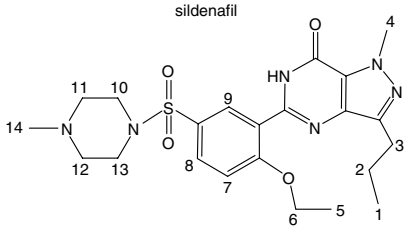


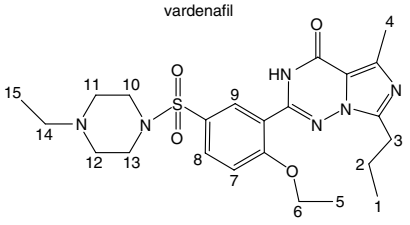
Figure 3. ^{1}H NMR spectra of genuine formulation of sildenafil (Viagra, formulation 1) (A) and Chinese formulations **16** (B) and **17** (C) in D_2O. (I): 7.2–8.2 ppm; (II): 2.4–4.4 ppm; (III): 1.2–1.9 ppm; (IV): 0.8–1 ppm. The numbering of sildenafil (S), vardenafil (V) and homosildenafil (H) protons is indicated in Table 2. (★) hypromellose.

Table 2. ^{1}H NMR data (500 MHz) of sildenafil, vardenafil and homosildenafil

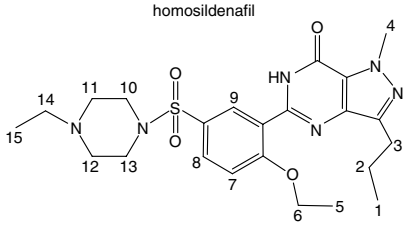
sildenafil



vardenafil



homosildenafil



δ (ppm)[a]	Multiplicity[b] (J Hz)	Position no.	δ (ppm)[a]	Multiplicity[b] (J Hz)	Position no.	δ (ppm)[a]	Multiplicity[b] (J Hz)	Position no.
8.08	1H, d (2.5)	9	8.05	1H, d (2.5)	9	8.14	1H, d (2.5)	9
7.98	1H, dd (8.9, 2.5)	8	7.97	1H, dd (8.9,2.5)	8	7.93	1H, dd (9.5, 2.5)	8
7.38	1H, d (8.9)	7	7.32	1H, d (8.9)	7	7.33	1H, d (9.5)	7
4.29	2H, q (7.0)	6	4.28	2H, q (6.9)	6	4.26	2H, q (7.0)	6
4.20	3H, s	4				4.13	3H, s	4
3.94, 3.29	4H, 2 broad s	10/13	3.38	8H, broad s	10/13/11/12	3.38	8H, broad s	10/13
2.86	2H, t (7.4)	3	2.92	2H, t (7.4)	3	2.79	2H, q (7.2)	3
			2.53	3H, s	4			
3.61, 2.89	4H, 2 broad s	11/12						
2.89	3H, s	14	3.18	2H, q (7.2)	14	3.20	2H, q (7.0)	14
1.75	2H, sext (7.2)	2	1.78	2H, sext (7.4)	2	1.73	2H, sext (7.2)	2
1.42	3H, t (7.0)	5	1.44	3H, t (6.9)	5	1.48	3H, t (7.0)	5
			1.28	3H, t (7.2)	15	1.30	3H, t (7.0)	15
0.91	3H, t (7.2)	1	0.94	3H, t (7.4)	1	0.93	3H, t (7.2)	1

[a] Spectra were recorded in D_2O.
[b] s: singlet; d: doublet; dd: doublet of doublet; t: triplet; q: quadruplet; sext: sextuplet; m: multiplet.

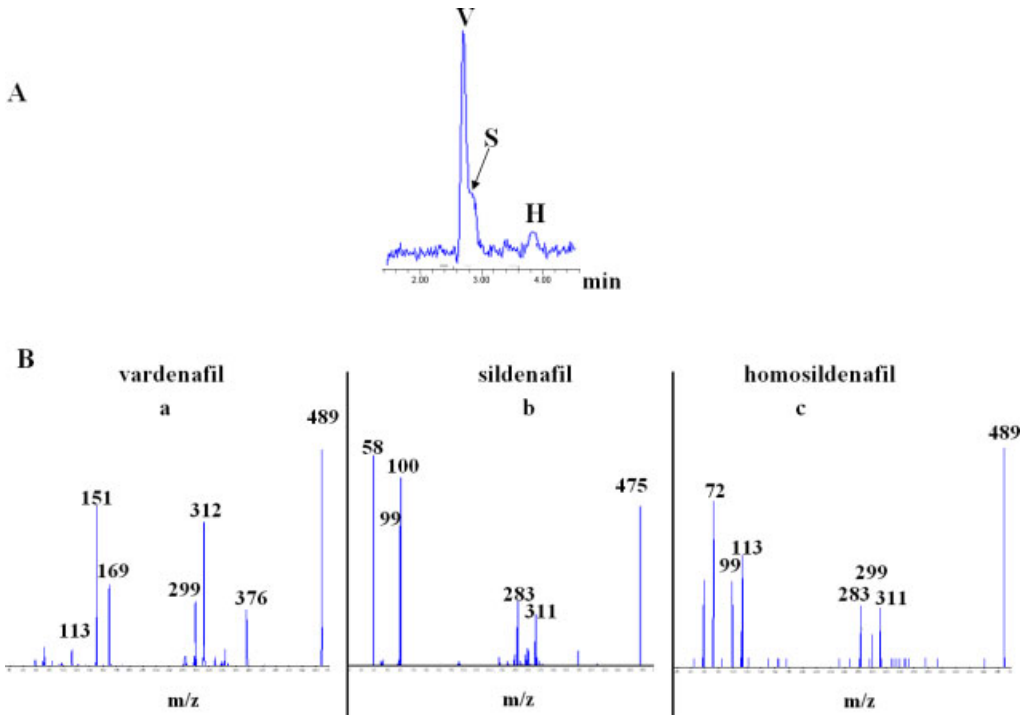


Figure 4. (A) LC-MS: total ion chromatogram (TIC) obtained from a solution of formulation **16** in methanol. (B) MS-MS fragmentation pattern of parent ion *m/z* 489 for vardenafil (a) and homosildenafil (c), and of parent ion *m/z* 475 for sildenafil (b).

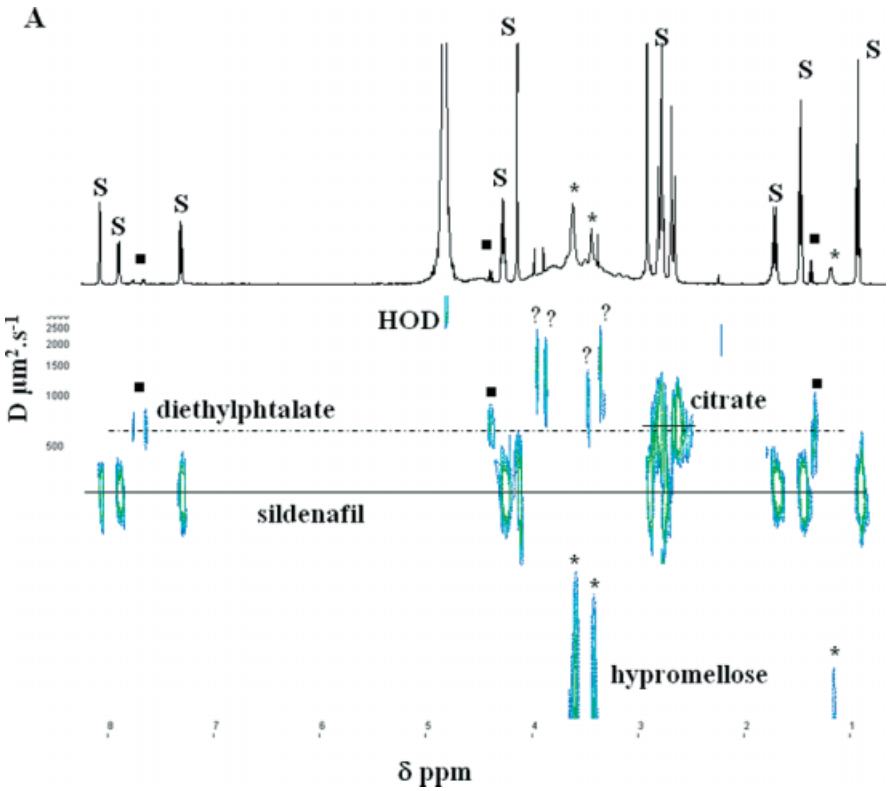


Figure 5. NMR spectra of the Indian formulation **5** in D_2O. (A) 2D DOSY ^1H spectrum; (B) COSY-DQF spectrum; COSY extractions from 3D DOSY-COSY experiment at (C) $D = 310\ \mu m^2\ s^{-1}$, and (D) $D = 510\ \mu m^2\ s^{-1}$. (S) sildenafil; (★) hypromellose; (■) diethylphtalate; (?) unknown.

from a selected line in the DOSY spectrum. Figure 5 illustrates the different NMR spectra obtained in the 3D DOSY-COSY ^1H NMR experiment applied to formulation **5**. First, the classical 2D DOSY (Fig. 5A) and COSY-DQF (Fig. 5B) spectra are shown. On the whole COSY spectrum, diagonal signals and off-diagonal cross peaks of sildenafil (S), citrate and diethylphtalate (■) are observed. The other spectra (Fig. 5C and D) are those of the projections from the 3D DOSY-COSY experiment. Figure 5C shows the COSY projection of sildenafil at a diffusion coefficient (D) of 310 $\mu m^2\ s^{-1}$; typical cross peaks $H_{1/2}$, $H_{1/3}$, $H_{2/3}$, $H_{5/6}$, $H_{7/8}$ and $H_{8/9}$ are exclusively detected. The COSY projection corresponding to $D \approx 510\ \mu m^2\ s^{-1}$ (Fig. 5D) shows the signals of diethylphtalate together with residual signals of citrate at around 2.6 ppm. These spectra clearly highlight the interest of 3D experiment, as virtual separation provided by DOSY acquisition can lead to real structural determination by extraction of COSY spectra.

Quantitative analysis by ^1H NMR

The amounts of sildenafil measured in the various formulations are reported in Table S2 (Supporting Information). The NMR procedure for quantification of sildenafil and analogs was validated by analyzing genuine Viagra. The recovery obtained for sildenafil content was 101 ± 5 ($n = 4$) in complete accordance with HPLC measurement (101 ± 3 ($n = 4$)).

Twelve out of the 17 commercial formulations of sildenafil analyzed (**1–9,11,13,15**) contain the active ingredient within 100 ± 10% of stated concentration. Three formulations **10**, **12** and **14** contain more than 110% of the stated amount (111 ± 1, 113 ± 4 and 114 ± 3%, respectively). Formulation **17** does not contain sildenafil, and formulation **16** contains only 2 mg of sildenafil per tablet. In addition, 8.9 ± 0.8 mg of vardenafil and 1.3 ± 0.1 mg of homosildenafil were found in the latter. Similar data were obtained for formulation **17,** which contains 9.2 ± 0.5 mg of vardenafil and 1.9 ± 0.2 mg of homosildenafil.

Discussion

The ability of NMR to provide valuable information regarding mixture analysis has created broad applicability across chemistry, biochemistry, biology and medicine. As drugs can be considered as complex mixtures (composed of many different substances or including simultaneously large and very low quantities of compounds), NMR is a great tool for studying such formulations. Unfortunately, NMR has traditionally been sensitivity-limited compared with other analytical techniques. Nevertheless, technological advancements in the field of magnetic resonance have made significant strides in improving sensitivity levels, developing new acquisition/processing tools and implementing innovative NMR experiments/sequences.

For a long time it has been preferred, when possible, to isolate each component of a mixture prior to its study by NMR rather than to analyze the mixture. For most analysts, the preferred methods for mixture analysis are still the chromatographic methods, generally coupled with spectroscopic methods like MS or NMR. But there is 'a pure NMR' method that allows precise analysis of a complex mixture without any prior separation of the different components: the DOSY method. It allows measurement of the translational self-diffusion of molecules in solution. Based on the analysis of mono- and multi-exponential decays, spectra of the components of a mixture can

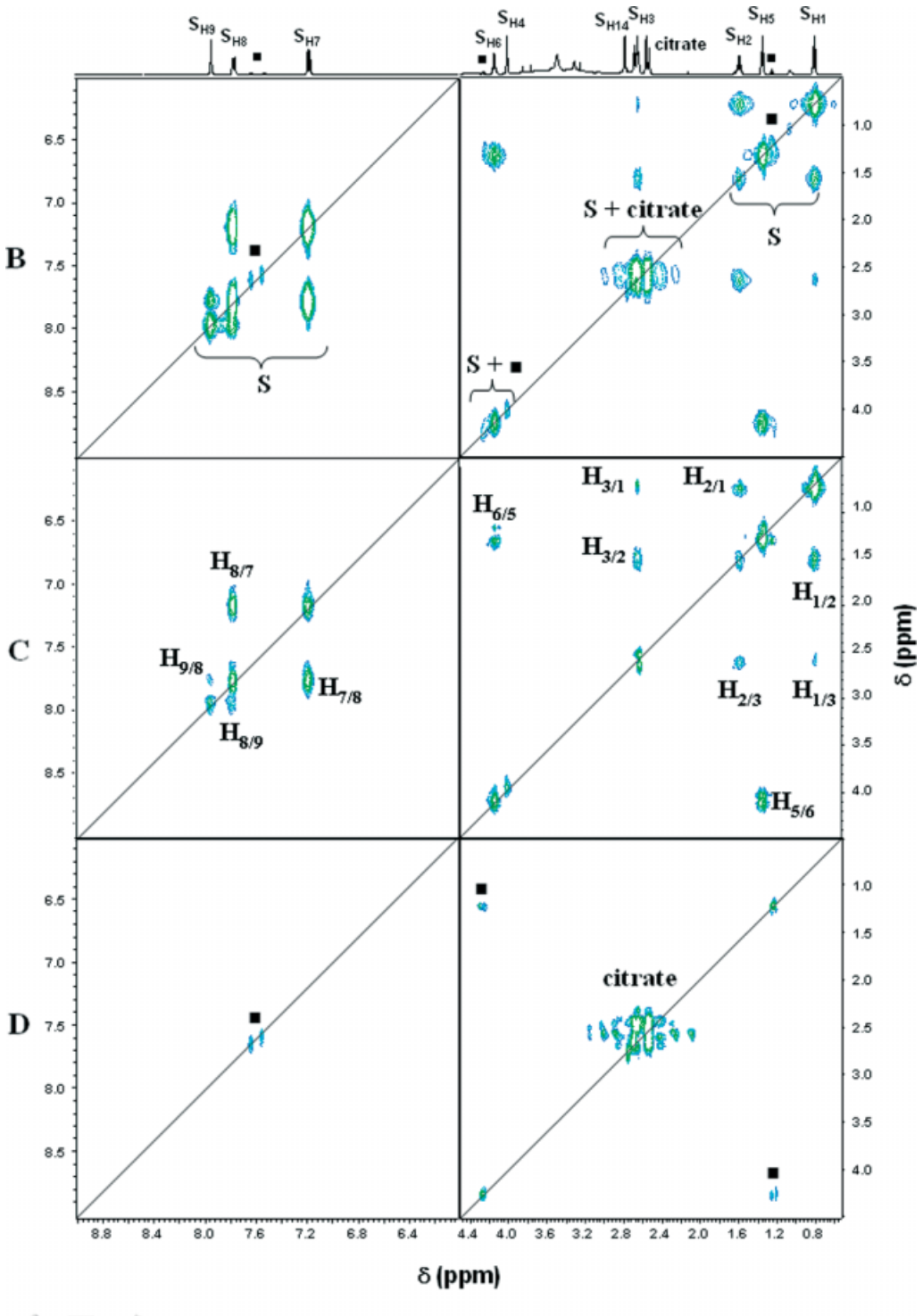


Figure 5. (*Continued*).

be separated depending on the value of their apparent diffusion coefficients. Furthermore, DOSY experiments do not need complicated set-up procedures and the method can be used routinely.

The use of NMR for measuring the self-diffusion of molecules in solution was originally proposed in 1965 by Stejskal and Tanner.[28] The principle of the measurement lies in the fact that the phase of the magnetization is spatially encoded at the beginning of the experiment. Brownian motion in the liquid results in translational diffusion of the various solutes, and a mean molecule displacement is observed at the end of the delay Δ. This displacement has the effect of reducing the signal intensity with an exponential law $I(q) = I_0 \exp(-D\Delta q^2)$ with $q = \gamma g \delta$, where D is the diffusion coefficient, γ the gyromagnetic ratio and g and δ the intensity and the duration of the pulsed field gradient, respectively. The DOSY experiment relies on this evolution law,

leading to a multidimensional spectrum on which the diffusion coefficient *D* is displayed along one of the spectral axes.

The DOSY experiment differs from the usual modern NMR experiments in two important ways. First, while modern NMR experiments are based on the analysis of the time dependence of the signal, here all the delays are kept strictly constant in order to abstract the measure from the relaxation phenomenon. In contrast, only the intensities of the coding and decoding gradients are varying. Second, the time dependence of modern NMR experiments is usually analyzed by some spectral analysis methods, Fourier transform being by far the most common one. In the case of DOSY, the signal variation follows an exponential law and Laplace analysis is applied.

A few analytical methods have been described for the analysis of the composition of counterfeit or imitated Viagra tablets. Using X-ray powder diffraction, Maurin *et al.*[16] could identify the active sildenafil and the two excipients with the highest concentrations, microcrystalline cellulose and anhydrous calcium hydrogenophosphate. The method can easily discriminate fake and original samples, even by visual examination of diffraction patterns, which can be done by not highly experienced employees. It does not require any prior preparation of samples, does not need application of advanced mathematical and statistical methods to process the data and is fast (45 min). Its main disadvantages are that it is insensitive and not quantitative. Screening between imitation and authentic Viagra was also described using NIR spectroscopy combined with wavelength correlation and principal component analysis.[15] The time of analysis is very short, the technique is nondestructive and no pretreatment of the formulation is required. However, it does not provide information on the excipients (except talc), is not quantitative and needs classical analytical methods (TLC, UV-vis spectrophotometry, HPLC-DAD, LC-DAD-MS^2) for identification and quantitation of actives. To detect counterfeit Viagra, Raman spectroscopy is more informative, as the excipients microcrystalline cellulose, magnesium stearate, lactose, calcium sulfate, calcium carbonate, mannitol and sucrose can be detected.[17] However, the discriminating power of the technique to assess the presence of actives other than sildenafil could not be demonstrated, as all the formulations analyzed contained this pharmaceutical ingredient. The technique is nondestructive, fast, and requires no sample preparation. Two downsides of this method are that it is only qualitative and a Raman spectrum of a genuine Viagra tablet is necessary for differentiating the various formulations. The best methods for identification and quantification of active pharmaceutical ingredients are chromatographic ones[2–8]; however, they do not provide any information on excipients.

A comparison of DOSY NMR with these methods highlights the interest of the technique for a global qualitative analysis of counterfeit pharmaceutical formulations of sildenafil, as different active ingredients (sildenafil, homosildenafil, vardenafil) and also various excipients (lactose, hypromellose, triacetine, propylene glycol, isopropanol, diethylphtalate, methyl paraben, polyethylene glycol, sucrose, menthol, mannitol, aspartame) were detected in a single experiment (Figs 1 and 2). Moreover, the method is nonselective and requires no prior knowledge of the structures of the various components present in the mixture. Even though DOSY NMR itself is so far not quantitative, the conventional ^{1}H NMR spectrum recorded independently from the DOSY experiment can be used for quantitation. The disadvantages of the method are that only compounds with protons and soluble in the NMR solvent are detected, which excludes mineral excipients to be observed. The duration of the 2D DOSY ^{1}H NMR experiment is ~2 h using a high-sensitivity cryoprobe, which precludes its use on the field but is not too disadvantageous with respect to the total chemical information gained in one DOSY NMR experiment. Also, no pretreatment of the sample is necessary. Nevertheless, DOSY NMR is a powerful analytical method which allows the fingerprinting of pharmaceutical formulations and can be used to determine the similarities or differences between samples. Not only can it distinguish between genuine and counterfeit tablets, but it is also helpful in determining the relationships between different samples and thus assists in the investigation of the sources of these drugs.

The two wrong active ingredients found in the fake Chinese formulations **16** and **17** are vardenafil and homosildenafil. As sildenafil, vardenafil is a PDE-5 inhibitor, and indications and contraindications for this medicine are close to those of other PDE-5 inhibitors. The amounts of vardenafil recovered in the two Chinese formulations **16** and **17** (8.9 and 9.2 mg per tablet, respectively) are in the range of therapeutic level, as the brand formulation of vardenafil (Levitra) is available in 2.5, 5, 10 and 20 mg doses with a normal starting dose of 10 mg. However, to the best of our knowledge, toxicological data on homosildenafil 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 these drugs via the Internet.

Conclusion

Detection and analysis of counterfeit medicines constitute a challenge to the analyst. The variety of unknown compounds present in these samples makes their identification difficult. The approach described in this paper shows that DOSY ^{1}H NMR, which relies on differences in translation diffusion as a means to separate components in a solution mixture, allows efficient characterization of genuine or counterfeit sildenafil tablets. Among the different methods available for screening of drug quality,[29] the tools and approach to be employed depend greatly on the type of information desired and the time taken to perform the analysis. This study demonstrated that DOSY NMR could be part of the 'analytical toolbox' of any analyst dealing with complex mixture analyses in quality control of drugs.

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Supporting information

Supporting information may be found in the online version of this article.

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