Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 679–687

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Review

Analytical challenges in drug counterfeiting and falsification—The NMR approach

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ARTICLE INFO

Article history:
Received 14 October 2010
Received in revised form 9 December 2010
Accepted 11 December 2010
Available online 21 December 2010

Keywords:
qNMR
DOSY NMR
Drugs
Impurities
Counterfeits

ABSTRACT

Counterfeiting of products is a global problem. As long as clothes, clocks, leather wear, etc. are faked there is no danger, but when it comes to drugs, counterfeiting can be life-threatening. In the last years sub-standard active pharmaceutical ingredients (APIs) were found more often even though the use of the quality-ensuring methods of international pharmacopoeias should have detected additional impurities and the low content of the API. Methods orthogonal to the separating methods used in the pharmacopoeias are necessary to find counterfeits. Beside Raman and NIR spectroscopies as well as powder X-ray analysis, NMR spectroscopy being a primary ratio method of measurement is highly suitable to identify and quantify a drug and its related substances as well as to recognize a drug of sub-standard quality. DOSY experiments are suitable to identify the ingredients of formulations and therefore to identify wrong and/or additional ingredients. This review gives an overview of the application of quantitative NMR spectroscopy and DOSY NMR in anticounterfeiting.

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1. Introduction

Counterfeiting has become a global problem with regard to almost all products, e.g. cosmetics, jewellery, clothes, aircraft and car industries and many more. Logically, the problem of counterfeit drugs is also globally increasing. A counterfeit medicine is one which is deliberately and fraudulently mislabeled with respect to identity and/or source. All kinds of medicines are counterfeited, generic ones as well as branded, ranging form medicines for the treatment of life-threatening conditions to inexpensive generic versions of painkiller, antibiotics, cancerostatics, and antihistamines, to name only a few. The World Health Organizations' website [1] gives some recent events, i.e. in 2009 in China, glibenclamide containing six times the normal dose (two people died, nine were hospitalized), in 2009 in Tanzania, an antimalarial drug (Metakelfin) was discovered in a pharmacy lacking sufficient active ingredient, and in 2008, Viagra and Cialis were smuggled into Thailand from an unknown source in an unknown country. In 2009, the UK's licensing authority MHRA reported on Seretide 250

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doi:10.1016/j.jpba.2010.12.017

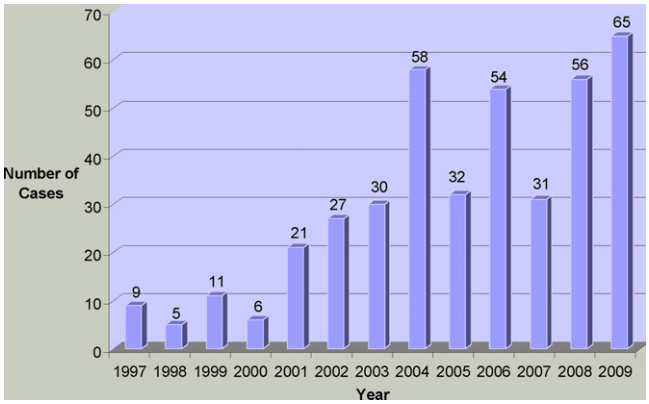

Fig. 1. Number of counterfeit cases; investigation per fiscal year.

Evohaler® (containing 25 μg of salmeterol xinafoate and 250 μg of fluticasone propionate per actuation) where performance tests have demonstrated that there could be a reduced patient dose if patients have obtained and used a counterfeit inhaler, in addition to problems with Zyprexa® (Olanzapine), and Plavix® (Clopidogrel), just to mention again a few cases only.

The growth of the problem can also be seen by the enhancing numbers of cases, opened by the FDAs' office of criminal investigation (see Fig. 1). We do not know the exact numbers because we see the tip of the iceberg only. In 2005, the Organization for Economic Co-operation and Development estimated the trade was worth at least US$ 32 billion per year, adding that by 2010 this figure might reach $75 billion [2].

Five categories of counterfeits are defined by the WHO [3]: 1. mislabeled counterfeits, 2. counterfeits containing less API, 3. counterfeits containing a wrong API, 4. counterfeits with no API, and recently the fifth category was introduced as drugs of substandard quality, consisting of new related substances or high amounts of old and/or new related substances, high amounts of residual solvents, or high amounts of heavy metals (for percentage of each category, see Fig. 2). In contrast to the first four categories which are mostly occurring in developing countries the substandard drugs are often found in developed countries, e.g. in 2007, unfractionated heparin was contaminated with oversulfated chondroitin sulfate (ca. 100 deaths in the USA), heavy metals were found in herbals for Traditional Chinese Medicine (TCM) originated from China, diethylene glycol in glycerin was found in tooth paste in UK in 2007 and in syrup for teething children in Nigeria in 2009. Simple one-dimensional ^{1}H or ^{13}C NMR spectra are often able to unravel such counterfeits.

Besides having a look at the APIs and its quality, the final formulation of a drug has to be analyzed in depth in order to check whether it contains the right API(s) and the right content of the API(s). This is especially of interest for TCM which mostly claim to consist of plants and corresponding extracts only but are often curative because of an unlabeled API, e.g. in the case of pain killers or Viagra-type drugs. Here 2D diffusion-ordered spectroscopy (DOSY) NMR experiments are extremely useful for elucidation of the ingredients.

This review will focus on the counterfeits with regard to substandard APIs and assignment of formulation ingredients, and the challenge to unravel and avoid such cases with a special emphasis on NMR techniques.

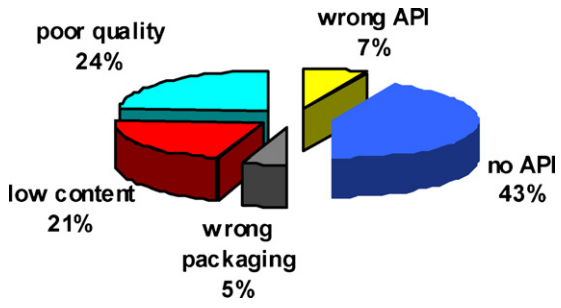

Fig. 2. WHO estimation of five categories of counterfeits.

2. Additional methods of quality assessment

In 1955, the WHO has given the following definition for Pharmacopoeias [4]. They contain "Pharmaceutical norms intended to ensure, within a given political entity the uniformity of quality, nature, composition and concentration of medicines approved by medical representatives." In other words, a pharmacopoeia is a collection of norms for quality control. They are dealing with the methods of quality assessment of drugs and/or drug products. The monographs of the drugs consist of information on physicochemical properties, identification methods, tests for the impurity quantifications and assays for the content determination. The European Directorate for the Quality of Medicines and HealthCare (EDQM) claims a guaranteed standard not only for quality but also for efficacy and for safety for all medicines [5]. The standards are defined by the three leading pharmacopoeias, i.e. European Pharmacopoeia (PhEur), the United States Pharmacopoeia (USP), the Japanese Pharmacopoeia, and the International Pharmacopoeia (IP published by the WHO) as well as many other regional pharmacopoeias. Nevertheless we see sub-standard drugs on the market. This might be caused by the fact that not all monographs in the pharmacopoeias are as modern as they should be, and that there are ways to circumvent the purity control, i.e. to hide new impurities.

The international pharmacopoeias assess the quality mainly by means of separating techniques such as high performance liquid chromatography (HPLC), and capillary electrophoresis (CE), mostly connected to UV detection, in rare cases with a fluorescence detector or a chemiluminescence detector. These techniques are not sufficient in the case the impurities do not have a chromophore or fluorophore, or do have a chromophore absorbing at a different wavelength. In addition, the separating methods are developed and validated for a special synthesis/production pathway. In the case the synthesis or production has changed (different synthesis routes, new purification methods, etc.) new related (toxic) substances may occur which may be not separated from the main peak or the peaks of other impurities. Thus, a new separation method has to be developed and validated in order to assess the quality of an API.

In order to recognize changes in the production, substandard APIs or counterfeiting with utmost probability an orthogonal method has to be performed additionally. The importance of this approach became clear when the heparin case occurred in the USA and parts of Europe (see below). Even though the low-molecular-mass heparins were identified by simple ^{1}H NMR spectroscopy since a long time, the corresponding test was not introduced to unfractionated heparin sodium and calcium, but would easily have found the additional impurity (see below).

Initiated by the heparin case the Food and Drug Administration (FDA) in the USA started to discuss the application of Raman and near infrared (NIR) spectroscopy as additional or alternative tests for drugs more intensively [6]. However, many pharmaceutical companies already use NIR spectroscopy for an initial API control. Raman spectroscopy would have a couple of advantages, namely non-invasive, non-destructive, water insensitive, signature providing, etc., but is less developed. Both methods need a statistical

analysis (mostly principal component analysis (PCA) or partial least square (PLS) methods) of the spectra in order to recognize changes and counterfeits which makes the application a little more complicated. Nevertheless NIR and Raman spectroscopies are cheap and fast methods and therefore suitable as an additional, orthogonal method for quality assessment.

Additionally, the power of X-ray analysis should be mentioned. It is not only suitable to characterize the crystallinity of a powder but also to characterize the composition of a tablet [7] by simply comparing the diffraction pattern of the original and the copy. Thus, it is used in the same way as NIR and Raman spectroscopies. However, the facts that the X-ray technique is non-destructive and it does not require the removal of the tablet from the blister packaging makes it to an important method in unraveling counterfeit formulations.

NMR spectroscopy is highly suitable for quality assessment of an API or excipient, because it can be used for a lot of purposes including both identification and quantification [8–10] and if necessary simultaneously:

1. to identify a drug or an excipient,
2. to evaluate the level of impurities (and to elucidate the structure),
3. to observe the course of a decomposition,
4. to evaluate residual solvents,
5. to determine the isomeric composition, i.e. the ratio of diastereomers and the enantiomeric excess by means of chiral additive,
6. to assess a single drug or drug composition,
7. to characterize a polymer mostly being a mixture and used as excipients,
8. to identify counter ions (if of organic origin and having protons),
9. to control a production process (see process analytical technology), and
10. to characterize an entire formulation, e.g. a tablet.

3. Fundamentals of quantitative NMR spectroscopy

NMR spectroscopy can be considered as a *primary ratio method of measurement* [11,12] being characterized by the fact that the ratio of substances can be determined directly from the physical context of the measurement *without* referencing to another substance. The absolute amount of substances can be determined by using simple reference substances, which holds also true for coulometry, gravimetry or titrimetry.

Since the intensity I_A of a signal is directly proportional to the number of nuclei N evoking the signal, the intensities of NMR signals (=areas under specific signals) can be taken for quantitative investigations. The linear relationship between the signal intensity I and the number of observed nuclei (in case of single pulse excitation) is given by

$$I = c_S \cdot N \quad (1)$$

The proportionality constant c_S results from parameters of the spectrometer, termed "spectrometer constant", and the sample. The precision of the integrals determines the accuracy of quantification depending on

1. the noise level of the spectrum,
2. the line shape,
3. the quality of shimming,
4. the choice of the window function,
5. the phase-, baseline- and drift corrections which are often performed manually because the software does not provide suitable results,
6. the relaxation time T_1 of the signal considered for integration (T_1 has to be determined for each signal considered), and
7. the correct choice of the integral interval, which should be 64 times the full width at half signal height.

Considering and stringently controlling these parameters qNMR methods can easily be validated. Details of parameter setting and optimization are given in Refs. [13,14].

NMR spectroscopy is known to be not very sensitive. However, for quality assessment of an API the sensitivity is sufficient as long as the signals considered are separated. The sensitivity has been enhanced in the past years by the development of high-field spectrometers (>400 MHz) for routine purposes, invention of gradient shimming techniques, and inverse or cryo probes as well as the microcoil technology. In addition the sensitivity can be enhanced by increasing the number of scans. In order to achieve a standard deviation (SD) <1% necessary for quantification purposes, the signal-to-noise ratio (S/N) needs to be >250:1 for ^{1}H, >300:1 for ^{19}F and >600:1 for ^{31}P [15].

3.1. Signal separation

The signal considered for integration and quantification has to be "pure", i.e. free of any other signal or side band (rotational side bands or ^{13}C satellites). Two-dimensional experiments such as homo- and heteronuclear correlation spectra can be indicative of signal purity [13].

Clearly separated signals are one of the most important prerequisites for the quantification of a substance in a mixture as is the case for the impurity evaluation of an API or characterization of a drug mixture, e.g. natural products, plant extracts, TCMs or excipients. In contrast to separation methods such as HPLC the possibilities to achieve a separation of signals which are initially not separated are limited. Nevertheless, the choice of the solvent plays a pivotal role. Beside the application of aromatic solvents (aromatic solvent induced NMR shift = ASIS) which produce high-field shifts due to diamagnetic anisotropy other solvents and even mixtures of solvents can be useful [13]. This was recently demonstrated for ergot alkaloids [16] where a mixture of chloroform/DMSO, chloroform/methanol or benzene/DMSO was employed for signal separation and eventually the quantification of the components of codergocrine components. The results obtained by ^{1}H and ^{13}C qNMR were in perfect agreement with the HPLC findings on the same sample.

For separation of signals the pH value of the solution measured might be varied in case of acids or bases as well as the ion concentration [e.g. 17]. The increase or decrease of the temperature is also sometimes an option [18]. Even the sample concentration influences the positions of the signals and therefore the separation of two signals [19]. And last but not least the addition of a shift reagent such as cyclodextrins, solvating agents (such as chiral agents for enantiomer separation, e.g. such as α-methylbenzylamine, α-methylbenzylisocyanate, *threo*-2,3-butanediol, ω-camphanic acid or phenylglycinol [20]) and lanthanide shift reagents (not with very high-field instruments) can be used [13].

3.2. Quantification

Principally a relative and an absolute method can be applied for quantification. The determination of the ratios of the components of a drug, synthesized compound mixture or natural product is easy to determine by qNMR using the integrals. The molar ratio n_X/n_Y of two compounds X and Y can be calculated straightforward using the integrals I of a pair of separated signals of a defined number of

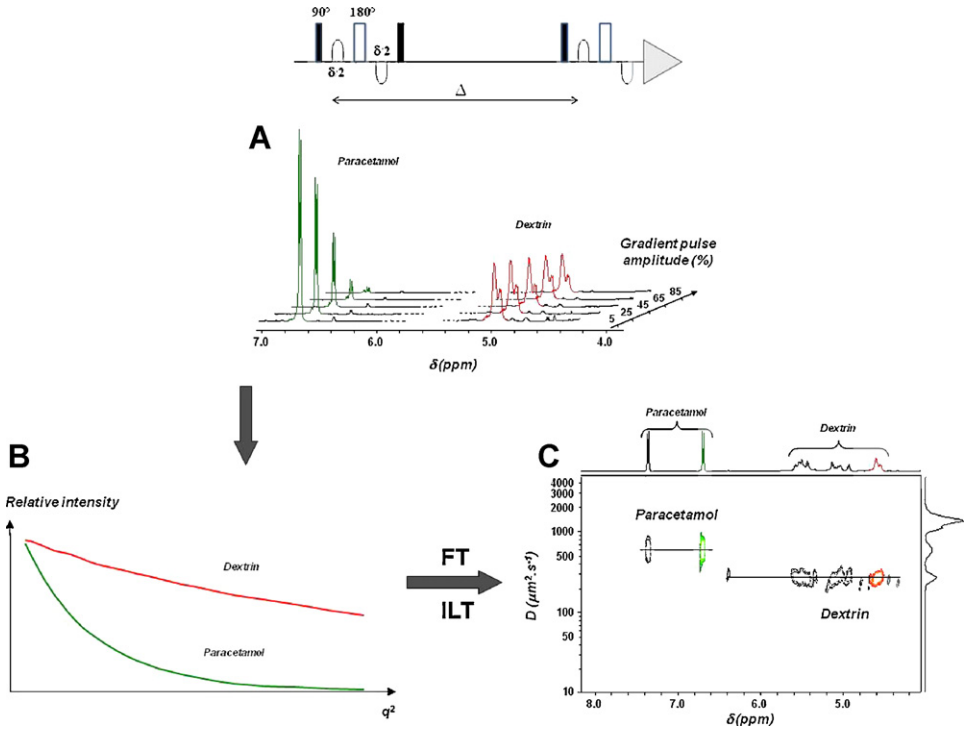

Fig. 3. Schematic principle of the basic DOSY NMR experiment. A BPP–STE sequence is represented. FT: Fourier transformation; ILT: inverse Laplace transformation.

nuclei:

$$\frac{n_X}{n_Y} = \frac{I_X}{I_Y} \cdot \frac{N_Y}{N_X} \tag{2}$$

Since c_S has to be constant, it is cancelled in this equation. Consequently, the amount fraction of a compound X in a mixture of m components is given by

$$\frac{n_X}{\sum_{i=1}^{m} n_i} = \frac{I_X/N_X}{\sum_{i=1}^{m} I_i/N_i} \times 100\% \tag{3}$$

The solvent signal has to be disregarded. The method can be regarded as a normalization procedure used in international pharmacopoeias for evaluation of drug mixtures by means of HPLC. It is often important for the assessment of the ratio of isomers.

qNMR offers three different absolute methods for quantification of a content or concentration:

a. The so-called 100% method: if all impurities show up in the NMR spectrum, if they can be assigned structurally and if they can be measured quantitatively, the assay is simply the difference to the 100% value. However, this approach is not applicable for samples consisting of impurities not containing the observed nucleus (e.g. inorganic impurities).
b. The main component P_X can be calculated directly from the NMR using a standard of known content P_{Std}:

$$P_X = \frac{I_X}{I_{Std}} \cdot \frac{N_{Std}}{N_X} \cdot \frac{M_X}{M_{Std}} \cdot \frac{m_{Std}}{m} \cdot P_{Std} \tag{4}$$

with M_X and M_{Std} being the molar masses of analyte and standard, m and m_{Std} the weights of the sample and standard, and P_X and P_{Std} the assays of analyte and standard, respectively. A one-point calibration has to be carried out by gravimetric addition of an internal standard in order to measure and calculate the ratio of the intensities of a signal from the analyte and from the standard. Both signals should be of comparable height which can be achieved by a corresponding concentration of the internal standard in the sample solution.
c. The standard addition method is a third possibility of absolute value determinations. If known amounts of the active compound are added to the solution in several steps the content can be calculated without the knowledge of the molar mass of the analyte.

3.3. Fundamentals of the DOSY method

DOSY NMR is a method particularly well adapted for uncovering counterfeit drugs or adulterated herbal medicines as it provides both comprehensive information on the formulation and a virtual separation of the components of the mixture based on the difference in their translational self-diffusion coefficients in solution.

Here we provide a basic and brief description of the DOSY experiment. For a more detailed description of the method, the reader is referred to [21]. The use of NMR for measuring self-diffusion coefficients of molecules in solutions is based on a pulsed field gradient (PFG) stimulated spin-echo (STE) experiment [22,23], which is particularly well suited for the analysis by high-resolution DOSY of complex mixtures where many signals are observed with a wide dynamic range. Typically, a series of 1D PFG–STE experiments is acquired with systematic variations of the gradient pulse amplitude (Fig. 3A). 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, which is the delay between coding and decoding gradients. This displacement has the effect of reducing the signal intensity with an exponential law:

$$I(q) = I_0 \exp(-D\Delta q^2) \tag{5}$$

with $q = \gamma g \delta$ where D is the diffusion coefficient, γ the gyromagnetic ratio, g and δ the intensity and the duration of the PFG, respectively. D, which depends on the molecular weight and other hydrodynamic properties (size, shape, and charge) of the solute as well as on its surrounding environment (temperature and aggregation state), can thus be estimated by analysis of the exponential signal decay. Signals from small molecules (large D) (e.g. paracetamol in Fig. 3B)

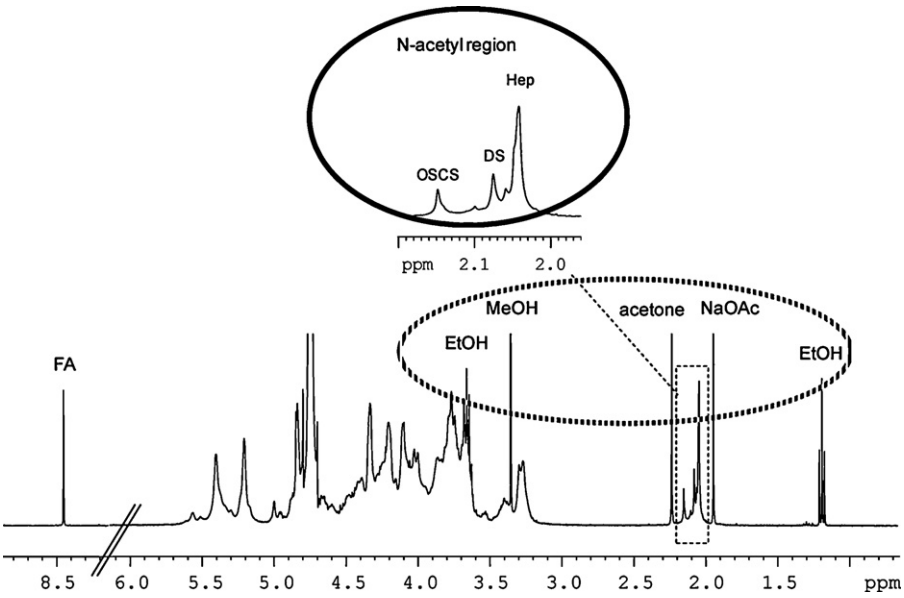

Fig. 4. ^{1}H NMR spectrum (400 MHz, D_2O, 300 K) of unfractionated heparin contaminated with oversulfated chondroitin sulfate (OSCS) and dermatan sulfate (DS) as well as residual solvents, i.e. ethanol (EtOH), methanol (MeOH), acetone, sodium acetate (NaOAc) and formic acid (FA), modified after [32].

decay more rapidly than those from large molecules (small D) (e.g. dextrin in Fig. 3B) as the PFG is incremented. The Fourier transformation of the NMR signal and the inverse Laplace transformation of the decaying signals leads to the 2D DOSY spectrum: a mixed Fourier–Laplace spectrum on which the chemical shifts (δ in ppm) lie on the horizontal axis while the diffusion coefficients are on the orthogonal axis (D in $\mu m^2\ s^{-1}$) [24]. All signals (spots) belonging to the same species are aligned and the diffusion coefficient can be measured (Fig. 3C).

The temperature must be stable during the DOSY experiment because the diffusion process is very sensitive to this parameter and temperature gradients may initiate convection movements in the NMR tube that impair a reliable measure of the diffusion. The presence of eddy currents also hampers the measurement. Eddy currents build up in the presence of the fast varying field gradients used in the DOSY experiments and can lead to strong distortion of the recorded signal. To reduce the intensity of the eddy currents generated by the PFG and to minimize their impact on the observed signal, one may resort to optimized STE sequences including bipolar gradient pulses (BPP) and/or longitudinal eddy current delay (LED) [23,25]. The linearity of the PFG amplitude is another important parameter that must be checked from time to time.

4. Uncovering counterfeits

4.1. What can be done by NMR spectroscopy?

There are innumerable examples reported where the structure of an impurity, being normally not present in a drug, is elucidated by means of NMR spectroscopy. Here, it is not always necessary to separate an impurity for structure elucidation and quantification, as long as enough signals are not covered by the main component.

Mostly ^{1}H NMR spectroscopy is first applied to characterize a sample. The spectrum will provide signals of the other components present in an API having protons. Thus, the API, an organic compound, and its related substances as well as residual solvents can be seen, remarkably, in one run if the API structure is not too complicated and the related substances are present not less than 0.05%. This might take about half an hour. The spectrum can be inspected visually by an experienced person (maybe the operator of the instrument). However, an automated evaluation using statistical methods, e.g. PCA or PLS, is also possible. The latter has the advantage that it might "see" impurities which are hidden under other signals, e.g. of the main component. A statistical analysis is also advantageous in the case of the analysis of more complicated spectra, e.g. originated from biologicals and polyherbal samples.

^{13}C NMR spectra, which will take a bit longer to measure due to the low natural abundance of the ^{13}C isotope, provide normally better separated signals because of the wide spectral range of more than 200 ppm. The spectra can be used for structure elucidation and recognition of impurities that should not be present, but quantification is difficult with ^{13}C NMR spectroscopy because each carbon atom might have a different nuclear Overhauser effect and the relaxation time T_1.

Even though NMR spectroscopy is a powerful tool for quality control it is rarely used in international pharmacopoeias [8]. Nevertheless, the pharmaceutical industries, especially "big pharma", started to use NMR spectra more often [26] in routine quality analysis. However, it will take quite some time till qNMR spectroscopy will find its way into the monographs of the international pharmacopoeias.

The power of qNMR spectroscopy and DOSY NMR for counterfeit detection will be demonstrated exemplarily by the heparin case and some other API samples as well as by some formulations of sildenafil.

4.2. The heparin case and application of ^{1}H NMR spectroscopy

In the beginning of 2008 the Center for Disease Control and Prevention (CDC) in USA was informed by health authorities about a cluster of anaphylactoid reactions among hemodialysis patients treated with unfractionated heparin, which occurred in November 2007. One month later Baxter Healthcare recalled suspicious batches of one-dose and multiple-dose heparin from the market and stopped the production of unfractionated heparin. In April, the adverse reaction could be attributed to oversulfated chondroitin sulfate (OSCS) [27] whose structure was elucidated by Guerrini et al. [28]. The regulatory authorities in the USA and in Europe started to revise the old monograph in the PhEur and the USP which had not been touched for many years. In the first and second stages of revision NMR spectroscopy was the first choice for quality assessment because the acetyl signals of OSCS and heparin were separated in a

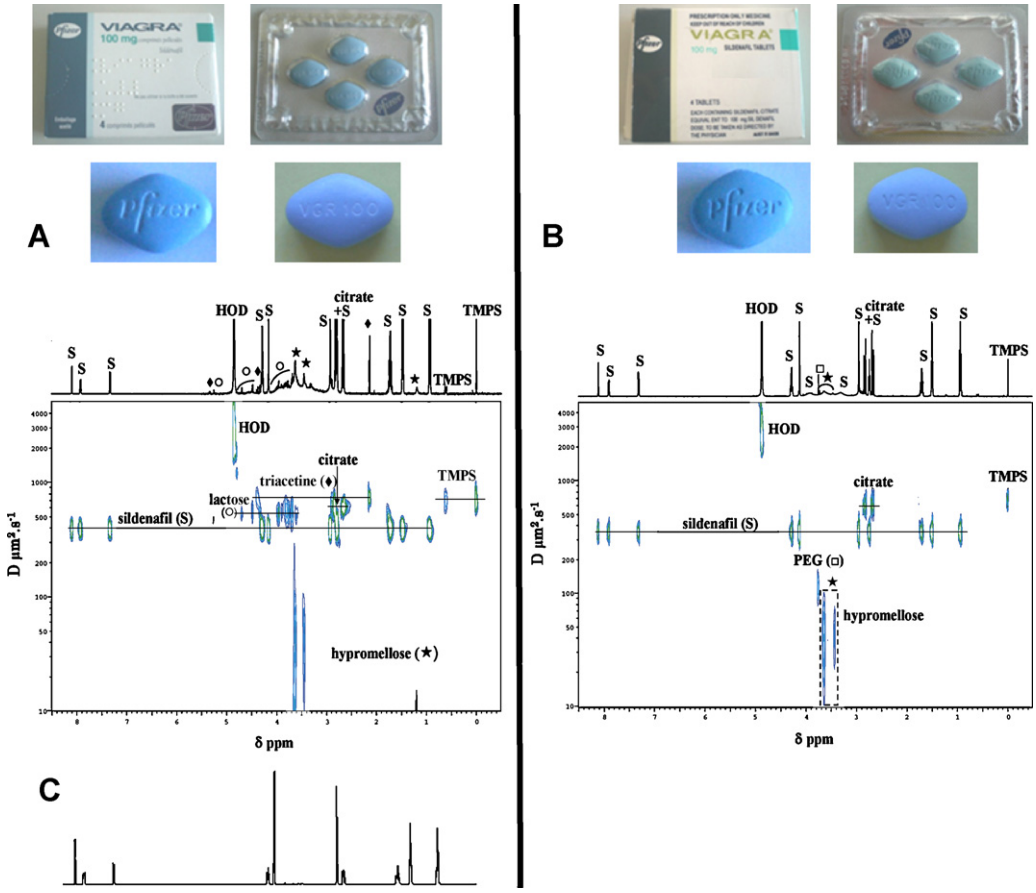

Fig. 5. 2D DOSY ^{1}H NMR spectra in D_2O of tablets from (A) genuine Viagra® and (B) a counterfeit formulation. (C) 1D ^{1}H NMR spectrum of sildenafil extracted from (A) at $D = 390\ \mu m^2\ s^{-1}$. Adapted from [37]. (S) sildenafil; (★) hypromellose; (♦) triacetine; (○) lactose; (□) polyethylene glycol (PEG). TMPS (trimethylsilylpropane sulfonic acid) is the internal reference. In part B, a deeper section of some signals is shown in boxes.

way that the quantification of OSCS was possible [29], most interestingly not by integration of the signal but by measuring the peak height, because the OSCS signal was sitting on starting point of the corresponding heparin signal (cf. Fig. 4) [30]. OSCS was found up to 25% in contaminated samples collected in Germany.

Beside the limitation of the toxic OSCS the intensive investigations on unfractionated heparin unraveled a couple of other ingredients which should not have been present in the API. Heparin normally isolated from intestinal porcine mucosa is always accompanied by the natural compound dermatan sulfate (DS) [31]. Although DS has the same pharmacological properties it should not be present in heparin. The amount of DS remaining in heparin is indicative of the quality of the purification procedure. Screening of about 200 batches collected from the German market by the Federal Institute of Drugs and Medical Devices (BfArM) revealed the presence of DS in almost all batches in concentrations between less than 1–8% [32]. The quantification was performed from the ^{1}H NMR spectra (cf. Fig. 4), using the standard addition method of DS to heparin [29].

In addition to heparin-like components the batches consisted of substantial amounts of residual solvents originated from the precipitation procedure which is one of the last steps of heparin purification. Ethanol was found in 94 batches in amounts up to 10%, the other solvents sodium acetate (53 batches), acetone (17), methanol (5), and formic acid (11) were detected in traces only [32].

The advantage of the qNMR spectroscopy is clearly shown here, because all quantifications, OSCS, DS and residual solvents, could be measured in one run, i.e. from one spectrum. Further statistical analysis, mainly principal component analysis, was performed in order to find out whether the ingredients are related to each other, but no correlation could be found. Additionally, all batches were evaluated by means of IR spectra, using an ATR unit, and Raman spectra. Both techniques were able to discriminate between OSCS-contaminated and non-contaminated batches but did not give any further information. Additionally, a chemometric evaluation by statistical classification techniques revealed the qNMR to perform best for OSCS detection [33].

It is worth mentioning that it is possible to discriminate between porcine and bovine heparin as well as sodium and calcium heparin [9]. Thus, the ^{1}H NMR spectroscopy can be applied for identification purposes, too. The application of ^{1}H NMR spectroscopy in the USP and PhEur monographs is therefore highly justified.

4.3. The example of Viagra: application of 2D DOSY ^{1}H NMR

The market success of the three approved phosphodiesterase type 5 (PDE-5) inhibitors for treating erectile dysfunction, sildenafil (Viagra®, Pfizer), tadalafil (Cialis®, Eli Lilly), and vardenafil (Levitra®, Bayer) has led to an explosion in counterfeit versions of these drugs. From 2004 to 2008, 35.8 million counterfeit sildenafil tablets had been seized in Europe [34]. In 2006, the number of users of legal sildenafil in the European Union was estimated to be 2.5 million, while 0.6–2.5 million men could have been exposed to illicit sildenafil [34]. PDE-5 inhibitors are a prime target for counterfeiting because of their high cost and the embarrassment associated with the underlying condition leading people to turn to the internet to buy these medicines easily, anonymously and often cheaply.

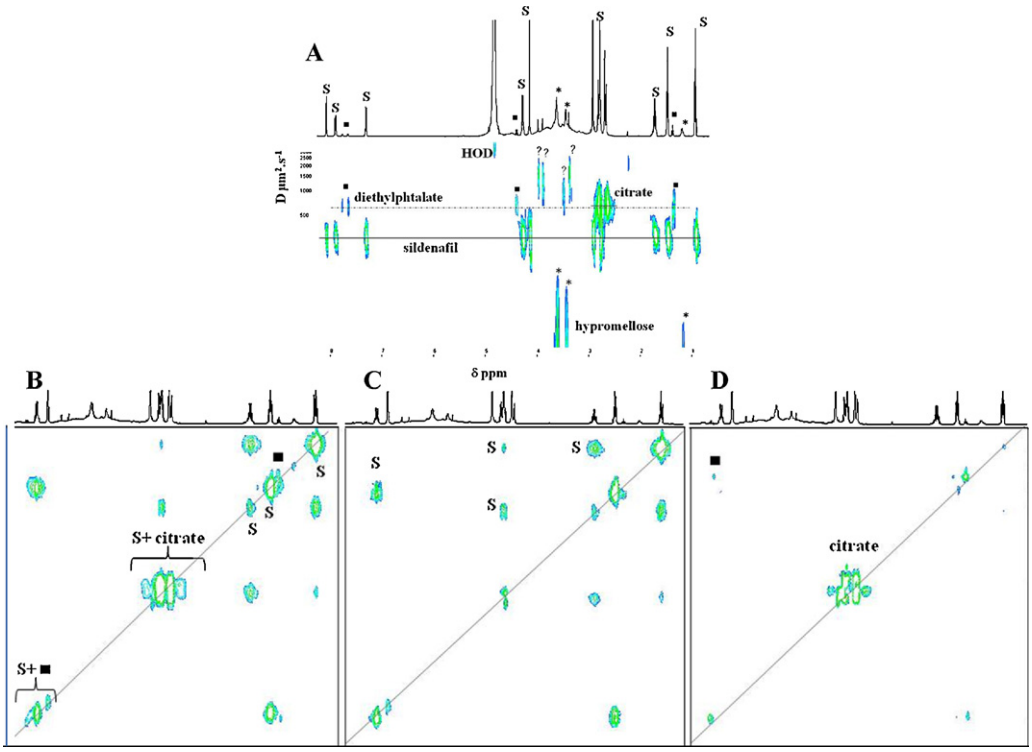

Fig. 6. NMR spectra of Kamagra, an Indian formulation of sildenafil in D_2O. (A) 2D DOSY ^1H spectrum; (B) COSY–DQF spectrum; COSY extractions from 3D DOSY–COSY experiment at (C) $D = 310\ \mu m^2\ s^{-1}$, and (D) $D = 510\ \mu m^2\ s^{-1}$. Adapted from [37]. (S) sildenafil; (⋆) hypromellose; (■) diethylphtalate; (?) unknown.

Moreover, a large market has developed for herbal medicines (HMs) as a safe alternative to synthetic PDE-5 inhibitors. Indeed, in contrast to conventional pharmaceuticals, HM are regarded by many as being harmless and free from any side-effects because of their natural origin. However, their adulteration with conventional drugs is a growing trend and poses a health threat to patients who unwittingly consume a synthetic drug [35]. HM marketed for enhancement of sexual function are the most affected.

Another major issue is that counterfeiters, in an attempt to evade regulatory inspection, use not only the three approved PDE-5 inhibitors but also unapproved analogues in which minor modifications were brought to the parent structure to falsify drugs or HM. In this context, the power of NMR for structural elucidation is particularly useful.

Fig. 5 shows a comparison of genuine and counterfeit Viagra. For the consumer, the box of the counterfeit formulation is "authenticated" by the Pfizer logo. The blister is very similar to the genuine one and the tablets are identical. However, the DOSY NMR spectrum proves that the tablet composition is different. The counterfeit formulation indeed contains sildenafil citrate and hypromellose but lactose and triacetin are absent, whereas polyethylene glycol is found. To help substantiating compound identification, the

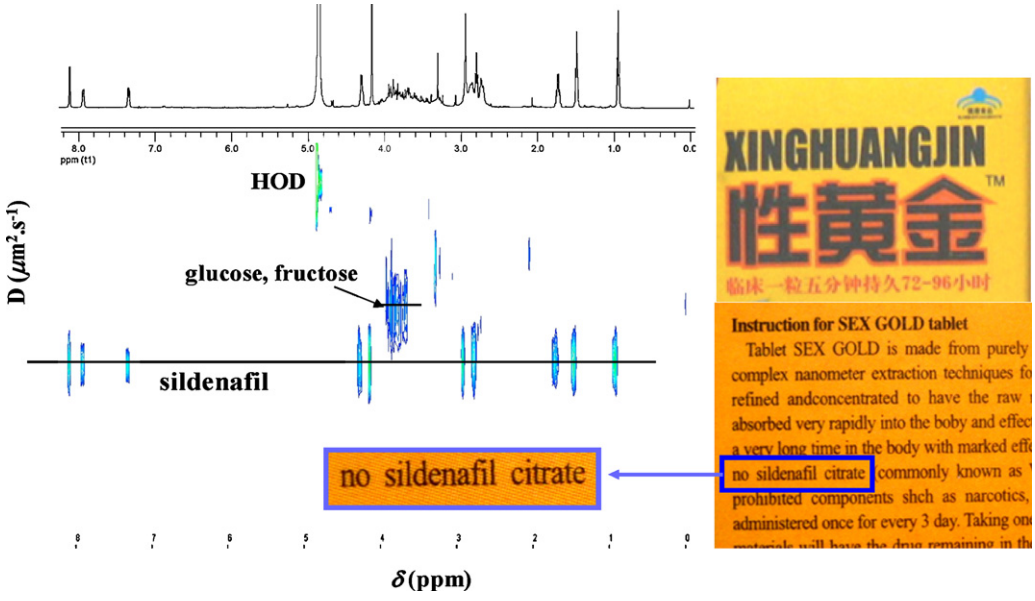

Fig. 7. 2D DOSY ^1H NMR spectrum of a Chinese "natural" formulation marketed for sexual dysfunction in D_2O.

"pure" 1D ^{1}H NMR spectrum of a component of the mixture can be extracted from the 2D DOSY spectrum, as shown for sildenafil in Fig. 5, provided that the compounds do not have too close D values. If further structural information is required, one can resort to 3D DOSY–COSY experiments where, here again, the COSY spectrum of each component of the mixture can be obtained from a selected line in the DOSY spectrum (Fig. 6).

The last example relates to a HM formulation purchased on the internet and marketed for sexual dysfunction which the manufacturer advertised as "all natural" and "containing no sildenafil citrate". However, the DOSY spectrum presented in Fig. 7 clearly demonstrates the presence of sildenafil along with natural sugars. Eight of the 17 herbal formulations analyzed were adulterated, four with sildenafil, one with tadalafil, one with both sildenafil and vardenafil, one with both tadalafil and hydroxyhomosildenafil, and one with a newly identified analogue, thiomethisosildenafil [36].

The examples described here and many others, such as the characterization of the contaminated heparin [38], clearly show that 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 it can distinguish between genuine and counterfeit formulations, but it is also helpful in determining the relationships between different samples and so assists in the investigation of the sources of these drugs. Moreover, the method is non-selective and requires no prior knowledge of the structures of the various components present in the mixture which is a major advantage for screening of counterfeit drugs or adulteration of HM. One can argue that all the information is already included in the 1D ^{1}H NMR spectrum, which is perfectly true. However, the virtual separation obtained by adding a second dimension based on diffusion coefficients enables a better visualization of the composition of the formulation analyzed and thus a more precise identification of its constituents. If 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 a pre-treatment of the sample is necessary and only compounds having 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 ≈1 h using a high-field spectrometer equipped with a cryoprobe, which precludes its use on the field but is not too disadvantageous with respect to the sum of chemical information gained in a single DOSY NMR experiment.

5. Conclusions

Using NMR spectroscopy as an additional orthogonal method to e.g. HPLC seems to be a powerful technique for APIs and excipients as well as formulations because

1. is not optimized for one synthesis pathway,
2. it cannot be manipulated,
3. gives normally more than one signal for an additional component/impurity,
4. deviations from a typical signature of a drug can be easily detected by simple inspection (small molecules) or statistical methods (e.g. PCA, PLS) in the case of "biologicals", and
5. DOSY NMR provides a virtual separation of components of a drug formulation

Thus, it is difficult to hide an impurity of an API or excipient or an additional component in a drug formulation indicating that NMR spectroscopy with its whole range of different techniques is perfect tool to unravel all kinds of counterfeits.

Acknowledgement

Thanks are due to the Federal Institute for Drugs and Medical Devices for providing financial support for the heparin investigations.

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