Journal of Pharmaceutical and Biomedical Analysis 47 (2008) 683–687

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Improved solid-state NMR quantifications of active principles in pharmaceutical formulations

Stéphanie Sanchez [a], Fabio Ziarelli [b,*], Stéphane Viel [a], Corinne Delaurent [a], Stefano Caldarelli [a]

[a] Aix-Marseille Université, ISM2-UMR-6263, Equipe CES, case 512, av. Escadrille Normandie Niémen, 13397 Marseille Cedex 20, France
[b] Aix-Marseille Université, Fédération des Sciences Chimiques de Marseille, Spectropole (CNRS-FR1739), case 511, av. Escadrille Normandie Niémen, 13397 Marseille Cedex 20, France

ARTICLE INFO

Article history:
Received 17 December 2007
Received in revised form 21 March 2008
Accepted 21 March 2008
Available online 4 April 2008

Keywords:
Solid-state NMR
External standard
Quantitative NMR
Optimized rotor packing
Pharmaceutical formulations

ABSTRACT

The facility of implementation reached by solid-state nuclear magnetic resonance (ssNMR) spectroscopy makes this technique increasingly popular in pharmaceutical sciences, and more specifically for the dosage of active principles in pharmaceutical formulations, since about 80% of the formulations currently available on the market are present in the solid form. In this case, analysis by MAS NMR allows faster and simplified protocols, as a solubilization step is not required. However, the specificity of the ssNMR experiments should be explicitly taken into account when designing an accurate measurement procedure. In this work we show that, by using a combination of external concentration referencing and a properly designed sample preparation optimized for quantitative determinations, quantification of active principles in pharmaceutical formulations can be performed with both speed and precision. The method is illustrated by reinvestigating the dosage of Meprobamate, an anxiolytic agent typically prescribed in case of anxiety or muscular soreness, present in a commercial formulation (Equanil®). Specifically, with respect to previously proposed analytical protocols, the procedure outlined here allows fast quantification with excellent precision.

1. Introduction

Solid-state nuclear magnetic resonance (ssNMR) has recently attracted growing interest for the characterization of pharmaceutical compounds in the solid state [1,2]. This is primarily due to considerable improvements in the instrumentation and to the fact that about 80% of the drugs currently available on the market are present in the solid form. ssNMR allows not only the chemical structure [3] but also the physical properties (polymorphism, multiple molecules per asymmetric unit cell, disorder) of the pharmaceutical substances to be investigated [4,5]. These properties are of the utmost importance because they influence not only the solubility but also the biocompatibility and physico-chemical stability of the drugs.

In parallel, many studies have reported on the use of NMR for quantitative analyses [6,7], showing that NMR is a very linear and robust technique that allows both for precise and accurate quantifications [7,8]. In this context, the use of ssNMR for quantification bears many advantages [9], as the technique is fully non-destructive and requires no preliminary sample manipulations [10]. In addition, the analysis is performed on the bulk on significant amounts of sample, which allows the effects of possible heterogeneities to be averaged out.

A few ssNMR quantitative studies have already been reported [11], devoted for instance to the relative quantification of various crystalline or amorphous forms [9,12], or to the quantification of active principles in pharmaceutical formulations [11,13,14]. In this latter case, the methods described in the literature have almost exclusively relied on the use of an internal standard.

The use of an internal standard reduces the effects of instrumental instabilities and allows direct quantifications to be achieved [15–17]. The most significant drawbacks are the difficulty of preparing homogeneous mixtures of sample and standard, an especially critical step in the solid state, and the reduction in sensitivity caused by sample dilution. This point is especially important for NMR because of its relatively low sensitivity with respect to other analytical techniques.

Recently, we have shown that it is possible to increase the sensitivity, and hence the precision, of ssNMR quantifications based on the external standard method, by using a rotor packing optimized specifically for quantitative determinations [18].

In this work, we will show an application of this method for the precise quantification of active principles in pharmaceutical formulations. As an illustration, we chose to work on Meprobamate

* Corresponding author. Fax: +33 491 282 897.
E-mail address: fabio.ziarelli@univ-cezanne.fr (F. Ziarelli).

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doi:10.1016/j.jpba.2008.03.030

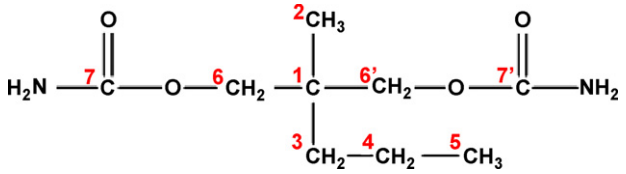

Fig. 1. Molecular structure and atom numbering of Meprobamate ([2-(carbamoyloxymethyl)-2-methyl-pentyl] carbamate).

(Fig. 1), an anxiolytic agent prescribed in case of anxiety or reflexive muscular contractions, by reinvestigating its dosage in commercial formulations; in fact, Meprobamate is commercially available as a solid formulation (Equanil®) containing other excipients such as starch, talc, magnesium stearate, etc.

While a few distinct analytical protocols have indeed been proposed for the dosage of Meprobamate, based either on chromatographic [19] or spectroscopic techniques [15], a trusted method has not been singled out. The sole official suggestion is to be found in the United States Pharmacopeia monograph which recommends the use of reversed phase liquid chromatography at 200 nm to assay Meprobamate in tablets [20]. As a matter of fact, typical chromatographic methods cannot be used in this case because this aliphatic compound does not exhibit any useful UV absorption. On the other hand, gas chromatographic methods are faced with the difficulty of thermal degradation as well as other experimental artifacts (peak tailing), which dramatically reduce the detection limit and decrease the analytical reliability.

In any case, the main drawback of most previously reported methods is the required sample manipulations (*e.g.* solubilization, extraction, derivatization, etc.) which, in addition to being both time- and reagent-consuming, may potentially introduce additional sources of errors.

In contrast, the method proposed here does not require any sample treatment. Overall, the proposed method may routinely yield relatively fast measurements (from 20 to 60 min depending upon the available NMR instrumentation) with a precision higher than 1%.

2. Experimental

2.1. Samples

High-purity Meprobamate ([2-(carbamoyloxymethyl)-2-methyl-pentyl] carbamate) was provided by the Council of Europe as a Chemical Reference Substance (CRS). Its molecular structure together with the corresponding atom numbering used in this study, are shown in Fig. 1. Equanil® 400 mg and 250 mg were obtained from SANOFI-AVENTIS. While Meprobamate is commercially available as a fine powder, Equanil® is provided in the form of tablets, which were finely grounded with a mortar and pestle prior to analysis. For the ssNMR analysis, 10 tablets were used giving an averaged weight of 615.9 and 398.5 mg per tablet for Equanil® 400 and 250, respectively.

Two synthetic mixtures of Meprobamate with starch (Sigma–Aldrich, starch from potato suitable for electrophoresis) were also prepared by grounding precisely known amounts of both substances (determined by weight) with a mortar and pestle. Mixture A was obtained by mixing 300.0 mg of Meprobamate and 150.3 mg of starch, whereas mixture B was obtained by mixing 60.0 mg of Meprobamate and 40.2 mg of starch. The proportion of these mixture components were chosen to respect those found in the commercial formulations. In addition, to perform the liquid-state NMR assignment, Meprobamate was dissolved in deuterated acetone purchased from Eurisotop. All materials were used as received.

2.2. Liquid-state NMR

All liquid-state NMR experiments were conducted at 300 K on a BRUKER AVANCE400 DPX spectrometer operating at 400 MHz for the ^{1}H Larmor frequency, and equipped with a BRUKER 5 mm liquid-state ^{1}H/X BBI probe. The liquid-state NMR assignment was performed in acetone-d_6 using a combination of conventional 1D (^{1}H, ^{13}C{^{1}H}) and 2D (^{1}H–^{13}C HSQC and HMBC) experiments, for which typical acquisition parameters were used as summarized in Ref. [21].

2.3. Solid-state NMR

All ssNMR experiments were conducted at 300 K on a BRUKER AVANCE400 DSX spectrometer operating at 400 MHz for the ^{1}H Larmor frequency, and equipped with a BRUKER 4 mm ^{1}H/X solid-state CP MAS probe. The spinning rate was set to 10 kHz. For the ^{13}C CP MAS experiments, a ramped ^{1}H pulse starting at 100% power and decreasing until 50% was used during the contact time (4 ms) in order to circumvent Hartman–Hahn mismatch [22,23]. ^{1}H decoupling during signal acquisition was ensured by the GT8 scheme [24]. The recycle delay was set to 13 s, five times the largest T_1. The number of scans was adjusted to ensure a signal-to-noise ratio of at least 150 [8].

In addition, to facilitate the assignment of the solid-state ^{13}C chemical shifts of Meprobamate, a CPPI (cross-polarization polarization inversion) experiment [25] was performed with a pulse inversion length of 40 μs. With this pulse length, CH_3 and quaternary carbons appear positive, CH_2 negative, and CH signals are nearly zero.

As evidenced later on in the text (Section 3.2), when using the external reference method, the quantitative coil volume of the probe must be known. The probe used here was equipped with a 12-turn coil and its quantitative volume was 30 μl. Accordingly, all ssNMR quantitative measurements were performed using a 4 mm rotor prototype provided by ROTOTEC–SPINTEC [18]. Importantly, because the quantitative coil volume is only probe-dependent, it can be used for the quantification of all types of solid samples provided that the same probehead is used. Finally, note that the integrals were measured and compared from one spectrum to another by using the INTERSCALE function of the XwinNMR 3.5 software from Bruker. This function allows the value of a signal integral in a given spectrum to be used as an intensity reference for all the other spectra.

3. Results and discussion

3.1. Assignment of the ^{13}C NMR spectra of Meprobamate

To the best of our knowledge, although Meprobamate is a rather well known active principle, the assignment of its ^{13}C NMR spectrum has not been reported yet. Therefore, to ease the assignment of the solid-state NMR spectrum, we first assigned the liquid-state ^{13}C spectrum (Table 1).

Specifically, the latter assignment was performed in acetone-d_6 using conventional experiments (see experimental section). This assignment served as a basis to interpret the ^{13}C CP MAS spectrum of the solid form of Meprobamate, shown in Fig. 2a. The solid-state NMR assignment was further confirmed using a CPPI experiment recorded on pure Meprobamate (Fig. 2b). Note that, in this spectrum, signals due to CH_3 and quaternary carbons are positive whereas CH_2 signals appear negative.

Overall, the CPPI experiment allows the solid-state assignment derived from liquid-state data to be confirmed, with the notable exception of the C-1 and C-3 groups, whose ^{13}C chemical shifts

Table 1
Solution and solid-states ^{13}C chemical shifts obtained for pure Meprobamate

^{13}C atom[a]		Solution state ^{13}C, δ (ppm)[b]	Solid-state ^{13}C, δ (ppm)[c]
1	C	38.27	37.4
2	CH_3	19.26	22.4
3	CH_2	37.63	38.9
4	CH_2	17.08	15.8
5	CH_3	15.25	15.5
6	CH_2	68.45[d]	65.3[e]
6′	CH_2	68.45[d]	71.1[e]
7	CO	157.7[d]	158.9[f]
7′	CO	157.7[d]	159.3[f]

[a] Atom numbering refer to Fig. 1.
[b] Obtained in acetone-d_6 and referenced with respect to the residual proton of the deuterated solvent at 2.03 ppm downfield of TMS.
[c] Obtained for pure Meprobamate and referenced with respect to the carbonyl signal of glycine, used externally, set at 176.5 ppm downfield of TMS.
[d] In the liquid state, 6 and 6′, and 7 and 7′, are chemically equivalent.
[e,f] These assignments could be interchanged with one another.

are inverted in the solid state with respect to the liquid state. This is evidenced in the CPPI spectrum by the small negative signal at 38.9 ppm, downfield of the large signal at 37.4 ppm. These are hence assigned to C-3 and C-1, respectively.

Finally, note that the signal due to C-4 is not detected in the CPPI spectrum, as evidenced by the inset shown in Fig. 2(bottom). This is one shortcoming of this pulse sequence, because signals of opposite phase may cancel whenever the difference in Hz between their chemical shifts is comparable to their linewidths.

3.2. Protocol for the quantification of Meprobamate

An exhaustive protocol has been recently published by Harris et al. [13] describing all relevant acquisition and processing parameters required to achieve optimal results in ssNMR. Here, we shall merely focus on the experimental parameters relevant to the proposed method.

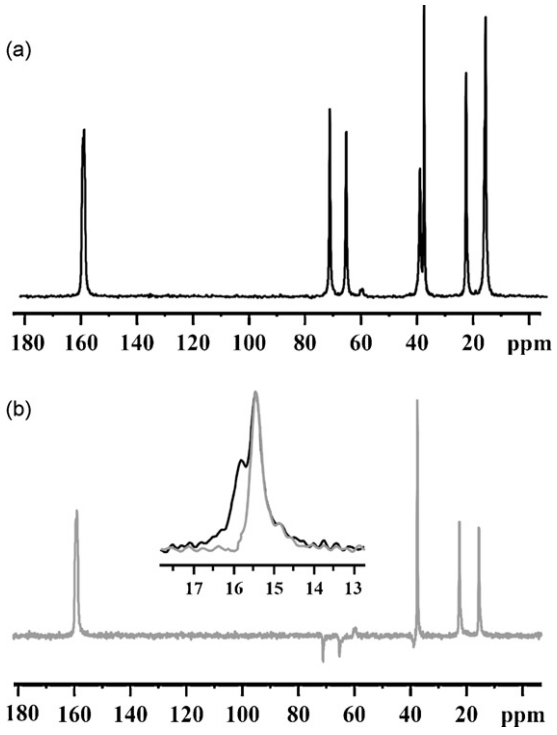

Fig. 2. (a) CP MAS and (b) CPPI spectra recorded for pure Meprobamate on a $^1H/X$ CP MAS probe at 10 kHz. In the CPPI spectrum, the signals due to CH_2 groups are negatively phased. The inset in (b) shows an expanded view of the C-4 and C-5 region of the CPPI (grey) and CP MAS (black) spectra, showing the absence of the C-4 signal at 15.8 ppm (see text).

3.2.1. Sample preparation

The signal intensity in NMR is a direct count of the number of nuclei excited by the radiofrequency scheme, multiplied by a factor measuring the effectiveness of the excitation. This latter factor is equal to one for a perfect 90° excitation pulse. The radiofrequency field produced by a typical coil in a NMR probe is only homogeneous on a fraction of its volume. Thus, if the analyzed sample extends over this specific volume, the NMR response will not be homogeneous for all molecules. When analyzing a solution, this effect is not a nuisance, as the perfectly homogeneous state of the sample and the internal dynamics allow the use of an average effectiveness factor for the radiofrequency excitation. In contrast, when analyzing a solid, molecules inside or outside the homogenous excitation volume, will contribute differently to the build up of the signal, thus degrading the quantitative response. We shall thus refer in the following to the volume of homogeneous response of the coil as to its "quantitative volume". While all of the above has been known for long time, a procedure to fully exploit the quantitative volume was proposed only recently [18], which showed, with respect to most common preexisting laboratory practices, an excellent improvement in accuracy at no significant cost in sensitivity. Details of the method are described in reference [18]. In short, the quantitative volume which is a function of a given probe/coil assembly, must be determined once by using adapted rotors and precisely designed inserts. In this work, the quantitative volume was of 30 μl, which corresponded to 30% of the full rotor volume.

3.2.2. Intensity referencing

An internal or external concentration standard is required to translate NMR signal intensities into actual number of molecules detected. While internal referencing provides the closest homogeneity of response with the observed sample, its introduction/removal in the sample can be problematic, for the homogeneity issues described above or when the sample is precious, or just because its diluting the sample reduces the intensity of the interesting signal. The external reference method can provide excellent accuracy and precision, if the sample volume is restricted to the quantitative volume and if the spectrometer stability is not an issue. A viable choice as external reference is the product under analysis itself, used for a calibration curve.

It should be noted that a mixed internal/external reference technique, the so-called ERETIC™ pulse, is finding a growing interest [26–28]. Since the external and the ERETIC™ method provide comparable results in ssNMR, we opted in this study for the former using high-purity Meprobamate provided by the Council of Europe.

3.2.3. Optimized ^1H–^{13}C cross-polarization experiments

In this work, ^{13}C CP MAS experiments were used in place of single pulse excitation (SPE) MAS experiments in order to maximize experimental sensitivity. As a matter of fact, because of the polarization transfer through the dipolar interaction from the abundant spins (^1H) to the rare spins (^{13}C), the signal intensity of the rare spins in CP experiments is enhanced with respect to SPE. Moreover, because relaxation in CP MAS experiments is primarily dictated by ^1H instead of ^{13}C, much faster repetition rates can be used, as the former relaxes more efficiently than the latter. This leads to overall improvement in signal-to-noise ratio per unit of time.

On the other hand, the signal intensity in a CP MAS experiment stems from the combination of several parameters, related to all species relaxation times and to the size of the proton–carbon dipolar couplings [29]. Among the relaxation parameters, only the

Table 2
^{1}H spin-lattice relaxation times (T_1) of the ^{13}C CP MAS signals measured for pure Meprobamate and Meprobamate in Equanil®

^{13}C atom		Pure Meprobamate ^{1}H, T_1 (s)	Meprobamate in Equanil® ^{1}H, T_1 (s)
1	C	2.4	2.6
2	CH_3	2.3	2.4
3	CH_2	2.4	2.6
4	CH_2	2.3	2.4
5	CH_3	2.6	2.4
6	CH_2	2.4	2.5
6′	CH_2	2.5	2.2
7	CO	2.5	2.4
7′	CO	2.5	2.4

values of the longitudinal relaxation time constants, T_1, of the protons feeding polarization to the carbons are required for quantitative analysis, since these dictate the time that must be left between each scan. This measurement was performed directly on the carbon CP MAS spectrum, thus using the carbon signal as a spy of the relaxation of the protons from which they are receiving polarization.

To assess possible effects of the environment on relaxation, these measurements were performed both on pure Meprobamate and on Meprobamate in the Equanil® formulation. The results, also reported in Table 2, show that similar T_1 values were obtained in both cases. It is worthwhile noting that all signals have roughly the same relaxation characteristics, typical of a system dominated by a single source of relaxation [29].

To establish the calibration curve, a signal must be selected in the CP MAS spectrum, whose intensity will hence be related to the amount of Meprobamate present in the rotor. The CP MAS spectrum of Meprobamate in Equanil® is shown in Fig. 3. As can be seen, all expected resonances due to Meprobamate are present, plus a series of broad resonances due most probably to the excipients.

In order for the integral of the selected signal not to be biased by the presence of these extra resonances, we selected the C-7,7′ signals, which resonate in a spectral region (about 159 ppm) where no peak due to the excipients is expected. Subsequently, the CP build up curve for this specific signal must be established, in order to optimize the experimental contact time, namely the time during which the magnetization is transferred from ^{1}H to ^{13}C.

To rule out the presence of matrix effects on the CP transfer all together, the full CP buildup curve was constructed for Meprobamate pure and in the Equanil® formulation. The results are reported in Fig. 4 for the C-7,7′ signals. As can be seen in Fig. 4, pure Meprobamate and Equanil® curves are almost perfectly identical, suggesting

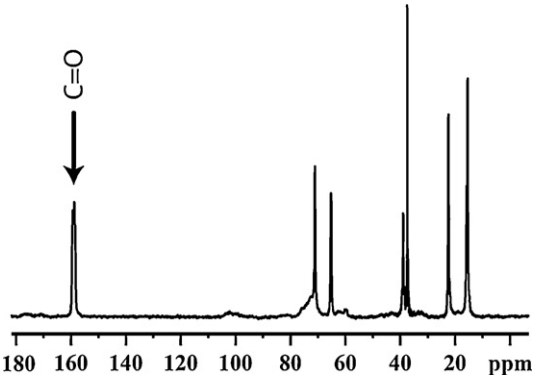

Fig. 3. ^{13}C CP MAS spectrum of Equanil®. All the resonances due to Meprobamate are present, plus a series of broad resonances of low intensity, probably due to the excipients.

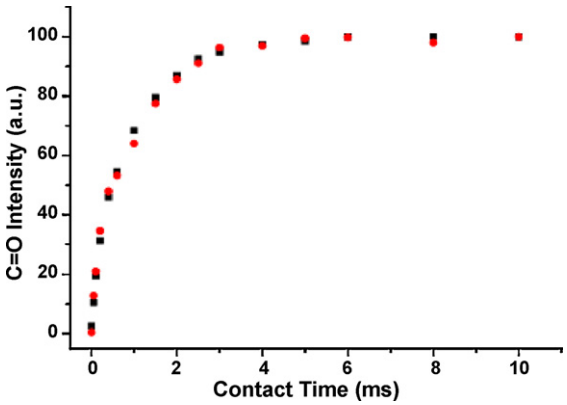

Fig. 4. ^{13}C CP MAS buildup curves obtained for the C-7,7′ signal in pure Meprobamate (●) and Meprobamate in Equanil® (■). Both curves are rigorously identical, which proves the absence of any matrix effect. From these curves, a contact time of 4 ms was selected.

very similar CP dynamics in both cases. Finally, from these data, an optimal contact time of 4 ms was chosen.

3.2.4. Calibration curve and quantitative measurements

Once all relevant experimental parameters have been optimized, including the contact and T_1 relaxation times, and the quantitative coil volume of the probe has been determined, the calibration curve can be established. This can be simply done by recording the CP MAS spectrum of a precisely known amount of Meprobamate, and reporting the signal integral of the CO signals as a function of the introduced amount of sample. This is illustrated in Fig. 5, which shows the excellent linearity of the technique over the range of weights considered in this study (from 0 to 20 mg). Clearly, the largest amount of pure Meprobamate analyzed is limited by the quantitative volume of the rotor in use (here 20 mg).

To estimate the accuracy of this curve, two synthetic mixtures, hereafter referred to as A and B, were prepared by grounding together known amounts of pure Meprobamate with starch (see Section 2). Then, for both mixtures, five distinct rotors each containing 20.0 mg of one the corresponding mixture were analyzed by ssNMR. The calibration curve reported in Fig. 5 allowed us to determine an average Meprobamate amount for these five rotors, and hence derive estimations for the corresponding amounts contained in the whole mixtures. The results reported in Table 3 suggest that

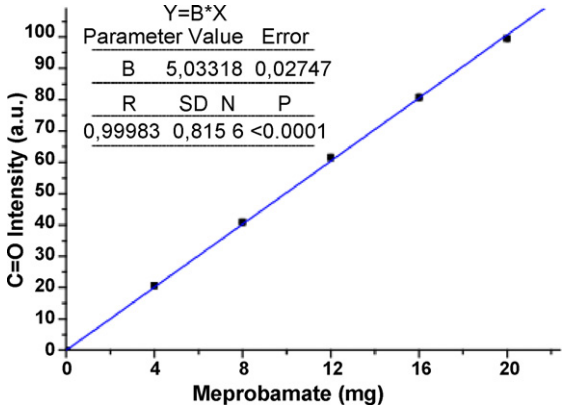

Fig. 5. Calibration curve obtained using ^{13}C CP MAS and a quantitative rotor packing showing the relationship between the amount of Meprobamate (weight, mg) and the corresponding intensity (area, arbitrary units) of the C-7,7′ resonance signal. The value of the regression R^2 parameter (0.9998) shows the excellent linearity of the obtained calibration curve. The standard deviations are included within the symbols size.

Table 3
Determination of the amount of Meprobamate in synthetic (mixtures A and B) and commercial samples (Equanil® 400 and Equanil® 250)

	Meprobamate theoretical weight (mg)	Meprobamate experimental weight (mg)	Deviation (%)[a]
Mixture A	300.0[b]	297.6 (2.2)[c]	−0.8
Mixture B	60.0[b]	60.4 (0.6)[c]	+0.7
Equanil® 400	400[d]	384.6 (4.5)[c]	−3.8
Equanil® 250	250[d]	240.2 (2.4)[c]	−3.9

[a] Relative deviation between the theoretical and experimental Meprobamate amounts, expressed with respect to the theoretical amount.
[b] As expected from our sample preparation.
[c] Standard deviation.
[d] As expected from the values indicated by the manufacturer.

the method is accurate, as the deviation between the theoretical and experimental Meprobamate amounts is lower than 1%.

The same procedure was then applied to quantify the amount of Meprobamate in Equanil® 250 and Equanil® 400. Here, for each formulation, 10 tablets were ground together and the same procedure as the one used for the synthetic mixtures was applied. The results, also shown in Table 3, illustrate the good agreement between the experimental data and the value reported by the manufacturer. These data confirm the reliability of the proposed method, and show that ssNMR yield quantitative results with a good precision.

4. Conclusion

We showed that a recently proposed ssNMR protocol for quantitative measurements could be fruitfully applied to the quantification of active principles in pharmaceutical formulations. The procedure provides optimal sensitivity and thus, combined with the external intensity reference method, allows fast analysis with excellent precision and accuracy.

Acknowledgements

Thanks are due to *Spectropole* for providing access to the NMR facilities of the *Fédération des Sciences Chimiques de Marseille*. The authors thank D. Cross from ROTOTEC-SPINTEC (Biebesheim, Germany) for providing them with the rotor prototype mentioned in the text.

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