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The Well-Characterized Synthetic Molecule: A Role for Quantitative ^{1}H NMR

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Abstract:

Experimental evidence is reported to illustrate the role of quantitative ^{1}H NMR in the analytical characterization of new synthetic molecules. This evidence includes comparison with results obtained for reference materials and for extensively characterized substances, precision data on a statistically significant set of different molecules, elemental analysis data. These data prove that ^{1}H NMR estimates quantitative purity with a precision of about 1% and with a comparable or better accuracy. Moreover, many of the limitations that generally afflict other methods (variability of response factors, incomplete accounting for inorganic or volatiles) do not apply to the quantitative ^{1}H NMR method, whose only requirement is the presence of at least one integrable signal in the spectrum. Because of the widespread usage of ^{1}H NMR in modern synthetic chemistry, it is suggested that spectra are routinely run under quantitative conditions, with a corresponding reduction of other quantitative characterization tools (elemental analysis, HPLC, loss on drying and residue on ignition determination) and speed-up of the whole analytical process. For a budget-conscious laboratory either connected to a discovery environment or in the field of process chemistry, this could be a considerable economic advantage, without a compromise on quality.

Introduction

Under the pressure of the constantly increasing productivity of synthetic organic chemists, many revised processes for the characterization of new molecules have emerged. LC−UV/MS is currently firmly established as a high-throughput investigative tool which maximizes the information/cost ratio and provides in reasonable time identity and purity data for chemical libraries composed of thousands of single compounds.[1] More recently, with the use of chemiluminescent nitrogen detector (CLND), the concentration of each compound has become accessible as another piece of data in the high-throughput characterization process.[2,3] However, these methods have limitations: UV-based purity and CLND-based quantitation suffer from response factor dependence on molecular chromophore and on chemical bond type, respectively, and even after appropriate correction, accuracy of CLND as a generic detector is not better than 10%;[4] MS cannot distinguish isomers, and sometimes accidental coincidence at unit mass resolution occurs for molecules with different atomic constitution. For these reasons, well-characterized synthetic compounds generally undergo a multianalysis process including MS, ^{1}H NMR, elemental analysis, HPLC, and determination of loss on drying (LOD) and of residue on ignition (ROI) for an accurate evaluation of compound purity. The scope of this article is to demonstrate that a combination of LC−UV/MS and quantitative ^{1}H NMR can provide information on identity and purity at the same (or higher) quality level as the above process, with considerable sample- and time saving and with great simplification of the analytical characterization process.

Our laboratory has regularly introduced quantitative ^{1}H NMR for purity and concentration assessment of small chemical libraries (<300 compounds), and with a fully automated sample preparation procedure, we demonstrated the feasibility of both accurate and precise measurements of purity.[5] The effort required for ^{1}H NMR spectra interpretation prevents the general applicability of this method to fulfill the needs of high-throughput chemistry,[6] at least until methods for the automatic spectra assignment grow out of their infancy. Under carefully controlled sample preparation, precision of ^{1}H NMR quantitation proved comparable with that of well-established methods such as HPLC.[7,8] We extend here the comparison to elemental analysis—another classical tool of the synthetic chemist—and we show that for both precision and accuracy ^{1}H NMR quantitation may compete with much more extensive characterization protocols.

Experimental Section

Materials. The ^{1}H NMR internal standard 1,4-bis-(trimethylsilyl)benzene was obtained from Aldrich at a nominal 96% purity. Purification through sublimation in a coldfinger apparatus at 80°C and under reduced pressure (∼20 mmHg) afforded a crystalline product of high purity (99.9% according to GC−MS, >99.9% from melting profile analysis) with melting point of 94.9 °C.

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(1) (a) Snyder, L. R.; Kirkland, J. J.; Glajch, J. L. *Practical HPLC. Method Development*, 2nd ed.; J. Wiley & Sons: New York, 1997. (b) Kyranos, J. N.; Hogan J. C. *Anal. Chem. News Features* **1998**, *June*, 389A−395A.

(2) Fitch, W. L.; Szardenings, A. K.; Fujinari, E. M. *Tetrahedron Lett.* **1997**, *38*, 1689−1692.

(3) Taylor, E. W.; Qian, M. G.; Dollinger, G. D. *Anal. Chem.* **1998**, *70*, 3339−3347.

(4) Arangio, M.; Riccardi Sirtori, F.; Marcucci, K.; Razzano, G.; Colombo, M.; Biancardi, R.; Rizzo, V. Evaluation of an integrated procedure for the characterization of chemical libraries on the basis of HPLC-UV/MS/CLND. In *HPLC Troubleshooting*; Kromidas, S., Ed., Springer: Berlin. In preparation.

(5) Pinciroli, V.; Biancardi, R.; Colombo, N.; Colombo, M.; Rizzo, V. *J. Comb. Chem.* **2001**, *3*, 434−440.

(6) Yan, B.; Fang, L.; Irving, M.; Zhang, S.; Boldi, A. M.; Woolard, F.; Johnson, C. R.; Kshirsagar, T.; Figliozzi, G. M.; Krueger, C. A.; Collins, N. *J. Comb. Chem.* **2003**, *5*, 547−559.

(7) Maniara, G.; Rajamoorthi, K.; Rajan, S.; Stockton, G. W. *Anal. Chem.* **1998**, *70*, 4921−4928.

(8) Wells, R. J.; Hook, J. M.; Al-Deen, T. S.; Hibbert, D. B. *J. Agric. Food Chem.* **2002**, *50*, 3366−3374.

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Of the five certified standards used for accuracy and precision of the ^{1}H NMR quantitative method, doxorubicin hydrochloride (97.6%), niacinamide (99.9%), and penicillin G-potassium salt (98.9%) are Pharmacia Corporate Reference Standards, whereas acetanilide (100.0%; standard for C, H, N, O) and caffeine (99.1%; testing and handling according to European Pharmacopoeia) were purchased from Aldrich and Fluka, respectively. Samples whose ^{1}H NMR strength was compared with elemental analysis or HPLC purity were synthesized in the API (Active Pharmaceutical Ingredient) and DRO (Discovery Research Oncology) departments of Pharmacia S.p.A., Nerviano, Italy.

DMSO-d_6 (99.95% D) was purchased from Merck, and Wilmad 528PP 5-mm NMR tubes were used for spectra acquisition.

Routine Methods. *Elemental analyses* were performed by an external company (REDOX snc, Monza, Italy) with a Pfizon EA1108 instrument.

Traditional determination of strength for new chemical entities (no reference standard available) were obtained according to guidelines for pharmaceutical products:[9]

$$\text{strength} = 100 - (\text{impurities} - \text{solvents} - \text{water} - \text{ROI}) \quad (1)$$

Impurities were evaluated by HPLC analysis as the sum of normalized areas of chromatographic peaks excluding the one of the chemical species under consideration. A specific HPLC method was developed for each individual compound.

Solvents and water were estimated from loss on drying (LOD) by thermogravimetric analysis (TGA) on Mettler-Toledo TGA 851 (Star System Software). The sample (about 25−30 mg) was accurately weighed in a 150-μL aluminum pan and generally heated from 25 to 120 °C at 5 °C /min. Weight loss is calculated as percentage with respect to the initial sample weight.

Residue on ignition (ROI) was determined as total ash after ignition on a Mylestone Pyro 1200 apparatus. The sample (about 25−30 mg) was accurately weighed in a 150-μL aluminum pan and ignited in the muffle furnace up to 650 °C. ROI is calculated as the percentage of inorganic residue with respect to the initial sample weight.

Quantitative ^{1}H NMR Method. *Solutions Preparation.* The internal standard 5/9 mM DMSO-d_6 stock solution was prepared by accurately weighing both the standard and the solvent. The suspension was sonicated and regularly shaken at 50 °C for 15 min to reach complete dissolution. The solution can be stored for 45 days at room temperature in a desiccator as demonstrated by periodically assaying the solution against freshly prepared solutions of acetanilide. After this time, the concentration measurably decreases, reaching 96.5% of the initial value at the three-month time point.

DMSO-d_6 specific weight at 24.0° C (average room temperature) was determined with a 25-mL pycnometer that was precisely calibrated with distilled water at known temperature. Four determinations gave an average value of $^{24.0}d_{\text{DMSO}-d_6} = 1.183 \pm 0.001$ g/cm^3.

Solutions to be assayed were prepared by weighing a few milligrams of each substance (± 1 μg precision) and adding the computed amount of the internal standard stock solution to obtain a 10 mM solution of the sample by means of a 1-mL micropipet. If necessary, sonication was applied to obtain a clear solution, and a further amount of DMSO-d_6 was added to obtain the minimum volume (0.6 mL) needed in the ^{1}H NMR tube. Five replicate solutions of every sample were prepared for method validation and two replicates for routine samples analysis. In the final solution, the mole ratio of internal standard to test molecule (assumed 100% pure) is 1:18, which exactly compensates for the 18 protons of the two trimethylsilyl moieties corresponding to the reference signal.

^{1}H NMR Data Acquisition and Processing. All ^{1}H NMR spectra were acquired on a Varian INOVA 500 instrument operating at 499.76 MHz and equipped with a 5-mm double resonance ^{1}H{^{15}N−^{31}P} ID-PFG Varian probe with single-axis (z) gradient coil. Samples were automatically loaded into the magnet with a nine-place Varian carousel autosampler. The standard software (VNMR 6.1C) provided by Varian was used for processing and automatic data acquisition.

The main acquisition parameters are as follow: sample temperature 28 °C, pre-acquisition delay of 2 min, no sample spinning, relaxation delay 30.0 s, 90° flip angle corresponding to a pulse duration of 7.1 μs, 48 transients, 45890 complex fid data point acquired over a spectral width of 9980 Hz (acquisition time 4.6 s). To obtain a perfectly flat baseline data were acquired using real-time digital signal processing,[10−12] and the first three fid data points were linearly predicted before Fourier transformation.[13,14] In these conditions the intensity response of digital filters was found perfectly flat in the region between −0.5 and 9.5 ppm. No weighting functions were applied, and fids were zero filled to 128 K complex data points, affording a final spectrum digital resolution of 0.0762 Hz/point. Spectra were referenced with respect to the residual solvent signal (DMSO-d_6, 2.53 ppm).

Integral reset points were optimized for baseline correction before selection of the integration regions. Whenever possible isolated and sharp peaks were chosen for purity calculation, and an integration tail of at least 30 Hz was added on each side of the integrated signals unless other resonances had to be excluded. Signals very near to intense water signal were never used. Broad signals were considered only when an integration tail of at least 20−30 times the half bandwidth could be added without including nearby peaks.

^{13}C satellite peaks were not included in the integral regions both of the internal standard and of the analyzed molecule since our method is intended to be general for purity evaluation, and signals overlapping does not allow their integration in crowded spectra. The effect on accuracy of

(9) *General guidelines for the establishment, maintenance, and distribution of chemical reference substances*; WHO Technical Report Series, No. 885; Annex 3, World Health Organization: Geneva, 1999.

(10) Boban, J. *Magnetic Moments (Varian Newsletter)* **1995**, *VII(1)*, 16−18.
(11) Smallcombe, S.; Patt S.; Wurl, J.; Provost, J. *Magnetic Moments (Varian Newsletter)* **1996**, *VIII(1)*, 6−12.
(12) Wider, G. *J. Magn. Reson.* **1990**, *89*, 406−409.
(13) Gray, G. A. *Magnetic Moments (Varian Newsletter)* **1995**, *VII(1)*, 30−32.
(14) Hoult, D. I.; Chen, C. N.; Eden, H.; Eden, M. *J. Magn. Reson.* **1983**, *51*, 110−117.

Table 1. **Method validation: certified and measured strength of reference compounds**

compound	source	certified strength, %	^{1}H NMR strength, %	^{1}H NMR RSD, %
acetanilide	Aldrich	100.0	100.0	0.3
doxorubicin hydrochloride	Pharmacia Standard	97.6	97.7	0.3
caffeine	Fluka	99.1	98.9	0.2
niacinamide	Pharmacia Standard	99.9	100.0	0.1
penicillin G, K salt	Pharmacia Standard	98.9	99.4	0.4

^{13}C satellites exclusion is minimized when the integral ratios between the internal standard and the compound of interest are calculated. On the other hand, ^{13}C satellites of nearby peaks are sometimes included in the integral regions used for purity calculation, but this is considered to be part of the method error.

Strength is computed on any integrated peak as follows:

$$\text{strength} = \frac{100 \cdot I_{\text{peak}}}{[n_{\text{H}} I_{\text{st}}]} \qquad (2)$$

where n_{H} = number of hydrogen atoms associated with the signal, I_{peak} = integral of the test molecule signal, and I_{st} = integral of the standard resonance at 0.22 ppm. Purity values obtained from the integration of a few signals are usually averaged to give the final sample strength.

Results and Discussion

Precision and Accuracy from Comparison with Well-Characterized Standards. Table 1 reports ^{1}H NMR data of strength for a few highly purified reference materials, which were obtained either commercially or from the internal Pharmacia service. With highly pure compounds, ^{1}H NMR delivers results which are perfectly comparable with those obtained with much more extensive and lengthy characterization. The relative standard deviations, always below 0.5%, are obtained from five different solutions per compound and witness the very high accuracy and precision which may be obtained with this method. This conclusion agrees with results reported by Maniara et al.,[7] although the concentration used in our study was significantly lower: between 5 and 10 mM (according to sample dilution) vs a minimum of 20 mM.

Figure 1 shows a comparison of strength determined with ^{1}H NMR and with the customary process (100 HPLC%−LOD−ROI). In this case, 53 compounds with various levels of purification are collected, including some synthetic intermediates and raw materials. Notably, the two methods agree better for highly pure compounds (strength > 90%), whereas a tendency at overestimating strength with the traditional approach is apparent for the low-purity compounds (strength < 90%). This is probably a result of the approximations contained in the traditional approach (equal response factor for all related impurities in HPLC, precise estimation of residual solvents from LOD, and of inorganic contaminants from ROI), which are expected to fail when excessive contamination is present.

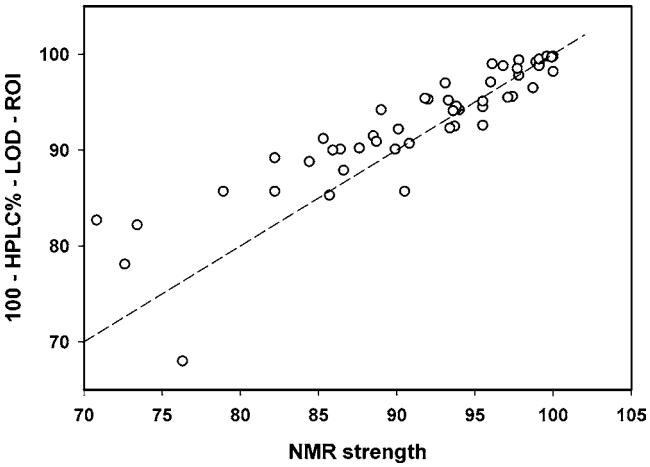

Figure 1. **Comparison of ^{1}H NMR purity with strength as determined with the traditional procedure (a combination of HPLC area %, LOD = loss on drying and ROI = residue on ignition assessment). Data for 53 different compounds are shown, including some reaction intermediates. The unitary slope, zero intercept line, is depicted as a guide for the eyes (dashed line). Results agree better for high-purity compounds, and the traditional method tends to overestimate purity with respect to ^{1}H NMR at strength <90%.**

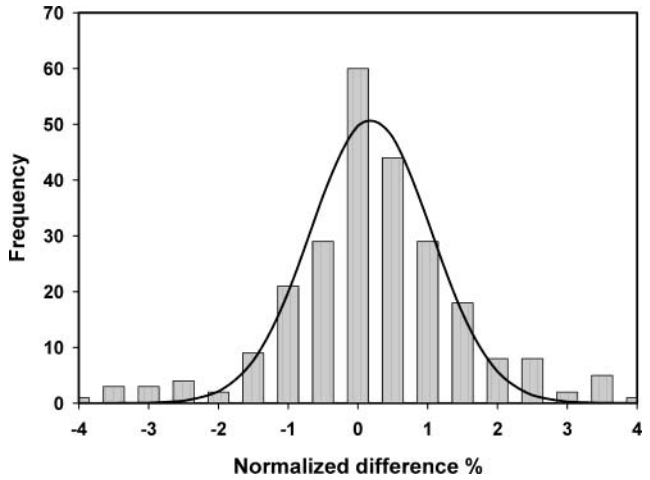

Figure 2. **Distribution of measured normalized differences between pairs of ^{1}H NMR purity determinations for a set of 248 different compounds. The data are best fit with a Gaussian curve centered around the origin, with a spread of 0.9%. This is, however, an underestimation of the true relative standard deviation, which is obtained numerically as 1.3%.**

Precision from Difference of Measurement Pairs. For the determination of routine sample strengths, two independent solutions were prepared with separate weighing, and these were independently assessed with ^{1}H NMR. Therefore, a considerable record of such paired determinations is available in our laboratory: 248, wherefrom 103 have been carried out with the sample-preparation procedure described in the Experimental Section; the other 145 have been collected with a former procedure, which we consider less accurate and precise. If we assume a normal distribution with a relative standard deviation (RSD) independent of the nature of the compound, then the normalized differences of measured strength can be used to estimate the relative standard deviation of the method. Figure 2 reports the distribution of normalized differences for the whole data set,

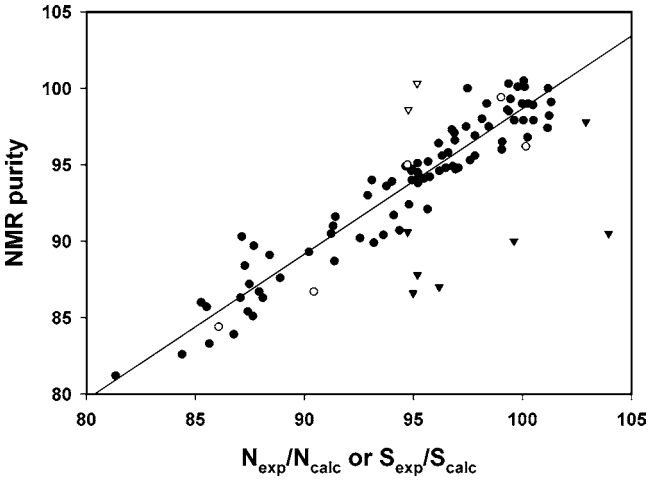

Figure 3. **Correlation between ^{1}H NMR purity and estimates from elemental analysis. Filled circles denote data for N_{exp}/N_{calc}, empty circles are data on S_{exp}/S_{calc}. Triangles are used for data which were not included in the correlation analysis: either because of poor ^{1}H NMR estimate (empty triangles, impurities signals overlapping integrated signals), or because of apparent presence of nitrogen-containing contaminants (filled triangles).**

together with the best-fitting Gaussian curve as obtained from nonlinear regression with the Sigma-Plot statistical package. Remarkably, the distribution corresponds to a sharp curve, closely centered around zero ($x_0 = 0.2\%$, $\sigma = 0.9\%$). When standard numerical calculation is applied to the set of 103 measurements obtained with the improved sample-preparation procedure, RSD = 0.9% is obtained, whereas RSD = 1.3% for the full set of 248 data.

Comparison with Results of Elemental Analysis. Elemental analysis results are often used as a crude estimation of purity, since the ratio of measured vs calculated amount of hetero-elements (N, S) may be taken as a measure of contamination by nonrelated substances (i.e.: solvents, salts, etc.) as long as these contaminants do not contain the hetero-element. Since the availability of elemental analysis results was more frequent than the complete characterization of strength, the comparison with ^{1}H NMR data was accomplished for 98 different compounds (a portion of the samples reported in the previous section) with a range of strength extending from 80 to 100% (^{1}H NMR value). The results are shown in Figure 3. Two groups of data were excluded from the correlation analysis; in one case, compounds with ^{1}H NMR evidence of nitrogen-containing impurities (seven compounds, represented as black triangles in the figure) are not expected to have a correct estimate of purity in elemental analysis; in the other group, compounds are included where insufficient signal resolution between impurities and the test molecule prevented accurate assessment of purity in the ^{1}H NMR spectrum (two compounds, empty triangles in the figure). For all other compounds (89), a correlation coefficient $r^2 = 0.907$ is obtained, and the best-fitting straight line has a nearly unitary slope (0.95). These figures may appear crude, but the limited value of elemental analysis as a criterion of purity should not be overlooked. Actually, the application of quantitative ^{1}H NMR offers a novel opportunity to organic chemistry, since the determination of quantitative purity, currently applied only to a few, well-characterized compounds, may well become routine.

Interestingly, Figure 3 highlights the success rate of our ^{1}H NMR method in a typical environment of medicinal chemistry laboratory: only two out of 98 cases gave evidence of signal overlap preventing a reliable determination of purity. Nevertheless, the difference with elemental analysis amounts to just a few percent. Much more common is the case of nitrogen-containing impurities (e.g., dimethylformamide, ammonium salts), where the N_{found}/N_{calc} ratio is totally misleading at measuring purity.

Conclusions

The use of quantitative ^{1}H NMR proves to be both simple and highly reliable for the determination of quantitative purity or strength of generic synthetic small molecules. In comparison to traditional methods, ^{1}H NMR requires less sample, is nondestructive, and is a truly universal detection method. Moreover, high-field ^{1}H NMR is adequately selective to distinguish signals of badly contaminated samples, where HPLC would require a lengthy setup. We have been routinely applying quantitative ^{1}H NMR in the last three years, and in our experience this approach is very profitable both to support discovery chemistry, where the available amount of material is usually limited, and also to support process development or scale-up chemistry where the very fast response time can be highly valuable.

The reason this technique, so much beloved by the synthetic chemist for qualitative structure confirmation, is not equally applied to purity assessment probably is based on an erroneous underestimation of its precision and an overestimation of measurement complexity. As a matter of fact, quantitative purity determination with ^{1}H NMR is taken into account by the U.S. Pharmacopoeia,[15] although sample preparation includes separate weighing of both active ingredient and reference substance to prepare a solution with concentration well above the value used in our method.

We hope that data presented in this paper may convince the general audience of synthetic and analytical chemists to extensively practice this technique, with much satisfaction for the laboratory efficiency and budget. The transition from qualitative to quantitative ^{1}H NMR simply requires a weighing step in sample preparation and a precise solvent delivery with a calibrated micropipet. These are very simple tasks which can be introduced in any laboratory where NMR is accessible either as a walk-up or as a specialist service.

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(15) Nuclear Magnetic Resonance. In *The United States Pharmacopeia 27, Physical Tests and Determinations*. The United States Pharmacopeial Convention Inc.: Rockville, MD, 2003; Chapter 761.