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Short communication

Determination of relative response factors for chromatographic investigations using NMR spectrometry

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ABSTRACT

In the absence of suitable reference materials for impurity quantitation, laboratories have developed techniques using mass detectors such as the chemical luminescence detector (CLND) and the charged aerosol detector (CAD) to normalize the UV response of each impurity of interest by their molar ratios and thus generate relative response factors without requiring isolated and purified compound-specific standards. While effective, these detectors are limited in response and are effective only with specific mobile phase requirements. Nuclear magnetic resonance (NMR) spectrometry has the advantage of allowing the universal detection of protons while not suffering from the limitations observed for CLND, CAD, and other common detectors. The determination of relative response factors using NMR has been successfully applied to several LC methods. An overview of this technique and representative results are presented.
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1. Introduction

In the early stages of pharmaceutical development, regulatory agencies allow the impurities to be quantitated with the assumption that the impurities/degradation products present have chemical properties similar to those of the active pharmaceutical ingredient (API) [1]. In other words, for liquid chromatographic detection the analyst can initially assume impurities present in early lots of API to have the same molar absorptivity and response as the API at the chosen analytical wavelength of interest using UV–vis detection. Thus, impurities are often estimated using an area-normalization approach since the response factor of each impurity is assumed to be identical to that of the parent compound. In fact, this lack of well-characterized impurity reference standards in early stages of development requires that area-normalization be used with spectroscopic detection. Accurate determinations on a weight–weight basis during early stages of development would require certified reference standards of known purity. Yet, because the drug is still in early development, processes are still changing and reference standards for impurities are limited, not readily available, or have uncertain purity. Generating these standards is a cost that most companies feel is not warranted at early stages; so, most impurities are usually not well characterized or available in sufficient quantities.

While area-normalization often is feasible for early stage investigations, there are times, such as for stability investigations, where the actual relative response factor of the API is needed. When a standard of known purity is available for both the impurity and the API, the response factor for each standard is established by normalizing the response factor at a specific wavelength by the mass used to produce this absorbance. The relative response factor (RRF) is the ratio of the response factor of the impurity of interest to the response factor of the API at a specific wavelength. The challenge for this investigation at early stages of development is actually having qualified impurity standards for the component(s) of interest. When standards are not available, an alternative procedure to normalize the absorbance by mass must be used. Nussmaum et al. first showed that using chemical luminescence detector (CLND) as a mass detector, the response factors for impurities in Fluoxetine HCl (Prozac) could be established using nitrogen-specific detection [2]. Jackson et al. expanded this application to other compounds of pharmaceutical interest [3]. Subsequently, Sun et al. showed that the charged aerosol detector (CAD) also provided a suitable mass response for determining the relative response factors for the liquid chromatographic analysis of paclitaxel-related substances [4]. Generating relative response factor values using chromatographic mass detection allows for more exact quantitation of contaminants, synthesis impurities, reactants and degradants, all without the previously necessary investment in developing suitable reference standards for these materials. This is a great tool for early stage pharmaceutical development to enable generating relative response factor information

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quickly without the investment of generating suitable reference materials.

While CLND and CAD detection for liquid chromatography covers a broad array of pharmaceutical applications, both detectors have significant limitations. CLND detection only responds to nitrogen containing compounds and are useful with only a limited number of mobile phases and volatile buffers [4]. The CLND also fails to respond to adjacent nitrogen pairs with a double bond (–N=N–) and does not have a uniform response for adjacent nitrogens having a single bond (–N–N–). Guanabenz acetate, Phenazopyridine HCl and Cefazolin and Cefmetazole are examples of marketed drugs that have this type of bonding. CAD detectors, like mass spectrometers, also must use volatile buffers and are suitable only for compounds that can carry a charge under electrospray type ionization conditions. Because of these limitations, a more universal method of mass detection/selection was sought for relative response factor determinations.

Recently, quantitative ^{1}H nuclear magnetic resonance (q-NMR) spectroscopy has been gaining acceptance as a tool for rapid potency determinations [5–8]. The technique is now accepted in ICH and USP compendial guidances [5,9]. One of the prime advantages of q-NMR is that for compounds containing a proton, NMR can act as a universal mass detector for the relative response factor application. The earlier relative response factor technique used a mass detector in series with the spectroscopic detector. While this can be done with NMR as well using commercially available LC-NMR systems, the technique presented here works offline from the LC system. This is a significant advantage not only in terms of instrumentation, but also allows any chromatographic technique or mobile phase to be used. No longer does the nature of the mobile phase such as volatile buffers, volatile modifiers, or acetonitrile present an issue with the relative response factor determination as in the case when using CLND or CAD.

2. Materials and methods

2.1. Chemicals and reagents

For NMR potency and relative response factor determinations, deuterated dimethyl sulfoxide (DMSO-d6), D_2O, and maleic acid internal standard were used as received from Sigma (St. Louis, MO). Maleic acid internal standard preparations were standardized against a benzoic acid certified standard (Lot 350b) from NIST (Gaithersburg, MD).

The drug and herbicide compounds for relative response factor determinations were all obtained from Sigma. The methanol and acetonitrile were HPLC grade from EMD (Gibbstown, NJ). The water used was UPS grade from an in-house supply. The H_3PO_4 was ACS reagent grade from J.T. Baker (Phillipsburg, NJ).

2.2. Instrumentation

2.2.1. Nuclear magnetic resonance spectroscopy

A Varian Inova 600 MHz NMR spectrometer controlled by Vnmr, ver. 6.1C software (Varian, Palo Alto, CA) was used for this investigation. The resulting spectra were processed at the desktop using MestReNova (Mesterlab Research SL, Santiago, Spain) version 5.1.1-3092. The operating conditions used for the NMR analysis were consistent with earlier references [5,6].

2.2.2. Liquid chromatography

A Waters (Milford, MA) Alliance 2695 Module equipped with a Waters 996 photodiode array detector was used for this investigation. The LC was controlled using Waters Millennium software version 4.0 and the data collected and processed using Atlas Version 8.20.2.7047, Thermo Electron Corporation (Waltham, MA). The analysis of Fluoxetine HCl impurities used a Zorbax Rx-C8 column from Agilent (Santa Clara, CA) that was 150 cm × 4.6 mm and consisting of 5 μm particles. The impurity analysis for the herbicides used a Supelcosil LC-PAH column from Sigma (Supelco) that was 25 cm × 4.6 mm and consisting of 5 μm particles.

2.3. RRF determinations by mass

In order to generate the “standard” relative response factor by mass for each analyte to the reference analyte, the linearity method used by Sun et al. [4] was used. Triplicate standard curves centered on a 0.1 mg/mL midpoint were generated for each analyte at the designated wavelength of the LC method. To simulate the preparation that would later be used in the NMR determination, 20 mg of the analyte neat or as a mixture was dissolved in nondeuterated DMSO and subsequently diluted to the range of interest in the LC method diluent. Each LC run used was required to post a typical system suitability precision of less than 1%RSD for the midpoint standard. In addition, the correlation curve for the linear regression was required to be $r \geq 0.999$. These typical LC suitability requirements were used to ensure the integrity of the regression value that would be used for the relative response factor. From Sun et al. [4], the relative response factor is simply calculated as the ratio of the corresponding slopes from the regression analysis of each analyte.

2.4. RRF by q-NMR method

The NMR procedure used for relative response factor determinations is based on our current procedure for q-NMR [5–8]. The internal standard/diluent used for NMR determinations was a preparation of maleic acid in DMSO-d6 that is spiked at approximately 1% with D_2O to improve the baseline. API samples and impurities are typically weighed and dissolved in the diluent to a concentration of approximately 10 mM.

To generate the relative response factor by NMR for a specific analyte, each of the analytes were spiked in triplicate at ~10% into a 20 mg sample of reference analyte (2-quinoxalinol or 2,4-D). The combined sample was dissolved in 5 mL of the maleic acid internal standard using DMSO-d6 spiked with 1% D_2O as the solvent. An aliquot of this stock was processed directly for NMR analysis and the rest further diluted to a nominal sample concentration of 0.1 mg/mL in the LC method diluent for LC analysis at the method wavelength.

3. RRF calculation

The calculation used with determining the relative response factor by NMR was adapted from the CLND work of Jackson et al. [3] and presented as the following equation:

$$\mathrm{RRF}^{\mathrm{UV}} = \frac{\mathrm{Area}_1^{\mathrm{UV}}}{\mathrm{Area}_2^{\mathrm{UV}}} \times \frac{I_2^{\mathrm{NMR}}}{I_1^{\mathrm{NMR}}} \times \frac{\mathrm{MW}_2}{\mathrm{MW}_1} \times \frac{N_1^{\mathrm{H}}}{N_2^{\mathrm{H}}} \quad (1)$$

where $\mathrm{Area}_{1,2}^{\mathrm{UV}} = \mathrm{HPLC} - \mathrm{UV}$ area count for analytes 1 and 2, $I_{1,2}^{\mathrm{NMR}} =$ ^{1}H NMR integral for analytes 1 and 2, $N_{1,2}^{\mathrm{H}} =$ number of hydrogen atoms in the integral response used for analytes 1 and 2, and $\mathrm{MW}_{1,2}$ = molecular weight for analytes 1 and 2.

As with CLDN, the equation requires the analyst to know the structure of the analytes of interest, in this case the designated impurity and API. This, of course, also leads to knowing the molecular weight of the impurity as well. The UV response at each wavelength of interest is normalized by the mass response represented by the NMR integral response. With CLND detection, the structure of each analyte was needed in order to account for the number of nitrogen atoms present in the molecule producing the CLND response. Likewise in NMR, the structure of each analyte is

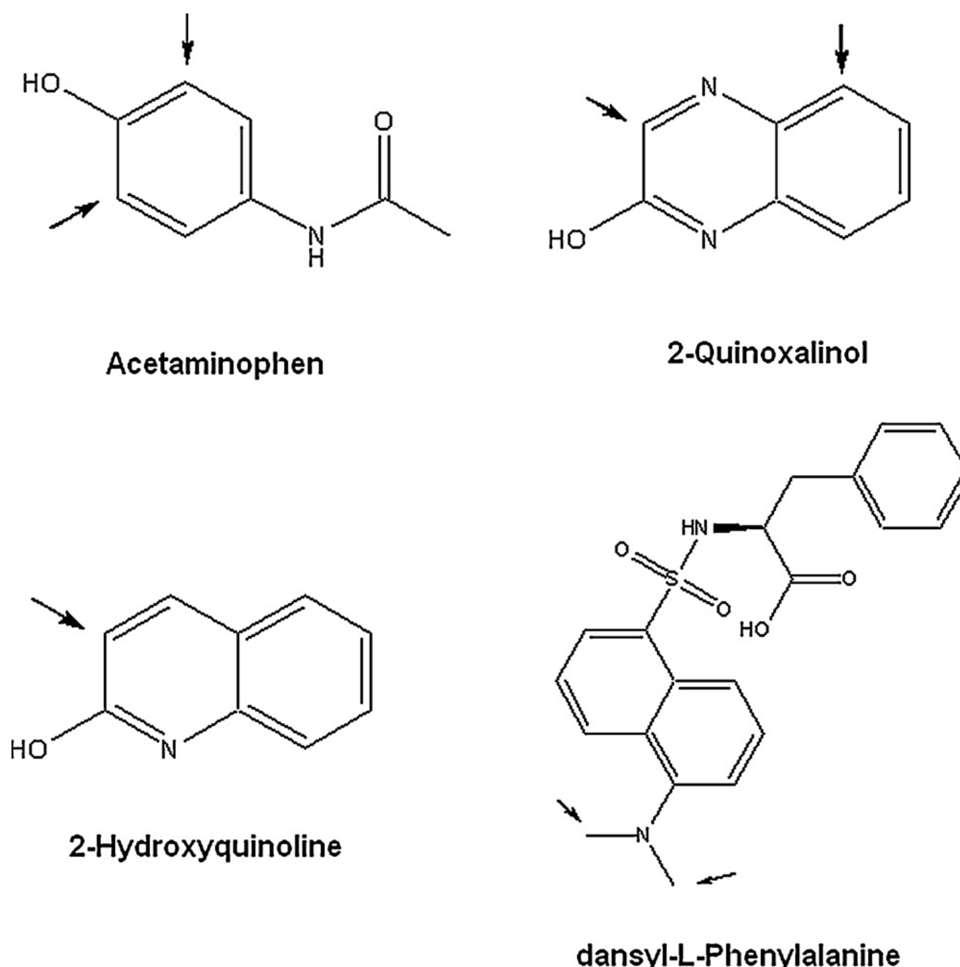

Fig. 1. Structure of Fluoxteine HCl impurities investigated.

needed in order to assign the number of protons present in the integral response assigned for both analytes.

4. Results and discussion

4.1. RRF determination of Fluoxetine HCl impurities

Our investigation into using NMR for relative response factor determination began with reproducing the CLND work of Nussmaum et al. [2] with the exception of Fluoxetine HCl. It was not used in order to avoid the expense of using this drug. Instead, the relative response factors of the other analytes were used. The dansyl-L-phenylalanine form was chosen simply due to availability from Sigma. By arbitrary choice, the relative response factor to 2-quinoxalinol was determined for this study. The LC method used was consistent with the set-up used in the original study. The structures of the analytes of interest for this portion of the investigation are illustrated in Fig. 1. The arrows denote the protons used for NMR quantitative analysis.

Using Eq. (1), the resulting peak area responses for the analyte under study were normalized by the integral of the NMR analysis. No significant difference between the RRF determination by mass or NMR is seen (Table 1). Because the NMR and chromatographic analysis are from the common stock sample preparation, the RRF determination is free of the chromatographic limitations of CLND and CAD detection and as a rule, can be applied to RRF determination of any chromatographic or electrophoretic separation technique through the proper choice of deuterated extraction solvent(s).

The samples were spiked individually to confirm the accuracy of the determination of relative response factors by NMR. However, had the samples been spiked simultaneously with all the analytes under study, the selectivity of the NMR response would have been maintained, as illustrated in Fig. 2. Resolution of the NMR signals is proportional to the field strength of the magnet configured with the individual instrument used.

4.2. RRF determination of acid herbicides

To illustrate the NMR determination of relative response factors using a chromatographic system that would not be compatible with either CLND or CAD detection, the technique was applied to the analysis of nonproprietary acid herbicides that are analyzed in the literature using a phosphate buffer [10]. The relative response factors were determined at 240 nm to pronounce the absorptivity differences of the analytes. The structures of the analytes of interest for this portion of the investigation are illustrated in Fig. 3.

Table 1
RRF determinations at 230 nm for Fluoxetine HCl impurities.

Analyte	RRF to 2-quinoxalinol using mass	RRF to 2-quinoxalinol using NMR
Acetaminophen	0.38	0.37
2-Hydroxyquinoline	1.02	1.10
Dansyl-L-phenylalanine	0.38	0.40

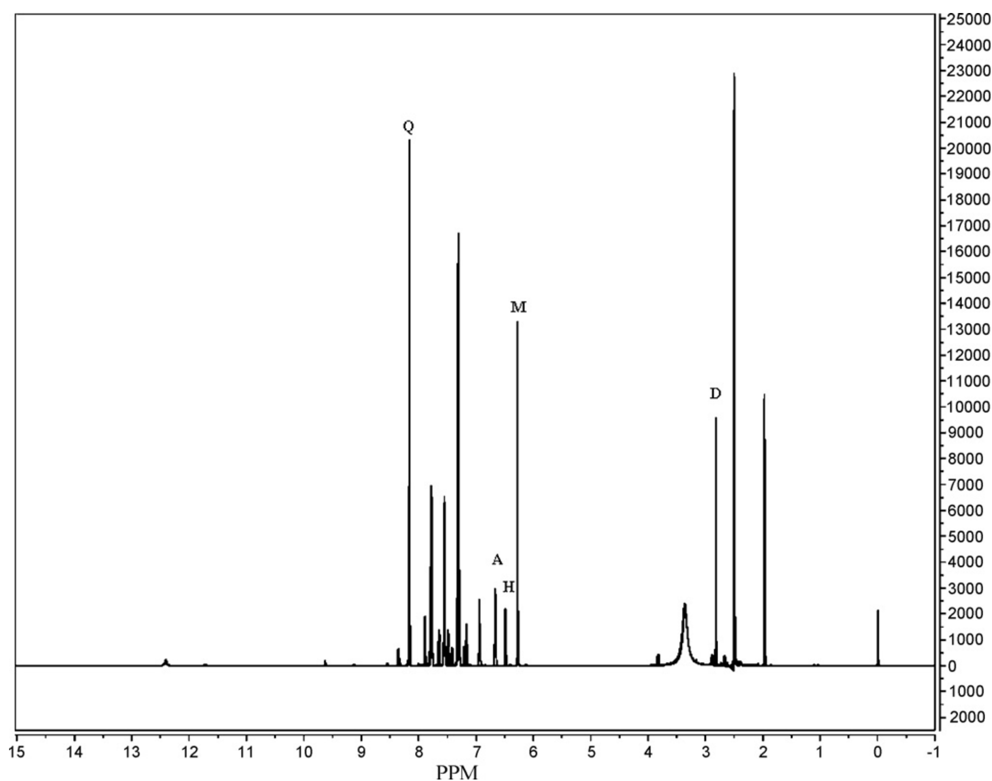

Fig. 2. Overlay of NMR spectra for RRF determination of Fluoxteine HCl impurities. "Q" 2-quinoxalinol proton assignment used; "A" acetaminophen proton assignment used; "H" 2-hydroxyquinoline proton assignment used; and "D" dansyl-L-phenylalanine proton assignment used. "M" is for the maleic acid internal standard proton assignment used.

The arrows again denote the protons used for NMR quantitative analysis.

During the course of this part of the investigation, it was discovered that the herbicide samples were not as pure as labeled. This is not uncommon for materials received from supply houses. However, RRF determinations with this technique are only accurate when the correct mass term is applied. The potencies for these materials had to be determined using quantitative NMR and the resulting potency was used to correct the mass determination. This was done on separate sample preparations independent of those used for the NMR RRF determinations in order to reduce bias. This correction allowed the study to confirm that unlike mass determinations, the RRF technique using mass detection does not need a certified potency value for the impurity. It also illustrated for the analysts the lost time required to post the RRF value using the mass determination technique. The mass determinations for this investigation were run by both the linearity method used as before, as well as direct mass normalization of the peak area result from the LC run.

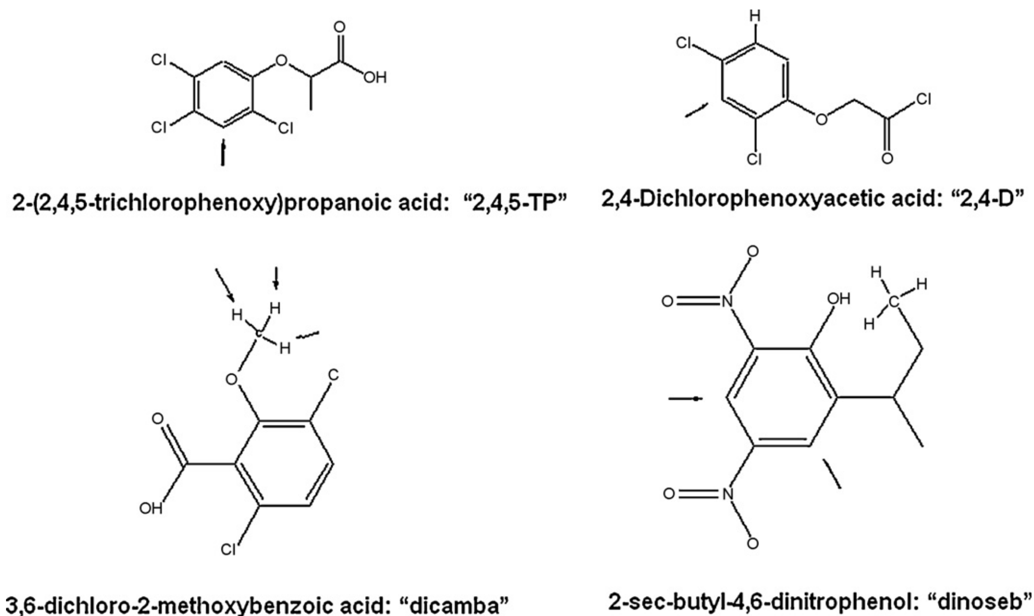

Fig. 3. Structure of herbicides investigated.

Table 2
RRF determinations at 240 nm for acid herbicide analytes.

Analyte	RRF to 2,4-D using mass (Slope method)	RRF to 2,4-D using mass (LC peaks)	RRF to 2,4-D using NMR
Dicamba	0.99	1.12	1.13
2,4,5-TP	3.01	3.03	2.93
Dinoseb	3.77	3.46	3.51

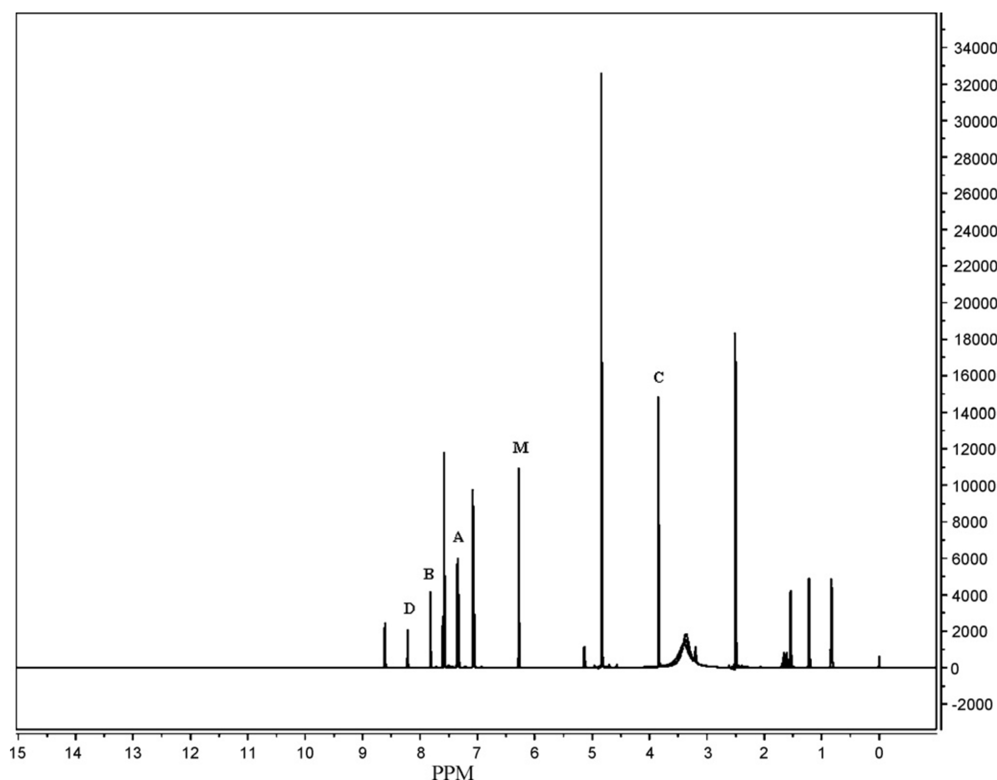

Fig. 4. Overlay of NMR spectra for RRF determination of acidic herbicide impurities. "A" 2,4-D proton assignment used; "B" 2,4,5-TP proton assignment used; "C" Dicamba proton assignment used; and "D" Dinoseb proton assignment used. "M" is for the maleic acid internal standard proton assignment used.

However, these values had to be corrected for their true potency prior to finalizing the results. A summary of the mass determinations is presented in Table 2.

The selectivity of the NMR response for the herbicide study is illustrated in Fig. 4. The results of the RRF determinations are presented in Table 2. No significant difference to the RRF determination by mass is seen.

5. Conclusion

The method presented generates relative response factors for chromatographic profiles without the need for impurity isolation and determination of purity of the isolated material. The method is universal for proton containing analytes and is not limited by the structure and mobile phase limitations seen with CLDN and CAD detection. Relative response factors generated using NMR to normalize peak area responses by mass yield values that are not significantly different than those posted by traditional mass determinations. While the method has been demonstrated to be effective for liquid chromatography, the procedure is easily transferable to gas and supercritical fluid chromatographic as well as capillary electrophoresis techniques since the technique simply uses a common stock preparation.

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