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The practical application of implementing the equimolar response principle of chemiluminescent nitrogen detection in pharmaceutical analysis

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ABSTRACT

Since nearly 90% of all developmental and marketed drugs contain at least one nitrogen atom, the implementation of chemiluminescent nitrogen detection (CLND) in pharmaceutical analysis is intriguing due to its equimolar response for nitrogen. Although the documented accuracy of CLND when using a surrogate nitrogen-containing standard may be inadequate for purity and potency determinations, it is acceptable for the quantitation of low-level impurities.

A comparison of the quantitative results obtained using both CLND and UV detection for a developmental drug and its related impurities is presented. The results indicated that the impurities can be accurately quantified using a surrogate standard, based on the equimolar response principle of CLND, when the concentrations of the surrogate standard and the component of interest are similar. When the impurities are present at much lower levels than the surrogate standard however, the common practice of direct conversion of area percent to weight percent can result in significant errors using CLND, due to the limited linear dynamic range for CLND. To increase quantitation accuracy, the authors propose that a secondary dilution of the surrogate reference standard solution should be used for the quantitation of low-level impurities. The practical application of this approach for impurity quantitation or as a means to determine impurity relative response factors for use with a traditional UV based method is discussed.

1. Introduction

In a relatively short time after its introduction, high performance liquid chromatography (HPLC) quickly became the method of choice in modern pharmaceutical analysis [1,2]. Coupling today's high-efficiency columns and variable wavelength UV detectors, HPLC–UV is the standard method for quantitation in nearly every pharmaceutical laboratory, due to its convenience in application and robustness in performance [3–5]. It is well known that the UV molar absorptivity is an intrinsic property of the compound being detected. Thus, the accurate quantitation of impurities in a sample matrix would theoretically require individual reference standards of each impurity of interest [6]. In practice however, the quantitation of impurities are generally performed using the relative response of the main component of interest in the sample for ease of analysis and limited availability of impurity standards. To compensate for any significant molar absorptivity differences, relative response factors (RRF) of the impurities vs. the main component of interest are determined to ensure accurate quantitation of the impurities. The RRF is defined as the area percent of the impurity vs. the main component of interest after normalizing the concentration differences of the two components. However, authentic impurity standards of known quality are required for RRF determination. Determining RRF can be very resource intensive as one has to isolate (or in some cases synthesize), purify and characterize each impurity [7,8]. For unknown impurities, or known impurities for which no authentic standard is available, the general practice is to use a RRF of 1.0, which can lead to a significant error in quantitation [6].

Over the years, pharmaceutical regulatory agencies have increased their scrutiny of the safety profile and risk benefits of new drug entities due in part to recent adverse events in both the clinic and the market place. As a result, an emphasis during the development of new drug entities has been placed on the identification, quantitation and evaluation of the fate of all observed impurities and degradants in the process [5,6,9–12]. Since the preferred means of quantitation for process quality control is weight percent, much interest has been placed on the development of an "universal" detector that will be sensitive to and provide equal response for all components in the mixture, eliminating the need for individual impurity standards. An "universal" detector of this type could be used directly with the LC system for quantitation of the impurities or coupled to an UV detector to accurately determine

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the RRF. These RRFs could then be applied to UV based methods for implementation in a wider range of applications. The significance of this approach is that the RRF for the impurities can be determined using only a fraction of the resources required compared to the current practice since no authentic reference material would need to be isolated, characterized and purified.

LC–MS had been considered an 'universal' detector. The development of affordable bench top instrumentation has significantly increased the popularity and availability of LC–MS, not only for routine structure elucidation investigations, but also, for quantitation in pharmaceutical analysis [13]. LC–MS is widely used for the analysis of high throughput combinatorial chemistry for its power in selectively detecting the analytes of interest in complex matrices [14,15]. Similarly, LC–MS-MS is widely used in pharmacokinetics and drug metabolism studies for its ability to provide insight into the identification of metabolites [16]. Although most organic compounds can be ionized using one of the various ionization sources available, the ionization efficiency can vary significantly from one molecule to the next due to differences in their chemical structures. In addition, other experimental parameters such as desolvation temperature and variations in the analyte matrix during gradient analysis can also impact the ionization efficiency. Though universally sensitive to most pharmaceutical compounds, LC–MS like LC–UV does not provide an equimolar response for the analytes in a sample.

Evaporative light scattering (ELSD) and refractive index (RI) detectors are two other commonly used detectors in LC that have been promoted as potential "universal" detectors [17,18]. ELSD has been shown to generate an "universal" response for most non-volatile compounds, if the compounds are of similar classes and detected under isocratic conditions. However, when ELSD is coupled with gradient elution analysis the changing mobile phase composition has been shown to have a profound effect on peak response [19]. It has been well documented that peak response in LC–RI can vary due to the eluent composition change in gradient analysis. Although certain empirical correction factors may be applied to compensate for changes in mobile phase composition and temperature, the poor sensitivity of the RI detector has rendered LC–RI of limited use in trace-level impurity quantitation in pharmaceutical analysis [19].

Corona-charged aerosol detector (CAD) has recently demonstrated its usefulness as an "universal" detector due in part to its sensitivity and equimolar response to non-volatile analytes [20]. For a gradient elution, however, a separate pump is required to compensate the organic content in the mobile phase by delivering exactly an inversed gradient prior to the detection. The organic content in the mobile phase impacts the efficiency of the nebulizer, which in turn affects the responses of CAD, as an aerosol based detector, to non-volatile analytes [21].

Since the peak response in chemiluminescent nitrogen detection (CLND) is directly proportional to the moles of nitrogen in the analyte, regardless of any structural differences. LC–CLND, in recent years, has been gaining acceptance as an "universal" detector for nitrogen-containing compounds [22,23]. The potential of LC–CLND in pharmaceutical analysis is intriguing, since nearly "90% of the >65,000 developmental and marketed drugs in the commercial database MDL Drug Data Report (MDDR) contain nitrogen" [24]. The use of LC–CLND for quantitation in combinatorial chemistry, pharmacokinetics and drug metabolism investigations using surrogate reference standards, which takes advantage of the equimolar response of CLND, has been well documented in the literature [25,26]. Recent improvements in the CLND instrumentation including, nebulizer design and ceramic pyrotube, have dramatically enhanced the robustness of the technique and its applicability to routine pharmaceutical analysis. In addition when coupled with an UV detector, CLND can provide accurate RRF determinations for any nitrogen-containing impurity in a sample eliminating the need for an authentic reference standard if the molecular formula of the compound is known. The obvious limitation of LC–CLND in pharmaceutical analysis, however, is its incompatibility with nitrogen-containing solvents and/or mobile phase modifiers. As a result, methanol has becomes the solvent of choice for the majority of the LC–CLND analyses.

The other limitation is the limited linear dynamic range of CLND. In this paper, the application of LC–CLND is evaluated for the accurate quantitation of low-level process related impurities in a new active pharmaceutical ingredient (API) by comparing the results to those generated using LC–UV. The practical aspects of the limited linear dynamic range of CLND for quantitating trace-level impurities in a sample matrix or determining RRFs for use in LC–UV analyses will also be discussed.

2. Experimental

2.1. Materials

HPLC-grade water used was from a Milli-Q system (Millipore, Bedford, MA, USA). HPLC-grade methanol (MeOH) was purchased from Burdick & Jackson (Muskegon, MI, USA). HPLC grade trifluoroacetic acid (TFA) and concentrated hydrochloric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Caffeine was purchased from Sigma–Aldrich (St. Louis, MO, USA). The active pharmaceutical ingredient (API) and related compounds (designated with BMS letters in Table 1 were all synthesized by

Table 1
Molecular information of the test compounds investigated and their relative response factors (RRFs) vs. the API (BMS-Compound A)

Compound	Molecular formula	Molecular weight	Number of nitrogen atoms	Percent of nitrogen	RRF[a]
BMS-Compound A	$C_{18}H_{25}N_3O_2$	315	3	13.33	API
BMS-Compound B	$C_{18}H_{25}N_3O_2$	315	3	13.33	0.66
BMS-Compound C	$C_{18}H_{27}N_3O_3$	333	3	12.61	1.10
BMS-Compound D	$C_{18}H_{25}N_3O_2$	315	3	13.33	1.01
BMS-Compound E	$C_{18}H_{25}N_3O_2$	315	3	13.33	1.15
BMS-Compound F	$C_{17}H_{25}N_3O_2$	303	3	13.86	1.57
BMS-Compound G	$C_{18}H_{25}N_3O_2$	315	3	13.33	1.27
BMS-Compound H	$C_{22}H_{34}N_2O_4$	390	2	7.18	1.03
BMS-Compound X	$C_{17}H_{26}N2O_2$	290	2	9.66	*
BMS-Compound Y	$C_{22}H_{26}ClN_7O_2S$	488	7	20.08	*
Caffeine	$C_8H_{10}N_4O_2$	194	4	28.87	*

BMS-Compound A to BMS-Compound H are structurally related compounds.
*The RRF of these compounds (vs. BMS-Compound A) were not determined, since they are not the related impurities or degradants of BMS-Compound A.
[a] RRF values reported were determined using quantitative NMR.

the Process R&D laboratories of Bristol Myers-Squibb Co. (New Brunswick, NJ, USA)). The purity of the synthesized compounds was evaluated using both HPLC and quantitative NMR.

2.2. Equipment

2.2.1. HPLC system

An HP-1100 LC from Agilent (Palo Alto, CA, USA) was used for all chromatographic analyses. The LC system was equipped with a solvent degasser, quaternary pump, heated column compartment, autosampler equipped with a Peltier cooling module, and a diode array detector (DAD). The HPLC system parameters were controlled using the Agilent ChemStation software version 08.03. The single wavelength UV response from the DAD and the CLND response were collected using a SAT/IN module (an analog to digital converter from Waters), which was connected to a LAC/E^{32} acquisition server from Waters (Milford, MA, USA). The Millennium^{32} software chromatography data management system from Waters was used for data acquisition and processing.

2.2.2. CLND system

The Chemiluminescence Nitrogen Specific Detector (Model 8060) was purchased from Antek Instruments (currently Petroleum Analyzer Company, Houston, TX, USA). The original quartz pyrotube was replaced with the newly developed ceramic pyrotube filled with Pyrochips. The furnace was set at 1050 °C. The inlet oxygen gas was set at 250 mL/min, and the inlet and make up argon gas supplies were both set to 50 mL/min and 90 psig. The reaction chamber vacuum was set at 26 Torr (18 Torr higher than when the splitter needle was completely closed). The instrument pressure reading was 35 psig and the ozone flow reading was 25 mL/min.

2.3. Chromatographic conditions

Reversed-phase HPLC was carried out using a Zorbax Eclipse AAA column, 3.0 mm × 150 mm, 3.5 μm particle size (Palo Alto, CA, USA). Column temperature was controlled to 40 °C. An injection size of 10 μL was used for all sample and standard solutions. The column was eluted using a double linear gradient. The first linear gradient was from 0% to 20% Mobile Phase B in 20 min. The gradient was then increased from 20% to 100% Mobile Phase B over the next 20 min and held for 5 min before cycling back to 0% Mobile Phase B in 1 min. Mobile Phase A was used to make up the gradient composition. Mobile Phase A was 10% MeOH in water containing 0.1% TFA and Mobile Phase B was 90% MeOH in water containing 0.1% TFA. The flow rate was set at 0.6 mL/min.

All sample solutions were prepared by dissolving the compounds of interest, either as individual components or together as a mixture, in Mobile Phase A.

2.4. Instrument setup

The entire HPLC column eluent was directed into the DAD and the absorbance at 215 nm was extracted for quantitation. The eluent after exiting the DAD was split using a micro-splitter from Upchurch Scientific (Oak Harbor, WA, USA). A portion of the eluent equivalent to 0.3 mL/min was directed into the nebulizer and pyrotube furnace of the CLND and the remaining eluent was directed to waste. To minimize the band broadening, 0.005″ i.d. PEEK tubing from Upchurch Scientific was used for all post column connections.

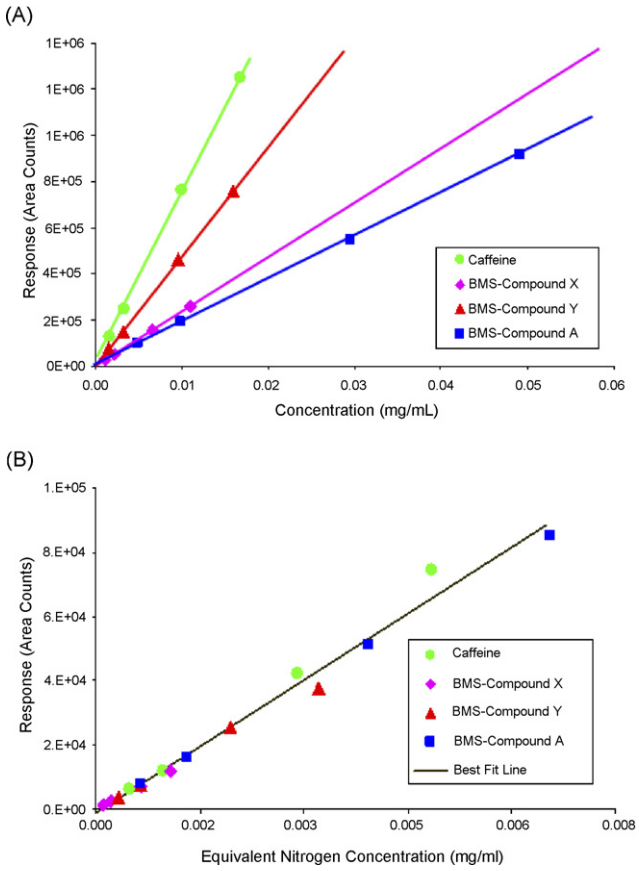

Fig. 1. Response vs. concentration plots. (A) UV response vs. concentration (mg/mL) and (B) CLND response vs. equivalent nitrogen concentration (mg/mL). The individual compounds were analyzed separately using the chromatographic conditions and instrument configuration described in Section 2. The 'equivalent nitrogen concentration' is calculated by multiplying the weight concentration by the percent nitrogen content of the molecule of interest. The UV response vs. weight concentration (mg/mL) for four different test compounds investigated are plotted in (A). The response for each individual compound is highly linear over the concentration range investigated as predicted by Beer's law. However, there is no overall linear correlation for the response of the four test compounds due to the differences in their individual molar absorptivities. In contrast, the same four test compounds demonstrate both individual and overall linear correlations when their CLND response vs. equivalent nitrogen concentration (mg/mL) are plotted (B). This overall linearity is a result of the equimolar response principle of CLND.

3. Results and discussion

3.1. Equimolarity of CLND vs. UV response

Over a limited concentration range, the UV absorbance of a compound at a specific wavelength is proportional to its concentration as described by Beer's Law, $A = \varepsilon bc$, where the molar absorptivity ε is compound specific. The UV responses vs. the weight concentrations of four different test compounds investigated are shown in Fig. 1A. As expected, the response for each individual compound is highly linear over the concentration range investigated. Due to the differences in their individual molar absorptivities however, there is no overall linear correlation for the four compounds. The similar response curves observed for BMS-Compound A and BMS-Compound X are due to the extreme structural similarities between these two compounds.

In contrast to the UV response, a good overall linear correlation is observed for the CLND response for the same four test compounds when the CLND response is plotted vs. the 'equivalent nitrogen concentration' (Fig. 1B), which is calculated by multiply-

ing the weight concentration by the percent nitrogen content of the molecule. Since each mole of nitrogen in the molecule is converted to one mole of NO_2*, the species detected in the CLND, compounds containing equal moles of nitrogen generate the same CLND response (equimolarity response) regardless of any structural differences in the molecules, except in some special cases [22,23]. The equimolarity of the CLND response has been widely documented in the literature since it permits the quantitation of nitrogen-containing compounds using any surrogate reference standard containing nitrogen [27–32].

3.2. Weight percent comparison of UV and CLND response

The equimolar response principle of CLND was further examined using a marker solution containing a highly characterized API (BMS-Compound A) currently in development and several of its potential process related impurities and degradants (Table 1). For this initial study, marker solutions containing approximately equal amounts of the API and individual impurities were prepared over the range of 0.001–0.05 mg/mL. These solutions were then analyzed using the chromatographic conditions and instrument configuration described in Section 2. Fig. 2 depicts the UV trace (A) and the CLND trace (B) for one of the marker solutions containing equal amounts of the API and the impurities investigated. The weight percent of each individual impurity relative to the API in the marker solutions were calculated separately using both the CLND and the UV data. For the CLND data, this was performed by taking the area percent of each component relative to the API and converting it to a weight percent by factoring the differences in molecular weight and the number of nitrogen atoms in each molecule as shown below:

$$\%(w/w)_{\mathrm{CLND}} = \frac{\mathrm{area}_{\mathrm{imp}} \times (\mathrm{MW}_{\mathrm{imp}}/(\#\,\mathrm{of}\,N)_{\mathrm{imp}})}{\mathrm{area}_{\mathrm{API}} \times (\mathrm{MW}_{\mathrm{API}}/(\#\,\mathrm{of}\,N)_{\mathrm{API}}) + \mathrm{area}_{\mathrm{imp}} \times (\mathrm{MW}_{\mathrm{imp}}/(\#\,\mathrm{of}\,N)_{\mathrm{imp}})}$$

Similarly for the UV response, the weight percent determinations were performed by correcting the area percent of each individual impurity relative to the API using the corresponding relative response factors (RRFs), which had been previously determined using quantitative NMR (internal BMS report).

$$\%(w/w)_{\mathrm{UV}} = \frac{\mathrm{area}_{\mathrm{imp}} \times \mathrm{RRF}_{\mathrm{imp}}}{\mathrm{area}_{\mathrm{API}} + \mathrm{area}_{\mathrm{imp}} \times \mathrm{RRF}_{\mathrm{imp}}}$$

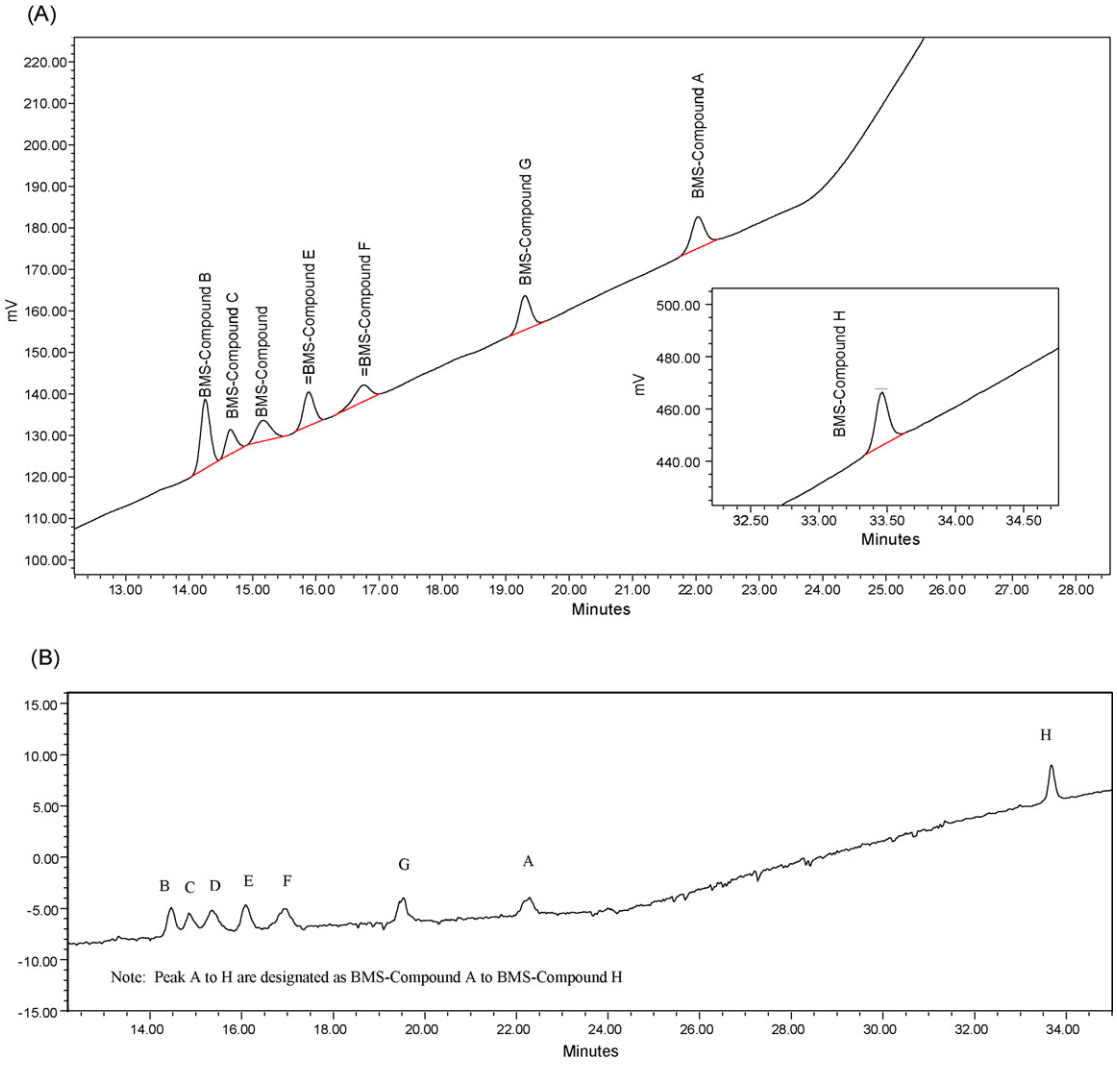

Fig. 2. Separation of API and related impurities investigated. (A) The Chromatogram of the UV trace and (B) the chromatogram of the CLND trace. Reversed-phase HPLC separation of the API and related impurities were investigated in this paper using UV (A) and CLND (B) as detectors. The separation was carried out using the conditions described in Section 2. The sample solution was prepared to contain the equivalent weight of each impurity, as well as, the API (BMS-Compound A). Note that the scales for the *X* and *Y* axes are different for the UV and CLND traces.

Table 2
Weight percent ratio of the UV vs. CLND responses determined from solutions containing equal concentrations of the impurities and surrogate reference standard

	Impurities (mg/mL)				
	0.001	0.005	0.01	0.03	0.05
BMS-Compound B	0.98	0.96	0.94	0.94	0.93
BMS-Compound C	1.07	0.97	0.98	0.97	0.96
BMS-Compound D	0.97	1.04	1.04	1.02	0.99
BMS-Compound E	1.02	1.02	1.00	0.99	0.97
BMS-Compound F	0.94	1.04	0.98	1.00	0.97
BMS-Compound G	0.90	1.00	0.97	0.98	0.97
BMS-Compound H	1.10	1.10	1.07	1.09	1.08
Average	1.00	1.02	1.00	1.00	0.98
R.S.D.	7.1	4.7	4.4	4.7	4.7

Marker solutions containing approximately equal amounts of the API (BMS-Compound A), used as the surrogate reference standard, and the related impurities were analyzed. The weight percent of each individual impurity vs. the API was calculated using both the CLND and the UV response. The weight percent determined by CLND was then divided by the weight percent determined by UV as discussed in the text. Based on the equimolarity response of CLND, a weight percent ratio of unity (1.0) was expected for all of the components investigated.

For comparison purposes, the weight percent values determined by CLND were divided by the weight percent values determined by UV weight percent. Based on the equimolarity response principle of CLND, a weight percent ratio of unity (1.0) was expected for all of the impurities investigated.

The weight percent ratios for each of the impurities evaluated ranged from 0.90 to 1.10 (±10%), as shown in Table 2. The results for this study are consistent with the literatures which report an accuracy of ±20% can be obtained using LC–CLND and a surrogate nitrogen-containing reference standard [19,23]. Although an accuracy of ±20% is insufficient for a purity or drug product potency assay, this accuracy is well suited in many cases for the quantitation of low-level impurities and degradants. The *USP* and *European Pharmacopoeia* indicate that an RRF of 1.0 can be applied to impurities when the true RRF has been determined to be between 0.8 and 1.2 [33,34]. Thus, the accuracy of CLND is well suited for the quantitation of impurities in pharmaceutical assays.

3.3. The dynamic linear range limitation of LC–CLND

As previously demonstrated in the literature and the investigation described above, LC–CLND can be used to determine the weight percent of a nitrogen-containing impurity by directly converting the area percent by factoring the molecular weight and the number of nitrogen atoms in the molecule. It is worth noting that this statement is generally based on results where the equivalent nitrogen concentration of the impurities and the surrogate standard (the API in this investigation) were approximately the same. In routine pharmaceutical analyses however, the impurities are typically present at levels much less than a percent, which is more than two orders of magnitude lower than the concentration of the main component of interest in the sample.

To further investigate the application of CLND in pharmaceutical analysis, new marker solutions were prepared using the same API, impurities and degradants as in the previous example. The concentrations of the impurities in the new marker solutions were varied over the range of 0.005–0.05 mg/mL. In this set of marker solutions the API concentration was kept constant at 1 mg/mL to simulate typical pharmaceutical sample solutions. These solutions were then analyzed using the same LC–UV–CLND setup. The weight percent of each individual impurity in the marker solutions was calculated using the UV and CLND responses, respectively. For the CLND response, area percent of the impurities was converted to weight percent by factoring the molecular weight and the number of nitrogen atoms in the individual impurities. Similarly for the UV response, the relative response factors were applied to correct the area percent for each individual impurity.

Based on the equimolar response principle of CLND and the results obtained in the previous experiment (Table 2), a weight percent ratio of unity (1.0) was expected for all of the impurities investigated. The results obtained, however, showed a weight percent ratio of only 0.6–0.8 at different concentrations. Thus the CLND would consistently and significantly underestimate the level of the impurities in the solutions. The only difference between the two data sets is the concentration of the surrogate standard (API) relative to the concentration of the impurities in the sample solutions. The results in Table 2 were generated when the surrogate standard (API) concentration was approximately equivalent to the concentration of the individual impurities. Whereas, the weight percent ratio of 0.6–0.8 were obtained when the surrogate standard (API) concentration was approximately two orders of magnitude higher than the concentration of the individual impurities in the sample.

Theoretically, the equimolar response principle of CLND is independent of the analyte concentration relative to the surrogate standard, as long as, the linear range of the detector is not exceeded. Since the equimolar response of the impurities investigated vs. the surrogate standard (API) had been demonstrated when the concentration of the individual impurity and surrogate standard were similar (Table 2), the significant deviation in the equimolar response using the same impurities and surrogate standard (API) was not anticipated. The experiment was repeated and the results were confirmed, indicating that the equimolar response principle of CLND is violated when the concentration differences between the analytes (impurities) and the surrogate standard (API) are significantly different. Since the objective of our work was to utilize the equimolar response principle of CLND for accurately quantitating trace-level impurities, an investigation into this concentration affect was undertaken.

Linearity is a critical parameter in method validation to confirm that a single point standard can be used to accurately quantitate samples over the entire concentration range anticipated. It ensures that the peak response ratio is a direct translation of the concentration ratio of the sample and standard solutions. Linearity evaluation also confirms that the instrument linear dynamic range is not violated within the anticipated concentration range of the method. The large dynamic linear range of the UV detector was demonstrated by preparing two separate linear curves of the surrogate standard (API) over significantly different concentration ranges: from 0.005 to 0.05 mg/mL (four equally spaced concentrations) and from 0.5 to 1.5 mg/mL (five equally spaced concentrations), respectively. When the two linear curves were plotted on the same graph as shown in Fig. 3A, which represent a concentration range of greater than two orders of magnitude, the curves are superimposable as predicted by Beer's law.

If the two curves had not been superimposable for the same compound due to a narrow linear dynamic range of the detector, a 'response factor' would have been required to convert the area percent to weight percent if the concentration of the analyte and the standard were significantly different. Thus, when applying a relative response factor (RRF) to trace-level impurity quantitation, the RRF could represents a combination of two correction factors: (1) an instrumental correction factor to adjust the response slope of the impurity to that of the main component of interest (linear dynamic range) and (2) a chemical response factor to correct for differences between the response of the impurity and the main component of interest due to differences in their chemical structures. In practice, RRFs applied in LC–UV analyses correct only for significant changes in the molar absorptivity values since commercial UV detectors possess dynamic linearity ranges of several orders of magnitude [35].

Unlike LC–UV analysis, LC–CLND does not require a chemical response factor correction. This is the equimolar response principle of CLND, which is based on the conversion of the nitrogen in the compounds of interest to NO*. The NO* response produced by Compound A is compared directly to the NO* response generated by Compound B, the surrogate standard. This equimolar response principle has been demonstrated for a wide range of structurally different compounds in the literature and is only violated by compounds that possess certain types of N=N bonds, due to the tendency of these bonds to form varying amounts of N_2 which does not chemiluminesence [22,23]. If the equimolar response principle is to be applied to accurately quantitate impurities in the sample mixture, then the slope of the data points generated from the low concentration components (impurities) must be identical to that of the higher concentration surrogate reference standard (API). If these slopes differ significantly, then an instrumental correction factor will need to be applied to adjust the two slopes for differences in the linear dynamic range of the detector.

Although a linear dynamic range of two orders of magnitude has been reported for LC–CLND in the literature [33], the results from our studies of structurally similar process related impurities and degradants do not appear to support these findings, assuming that the equimolar response principle was not violated. To understand any response variations that the compounds in these studies may have had on the equimolarity, a close inspection of the data was performed by looking at only the API response over the concentration range of interest. At first glance, the CNLD response of the API over the entire concentration range investigated, 0.005–1.5 mg/mL, appears to be linear and the correlation coefficient is 0.993. However, plotting the data points for the low and high concentration data sets separately, the two regression lines are not superimposable like the UV data sets that were generated from the same solutions, but rather a significant difference in the slopes of the two curves is readily evident, as shown in Fig. 3B. This phenomenon was reported in a study using CLND to analyze Oxazepam and Temazepam by Deng et al. [26]. In that study, the authors contributed the significant deviation of the equimolar response observed at the limit of quantitation (LOQ) to the use of a single-higher concentration calibration standard. This observation indicates that caution should be exercised when directly converting the CLND response to weight percent when the concentration of the components of interest differs significantly from the concentration of the surrogate reference standard.

The limited dynamic linear range of LC–CLND compared to LC–UV should not however, diminish the application of the equimolar response principle for the quantitation of low-level impurities. LC–CLND still possesses a distinct advantage in pharmaceutical and other analyses where synthesized or isolated reference materials may not be readily available for the quantitation of low-level impurities or where RRFs are not available to correct the UV responses of the impurities.

The current practice within the pharmaceutical industry is to prepare and make available well-characterized reference standards for the quantification of all APIs, intermediates and starting materials for use through out the drug development process. These well-characterized reference standards are ideal for use as CLND surrogate reference standards for impurity and/or degradant quantitation. All that is required to overcome the limited linear dynamic range of the CLND is a simple secondary dilution of the reference standard solution, approximating the anticipated level of impurities. Diluting the reference standard solution provides a rapid and convenient approach to accurately quantitating impurities that takes advantage of the structural similarity of the components of interest and the availability of existing reference standards.

The compounds investigated previously in this paper were re-evaluated using the secondary dilution protocol described above. Standard solutions of the API were prepared at concentrations of 1.0 and 0.01 mg/mL to quantify the API and the impurities in the

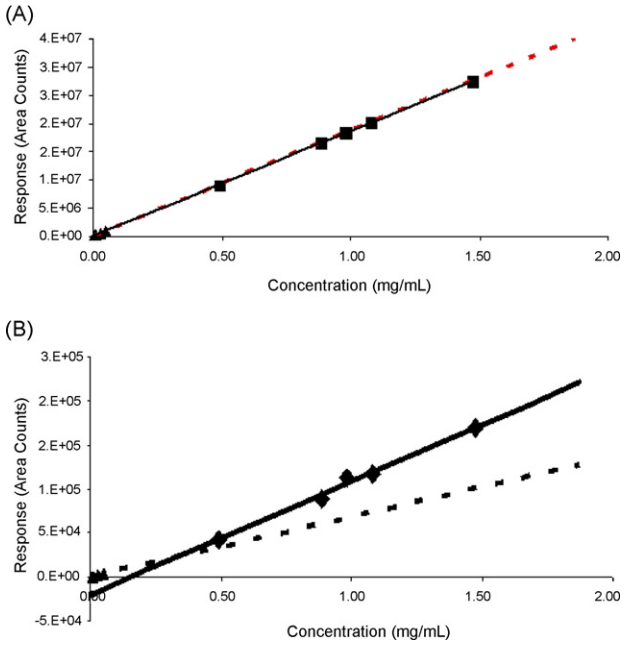

Fig. 3. Dynamic linear range of the CLND response for BMS-Compound A (API). (A) UV response vs. concentration (mg/mL) (the superimposable linear regression lines for the UV detector) and (B) CLND response vs. nitrogen concentration (mg/mL) (the non-superimposable linear regression lines for the CLND detector). The linear regression line for the CNLD response vs. concentration (mg/mL) generated using the same two sample solution sets of the surrogate standard (API) as in (A), is shown in (B). An acceptable correlation ($r^2 > 0.993$) was observed when plotted together. However, plotting the data points for the low and high concentration data sets: 0.005–0.05 mg/mL (dotted line) and 0.5–1.5 mg/mL (solid line) separately (B), the two regression lines are not superimposable as the UV data sets in (A). The differences in the slopes of the two data sets are due to the limited linear dynamic range of the CLND detector.

Table 3
Weight percent ratio of the UV vs. CLND response determined from solutions containing trace levels of impurities using the secondary dilution protocol

	Impurities (mg/mL)			
	0.005	0.01	0.03	0.05
BMS-Compound B	0.72	0.85	0.92	0.93
BMS-Compound C	0.96	0.99	1.12	1.04
BMS-Compound D	1.08	0.99	1.12	1.23
BMS-Compound E	1.53	1.17	1.21	1.17
BMS-Compound F	1.26	1.00	1.04	0.99
BMS-Compound G	0.83	0.92	0.94	0.97
BMS-Compound H	0.91	1.09	1.03	1.10
Average	1.04	1.00	1.05	1.06
R.S.D.	26.6	10.6	9.9	10.4

Marker solutions containing 1 mg/mL of the API (BMS-Compound A) and trace levels of the related impurities ranging from 0.005 to 0.05 mg/mL were analyzed by both CLND and UV. The area percent of the impurities determined from the UV data was converted to weight percent using individual relative response factors.
For CLND, the concentrations of the API and individual impurities were quantified using surrogate reference standard solutions of 1.0 and 0.01 mg/mL, respectively, to account for the limited linear dynamic range of the CLND. The weight percent determined by CLND analysis was then divided by the weight percent determined by UV analysis for each individual impurity. As expected based on the equimolarity response of CLND, a ratio of unity (1.0 ± 20%) was achieved for the impurities at all but the lowest level concentration investigated. The deviation from unity at the lower concentration is due to the poor detector sensitivity caused by the low multiplier setting used on the CLND to ensure that all components of interest were on scale.

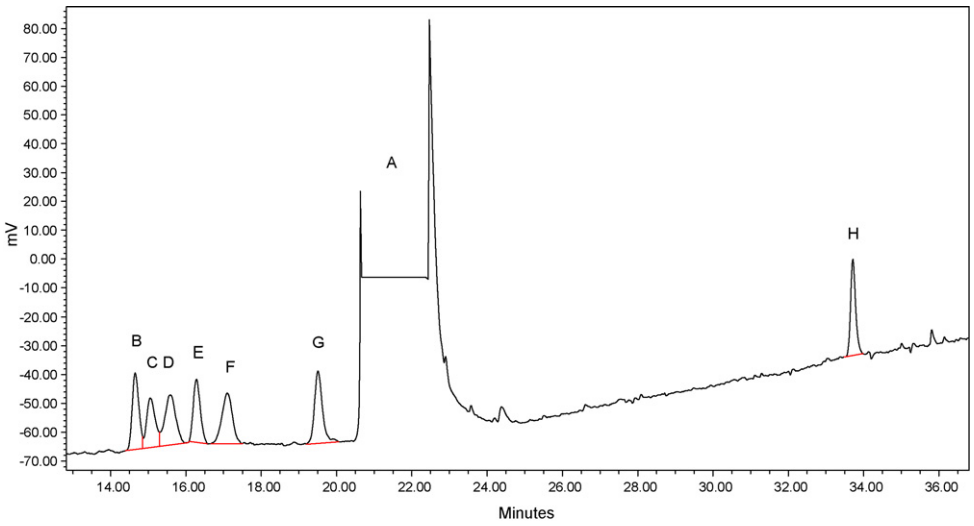

Fig. 4. Impact of higher multipler setting (25×) on CNLD signal. Note: Peaks A to H are designated as BMS-Compound A to BMS-Compound H. The CLND detector multiplier setting was raised to 25× to increase the peak response and the accuracy of peak integration. However, the use of this higher multiplier setting resulted in a "deformed" signal for the main component of interest (API) preventing its accurate integration and quantitation by CLND.

sample, respectively. The results of this study are shown in Table 3. In comparing the results in Table 3 to those obtained in the previous data set for the same compounds, it is evident that the secondary dilution protocol significantly improves the accuracy of CLND for the quantitation of low-level impurities in the sample. Using this approach, the weight percent values for the impurities determined from the CLND at the three highest concentrations were within ±20% of the values determined from the RRF corrected UV response.

Despite this significant improvement, the CLND response was still observed to deviate significantly at the lowest concentration level investigated. This deviation was of concern since it occurred at an impurity concentration equivalent to ~ 0.5% *w*/*w* in the sample, significantly higher then acceptable limits for pharmaceutical analysis. The source of this deviation was attributed to the difficulty in accurately and reliably integrating very small peaks in the LC–CLND chromatogram due to the low multiplier setting (1×), that was required to keep both the impurity and the main component peaks on scale. Increasing the detector multiplier setting from 1× to 25× or 50× will significantly increase the peak response and accuracy of low-level impurity peak integration however, when using these higher multiplier settings, the response for the main component of interest saturates the detector resulting in a "deformed" signal, preventing accurate peak integration as shown in Fig. 4. To resolve this issue and improve the sensitivity of CLND analysis, the authors propose that the following procedures and a tandem LC–UV–CLND system be utilized for impurity/degradant quantitation:

(1) Prepare a secondary dilution of the reference standard solution used for the quantitation of the main peak at a concentration approximately equivalent to the expected impurity or degradant concentration.
(2) Optimize the CLND multiplier setting for the quantitation of impurities (will generate a "deformed" signal for the main peak in the sample).
(3) Quantitate the main component of interest peak concentration using the UV response vs. the reference standard solution as in a typical LC–UV analysis.
(4) Quantitate the concentration of the impurities or degradants using the CLND response vs. the secondary dilution of the reference standard solution.
(5) The weight percent purity of the sample can then be calculated using the concentration of the impurities and main component of interest determined from the CLND and UV responses, respectively.

Using the above protocol, the results in Table 4 were obtained for the compounds of interest indicating that these impurities can

Table 4
Weight percent ratio of the UV vs. CLND response determined from solutions containing trace levels of impurities using the secondary dilution protocol and optimized multiplier setting (25×)

	Impurities (mg/mL)			
	0.001	0.005	0.01	0.03
BMS-Compound B	0.87	0.80	0.93	1.02
BMS-Compound C	0.90	0.88	1.10	1.15
BMS-Compound D	1.22	1.00	1.13	1.21
BMS-Compound E	0.87	0.94	1.09	1.20
BMS-Compound F	0.93	0.91	1.10	1.18
BMS-Compound G	0.73	0.81	0.95	1.04
BMS-Compound H	1.11	0.98	1.15	1.14
Average	0.95	0.90	1.70	1.14
R.S.D.	17.5	8.5	8.1	6.6

Marker solutions containing 1 mg/mL of the API (BMS-Compound A) and trace levels of the related impurities ranging from 0.001 to 0.03 mg/mL were analyzed by both CLND and UV. The area percent of the impurities determined from the UV data was converted to weight percent using individual relative response factors.
For CLND, the multiplier setting of 25× was optimized for the quantitation of the impurities, which generated a "deformed" signal for the API peak in the sample. The concentration of the impurities was determined using the optimized CLND detector response vs. the low concentration surrogate reference standard solution (0.01 mg/mL), whereas the API concentration was determined using the UV detector response vs. the higher concentration reference standard solution (1.0 mg/mL) as in a typical LC–UV analysis. The weight percent determined by CLND analysis was then divided by the weight percent determined by UV analysis for each individual impurity. As expected based on the equimolarity response of CLND, a ratio of unity (1.0 ± 20%) was achieved for all concentrations and impurities investigated, except for the lowest concentration of BMS-Compound G.

be accurately quantified by CLND. Although a 27% deviation was observed for BMS-Compound G at the 0.1 % w/w, the absolute concentration difference reported was only 0.03% (0.06% by CLND vs. 0.09% by UV), which is typically acceptable for impurities at this level in pharmaceutical analysis.

An added benefit of the tandem LC–UV–CLND system is the simultaneous determination of impurity RRFs. The RRF can be easily determined by comparing the weight percent results of the CLND and the area percent results of the UV detector from a single injection. The only additional information required is the molecular formula of the impurity or degradant, which is routinely collected in pharmaceutical development using high resolution LC–MS. This is a more efficient approach compared to the traditional method of isolating and characterizing impurity reference materials for RRF determinations. This simplistic approach to RRF determination is a significant benefit during the early phases of pharmaceutical development when the RRF of impurities are often assumed to be 1.0. It is well known that these assumptions can lead to gross over or under estimations of sample purity due to significant differences in its molar absorptivity of the impurities.

4. Conclusion

Since over 90% of all developmental and marketed drugs contain at least one nitrogen atom, the potential applications for CLND in pharmaceutical analysis are manifold. It is generally reported in the literature that the accuracy of the equimolar response principle of CLND is ±20%. Thus, CLND is well suited for the quantitation of degradants and synthetic process related impurities, eliminating the need for the preparation of individual reference materials. However, the limited linear dynamic range of CLND must be overcome to successfully apply this technique to the quantitation of trace-level impurities. This paper has outlined a useful protocol for the accurate quantitation of trace-level impurities in pharmaceutical analysis. Accurate quantitation is achieved by utilizing a secondary dilution of the reference standard solution at the approximate concentration of the impurities of interest. Quantitation of the impurities vs. this diluted surrogate reference standard solution is then performed, using a multiplier setting that is optimized for detection of the impurities. To permit the quantitation of the main peak of interest in a single injection, an in-line UV detector is utilized. Simultaneous collection of the CLND and UV responses allows one to quickly and accurately determine the RRFs of the impurities. These RRFs can be utilized in LC–UV based methods for routine analysis and transfer to other laboratories.

This technique is readily adapted to the quantitation of unknown impurities, eliminating the need for the full structural characterization and/or isolation of unknown impurities in early development. The only information needed is the molecular formula, which is easily obtained using commercially available high resolution MS instrumentation.

One significant limitation of the described technique is the incompatibility of LC–CLND with nitrogen-containing solvent and mobile phase modifiers, such as acetonitrile and ammonium acetate, which are commonly used in pharmaceutical analyses. The authors are actively working on practical solutions to overcome this incompatibility issue and extend the applicability of the technique.

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