Comparison of an Evaporative Light-Scattering Detector and a Chemiluminescent Nitrogen Detector for Analyzing Compounds Lacking a Sufficient UV Chromophore

The authors evaluated an evaporative light-scattering detector and a chemiluminescent nitrogen detector for high performance liquid chromatography analyses of compounds that lack a sufficient ultraviolet (UV) chromophore. They compared the detectors in three critical areas of performance: precision, linearity, and limit of detection. The four compounds used as test analytes included caffeine and three pharmaceutical compounds with weak UV chromophores. Based on these experiments, the authors discuss the advantages and disadvantages of these detectors.

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Ultraviolet (UV) detection has been the preferred detection technique for high performance liquid chromatography (HPLC) analyses of pharmaceutical compounds because of its ease of use, dynamic linear range, precision, and high sensitivity. However, one shortcoming of UV detection is the inability to detect compounds that lack UV chromophores. Chemists can use various derivatization methods to enhance UV absorption, but these manipulations tend to be tedious and introduce their own errors. To avoid the derivatization of these compounds, analysts need alternative detection schemes that are independent of radiative absorption by the compound yet still provide adequate linearity, precision, limit of detection, and ruggedness. Evaporative light-scattering detection (ELSD) and chemiluminescent nitrogen detection (CLND) are two commercially available detection techniques for HPLC, both of which operate independently of radiative absorption.

ELSD has been demonstrated successfully as an alternative to UV detection for weakly UV-absorbing pharmaceutical compounds within our laboratory (1–4). In ELSD, as depicted in Figure 1, the HPLC effluent is nebulized and then vaporized in a heated drift tube, which results in a cloud of analyte particles that pass through a beam of light. The analyte particles scatter the light and generate a signal at a photodiode (or photomultiplier tube, depending upon the manu-

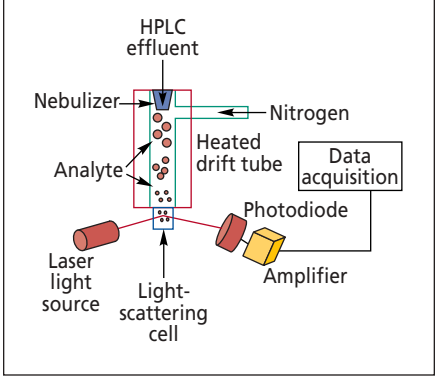

Figure 1: Schematic design of an evaporative light-scattering detector.

facturer) that is proportional to the amount of light scattered. The amount of light scattered is dependent upon the particle size and quantity, which in turn are functions of the analyte, analyte concentration, nebulizer characteristics, mobile-phase flow rate, mobile-phase composition, nebulizing-gas flow rate, and drift-tube temperature (5–8). ELSD is limited to the use of volatile mobile-phase components and the detection of relatively nonvolatile analytes.

In cases in which the weakly UV-absorbing compounds contain at least one nitrogen atom, CLND also can be used as an alternative to UV detection. The chemiluminescent nitrogen detector for HPLC first was described in 1988 and subsequently refined and described in its current incarnation in 1992 (9,10). Since its introduction, CLND has been used successfully for HPLC analyses of small organic molecules and peptides (11,12). Figure 2 depicts the principles of CLND. Oxidation of the nebulized HPLC effluent by combustion in a high-temperature furnace converts all nitrogen-containing compounds, with the exception of N_2, into nitric oxide. The vaporized water in the effluent is then removed in a membrane dryer. The dried gas is passed into a chamber where it reacts with ozone, which causes the conversion of the nitric oxide in the dried gas to excited-state nitrogen dioxide, which subsequently produces photon emission upon relaxation. Thus, the signal generated is proportional to the moles of nitrogen present in a given molecule. Because of the nature of the detection mechanism, CLND is limited to detection of analytes that contain nitrogen. Conversely, the CLND detection mechanism also imposes another limitation — all mobile-phase components must be free of nitrogen to keep baseline noise to a minimum. Common HPLC organic modifiers that contain nitrogen, such as acetonitrile, thus are unsuitable. The volatility of the sample is of no concern with the chemiluminescent nitrogen detector, as is the case with the evaporative light-scattering detector, because the sample is completely oxidized in the chemiluminescent nitrogen detector chamber. As with ELSD, CLND is limited to analyzing volatile mobile-phase components.

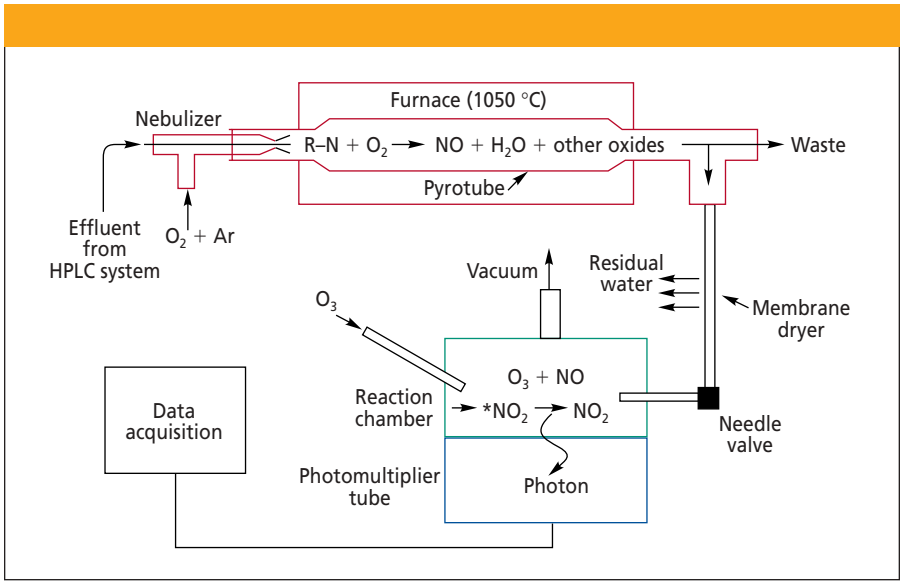

Figure 2: Schematic design of a chemiluminescent nitrogen detector.

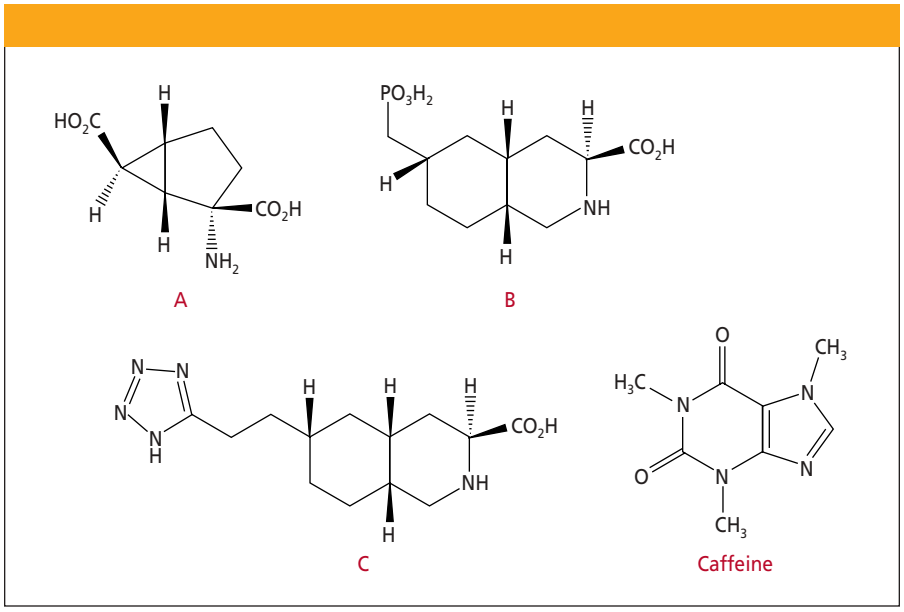

Figure 3: Structures of the test compounds.

We compared the analytical capabilities of evaporative light-scattering and chemiluminescent nitrogen detectors using caffeine and three additional pharmaceutical compounds as test analytes that lacked sufficient UV chromophores (Figure 3) and provided the desired variation in the number and bonding of nitrogen atoms in the molecule. We will report the precision, linearity, and limit of detection results for each detector and each compound based upon these experimental studies.

Experimental

The HPLC system included a model 1050 series autosampler and pump (Agilent Technologies, Wilmington, Delaware), a model 500 evaporative light-scattering detector (Alltech Associates Inc., Deerfield, Illinois), and a model 8060 chemiluminescent nitrogen detector (Antek Instruments Inc., Houston, Texas). We obtained a 150 mm × 2.1 mm Intersil C4 column from GL Sciences (Tokyo, Japan) and used it for analyses of the pharmaceutical compounds (see Figure 3). We obtained a 150 mm × 2.1 mm Zorbax RX-C8 column from Agilent Technologies and used it for the caffeine analysis. Caffeine was purchased from Sigma (St. Louis, Missouri) and the three pharmaceutical compounds were synthesized at Eli Lilly and Co. (Indianapolis, Indiana).

We chose isocratic mobile phases to eliminate possible effects of the gradient (for example, eluent viscosity changes) in ELSD nebulization. The mobile phase for the pharmaceutical compounds A and B was

99% water, 0.05% trifluoroacetic acid, and 1% methanol. The mobile phase used for compound C was 85% water, 0.1% trifluoroacetic acid, and 15% methanol. The mobile phase for caffeine was 60% water, 0.1% trifluoroacetic acid, and 40% methanol. The flow rate in all analyses was 0.2 mL/min with an injection volume of 10 µL. The same mobile-phase preparation, column, and sample preparations were used for each detector and for each sample.

The evaporative light-scattering detector settings were optimized for each mobile phase according to the manufacturer's recommendations (5). For compounds A, B, and C, the detector settings were as follows: 75 °C drift-tube temperature, 3.1–standard liters per minute (SLPM) nitrogen gas flow, and ¼ attenuation. For caffeine, the evaporative light-scattering detector conditions were as follows: 60 °C drift-tube temperature, 2.4-SLPM nitrogen gas flow, and ⅛ attenuation.

The chemiluminescent nitrogen detector settings for compounds A, B, and C were as follows: −10 °C photomultiplier-tube temperature, −0.780-kV photomultiplier-tube voltage, high × 10 sensitivity, 40-cm^3/min argon makeup gas flow, 150-cm^3/min inlet argon gas flow, 150-cm^3/min inlet oxygen gas flow, 25-cm^3/min ozone flow, 1050 °C oven temperature, and 85 °C membrane dryer temperature. The chemiluminescent nitrogen detector settings for caffeine were as follows: 0 °C photomultiplier-tube temperature, −0.770-kV photomultiplier-tube voltage, high × 1 sensitivity, 50-cm^3/min makeup argon gas flow, 180-cm^3/min inlet argon gas flow, 180-cm^3/min inlet oxygen gas flow, 25-cm^3/min ozone flow, 1050 °C oven temperature, and 85 °C membrane dryer temperature.

Results and Discussion

To evaluate the linearity of response for each detector, we prepared a stock solution in the range of 1–1.5 mg/mL for each compound. We performed serial dilutions of the stock until we found a concentration at the approximate limit of quantitation, a signal-to-noise ratio of 5:1. We made duplicate injections of each concentration. The results of the linearity comparison are shown in Table I for the evaporative light-scattering detector and Table II for the chemiluminescent nitrogen detector.

Figure 4 shows an example plot of the data for compound A. The data show that the chemiluminescent nitrogen detector exhibits a more linear response, with correlation coefficients closer to 1, *y* intercepts closer to 0, and a larger linear range compared with that obtained from ELSD. As expected, a nonlinear response in the ELSD data is evident for each compound, particularly at low concentrations: see Figure 4 (6,8). Unlike spectroscopic detectors, which operate within Beer's law ($A = \varepsilon bc$), the ELSD response is described with the nonlinear equation

$$A = am^x \qquad [1]$$

where A is the peak area response, m is the sample quantity, and a and x are coefficients reflecting properties of the sample and the parameters of the chromatographic and detector systems. A log–log plot of peak area

Table I: Linearity data for the test analytes analyzed using ELSD

Compound	Concentration Range Analyzed (mg/mL)	Response/ mg Concentration	*y* Intercept	Correlation Coefficient
A	0.0024–1.22	3.4×10^4	−1020	0.9987
B	0.0033–1.16	3.1×10^4	−1010	0.9984
C	0.0053–1.07	4.1×10^4	−1460	0.9978
Caffeine	0.021–1.10	2.7×10^4	−1990	0.9969

Table II: Linearity data for the test analytes analyzed using CLND

Compound	Concentration Range Analyzed (mg/mL)	Response/ mg Concentration	*y* Intercept	Correlation Coefficient
A	0.0012–1.22	2.6×10^4	−184	0.9994
B	0.0009–1.66	1.6×10^4	−199	0.9992
C	0.0008–1.07	4.2×10^4	−104	0.9999
Caffeine	0.0008–0.44	1.0×10^5	−256	0.9999

response versus sample quantity will be linear, which allows for quantitative results as described by the equation

$$\log A = x \log m + \log a \qquad [2]$$

Likewise, all nonlinear curves have a region of linear response; if calibration standards and samples are within this linear range, a linear equation can be applied.

The response of the chemiluminescent nitrogen detector ideally is equimolar with the amount of nitrogen in the sample. This response can be seen in the relative increase in slope for caffeine, which is caused by the large number of nitrogen atoms per molecule of caffeine (four nitrogens per molecule). However, compound C, with five nitrogen atoms per molecule, did not show as large a slope as that demonstrated by caffeine. The likely reason for this is the presence of the R–N=N–R bonding configuration in compound C. In this configuration, conversion to nitrogen oxide is less than optimal due to some conversion of the N=N moiety to elemental nitrogen (13). Thus, the signal per mole of nitrogen for compound C is less than would be observed for an equal number of nitrogen atoms that were not in a R–N=N–R configuration.

To evaluate the precision of each detector, we determined the relative standard deviation (RSD) of the measured peak areas of 10 replicate injections (Table III). The results for the evaporative light-scattering detector generally were better than those obtained with the chemiluminescent nitrogen detector. The improved precision of the evaporative light-scattering detector is attributed partly to the superior peak shape obtained versus that of the chemiluminescent nitrogen detector (Figure 5). The superior peak shape might be attributed to the less complicated design and detection mechanism of the evaporative light-scattering detector versus the chemiluminescent nitrogen detector. The lower RSD for caffeine versus compounds A and B, when analyzed using the chemiluminescent nitrogen detector, resulted from the greater number of nitrogens in caffeine. This nitrogen content generates a larger signal and makes for more-precise peak integration. As expected for the chemiluminescent nitrogen detector, the RSD generally decreased as the number of nitrogens in a given molecule increased.

We measured the limit of detection for the chemiluminescent nitrogen and evaporative light-scattering detectors by finding the lowest-concentration sample from serially diluted samples that yielded a peak roughly three times the baseline noise. The limit of detection is expressed as the mass of compound injected on the column, and, in the case of the chemiluminescent nitrogen detector, the mass of nitrogen injected on the column (Table III).

The chemiluminescent nitrogen detector was determined to be more sensitive than the evaporative light-scattering detector by a factor of approximately four. The evaporative light-scattering detector demonstrated

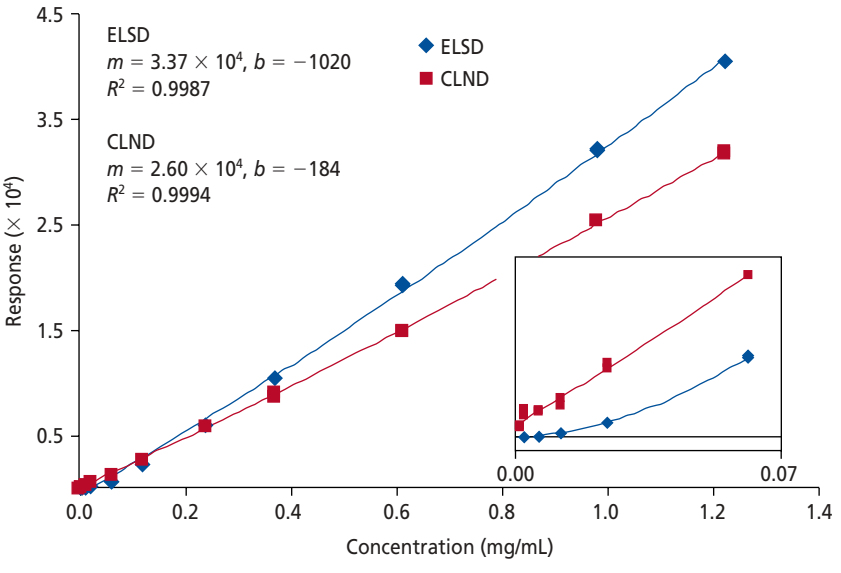

Figure 4: The linearity results for compound A. The slope, intercept, and correlation coefficient of the linear fit also are shown in Tables I and II. The boxed region is an enlarged view of the low-concentration range.

Table III: Precision and limit of detection* results for test analytes using ELSD and CLND

Compound	Parameter	ELSD	CLND
A	Analyte (ng)	22	5.4
	Nitrogen (ng)	—	0.4
	RSD (%)	0.37	3.31
B	Analyte (ng)	30	9.0
	Nitrogen (ng)	—	0.4
	RSD (%)	0.60	2.75
C	Analyte (ng)	19	5.4
	Nitrogen (ng)	—	1.3
	RSD (%)	0.34	1.56
Caffeine	Analyte (ng)	200	5.0
	Nitrogen (ng)	—	1.5
	RSD (%)	1.45	0.58

*Limit of detection results are expressed as nanograms of analyte and nanograms of nitrogen injected on column and detected.

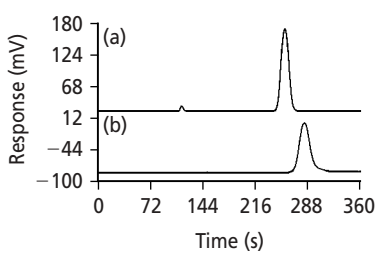

Figure 5: Sample chromatograms of compound A as detected by (a) ELSD and (b) CLND. Conditions are described in the text.

very poor sensitivity for caffeine. We used various instrument settings as well as methanol–water and acetonitrile–water mobile-phase systems to try to decrease the relatively large limit of detection obtained with the evaporative light-scattering detector for caffeine — without success. We believe that solute properties affected this relative increase in limit of detection. Cebolla and co-workers (14) demonstrated inaccuracy in ELSD responses using only molecular weight and boiling points of polyaromatic compounds. Kildew and colleagues (15) illustrated the importance of the sample vapor pressure with variations in response factors for ELSD.

Using an aerosol splitter for the evaporative light-scattering detector would have enabled lower drift tube temperature, which could have resulted in improved particle formation, increased sensitivity, and better sensitivity for caffeine. In general, ELSD response is proportional to concentration. However, these results confirm some nonuniversal response characteristics of the evaporative light-scattering detector with some variation to the chemical structure and physical properties of the analyte; therefore, ELSD is not a true mass detector in certain situations (7). The limit of detection of compounds A and B for the chemiluminescent nitrogen detector agree well with other literature reports (11,16). The presence of the R–N=N–R configuration in compound C likely explains the high limit of detection, as measured in nanograms of nitrogen, observed for compound C relative to compounds A and B (13). Caffeine was not run on our chemiluminescent nitrogen detector until after significant maintenance was performed on the detector, including rebuilding the nebulizer and replacing the pyrotube, membrane dryer, and ozone generator. These changes resulted in a higher limit of detection, an increase by nearly a factor of four, as measured by comparing data obtained before and after maintenance. If caffeine had been evaluated on the system before these changes, we expect that the limit of detection would have been comparable to the values obtained for compounds A and B.

Conclusion

In terms of performance, the chemiluminescent nitrogen detector provides a complementary tool to the evaporative light-scattering detector for detecting compounds that lack a sufficient chromophore. The chemiluminescent nitrogen detector clearly demonstrated a more linear response and lower limit of detection than the evaporative light-scattering detector. The precision of the chemiluminescent nitrogen detector generally was poorer than that of the evaporative light-scattering detector, likely because of the more complicated mechanism of detection of the chemiluminescent nitrogen detector. The evaporative light-scattering detector required no maintenance during the course of these experiments, but the chemiluminescent nitrogen detector required significant maintenance and troubleshooting. The chemiluminescent nitrogen detector currently is much less rugged and not readily amenable for routine use, but it will become a powerful analytical tool when the reliability issues are resolved. However, both detectors are extremely valuable analytical tools compared with UV detectors for analyzing compounds that lack chromophores.

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