HPLC Analysis of Non-volatile Analytes Using Charged Aerosol Detection

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A new detection method based upon aerosol charging was examined for its applicability and performance with high performance liquid chromatography (HPLC). Our results demonstrate universal detection of nonvolatile analytes with response magnitude that is independent of analyte chemical properties, four orders of magnitude dynamic range, low nanogram, lower limits of detection and < 2% relative standard deviation response variability. Broad applicability was demonstrated for a range of methods including those using gradient elution, reversed phase, hydrophilic interaction, and ion chromatography; normal and narrow bore column formats; and in combination with other detectors (for example, UV detectors, evaporative light-scattering detectors and mass spectrometers).

There continues to be strong demand for improvements in sensitivity, selectivity, throughput, qualitative content and many other performance characteristics of high performance liquid chromatography (HPLC). A major need is for methods that provide both universal detection and quantitative analysis. It is widely recognized that no single HPLC detector is capable of distinguishing all possible analytes from a given chromatographic eluent and the term “universal” is often used to describe detection of a diverse range of analytes.

A primary goal for most analyses that seek universal detection is to obtain a consistent relationship between the magnitude of response and quantity injected for a range of analytes. This “consistency of response factors” allows the use of global mathematical relationships to estimate quantity (for example, use of parent drug response factor to quantify metabolites and degradants). This characteristic is useful in many applications, where it is impractical or impossible to use individual standards to calibrate the response for each analyte such as drug library QC, pharmaceutical impurity testing, complex lipid analyses and many applications requiring mass balance assessment. Currently, performing such an analysis is difficult with available technologies.
Several techniques are employed for universal detection that are based upon measuring bulk properties or generic attributes among diverse analytes. These techniques include refractive index (RI), low-wavelength UV, evaporative light-scattering detection (ELSD) and chemiluminescent nitrogen detection. These techniques are in contrast to those geared towards selective detection, which is typically based upon measurement of specific properties or reactivity of analytes (for example, fluorescence at a specific wavelength, oxidation at a specific potential and mass-to-charge ratio \([m/z]\)). It should be noted that for most detectors, selectivity and universality are mutually exclusive. Universal detectors often require very selective sample prepurification and analytical separation techniques for analysis of biological matrices.

Various characteristics of commercially available instruments employed for universal detection have been compared previously.\(^1\)–\(^3\) RI, while widely used, has significant limitations in sensitivity and is not compatible with gradient elution. Low-wavelength UV provides higher sensitivity and improved gradient compatibility. However, detection is limited to chromophores, and response magnitude depends upon molar absorptivity, which can vary by orders of magnitude even among analogous structures. Although UV detection remains the primary technique for many HPLC analyses, it is unable to see compounds that lack a sufficient UV chromophore such as many underivatized amino acids, carbohydrates, lipids, polymers, surfactants, drug substances and natural products. ELSD has become widely used alone or to complement absorbance and mass spectrometry (MS) detectors.\(^3\) ELSD can see compounds lacking a chromophore provided they have low volatility. Also, ELSD response magnitude is often less dependent on analyte chemical properties than MS or UV. ELSD, however, has significant limitations in precision, sensitivity, dynamic range and the nature of calibration curves.\(^1\)–\(^5\) Chemiluminescent nitrogen detection is a relatively new technology for nitrogen-containing molecules and, while less universal, can detect a broad range of pharmaceutical compounds. It has been reported that this detection technique can provide a more linear response and lower limit of detection (LOD) than ELSD, but with poorer precision, higher maintenance and no compatibility with nitrogen-containing mobile phases such as those with acetonitrile and triethylamine.\(^1\) The demand for improved methods continues to drive novel approaches toward universal HPLC detection such as those involving condensation nucleation light scattering and inductively coupled plasma MS.\(^6\)

A new HPLC detection method has been developed based upon charged aerosol detection (CAD). This technique is fundamentally different from that of other detectors and is based upon the coupling of HPLC with widely used electrical aerosol analyser technology.\(^4\)–\(^7\) A similar HPLC detection method, termed aerosol charge detection, was described by Dixon and Peterson.\(^4\) The detection principle involves the charging of aerosol particles via corona discharge with subsequent electrometer-based measurement and thus has some commonality with atmospheric-pressure chemical ionization (APCI) MS. However, CAD operates by detecting charged particles that have a selected range of mobility rather than by measuring individual gas-phase ions that are differentiated based upon \(m/z\). Furthermore, previous studies have shown that the signal obtained with electrical aerosol technology depends primarily upon particle size across a wide range and does not depend significantly upon individual analyte properties.\(^9\) This more generic principle can thus theoretically provide consistent interanalyte response factors and complement atmospheric pressure ionization MS techniques such as electrospray and APCI.

Described in the following sections is our initial evaluation of this technique. We tested the general applicability of CAD using various HPLC systems and modes of separation and for a diversity of analytes. Particular emphasis was on detection of

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**Table 1: Peak area response for two CAD devices from flow injection analysis of 10 µg of each compound with 50% aqueous methanol as mobile phase and diluent. Values are the average from three injections.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Response CAD #1</th>
<th>Response CAD #2</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>1.90</td>
<td>1.87</td>
<td>MW – 65 000 Da</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>2.14</td>
<td>2.29</td>
<td>Weak base</td>
</tr>
<tr>
<td>Caffeine</td>
<td>1.88</td>
<td>1.94</td>
<td>Weak base</td>
</tr>
<tr>
<td>Dextrin</td>
<td>1.82</td>
<td>1.78</td>
<td>Polymer, no UV</td>
</tr>
<tr>
<td>Estradiol</td>
<td>1.87</td>
<td>1.94</td>
<td>Neutral</td>
</tr>
<tr>
<td>Fructose</td>
<td>1.95</td>
<td>2.10</td>
<td>Polar, no UV</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.99</td>
<td>2.02</td>
<td>Polar, no UV</td>
</tr>
<tr>
<td>Glutathione</td>
<td>1.96</td>
<td>2.04</td>
<td>Zwitterion</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>1.97</td>
<td>2.07</td>
<td>Zwitterion</td>
</tr>
<tr>
<td>Lactose</td>
<td>1.89</td>
<td>2.02</td>
<td>Polar, no UV</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.09</td>
<td>2.17</td>
<td>Zwitterion</td>
</tr>
<tr>
<td>Naphthol</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Neutral, volatile</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>2.20</td>
<td>2.34</td>
<td>Weak base</td>
</tr>
<tr>
<td>Propranolol</td>
<td>2.16</td>
<td>2.31</td>
<td>Weak base</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>2.15</td>
<td>2.26</td>
<td>Weak acid</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.98</td>
<td>2.03</td>
<td>Polar, no UV</td>
</tr>
<tr>
<td>Umbelliferone</td>
<td>1.91</td>
<td>2.00</td>
<td>Neutral</td>
</tr>
</tbody>
</table>

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% RSD 6.44% 8.31%
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non-chromophores and to assess dependence of response upon analyte chemical properties and chromatographic variables. We also evaluated CAD for its use, in parallel, with MS and photodiode-array detection for high-throughput analysis of diverse small-molecule pharmaceutical library compounds using typical methods for quality control and for support of synthesis.

Experimental

A diagram of the CAD device is shown in Figure 1. The detection method involves pneumatic nebulization of HPLC column eluent, exclusion of the largest droplets via an impactor and ambient T solvent evaporation to form an aerosol of analyte particles. A turbulent jet of positive ions, formed by passing a secondary stream of gas by a corona discharge and through an orifice, is mixed with the opposing analyte aerosol stream. In this process, charge is transferred diffusionally to analyte particles. Excess positive ions are trapped by a weak electric field and charged particles impinge on a conductive filter, which transfers the charge to an electrometer for signal transduction.

Several CAD devices (Corona CAD, ESA Inc., Chelmsford, Massachusetts, USA) were evaluated with a variety of HPLC systems, including Agilent 1100LC (Agilent Technologies, Wilmington, Delaware, USA) and ESA HPLC systems.

Results and Discussion

To study the chemical scope of CAD and dependence of response on analyte nature, we performed FIA of 17 chemically diverse compounds. Results and conditions, summarized in Table 1, show that all analytes, except for the volatile compound naphthol, were detected by CAD. Using these conditions, naphthol was detected by UV but did not respond with low-temperature ELSD (data not shown). These results suggest that CAD response depends upon analyte volatility. This is as expected based upon similar observations with other techniques that involve particle detection subsequent to solvent evaporation (for example, ELSD).

Peak area from FIA of a constant mass (10 µg) of each analyte ranged from 1.78–2.34 units across two devices with 6.44 and 8.31% RSD within each instrument (Table 1). Because these analytes represent a wide range of molecular weights, polarity, acid–base characteristics, and included several nonchromophores, these results demonstrate that response magnitude does not depend significantly upon analyte chemical structure or properties. This is consistent with the described independence of electrical aerosol measurement on the nature of analytes.

The detection scope was characterized further for 735 compounds from Millennium Pharmaceuticals (Cambridge, Massachusetts, USA). Nitrogen gas (ESA nitrogen generator or cylinder), regulated at 35 psi was introduced to the detector and the resultant gas flow-rate was regulated automatically and monitored by the CAD device. The only parameter that required user input was response range, which typically was set to 100 pA full scale with 1 V analogue output to the Agilent ChemStation or EZChrom Elite for ESA chromatography data systems. Initial studies of detection scope and response factors involved flow-injection analysis (FIA) of compounds representing a wide diversity of chemical properties. Precision, sensitivity, dynamic range and nature of calibration curves were evaluated for a variety of compounds and chromatographic conditions and included some comparison with UV and ELSD. Specific conditions are described with results.
Massachusetts, USA) small molecule library. Compound molecular masses ranged from 165 to 684 Da and included a wide range of chemical structures and properties from several drug discovery programs. CAD was used in combination with photodiode-array detection, single-quadrupole MS and ELSD, as shown in Figure 2. Using the described conditions, the same 733 compounds were detected by both CAD and ELSD. The only two compounds that did not respond were those with the lowest MW (165 and 178 Da). This provides further evidence of the dependence of response on analyte volatility and demonstrates that CAD, which uses ambient temperature evaporation, has a similar detection scope to low-temperature ELSD.

CAD response factors (area/micrograms) were highly variable with >50% RSD for the 733 library compounds detected. Because our FIA results demonstrated that there was no significant dependence of response upon chemical structure, we therefore studied the dependence of response upon analytical conditions. We performed FIA of six compounds of known purity using various mixtures of solvents A and B, as described in Figure 2. The results demonstrate that both ELSD (not shown) and CAD response were dependent upon eluent composition.

Figure 3 shows that CAD response was directly proportional to eluent percentage organic content, and that the magnitude of this effect was very similar for all compounds. These results are consistent with the theory of nebulization, specific droplet size selection (for example, impactor, syphon) and evaporation. This effect, in which higher solvent volatility favours delivery of analyte for subsequent detection, has been observed widely for many techniques that involve solvent evaporation (for example, atmospheric pressure ionization [API]–MS, ELSD). This is further demonstrated in Figures 4 and 5, which show the response obtained for a constant mass of several compounds separated by gradient reversed-phase and hydrophilic interaction chromatography (HILIC), respectively.

As shown, peak area either increases or decreases proportionally with retention time as a consequence of a corresponding increase or decrease in percentage organic content during gradient elution.

As shown in Figures 3–5, the dependence of CAD response upon eluent composition was predictable and uniform among diverse analytes. Based upon these observations, our ongoing efforts are to examine specifically the use of empirical and computational approaches as a means of normalizing CAD response. These approaches will be applied to a broader scope of Millennium Pharmaceuticals’ compound library (that is, more compounds, greater chemical diversity) to assess the practical capabilities of CAD to provide uniform response factors (that is, accurate quantification) across numerous
analytical runs and in support of several ongoing drug discovery programs. The chromatograms in Figures 4–8 show several examples of the use of CAD for a variety of separations. This includes use with several solvents, additives and conditions including gradient elution (Figures 4 and 5), reversed phase (Figures 4 and 8), hydrophilic interaction (Figures 5 and 6), and high-performance liquid chromatography (Figure 7). As expected, our data show that CAD has similar requirements for mobile phase volatility as API–MS and ELSD. In addition to that shown, the detection technique was also compatible with many other solvents and modifiers including acetonitrile, methanol, triethylamine, trifluoroacetic acid, triphobonic acid and triphosphoric acid. Figure 8 shows the use of CAD with several HPLC column formats. Use of a single nebulizer design and the described conditions, peak width increased and resolution only when using a column inner diameter less than 2 mm. These results are in accordance with our measurements of an effective detector volume of approximately 1.5–2 µL. This dimensional good compatibility with narrow and normal bore chromatography, and further study is required to address lower volume formats.

Figure 4 is an overlay of 10 replicate injections in which response variability (percent RSD) was 1.62, 1.75, 1.14 and 1.48 for glucose, caffeine, propranolol and chlorophenazine, respectively. Variability obtained with ELSD, using these same conditions, was 3.52, 3.61, 2.95 and 2.41 %RSD. for these compounds. This twofold or more improvement in precision over ELSD has been observed consistently in several applications in which we have compared CAD with ELSD. The lower limit of detection for the previously mentioned analytes was estimated using serially diluted standards and determined as the lowest mass injected that yielded a peak-to-peak signal-to-noise ratio of 3 or greater. The limit of detection ranged from 5 to 20 ng for CAD and 100 to 150 for ELSD. Figure 6 provides an example of CAD sensitivity for simple sugars. The 10-fold or better improvement in LOD obtained with CAD has been consistently observed again in several comparison studies with ELSD.

Figure 9 shows that a dynamic range of at least four orders of magnitude (typically 10 ng to 100 µg) was obtained with CAD with very similar calibration curves (that is, response factors) for all five phenolic compounds. CAD response was nonlinear and can be represented by \( y = ax^b \) where \( y = \text{peak response (height or area)} \), \( x = \text{analyte mass} \) and \( b = \text{the exponential response factor (that is, sensitivity).} \) The slope determined from a log–log plot of response versus concentration for these five phenolic compounds ranged from 0.48 to 0.50. This response behaviour is in substantial agreement with both theory and empirical data on pneumatic nebulization and aerosol charging. Because CAD and low-temperature ELSD use comparable techniques for aerosol formation, the observed improvements in precision, sensitivity and dynamic range most likely result from differences in particle detection technology. It is widely recognized that light-scattering efficiency is dependent upon particle diameter and involves exponential changes between scattering regimes (that is, Rayleigh, Mie, refraction, reflection). This has been shown to underlie the dramatic “drop-off” in response, limited sensitivity, and high response variability often observed with ELSD. In contrast, the response obtained with electrical aerosol detection technology used with CAD has been shown to have unimodal efficiency over a broad range of particle diameters. These performance characteristics can provide significant advantages in many applications, including those requiring detection of diverse compounds and trace analyses.

**Conclusions**

The described studies demonstrate that charged aerosol detection applies to a broad range of HPLC separations and appears to possess a number of very desirable characteristics. These include: universal detection of nonvolatile analytes, response independent of chemical properties, a wide dynamic response range with high sensitivity, good precision for a wide
diversity of analytes, and simple and reliable operation. The magnitude of CAD response was found to be proportional to eluent percentage organic content. Additional study is therefore required to determine if the observed predictable nature of this dependency can provide a means of normalizing response for accurate quantification of unknowns. The applicability of this technique to biological matrices also requires additional study.

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References

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