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ABSTRACT

An array of pharmaceutical compounds and impurities were used to investigate the applicability of atmospheric pressure ionization mass spectrometry (MS) to routinely detect coeluting impurities in HPLC (i.e. peak purity). Four drugs were individually tested against their related impurity set utilizing a straightforward HPLC-MS peak purity strategy. For the investigated set, which represents 24 unique drug-impurity permutations, 75% of the coeluting impurities were detected at levels $\leq 1.0\%$, including one-third at 0.1%. Factors that affect the applicability of this peak purity approach are also discussed.

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1. INTRODUCTION

The pharmaceutical industry routinely uses high performance liquid chromatography (HPLC) to quantitate levels of impurities at the mandated level of 0.1% [1, 2, 3]. However, pharmaceutical drug substances and their impurities are often structurally similar and therefore may coelute. The evaluation of peak purity is a key component of method development and validation (i.e. specificity). Analytical techniques typically used to increase the analyst's confidence in the purity of chromatographic peaks include orthogonal separations, selectivity testing using potential impurities and fraction collections followed by alternate testing. These off-line techniques can be time consuming and ineffective, especially at the early stages of drug development when impurities are unknown.

Diode array detection (DAD) has also been widely used to provide chromatographic peak purity determinations [4, 5, 6, 7, 8]. Although it was demonstrated that DAD can provide rapid on-line determinations, disadvantages, such as inability to consistently detect an impurity below 0.5% coeluting with a high level main analyte and a requirement for some separation of analytes, were acknowledged. Analytes must also have dissimilar UV spectra to achieve low detection levels. This shortcoming is important in the pharmaceutical industry as impurities typically have UV spectra similar to the main analyte.

Coupling HPLC with mass spectrometry (LC-MS) offers an alternative mode of detection which might be exploited for rapid on-line HPLC peak purity assessment. Unlike DAD, LC-MS has the potential, with the exception of isobaric impurities, to provide detection capabilities for all impurities. Mass spectrometric detection offers the added ability to provide mass and structural information on the coeluting impurity. LC-MS can provide high sensitivity for a wide range of compound classes relevant to the pharmaceutical industry while simultaneously providing chromatographic selectivity and mass selectivity.

Indeed, LC-MS has been used to demonstrate method specificity and to screen drug purity [9, 10]. MS has also been implemented as a tool to determine HPLC peak purity during the analysis of a few particular compounds [11, 12, 13, 14]. Bylund et al. applied mathematical modeling to LC-MS peak purity determinations using both real and simulated data [15]. Bryant et al. [16] compared electrospray ionization (ESI) LC-MS and LC-MS-MS with DAD for examination of coeluting impurities in famciclovir and ropinirole. This study demonstrated that for an impurity set with similar UV spectra, MS could be optimized in such a way to consistently detect semi-coeluting known impurities at 0.1% whereas DAD could not. All examined compounds contained amine groups, which typically provide good sensitivity by ESI [17]. Salau et al. [18] determined HPLC peak purity of pesticide mixtures by thermospray MS. This study demonstrated the suppression effects of exactly coeluting compounds in

MS and that mathematical modeling can detect coeluting compounds at the 5% level. This high detection limit is attributed to the fact that thermospray exhibits greater noise and lower sensitivity relative to other ionization techniques such as atmospheric pressure ionization (API).

Fisher et al. [19] examined the use of API LC-MS and LC-MS-MS techniques to be used as chromatographic peak purity tools using one drug coeluted with four impurities at levels ranging from 0.05% to 5.0%. Data indicated that, despite spectral comparison of the pure compound with that of the spiked samples, unambiguous detection of coeluting impurities was possible only at the 0.4% level, falling short of the mandated level of 0.1%.

The application of MS as a peak purity tool for additional separation techniques was also discussed by Fanali et al. [20]. A preliminary study examined ESI MS as a tool for peak identification, peak purity testing, and selective monitoring of overlapping peaks with capillary electrophoresis (CE). Other studies have interfaced MS with gas chromatography (GC) to determine peak purity [21, 22]. These GC and CE applications illustrate the potential flexibility of MS as a general tool for peak purity.

These studies referenced above provide some evidence for the use of MS as a chromatographic peak purity tool in specific instances. However, these

studies do not provide an evaluation of the general applicability of API LC-MS to detect low levels of unknown impurities coeluting with a high level main analyte. The impact of ionization technique (i.e. thermally assisted ESI and APCI) was not sufficiently explored. As it is widely known that response factors vary using mass spectrometric detection, it is important to test a diverse array of compounds and impurities to provide a robust evaluation.

This paper presents the evaluation of API MS as a general tool for detection of coeluting unknown impurities in HPLC. The investigation employed a single stage MS to facilitate broad applicability. Data was obtained on an array of drugs and impurities coeluting at levels ranging from 10% down to 0.1%. The compounds tested included acids, bases and zwitterions possessing a wide range of polarities, spanning a mass range typical of pharmaceuticals and with varying ionizabilities. Discussions of additional procedures and factors that can affect the application of API MS to HPLC peak purity investigations are also discussed.

2. EXPERIMENTAL

2.1. *Chemicals:*

All pharmaceuticals and impurities were obtained from Procter & Gamble Pharmaceuticals, Norwich, NY (Tables I, II and III) [23, 24, 25]. HPLC grade acetonitrile, acetic acid, ammonium acetate and ammonium formate were all purchased from Mallinckrodt Baker Inc., (Phillipsburgh, NJ). Water was purified to 18 m Ω using a Millipore Corporation (Bedford, MA) Milli-Q+ system. Formic acid (98%), sodium trifluoroacetate (99.5%) and pentafluoropropionic acid (PFPA) (97%) were purchased from Sigma-Aldrich, Inc. (Milwaukee, WI).

Table I: Azimilide and azimilide impurities

AZIMILIDE DRUG SUBSTANCE mass 457

AI-1 mass 443

AI-2 mass 303

AI-3 mass 501

AI-4 mass 375

AI-5 mass 206

AI-7 mass 815

AI-6 mass 800

Table II: 5-aminosalicylic acid (5-ASA) and 5-ASA impurities

5-ASA DRUG SUBSTANCE mass 153.04

n-acetyl-5-aminosalicylic acid mass 195.05

2, 5-dihydroxybenzoic acid mass 154.03

4-aminophenol mass 109.05

3-aminosalicylic acid mass 153.04

4-aminosalicylic acid mass 153.04

Table III: Digitalic drug substances and impurities. *Digoxin used as digitoxin impurity. **Digitoxin used as digoxin impurity

DIGOXIN DRUG SUBSTANCE*

mass 780.43

Digitoxigenin mass 390.24

Digitoxigenine mass 520.30
(mono-digitoxosid)

Alpha-acetyl-digoxin mass 822.44

DIGITOXIN DRUG

SUBSTANCE** mass 764.43

Digitoxigenin mass 374.25

Digitoxigenine mass 650.37
(bis-digitoxosid)

Beta-acetyl-digoxin mass 822.44

2.2. *Equipment:*

The HPLC system was comprised of a Thermoseparations Products (Fremont, CA) TSP P4000 gradient pump with an Applied Biosystems (Foster City, CA) UV detector. Both a Hewlett Packard (Wilmington, DE) HP1100 autosampler and a Rheodyne manual injection manifold (Rohnert Park, CA) were used for sample injection.

Mass spectrometry was performed on an Applied Biosystems (Foster City, CA) Perkin-Elmer-Sciex single quadrupole API-150 MCA Mass Chromatographic Analyzer which was calibrated using sodium trifluoroacetate [26]. The mass spectrometer was optimized specifically for the main analyte under investigation and was not optimized for impurities so as to mimic typical peak purity assessments for unknown impurities. For the 5-ASA studies, a Heated Nebulizer[®] atmospheric pressure chemical ionization (APCI) ion source was used. For azimilide, digoxin and digitoxin studies, a TurbolonSpray[®] thermally assisted electrospray ionization (ESI) ion source was used. Optimal sensitivity was achieved for all drugs when spectra were obtained in positive ion mode. Full scan data were obtained to include all potential impurities. A typical scan range of 100 – 1000 amu for 1.0 second in steps of 0.1 amu was employed.

2.3. *Chromatographic parameters:*

The HPLC method for both azimilide on-column and flow injection experiments used a mobile phase of 40/10/50, acetonitrile/ 100 mM ammonium acetate (pH 4.5 with acetic acid)/ water at a flow rate of 0.4 mL/minute. Azimilide on-column experiments used a Waters Corporation (Milford, MA) Symmetry Shield RP8, 2.1 x 150 mm HPLC column. The HPLC method for 5-ASA (on-column) used a Phenomenex (Torrance, CA) Primesphere 5 μ m, 250 x 4.6 mm, C18 HC (high carbon) HPLC column and a 13/87, acetonitrile/ water (with 2 mL pentafluoropropionic acid added per liter) mobile phase at a flow rate of 1.0 mL/minute. The method for digoxin and digitoxin (flow injection) used a mobile phase of 70/10/50, methanol/ 100mM ammonium formate buffer (pH 3.5)/ water at a flow rate of 0.4 mL/minute.

2.4 *Method:*

Coelution was generated in one of two approaches. The on-column coelution experiments were performed by autoinjecting pure main analyte, combined with a manual injection of the impurity at the time necessary to cause coelution. The flow injection coelution experiments used 15 feet of 0.01 inch i.d. HPLC tubing to simulate band broadening. The mass spectrometer parameters were then optimized on the main analyte to provide maximum signal and

minimum fragmentation. Nominal concentrations were calculated as 1000 times that of the limit of detection (LOD) of the main analyte (LOD = 0.1% based on a signal-to-noise ratio of 3 from full scan spectra). Next, background subtracted, full scan spectra of blank, sample, and available pure standard were collected and analyzed. Suspect impurity responses were then investigated by comparison of extracted ion chromatograms (XICs) of suspect responses to the main analyte. As the difference in retention times and peak shapes increase, so does the confidence that the suspect response is in fact an impurity. In the case of suspect responses that exhibited perfect coelution with the main analyte, spectral knowledge of the main analyte (e.g. fragmentation, adducts and isotopes) was utilized to determine if a suspect response was likely to be an impurity. In this latter case, additional experiments involving collision-induced dissociation were performed to develop a better understanding of the fragmentation for the main analyte. When an exactly coeluting impurity was isobaric to a fragment, signals from replicate analyses of a sample and standard were compared to determine if there were differences in average peak areas. A significant difference was deemed to arise from the presence of an impurity.

3. RESULTS AND DISCUSSION

The summarized LOD data observed for all experiments are presented in Table IV. The tabulated data show that only eight of the twenty-four impurities achieved the targeted 0.1% detection limit. Detailed discussions of these results, including factors affecting sensitivity, are included in the remainder of this paper.

Table IV: Limit of detection (LOD) of impurities coeluting with nominal level drug using LC-MS. The LOD for an analyte was defined as a peak observed in a profile mode full scan mass spectrum which had a signal to noise ratio of 3:1. Percent is calculated as 100 x (impurity concentration / drug concentration).

Drug	Impurity	LOD (% on wt/wt basis)
Azimilide	AI-1	0.1%
	AI-2	>10%
	AI-3	0.2%
	AI-4	0.5%
	AI-5	5%
	AI-6	0.5%
	AI-7	1%
5-ASA	n-acetyl-5-amino salicylic acid 2, 5-dihydroxybenzoic acid 4-aminophenol	0.1% Not Detected (ND) interfering fragment
Digoxin	Digoxigenin	1%
	Mono-digitoxosid	1%
	Bis-digitoxosid	2%
	Digitoxin	5%
	Alpha-acetyl-digoxin	0.1%
	Beta-acetyl-digoxin	0.1%
	Digitoxigenin	0.1%
Digitoxin	Digoxigenin	1%
	Bis-digitoxosid	0.1%
	Alpha-acetyl-digoxin	0.3%
	Beta-acetyl-digoxin	0.3%
	Digitoxigenin	0.1%
	Mono-digitoxosid	0.1%
	Digoxin	1%

3.1. *Azimilide:*

The azimilide structure contains basic groups that provided excellent potential for protonation in solution, and subsequently yielded good full scan sensitivity by MS (0.06 µg/mL LOD for $[M+H]^+ = 458$). Analysis of azimilide using the relatively soft ionization process of electrospray did not produce any significant system, impurity, or fragmentation response that interfered with the detection of any of the azimilide impurities tested (Fig. 1). The responses at 155, 253 and 358 were confirmed by LC-MS-MS to be fragmentation products and the response at 496 is the potassium adduct $[457+K]^+$ [27]. The sodium adduct $[457+Na]^+$ was also observed at 480 (Fig 2).

Fig. 1: Full scan, positive ion, mass spectrum of 0.06 mg/mL azimilide using the TurbolonSpray[®] ESI ion source and a mobile phase of 40/10/50, acetonitrile/ 100 mM ammonium acetate (pH 4.5 with acetic acid)/ water.

Azimilide impurities AI-1, AI-2, AI-3 and AI-4 were coeluted with azimilide using both the on-column and flow injection techniques. Both techniques resulted in identical detection limits. Impurities AI-5, AI-6 and AI-7 were coeluted with azimilide using flow injection only. The spectra (Figs. 2, 3, 4) of azimilide spiked with 0, 0.1, and 0.5% impurity AI-1 provide a positive example of the peak purity capability of this technique. The AI-1 impurity ion $[M+H]^+$ (444) was directly observable down to an LOD of 0.1%. Triplicate analyses of azimilide spiked with 0.1% AI-1 produced 444 peak heights with a relative standard deviation of 30%.

Fig. 2: Zoom view of full scan, positive ion, ESI mass spectrum of nominal level (0.06 mg/mL) azimilide coeluting with 0% impurity AI-1. A mobile phase of 40/10/50, acetonitrile/ 100 mM ammonium acetate (pH 4.5 with acetic acid)/ water was used.

Fig. 3: Zoom view of full scan, positive ion, ESI mass spectrum of nominal level (0.06 mg/mL) azimilide coeluting with 0.1% AI-1. A mobile phase of 40/10/50, acetonitrile/ 100 mM ammonium acetate (pH 4.5 with acetic acid)/ water was used.

Fig. 4: Zoom view of full scan, positive ion, ESI mass spectrum of nominal level (0.06 mg/mL) azimilide coeluting with 0.5% AI-1. A mobile phase of 40/10/50, acetonitrile/ 100 mM ammonium acetate (pH 4.5 with acetic acid)/ water was used.

Although the azimilide impurity AI-1 was detected at 0.1%, the detection limits for the other six impurities ranged from 0.5% to >10%. To investigate the causes of detection limits greater than 0.1%, azimilide impurities were injected separately and their spectral patterns were studied. The primary reason found for high detection limits was reduced signal intensity (i.e. limited ionization) of the impurity relative to the main analyte. Not surprisingly, another limiting factor was that performing peak purity experiments for unknowns did not allow further MS optimization to maximize signal for each impurity. For example, when the mass spectrometer was optimized specifically on the impurity, AI-4, the impurity response doubled (Figs. 5, 6).

Fig. 5: Zoom view of full scan, positive ion, ESI mass spectrum of 0.02 mg/mL azimilide impurity Al-4. A mobile phase of 40/10/50, acetonitrile/ 100 mM ammonium acetate (pH 4.5 with acetic acid)/ water was used. Mass spectrometer lens voltages were optimized on azimilide to produce an LOD for Al-4 of 0.5%.

Fig. 6: Zoom view of full scan, positive ion, ESI mass spectrum of 0.02 mg/mL azimilide impurity Al-4. A mobile phase of 40/10/50, acetonitrile/ 100 mM ammonium acetate (pH 4.5 with acetic acid)/ water was used. Mass spectrometer lens voltages were optimized on the impurity, thus, doubling the response of the impurity.

3.2. 5-ASA:

In contrast to azimilide, which presented basic properties, the 5-ASA compound represents analyses of a potentially zwitterionic compound. The polar properties of the zwitterionic 5-ASA yielded insufficient retention using typical reversed phase or multi-modal stationary phases (i.e. C18, CN, NH₂ and a dual mode carbamide-C8). Therefore, 5-ASA required the use of a volatile ion-pairing agent, PFPA, for chromatographic retention.

It is important to note that MS sensitivity is affected by the selection of mobile phase [28]. For example, ion pairing agents have been demonstrated to decrease sensitivity [29, 30]. In the case of 5-ASA, pentafluoropropionic acid (PFPA) did in fact contribute to poor sensitivity by MS when the ESI ion source was employed (Figs 7, 8). A 5-ASA peak intensity of 3.0e6 was achieved using an ammonium formate buffer whereas the 5-ASA peak intensity was reduced to 1.2e5 using PFPA.

Utilization of the APCI ion source, rather than electrospray, resulted in a five fold improvement in sensitivity to achieve an LOD of 0.5 µg/mL for [M+H]⁺ = 154. However, APCI also produced significant fragmentation (Figs. 8, 9).

Of the five 5-ASA related impurities tested, only one, n-acetyl-5-aminosalicylic acid, achieved the 0.1% LOD level. The low detection level for n-acetyl-5-aminosalicylic acid ($[M+H]^+ = 196$) was achieved despite the fact that the acetonitrile adduct for 5-ASA itself $[M+H+ACN]^+$ was observed at 195 (Figs. 9, 10). Conversely, while the response factor for 4-aminophenol was 4 times higher than that of 5-ASA, a 5-ASA fragment (110) at a 0.5% level significantly interfered with detection of 4-aminophenol.

Two additional impurities, 3-ASA and 4-ASA, could not be distinguished from 5-ASA with this approach as they are isobaric with respect to 5-ASA. Consequently, an HPLC separation would be required to observe isomers. The finalized HPLC conditions used for 5-ASA did in fact separate 4-ASA by 2.5 minutes. However, 3-ASA coeluted with 5-ASA. Analysis of these types of mixtures, thus, continue to represent a significant challenge.

Fig. 7: Full scan, mass spectrum of 0.5 mg/mL 5-ASA using electrospray and a mobile phase of 10/90, acetonitrile/ buffer (10mM NH_4COOH , pH 3.5 with formic acid). Optimum MS sensitivity for this compound, in this mobile phase was achieved in negative ion mode. This 5-ASA intensity of $3.0e6$ was significantly greater than with the use of the ion pairing agent pentafluoropropionic acid (see fig. 8).

Fig. 8. Full scan, positive ion, mass spectrum of 0.5 mg/mL 5-ASA using electrospray. A mobile phase of 13/87, acetonitrile/ water (with 2 mL pentafluoropropionic acid added per liter) was used. The ion pairing agent decreased electrospray 5-ASA intensity from $3.0e6$ using NH_4COOH buffer to $1.2e5$ (see fig. 7.) Electrospray eliminated the fragmentation which was observed using APCI (see fig 9).

Fig. 9: Full scan, positive ion, mass spectrum of 0.5 mg/mL 5-ASA using the Heated Nebulizer[®] atmospheric pressure chemical ionization (APCI) ion source illustrating significantly increased fragmentation as observed using electrospray (see fig. 8). A mobile phase of 13/87, acetonitrile/ water (with 2 mL pentafluoropropionic acid added per liter) was used.

Fig. 10: Full scan, positive ion, mass spectrum of 0.0005 mg/mL (0.1%) n-A-5-ASA coeluting with 0.5 mg/mL 5-ASA using the APCI ion source. A mobile phase of 13/87, acetonitrile/ water (with 2 mL pentafluoropropionic acid added per liter) was used.

3.3. *Digoxin:*

The evaluation of digoxin complements the azimilide and 5-ASA studies in that it represents a class of compounds that contains no basic groups for facile protonation. Consequently, ammonium adduct formation $[M+NH_4]^+ = 799$ was observed as the predominant ionization mechanism utilizing electrospray. With this approach, an LOD of 0.2 $\mu\text{g/mL}$ was obtained (Fig. 11).

Of the seven impurities coeluted with digoxin, only alpha-acetyl-digoxin, beta-acetyl-digoxin and digitoxigenin achieved the targeted LOD level of 0.1%. The impurity, digitoxigenin ($[M+NH_4]^+ = 392$), was detectable at 0.1%, despite a fragment response at 391 (Figs 12, 13).

The main reason that four impurities did not achieve the 0.1% detection limit was interference from fragments or adducts. Three main fragments of digoxin were confirmed by LC-MS-MS to result from the loss of one, two and three sugar units from the base structure [31]. The levels of these fragments, observed as the ammoniated adducts, were determined by MS to be 0.3% (408), 0.4% (538) and 1% (668) (Fig. 11). These fragment adducts were isobaric to digitoxigenin (408), mono-digitoxosid (538) and bis-digitoxosid (668). Digoxin also produced an isotope ion (782) at a 1% level which was isobaric with the impurity, digitoxin.

To determine the limits of detection for coeluting impurities which were isobaric to fragments, adducts or isotopes, signals from replicate analyses of a sample and standard were compared to determine if there were differences in average peak height intensities. A significant difference was deemed to arise from the presence of an impurity. The results were detection limits of 1% for digoxigenin (408), 1% for mono-digitoxosid (538), 2% for bis-digitoxosid (668) and 5% for digitoxin (782) (Table V).

Fig. 11. Full scan, positive ion, mass spectrum of 0.2 mg/mL digoxin using the TurbolonSpray[®] ESI ion source illustrating ammonium adduct formation $[M+NH_4]^+$ as the predominant ionization mechanism. A mobile phase of 70/10/50, methanol/ 100mM ammonium formate buffer (pH 3.5)/ water was used.

Fig. 12. Full scan, zoom view of positive ion, mass spectrum of 0.2mg/mL digoxin using the TurbolonSpray[®] ESI ion source illustrating a fragment at 391 Da. A mobile phase of 70/10/50, methanol/ 100mM ammonium formate buffer (pH 3.5)/ water was used.

Fig. 13. Full scan, zoom view of positive ion, mass spectrum of 0.2 mg/mL digoxin spiked with 0.1% digitoxigenin using the TurbolonSpray[®] ESI ion source illustrating MS detection of the coeluting impurity, digitoxigenin (392 Da). A mobile phase of 70/10/50, methanol/ 100mM ammonium formate buffer (pH 3.5)/ water was used.

Table V: Limits of detection for impurities isobaric to a digoxin isotope or fragment adduct. Replicate analyses of a sample and a standard were compared to determine if there were differences in average peak height intensities. A significant difference was deemed to arise from the presence of an impurity.

Impurity (Da)	Standard intensities	Sample intensities	LOD
Digoxigenin (408)	Ave = 45600 (0.3%) n = 5 s = 11171 RSD% = 25	Ave = 145000 n = 4 s = 43589 RSD% = 30	1.0%
mono-digitoxosid (538)	Ave = 33429 (0.4%) n = 7 s = 2299 RSD% = 6.9	Ave = 73000 n = 4 s = 6218 RSD% = 8.5	1.0
bis-digitoxosid (668)	Ave = 131250 (1%) n = 8 s = 12464 RSD% = 9.5	Ave = 190000 n = 3 s = 20000 RSD% = 11	2.0
digitoxin (782)	Ave = 125000 (1%) n = 4 s = 12910 RSD = 10	Ave = 276667 n = 3 s = 5774 RSD% = 2.1	5.0

3.4. *Digitoxin:*

Digitoxin is structurally similar to digoxin and therefore provides within-class comparison data. Analogous to digoxin, maximum signal occurred for digitoxin when the mass spectrometer was optimized on the $[M+NH_4]^+$ ion (783) with a lower observable level of $[M+H]^+$ (765) (Fig. 14). Digoxin and digitoxin utilized the same HPLC-MS conditions, had equivalent responses and similar respective fragmentation patterns. Fortuitously however, the digitoxin fragment responses did not interfere with this specific impurity set examined in this study. The only potentially interfering fragment was observed at a level of less than 0.1% (digitoxigenin, 392).

The reduced frequency of interferences observed for digitoxin relative to digoxin resulted in slight improvements in LODs (Table IV). Four of seven impurities did not achieve the targeted 0.1% LOD level due to reduced sensitivity relative to the main analyte. These results, not surprisingly, suggest that similar performance could be expected within a class of compounds.

Fig. 14. Full scan, positive ion, mass spectrum of 0.2mg/mL digitoxin using the TurbolonSpray[®] ion source illustrating ammonium adduct formation $[M+NH_4]^+$ as the predominant ionization mechanism. A mobile phase of 70/10/50, methanol/ 100mM ammonium formate buffer (pH 3.5)/ water was used.

4. CONCLUSION

This paper presented an extensive evaluation of API MS to be used as a general tool for detection of coeluting impurities in HPLC. To facilitate broad applicability this investigation employed a single stage MS, coupled with a thermally assisted ESI or an APCI ion source. As it is widely known that response factors vary using mass spectrometric detection, the compounds tested included acids, bases and zwitterions possessing varying ionizabilities, a wide range of polarities and a mass range typical of pharmaceuticals. Four drugs, were individually tested against their related impurity set to produce 24 unique drug-impurity permutations. Data was obtained on impurities coeluting at levels ranging from 10% down to 0.1%.

This simplified procedure frequently (75%) achieved peak purity performance of less than 1%. However, the technique cannot be expected to reliably detect all impurities at the regulatory required level of 0.1%. Therefore, this technique should not be employed as the sole method for peak purity assessment. It can, however, be a valuable adjunct tool during research and development such as monitoring reactions during chemical development syntheses. It can also speed and improve HPLC method development by replacing time consuming and ineffective off-line techniques such as orthogonal separations, selectivity testing

using synthesized proposed impurities and fraction collections followed by alternate testing.

Further examination of this study suggested that there are several important factors to consider when employing this LC-MS peak purity approach. In particular, knowledge of the functional groups of potential impurities, solution chemistry (i.e. compound ionization, mobile phase additives) and MS spectral interpretation were critical to the successful application of this technique for peak purity measurements. In early stages of drug development many impurities have not been synthesized, isolated or identified for proper HPLC selectivity testing. However, the functional groups of potential impurities will likely be known. It is also known that ionic abundance is directly related to MS sensitivity. It was observed that knowledge of the ionic state of these functional groups in solution can be used during mobile phase selection to maximize the probability that coeluting impurities are detected by this technique.

It is also well known that mobile phase characteristics that affect ion formation include pH relative to the pKa of the compounds (i.e. acid/base equilibrium), ionic strength, buffer selection and properties of ion pairing or derivatization agents present. Mass spectrometric mobile phase additives must also be volatile, which further limits selection during HPLC method development. However, while the chromatographer has some latitude for mobile phase

selection, the zwitterionic 5-ASA HPLC separation requirements demonstrated that the applicability of MS as an on-line detector can ultimately be limited for specific compounds.

Spectral interpretation also played a critical role in these studies as the full scan spectra contained many responses that were not actually coeluting impurities. Non-impurity responses observed in this study included isotopes, multiple ion adducts (i.e. $[M+NH_4]^+$ $[M+Na]^+$ $[M+K]^+$), in-source dimers and fragmentation products. The APCI ion source was shown to increase potentially interfering fragmentation levels versus ESI. Determination of the source of these responses can be a complex process. Collision induced dissociation experiments can be performed on a single stage MS to provide supporting data for identification of fragments. Analysis of a compound by MS-MS can provide more valuable information on which observed ions result from fragmentation.

Although it was demonstrated that spectral interpretation can be a complex process and that this technique cannot reliably detect all impurities at the regulatory required level of 0.1%, future technology advancements will likely improve peak purity performance. The evolution of MS has shown improvements in sensitivity, allowed the use of very small amounts of nonvolatile buffers, produced ion sources with more efficient nebulization and shown reductions in cost and size. Rapid developments in computers have improved

data processing speeds and software capabilities. New HPLC column packing materials are reducing the need for mobile phase additives that are not compatible with mass spectrometers. As MS advancements progress further, additional testing, such as described in this study, will most certainly show improvements in the applicability of mass spectrometry to detect coeluting impurities in high performance liquid chromatography.

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