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Applicability of mass spectrometry to detect coeluting impurities in high-performance liquid chromatography

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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 using 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 <1.0%, including one-third at 0.1% (%, w/w). Factors that affect the applicability of this peak purity approach are also discussed.

Keywords: Pharmaceutical analysis; Peak purity; Azimilide; Aminosalicylic acid; Digoxin; Digitoxin

1. Introduction

The pharmaceutical industry routinely uses high-performance liquid chromatography (HPLC) to quantitate levels of impurities at the mandated level of 0.1% [1–3]. 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 [4–8]. 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 isomeric impurities,

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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 [11–14]. 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 as 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 in the positive ion mode due to favorable protonated molecular ion formation [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.

This paper presents the evaluation of API-MS as a general tool for detection of coeluting impurities in HPLC. The investigation employed a single stage MS to facilitate broad applicability. Detection level data were obtained on impurities coeluting at concentrations ranging from 10% down to 0.1% versus the nominal concentration of their related drug. 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, USA) (Figs. 1–3) [23–25]. HPLC-grade acetonitrile, acetic acid, ammonium acetate and ammonium formate were all purchased from Mallinckrodt Baker (Phillipsburgh, NJ, USA). Water was purified to 18 mΩ using a Millipore (Bedford, MA, USA) Milli-Q+ system. Formic acid (98%), sodium trifluoroacetate (99.5%) and pentafluoropropionic acid (PFPA) (97%) were purchased from Sigma–Aldrich (Milwaukee, WI, USA).

2.2. Equipment

The HPLC system was comprised of a Thermoseparations Products (Fremont, CA, USA) TSP P4000 gradient pump with an Applied Biosystems (Foster City, CA, USA) UV detector. Both a Hewlett-Packard (Wilmington, DE, USA) HP1100 auto-

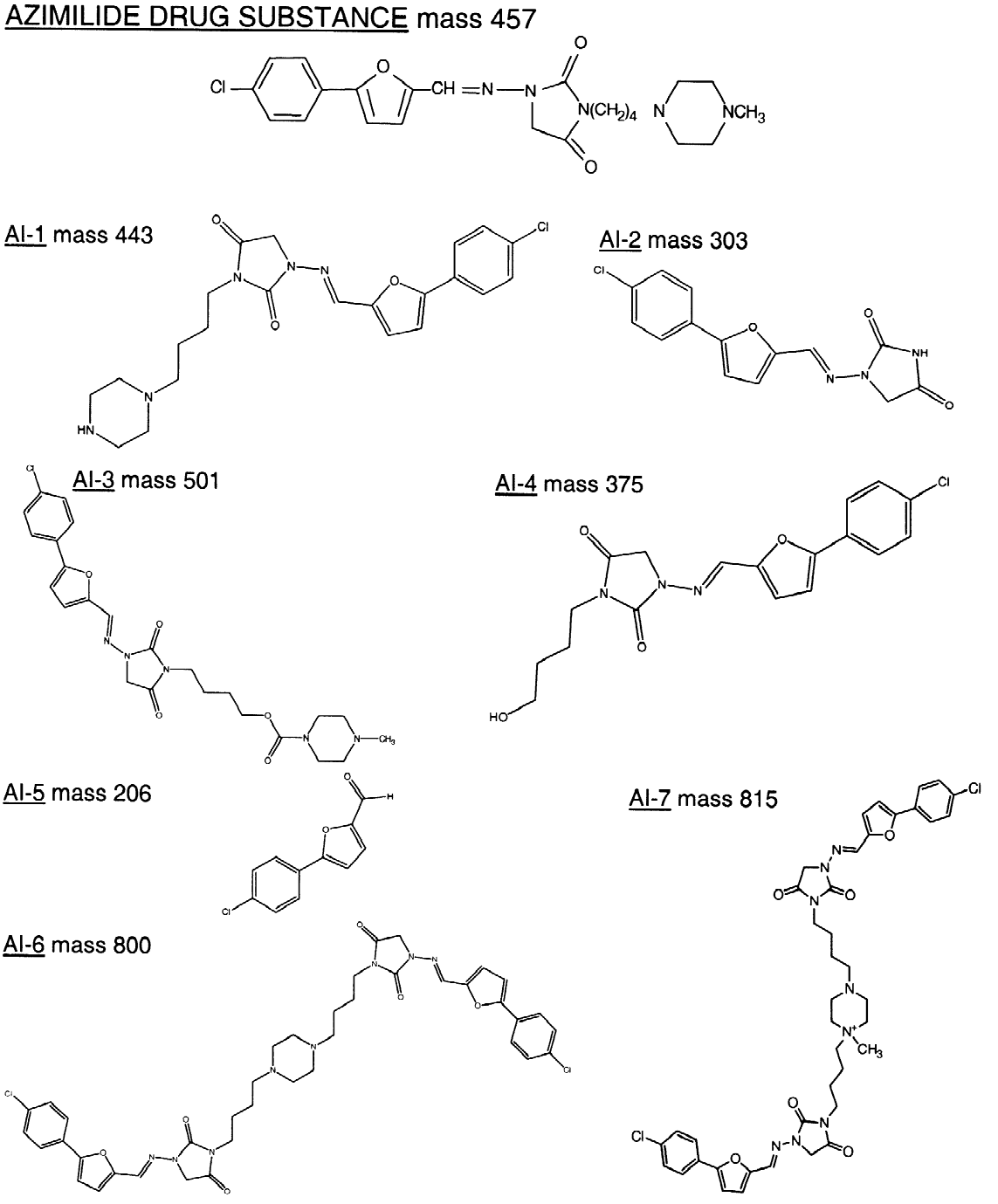

Fig. 1. Azimilide and azimilide impurities.

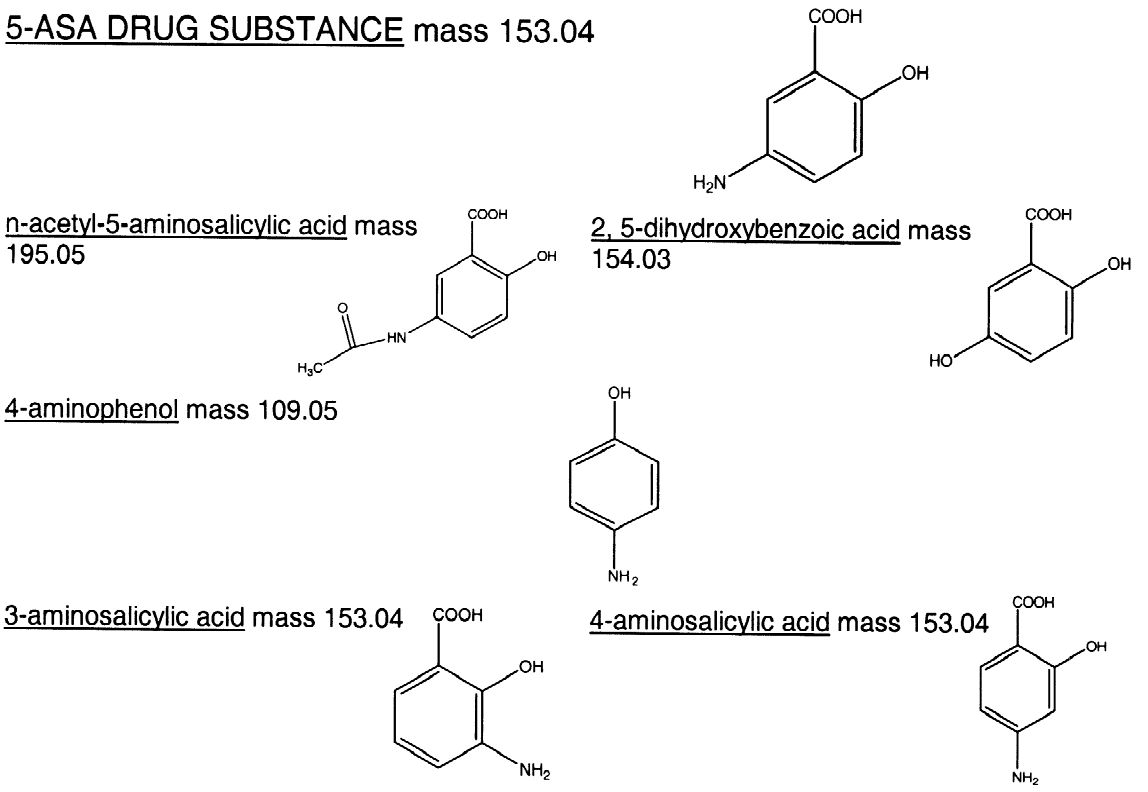

Fig. 2. 5-Aminosalicylic acid (5-ASA) and 5-ASA impurities.

sampler and a Rheodyne manual injection manifold (Rohnert Park, CA, USA) were used for sample injection.

Mass spectrometry was carried out on an Applied Biosystems 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 TurboIonSpray thermally assisted 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 u for 1.0 s in steps of 0.1 u was employed.

2.3. Chromatographic parameters

The HPLC method for both azimilide on-column and flow-injection experiments used a mobile phase of acetonitrile–100 m*M* ammonium acetate (pH 4.5 with acetic acid)–water (40:10:50) at a flow-rate of 0.4 ml/min. Azimilide on-column experiments used a Waters (Milford, MA, USA) Symmetry Shield RP8, 150×2.1 mm HPLC column. The HPLC method for 5-ASA (on-column) used a Phenomenex (Torrance, CA, USA) Primesphere 5 μm, 250×4.6 mm, C_{18} HC (high carbon) HPLC column and an acetonitrile–water (13:87) (with 2 ml pentafluoropropionic acid added per liter) mobile phase at a flow-rate of 1.0 ml/min. The method for digoxin and

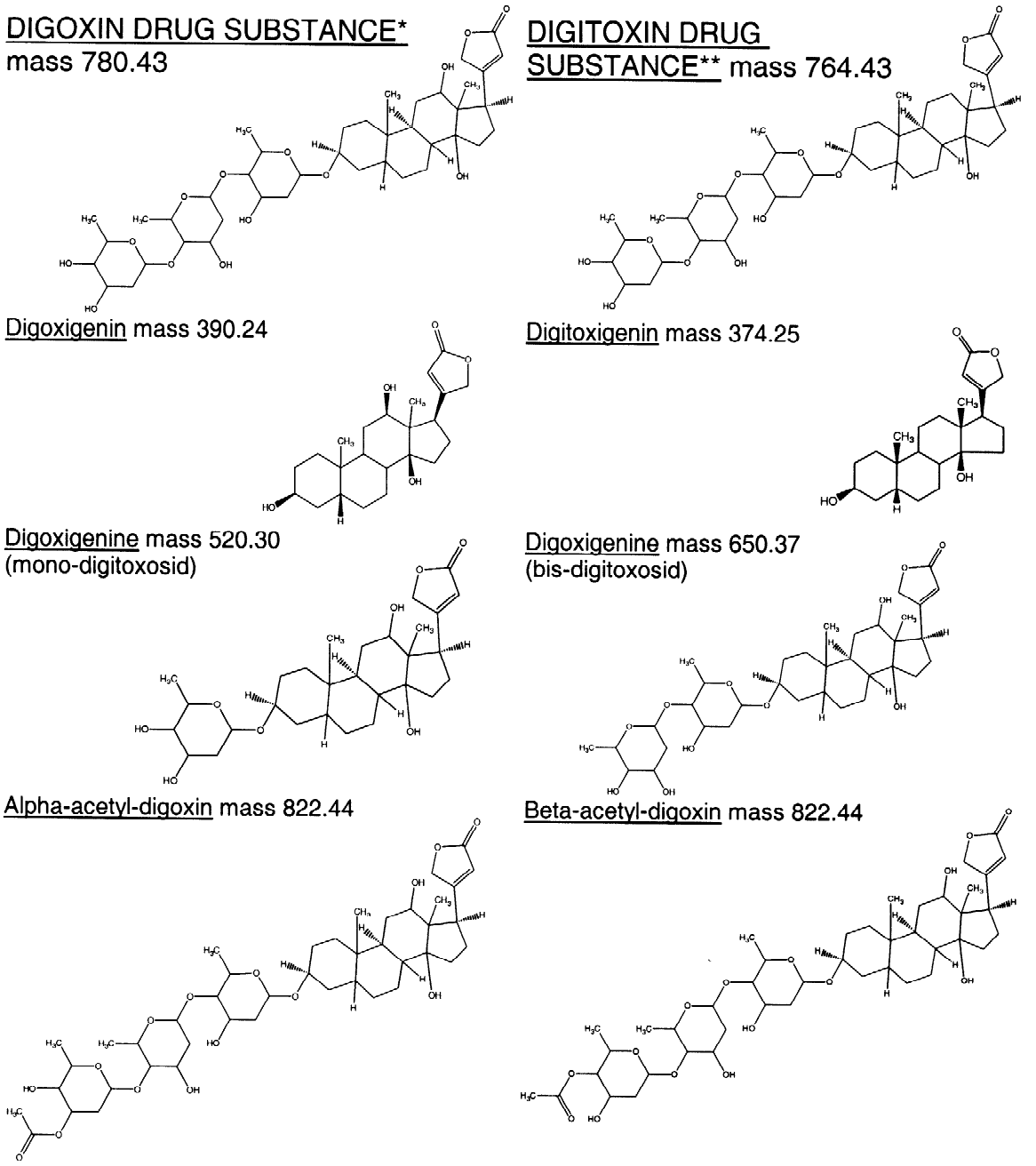

Fig. 3. Digitalic drug substances and impurities. *Digoxin used as digitoxin impurity. **Digitoxin used as digoxin impurity.

digitoxin (flow-injection) used a mobile phase of methanol–100 m*M* ammonium formate buffer (pH 3.5)–water (70:10:50) at a flow-rate of 0.4 ml/min.

2.4. Method

Coelution was generated in one of two ap-

proaches. 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 ft. of 0.01 in. I.D. HPLC tubing to simulate band broadening (1 ft.=30.48 cm; 1 in.=2.54 cm). 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). Data were obtained on impurities coeluting at concentration levels ranging from 10% down to 0.1% versus the nominal concentration of their related drug. 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 used to determine if a suspect response was likely to be an impurity. In this latter case, previous experiments involving collision-induced dissociation and MS–MS techniques were referenced, which developed 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 intensities. 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 1. The tabulated data show that only eight of the 24 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.

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. 4). 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. 5).

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. 5–7) 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%.

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.

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

Table 1
Limit of detection (LOD) of impurities coeluting with nominal level drug using LC–MS

Drug	Impurity	LOD (%, w/w)
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	0.1
	2,5-Dihydroxybenzoic acid	Not detected (ND)
	4-Aminophenol	Interfering fragment
Digoxin	Digoxigenin	1
	Mono-digitoxosid	1
	Bis-digitoxosid	2
	Digitoxin	5
	α-Acetyldigoxin	0.1
	β-Acetyldigoxin	0.1
	Digitoxigenin	0.1
Digitoxin	Digoxigenin	1
	Bis-digitoxosid	0.1
	α-Acetyldigoxin	0.3
	β-Acetyldigoxin	0.3
	Digitoxigenin	0.1
	Mono-digitoxosid	0.1
	Digoxin	1

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×(impurity concentration/drug concentration).

properties of the zwitterionic 5-ASA yielded insufficient retention using typical reversed-phase or multi-modal stationary phases (i.e. C_{18}, CN, NH_2 and a dual mode carbamide-C_8). 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, PFPA did in fact contribute to poor sensitivity by MS when the ESI ion source was employed. Utilization of the APCI ion source resulted in a fivefold improvement in sensitivity to achieve an LOD of 0.5 μg/ml for $[M+H]^+=154$. However, APCI also produced significant fragmentation (Figs. 8 and 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 and 10). Conversely, while the response factor for 4-aminophenol was four 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 min. However, 3-ASA coeluted with 5-ASA. Analysis of these types of mixtures, thus, continue to represent a significant challenge.

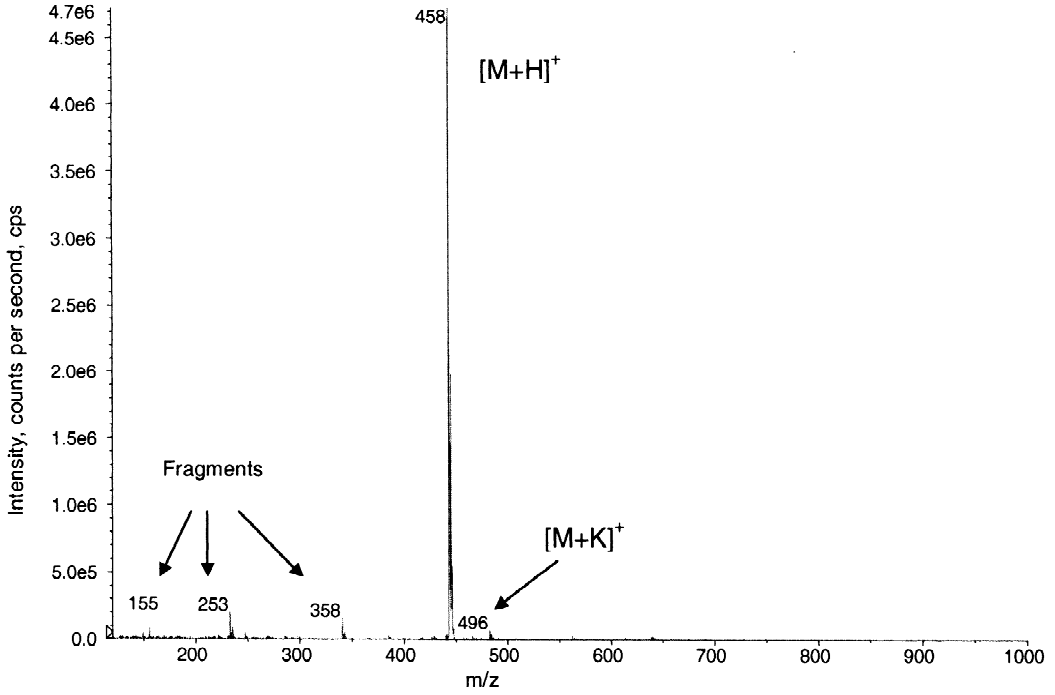

Fig. 4. Full scan, positive ion, mass spectrum of 0.06 mg/ml azimilide using the TurboIonSpray ESI ion source and a mobile phase of acetonitrile–100 m*M* ammonium acetate (pH 4.5 with acetic acid)–water (40:10:50).

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

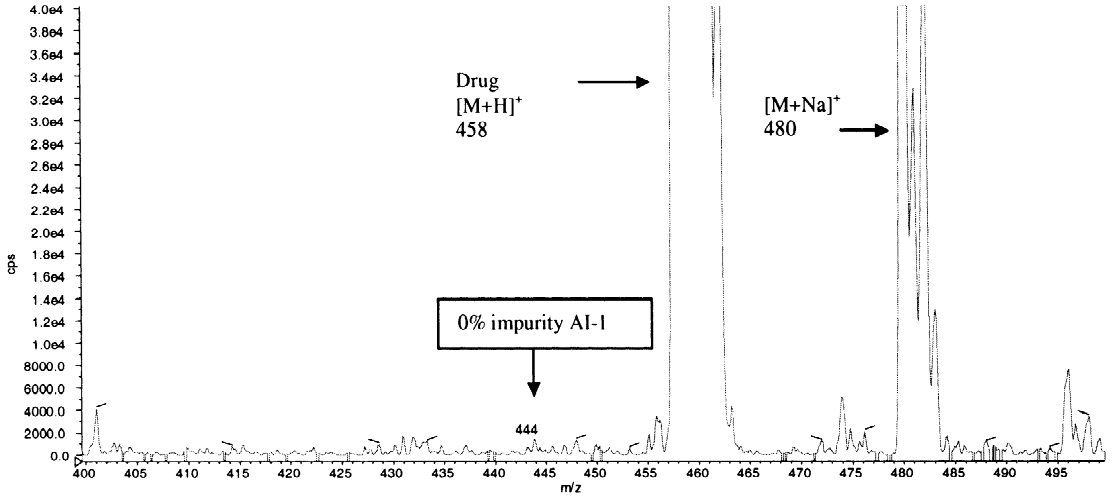

Fig. 5. Zoom view of full scan, positive ion, ESI mass spectrum of nominal level (0.06 mg/ml) azimilide coeluting with 0% impurity AI-1. A mobile phase of acetonitrile–100 m*M* ammonium acetate (pH 4.5 with acetic acid)–water (40:10:50) was used.

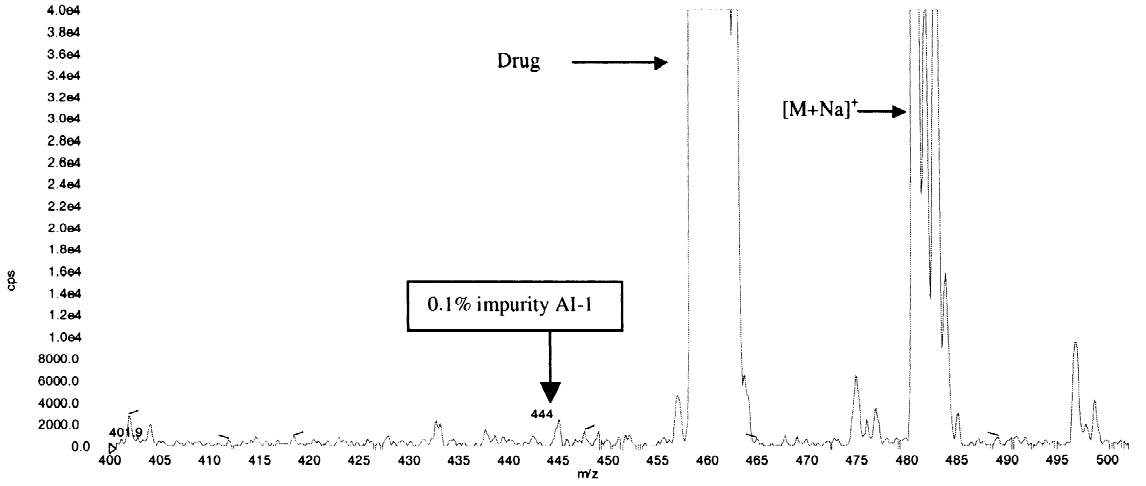

Fig. 6. Zoom view of full scan, positive ion, ESI mass spectrum of nominal level (0.06 mg/ml) azimilide coeluting with 0.1% AI-1. A mobile phase of acetonitrile–100 m*M* ammonium acetate (pH 4.5 with acetic acid)–water (40:10:50) was used.

formation $[M+NH_4]^+=799$ was observed as the predominant ionization mechanism using electrospray (note: the mass spectrometer measured digoxin $[M+NH_4]^+$ as 798.6, then rounded to 799 which is within the accuracy specification of 798.4+0.3). With this approach, an LOD of 0.2 μg/ml was obtained (Fig. 11).

Of the seven impurities coeluted with digoxin, only α-acetyldigoxin, β-acetyldigoxin 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.

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

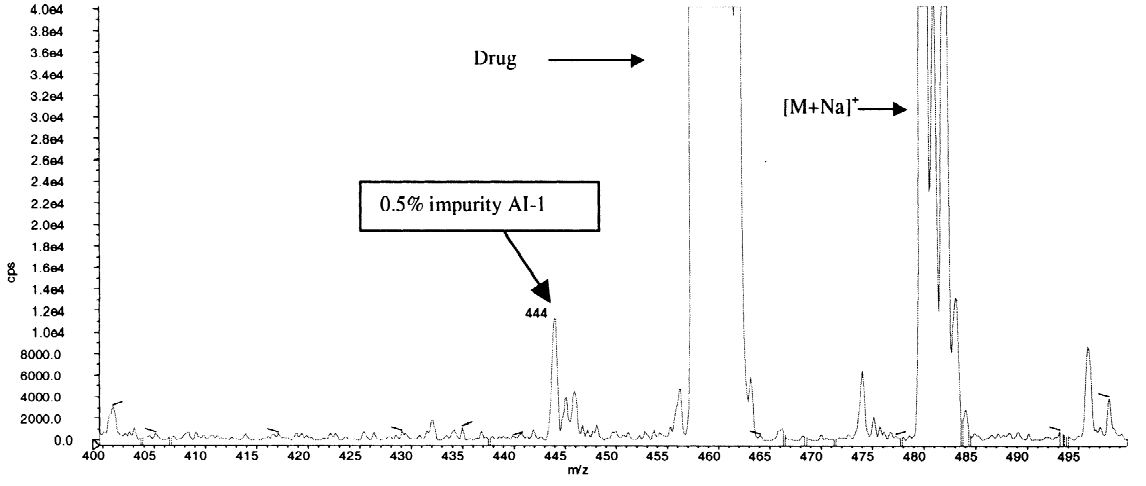

Fig. 7. Zoom view of full scan, positive ion, ESI mass spectrum of nominal level (0.06 mg/ml) azimilide coeluting with 0.5% AI-1. A mobile phase of acetonitrile–100 m*M* ammonium acetate (pH 4.5 with acetic acid)–water (40:10:50) was used.

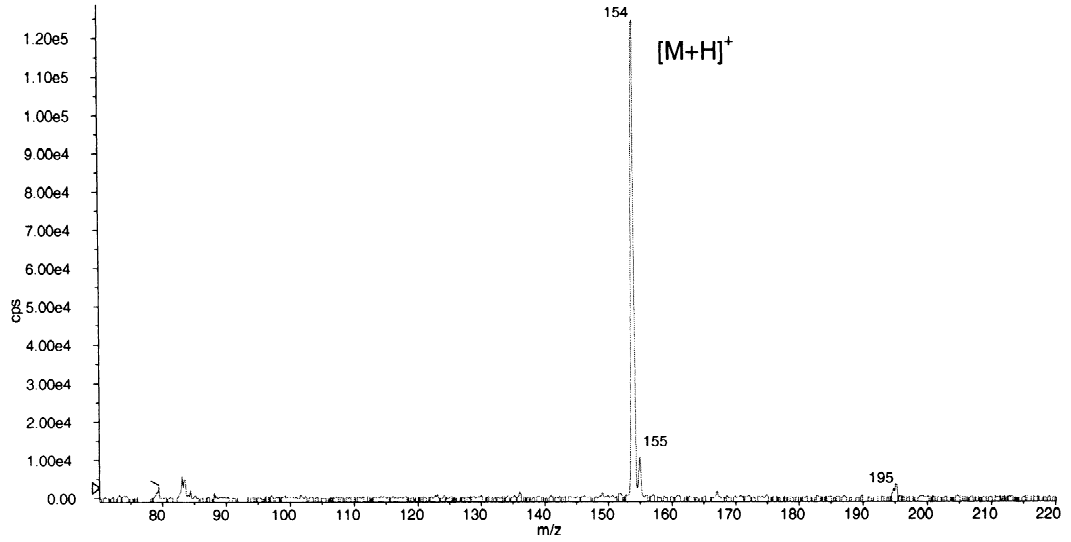

Fig. 8. Full scan, positive ion, mass spectrum of 0.5 mg/ml 5-ASA using the TurboIonSpray (thermally assisted electrospray) ion source illustrating elimination of fragmentation as observed using APCI (Fig. 9). A mobile phase of acetonitrile–water (13:87) (with 2 ml pentafluoropropionic acid added per liter) was used.

1% (668) (Fig. 11). This fragmentation limited the impurity LOD levels to 1% for digoxigenin (408), 1% for mono-digitoxosid (538) and 2% for bis-digitoxosid (668). Digoxin also produced an interfering isotope ion (782) at a 1% level, which raised the LOD for the impurity, digitoxin, to 5%.

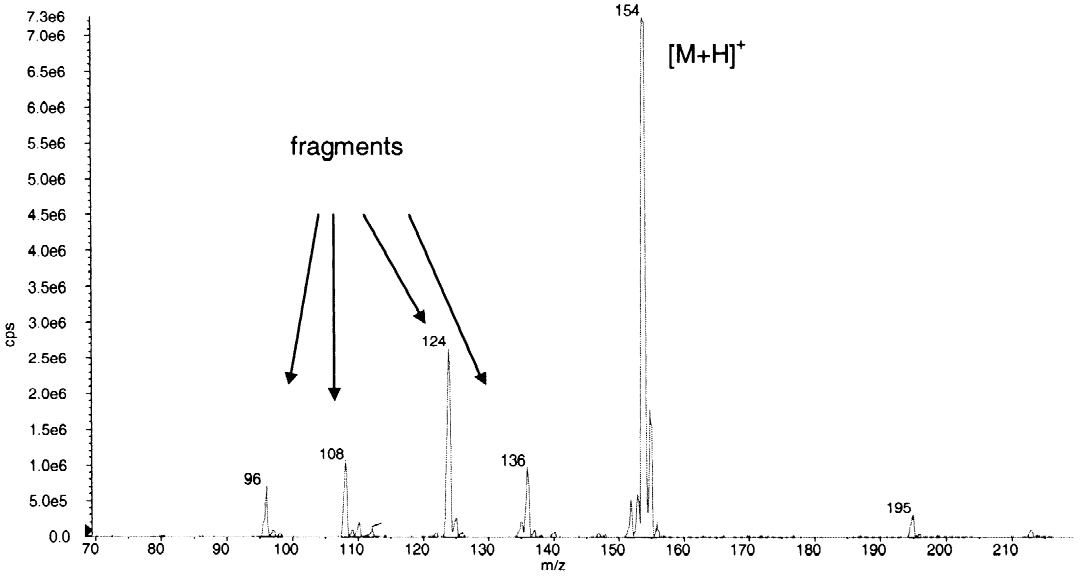

Fig. 9. Full scan, positive ion, mass spectrum of 0.5 mg/ml 5-ASA using the Heated Nebulizer atmospheric pressure chemical ionization (APCI) ion source illustrating significantly increased fragmentation as observed using electrospray (Fig. 8). A mobile phase of acetonitrile–water (13:87) (with 2 ml pentafluoropropionic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

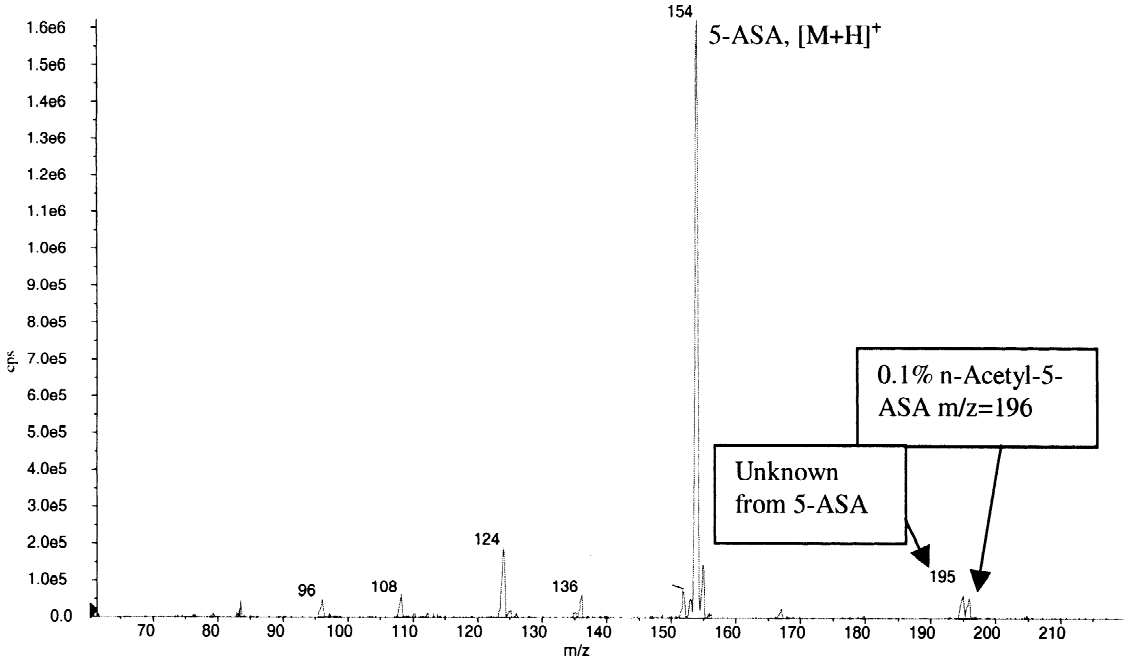

Fig. 10. Full scan, positive ion, mass spectrum of 0.0005 mg/ml (0.1%) n-A-5-ASA coeluting with 0.5 mg/ml 5-ASA using the APCI ion source. A mobile phase of acetonitrile–water (13:87) (with 2 ml pentafluoropropionic acid added per liter) was used.

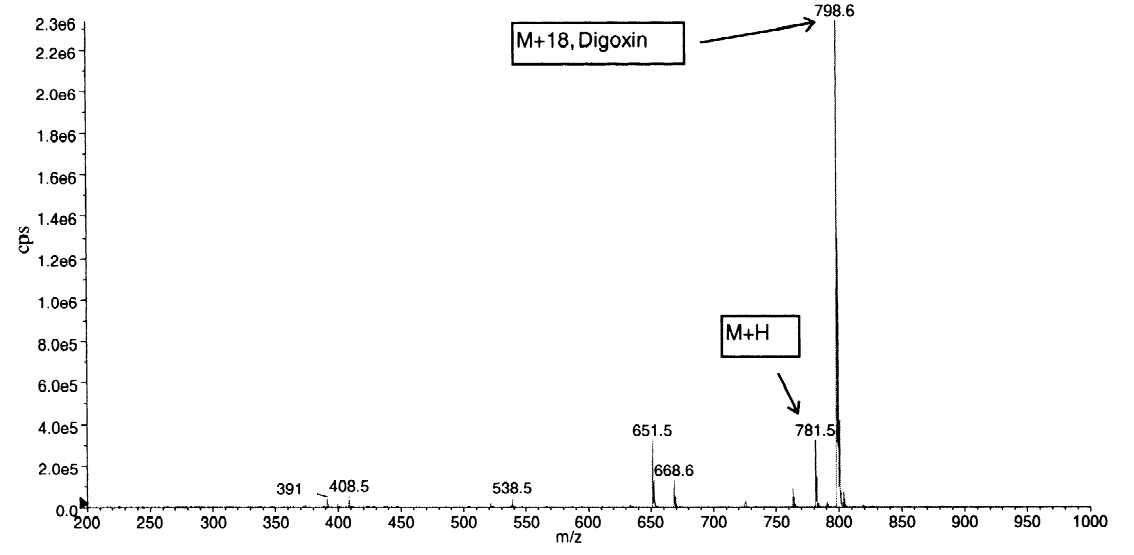

Fig. 11. Full scan, positive ion, mass spectrum of 0.2 mg/ml digoxin using the TurboIonSpray ESI ion source illustrating ammonium adduct formation $[M+NH_4]^+$ as the predominant ionization mechanism. A mobile phase of methanol–100 m*M* ammonium formate buffer (pH 3.5)–water (70:10:50) was used.

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 (782.4+0.3, measured as 782.5, rounded to 783) with a lower observable level of $[M+H]^+$ (765) (Fig. 12). Digoxin and digitoxin used 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 1). 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.

3.5. Considerations

The results from this study suggest that there are several important factors to consider when employing this LC–API-MS peak purity approach. In particular, knowledge of potential impurities, solution chemistry (i.e. compound ionization, mobile phase additives) and MS fragmentation of the main analyte should be applied to obtain the stated level of performance.

Proper selection of sample solution chemistry during HPLC method development can yield improved MS performance. Mobile phase characteristics that affect ion formation include pH relative to the pK_a of the compounds (i.e. acid/base equilibrium), ionic strength, buffer selection and properties of ion pairing or derivatization agents present. Mobile phase additives must be volatile, which limits selection during HPLC method development. However, while the chromatographer has some latitude for mobile phase selection, HPLC separation requirements often limit the applicability of MS as an on-line detector.

Spectral interpretation is also critical as full scan

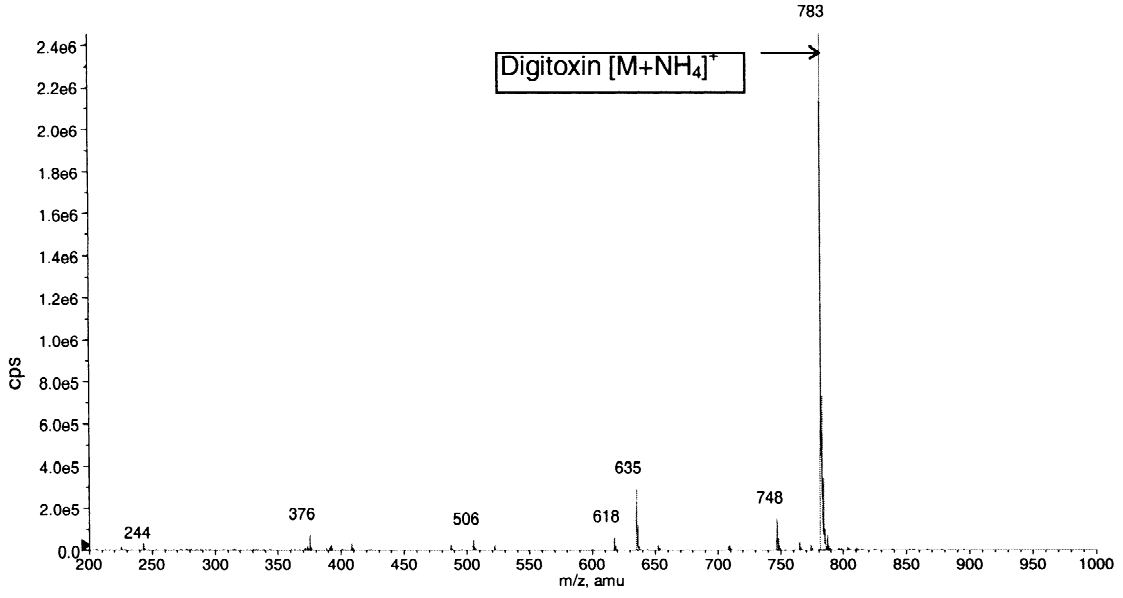

Fig. 12. Full scan, positive ion, mass spectrum of 0.2 mg/ml digitoxin using the TurboIonSpray ion source illustrating ammonium adduct formation $[M+NH_4]^+$ as the predominant ionization mechanism. A mobile phase of methanol–100 m*M* ammonium formate buffer (pH 3.5)–water (70:10:50) was used.

spectra may contain many responses that are not actually coeluting impurities. Determination of the source of these responses can be a complex process. Non-impurity responses observed in this study include 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. 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.

4. Conclusion

Single quadrupole MS coupled with a thermally assisted ESI or an APCI ion source has been demonstrated to be a useful adjunct tool for the detection of coeluting impurities in HPLC during research and development. 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 0.1% LOD level. Knowledge of solution chemistry and spectral interpretation were critical to the successful application of this technique for peak purity measurements.

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