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Ambient Pressure Desorption Ionization Mass Spectrometry in Support of Preclinical Pharmaceutical Development

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Abstract:

The use of ambient pressure desorption ionization mass spectrometry for the rapid analytical support of process and pharmaceutical development is demonstrated. The ability of direct analysis in real time (DART) technology to analyze both active pharmaceutical ingredients (APIs) and intermediates without sample preparation or the development of LC-based separations provided critical experimental results with minimal time required for method development. The utility and versatility of DART is shown for applications such as degradation studies, analysis of high-throughput catalyst screens, preparative-scale chromatography fractions, and impurity determination.

Introduction

In recent years, increasing competition and regulatory pressures have forced process and pharmaceutical chemistry to improve overall experimental speed and efficiency wherever possible. Analytical chemists have embraced a number of new technologies that offer the promise of faster characterization to solve critical problems.[1−7] Attempts to improve the throughput of analytical laboratories have often involved the utilization of faster high pressure liquid chromatographic or mass spectrometry systems to reduce the analysis time per sample.[8−13] Recent years have seen a variety of emerging mass spectrometry techniques that hold the promise of rapid and direct analysis without the need for sample preparation.[14] Of these techniques, ambient pressure desorption ionization method such as direct analysis in real time (DART) and desorption electrospray ionization (DESI)[15−17] have gained particular notice for fast analysis of chemicals present in solid, liquid, and gel-based formulations with minimal sample preparation.[18] A new analytical technology that potentially offers both faster analysis and decreased time for sample preparation is especially attractive in an environment where speed and cost savings are critical.

The DART ionization method utilizes both heat to differentially desorb molecules from the sample and Penning ionization to provide the charged species necessary for mass spectrometric analysis. By applying potentials to electrostatic lenses inside the DART source to a heated gas stream, metastable gas molecules are created from atmospheric water and oxygen. These molecules transfer the required energy to ionize analytes in the sample placed in the same gas stream. The displaced analyte ions are carried by the heated gas stream into the aligned sampling orifice of the mass spectrometer atmospheric pressure interface.[15]

DART analysis has previously been shown to be useful for supporting pharmaceutical discovery chemistry;[19] however, the requirements for analytical support of process development are different. In addition to supporting exploratory synthetic chemistry and purification, analytical support of development also entails the need to quickly and easily carry out high-throughput analysis, impurity/degradant identification, and determination of specific activity of isotopically labeled compounds. In this study the ability of DART to provide a rapid, simple, and general analytical tool for supporting product development is evaluated.

Experimental Section

A DART-100 CE ionization source (IonSense, Inc., Saugus, MA) was interfaced to several different LC-MS instruments including an Agilent 1100 LC-MSD (Agilent Technologies, Santa Clara, CA.) and a Thermo LTQ LC-MS (Thermo, Waltham, MA.). Unless otherwise indicated, the settings for the electrical components in the DART source were as follows: operating in positive ion mode: −3350 V on the HV needle, +300 V on the grid electrode, and +400 V on the center

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electrode. Helium was used as the ionization gas with the flow rate of 1.0 L min^{-1}, and the DART gas was heated to 350 °C for all experiments.

Two sample application methods were employed in this study, analysis of small volume liquid spots and analysis of solid tablets. For automated analysis of samples from 96-well plates, an HTC PAL autosampler (LEAP Technologies, Carrboro, NC) was used to position samples in the desorption ionization region for analysis. Sampling from the microplate wells consisted of dipping the closed end of a glass capillary melting point tube into the liquid sample approximately 2 mm below its surface.[20,21] This approach was automated using glass capillary tubes embedded in a plastic housing (DIP-it Sampler, IonSense, Inc.) and a CTC PAL autosampler, which immersed a tube in each sample (either vials or the wells of a microplate) and then passed the tube through the ionization source stream. Analysis of tablets was carried out by positioning intact tablets with tweezers so that the path of the flowing helium grazed the tablet surface. Mass spectra were continuously collected as the sample was held in the ionization region of the DART source.

Results and Discussion

In setting out to study the applicability of DART for supporting preclinical pharmaceutical research we chose to focus on previously unexplored areas that could be of greatest potential benefit in terms of increasing analysis speed or decreasing method development labor required for analysis. A variety of different analytical challenges were investigated across the preclinical space from drug substance synthesis and purification to drug product formulation and stability studies.

Liquid chromatography−mass spectrometry (LC-MS) is an important tool for carrying out rapid analytical support for process development research; however, some compounds are poorly ionized by this technique, necessitating the use of other analytical techniques which may be slower or more cumbersome. The potential ability of DART to offer orthogonal ionization to the most popular electrospray ionization approach could offer great value to analytical chemists for analysis of otherwise difficult to ionize compounds.

Reaction Optimization. Figure 1 shows an example of an analyte that is poorly ionized by LC-MS, but easily ionized using DART. In this example, high-throughput catalyst screening was carried out to produce the desired benzofuranone, **1**, for which no authentic standard was initially available. An inability to initially detect product using LC-MS means that,

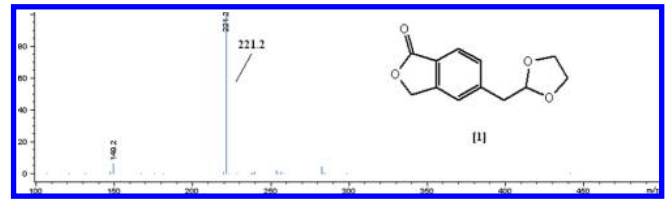

Figure 1. **DART mass spectrum of a benzofuranone compound. DART conditions: 325 °C, 1.8 L min^{-1} He, 3000 V needle voltage, +125 V discharge voltage, +300 V grid voltage. Agilent MSD conditions: 3000 V capillary voltage, 140 V fragmenter voltage, 0.4 L min^{-1} drying gas.**

when new product peaks are observed in LC-UV traces of catalyst screening reactions, purification and NMR analysis are required to determine product identity. Note that APCI-MS was able to adequately quantitate the compound in single ion monitoring mode after tuning with an authentic sample, which became available later in process development. In contrast, DART analysis of catalyst screening wells clearly showed successful formation of the desired product with the correct molecular weight, and allowed reaction optimization and scale-up to proceed.

Qualitative assessments of the extent of reaction are quite valuable in initial high-throughput experimentation screening. However, follow-up studies often require more quantitative analysis. Simply determining that product is present does not provide sufficient information for the chemist to identify the optimum reaction conditions. Given the reported capability of DART to quantitate chemical entities in complex sample matrices including serum and cell culture preparations, we were interested in evaluating the suitability of the technique for obtaining at least semiquantitative results for high-throughput reaction optimization studies. Initially, the linearity and precision of the DART technique was evaluated using the benzofuranone compound described in Figure 1.

Catalysis screening experiments typically yield solutions with a maximum concentration of 0.1 mg mL^{-1} analyte after sample workup (assuming 100% yield). A series of solutions containing the benzofuranone compound described earlier were therefore prepared within this range to evaluate the linearity and precision of the DART technique. The AutoDART was once again used for introducing the sample into the source. A plot of the relative abundance of the relevant ions of interest is shown in Figure 2. Initial analysis of the protonated molecular ion counts showed a marked negative deviation from linearity at the higher concentrations.

It seemed unlikely that a single quadrupole mass spectrometer would be overloaded at such a relatively low concentration. In addition to $m/z = 221$ $[M + H]^+$ ion, a significant dimer ion at $m/z = 441$ was also observed. Dimer formation commonly occurs with DART ionization, especially as analyte concentration increases.[22] The production of a single dimer ion requires 2 product molecules while generating only a single ion so the relative abundance was recalculated by adding twice the ion counts for the dimer ion to the ion counts for the $m/z = 221$ $[M + H]^+$ ion. This resulted in the more linear "$(m/z = 221) + 2 \times (m/z = 441)$" curve shown in Figure 2. Care should be taken when using this approach to ensure that observed dimer is indeed formed during the DART ionization process and that

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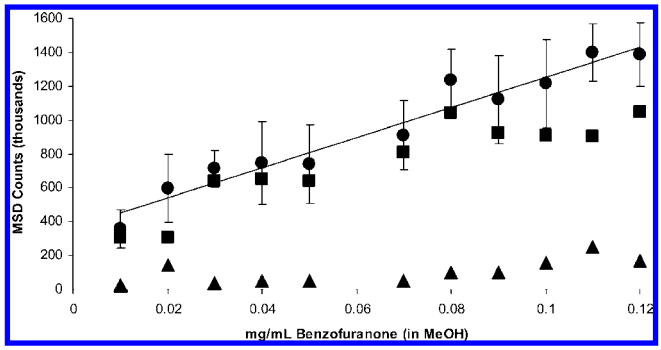

Figure 2. **DART linearity and dimer formation for a benzofuranone compound; (■) benzofuranone *m/z* 221 $[M + H]^+$, (▲) dimer ion, (●) sum of *m/z* 221 $[M + H]^+$ and 2 times the dimer ion. DART conditions: 325 °C, 1.8 L min^{-1} He, 3000 V needle voltage, +125 V discharge. Agilent MSD conditions: 3000 V capillary voltage, 140 V fragmenter voltage, 0.4 L min^{-1} drying gas.**

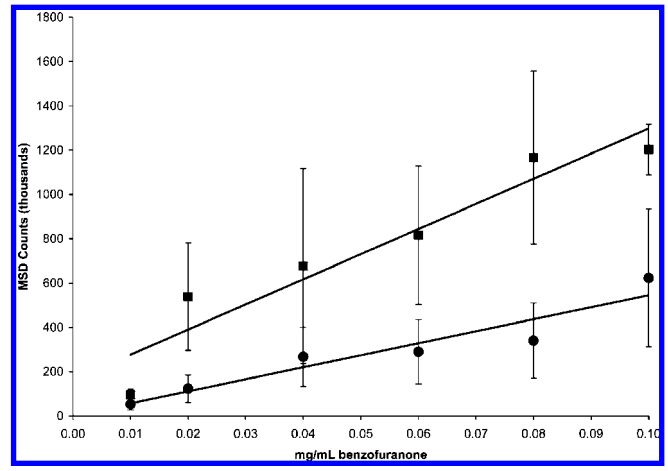

Figure 3. **Linearity of neutral research compound: (■) in methanol (●) in methanol with 10 mol % diethylamine. DART conditions: 325 °C, 1.8 L min^{-1} He, 3000 V needle voltage, +125 V discharge voltage, +300 V grid voltage. Agilent MSD conditions: 3000 V capillary voltage, 140 V fragmenter voltage, 0.4 L min^{-1} drying gas.**

the sample itself does not contain the dimer as an impurity. Although not definitive, dilution of the sample should reduce the relative response of the dimer ion to other fragments or the protonated molecular ion if it is being formed in the DART source.

Interestingly, the relative amount of dimer formed varies widely even across multiple determinations of the same solution. Error bars representing the standard deviation of five separate measurements at each concentration are shown for the combined ion counts. Despite the rather large imprecision at each concentration, the data provide sufficient information to distinguish between reactions with good yield (>75%) and those with poor yield (<25%).

Potential for Interference in Quantitative Measurement. The ability of DART to carry out semiquantitative analysis led us to undertake a systematic evaluation of the technique for rapid analysis of microplates from catalysis screening experiments normally analyzed by LC-MS. Of particular concern was the effect of the sample matrix on ionization of the product of interest and its potential impact on quantitative determination of the relative yield of the product in each screening plate well. The aim of catalysis screening is to identify the appropriate catalyst for a given reaction and to begin to understand the effects of catalyst loading, solvent composition, the role of base additives, and other relevant reaction conditions. It is therefore important that the analysis technique be relatively unaffected by the sample matrix, since such 'design of experiments' approaches often lead to substantial variation in sample matrix across the plate.

Sample interference is one of the banes of the flow injection analysis with mass spectrometric detection (FIA-MS) technique for catalyst screening, with ion suppression or enhancement frequently causing problems in quantitation, especially when amine additives are present. This represents a significant problem for high-throughput catalyst screening, as amine additives are often investigated as a part of many catalyst screening workflows. Ion suppression caused by amine additives is quite severe with FIA-ESI-MS approaches. We were interested in evaluating the severity of ion suppression caused by amine additives with DART, and carried out the study illustrated in Figure 3. In this experiment, 10 mol % diethylamine was shown to severely decrease the DART signal over a range of concentrations for the benzofuranone test compound.

The very small reaction volumes and the resulting variability in performing sample workups often significantly impact the ability to obtain accurate reaction yields in 96- and 384-well plate high-throughput experiments. Although the addition of an internal standard to each reaction well within the microplate to overcome these difficulties is common practice when UV detection is used, the requirement that the internal standard be completely inert with respect to the reaction often precludes the ability of ESI-MS to ionize it. In theory, the ability of DART to detect inert hydrocarbon compounds such as biphenyl should allow the use of internal standards to improve the technique's precision and accuracy and perhaps correct for the ion suppression noted in these studies.

The use of trimethoxybenzene as an internal standard in the mixtures was investigated, and while this afforded some improvement, the results were still unacceptable for use in quantitative reaction screening. The influence of solvents on the intensity of the measured DART signal was also investigated, with some solvents, notably DMSO, showing almost complete signal suppression even when diluted 10 times with methanol. Taken together, these results suggest that DART may be most useful for high-throughput analysis when the sample matrixes of all samples being investigated are comparable. In cases where the solvent or additives differ substantially from sample to sample, as in catalysis screening or reaction condition optimization studies, care must be taken to ensure freedom from signal suppression across the range of sample compositions.

DART as a High-Throughput Analysis Tool. Given the fast performance of DART, there is an obvious potential use of the technique for high-throughput analysis. In recent years, fundamental changes in the way that pharmaceutical development research is carried out have led to an increasing use of high-throughput experimentation[23−25] Flow injection analysis with mass spectrometric detection (FIA-MS) has been used extensively in our laboratories for the initial analysis of 96-well plates.[26,27] This technique has proven to be a quick and

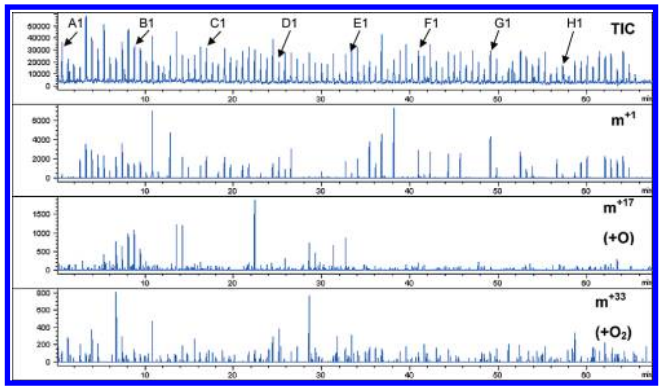

Figure 4. **DART analysis of a 96-well enzymatic oxidative study. DART conditions: 325 °C, 1.8 L min^{-1} helium, 3000 V needle voltage, +125 V discharge voltage, +300 V grid voltage. Agilent MSD conditions: 3000 V capillary voltage, 140 V fragmenter voltage, 0.4 L min^{-1} drying gas.**

reliable means of identifying which reaction wells in high-throughput catalysis screening have the greatest degree of product formation. The technique is especially valuable in those instances where no authentic standard for the desired product is available. Figure 4 clearly shows the value of DART for carrying out similar high-throughput analysis of microplates in support of catalyst screening. In this example, the use of the AutoDART-96 liquid sample handler allowed the rapid identification of wells in which a desired enzyme-mediated oxidation is observed. In this example the plate time (time required to analyze a complete 96-well microplate) is only 65 min, which compares favorably with time for LC-MS approaches.

Impurity Identification. A more challenging problem is the detection and identification of minor impurities and degradants during process development as well as in formulated products. Due to the increasing demands for higher purity in active pharmaceutical ingredients (APIs) as analytical techniques improve and the impact of low levels of impurities, such as genotoxic agents, are realized, rapid and accurate detection of such impurities in process samples is critical. Forced degradation studies allow one to quickly understand if a given compound or formulation may have a certain liability or propensity to decomposition. This information can lead to an important understanding of degradation pathways (e.g., a susceptibility to oxidation, hydrolysis, or elimination) and can be critically important for identifying proper storage and packaging conditions. A typical forced degradation study, with analysis by a generic HPLC method, is illustrated in Figure 5. In a set of parallel small-scale reactions, the API, **2**, is exposed to aqueous acid and base, as well as an oxidant (hydrogen peroxide, a free radical initiator (AIBN), and heat (100 °C). A substantial amount of a new degradant was observed with the hydrogen peroxide-treated sample, indicating a possible vulnerability of the API toward oxidation. DART analysis of these samples quickly (~1 min per analysis) showed the presence of a new, lower-molecular weight impurity, **4**, for the peroxide-treated sample corresponding to the loss of 28 mass units (Figure 5b), presumably originating from the initial formation of epoxide, **3**, followed by decarboxylation and rearrangement to afford the observed degradant **4** (Figure 5c).[28] Ultimately, DART analysis was completed without the use of any MS modifiers or sample pretreatments, and the results were obtained within minutes.

Preparative Chromatography Fraction Analysis. Preparative chromatography is routinely used to support synthetic chemistry investigations. An example illustrating this approach for the purification of a radioactive ^{14}C-labeled cholesterol derivative is shown in Figure 6. The DART analysis confirmed that fractions 2−5 contained the desired product of *m*/*z* 839 $[M + H]^+$, with a total analysis time of only 2 min. This fast turnaround time allowed rapid access to the purified product, eliminating the requirement for lengthy HPLC-MS analysis and reducing the generation of radioactive HPLC waste as well. Since disposal costs are rising dramatically, the potential of this method to reduce the production of radioactive HPLC waste is especially attractive.

Rapid Testing for API Identity and Drug Loading in Formulated Drug Product. The ability to directly analyze solid samples without the need for sample preparation prompted an evaluation of DART as a rapid identification tool for determining drug load in tablets. This is especially helpful for analyzing tablets used in clinical studies, where visually identical tablets are often used for varying dosages, and for placebo. Tablets weighing 100 mg each and containing either 0.25, 2.5, or 25 mg of an API were analyzed using DART. To maximize sensitivity for the low-potency tablets, analysis was conducted in single ion monitoring (SIM) mode, selecting for the API *m*/*z* 634 $[M + H]^+$ *m*/*z* 634. Replicate measurements of tablets ($n = 5$) were analyzed, and the average peak height was calculated to generate a linear calibration curve with a correlation coefficient of 0.9998. Using DART technology, no dissolution or other sample preparation was needed and the entire analysis was completed within 6 min. If conducted by traditional sample preparation and HPLC-UV methods, the same analysis would take several hours. This study convincingly shows that DART can be used to quickly confirm API identity and distinguish between potencies when the percent drug loading varies significantly (e.g., factor of 10).

Counterfeit Analysis. Several recent high profile cases of adulterated or fraudulent counterfeit drugs have raised worldwide awareness of this growing problem.[29−32] In order to investigate the potential of DART to facilitate fast API verification and counterfeit detection, we examined authentic and suspected counterfeit Arcoxia tablets

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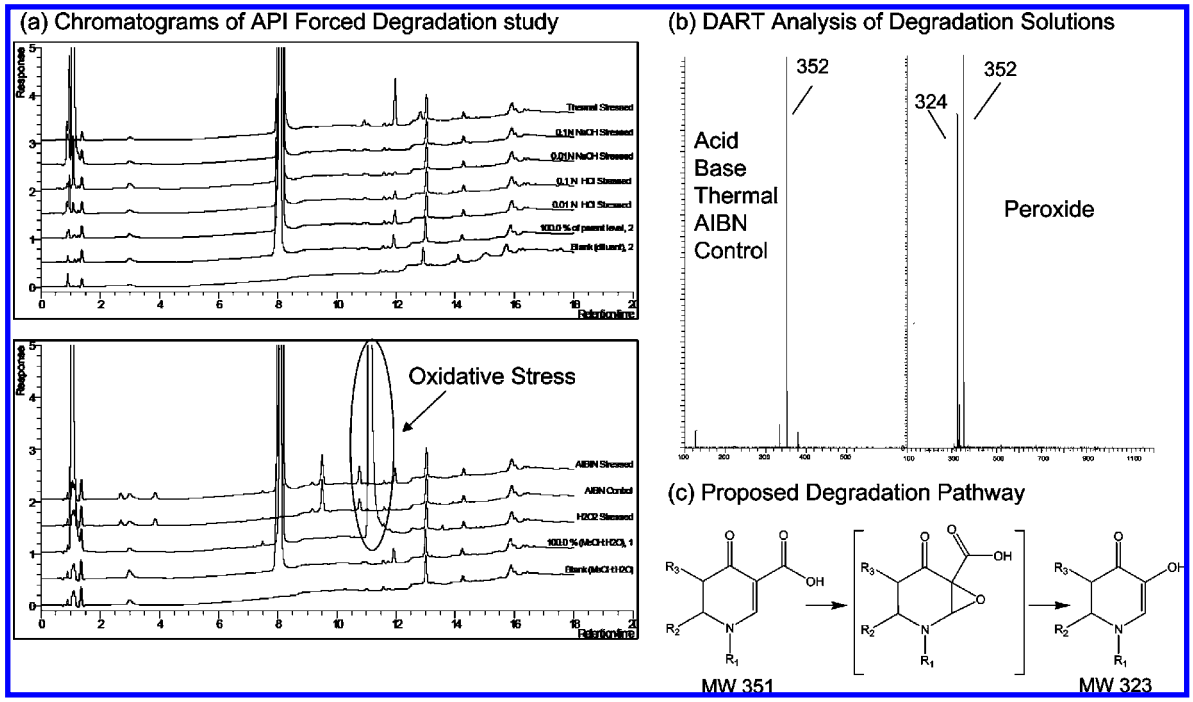

Figure 5. **Forced degradation of an API analyzed by HPLC and DART. (a) HPLC conditions: Agilent 1100; column HALO C18, 150 mm × 4.6 mm, 2.7 μm; temperature 40 °C; detection UV at 220 nm; injection volume 10 μL; flow rate 1.2 mL/min; sample preparation 0.1 mg/ml in 50/50 acetonitrile/water; binary RP gradient profile 40% to 60% acetonitrile in 7 min, then to 98% B in 8 min, hold for 5 min; aqueous MP: 0.1% perchloric acid. (b) DART conditions: 350 °C, 3 L/min of nitrogen, 4000 V discharge, electrode 1 and 2 were set at 150 V and 300 V, respectively. (c) Proposed degradation pathway.**

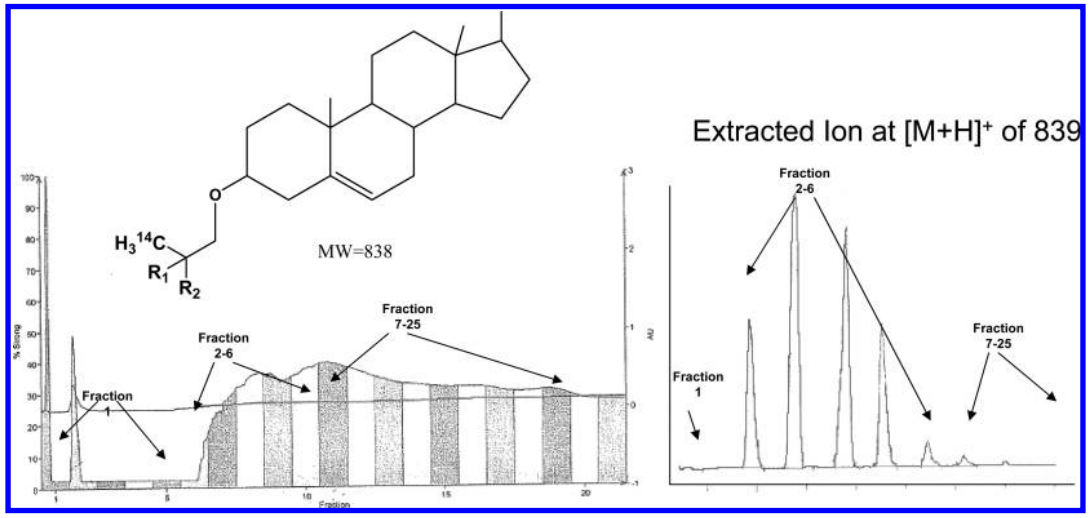

Figure 6. **Analysis of preparative chromatography fractions by DART. DART conditions: 350 °C, 3 L min^{-1} of helium, 4000 V discharge, electrodes 1 and 2 were set at 150 V and 300 V, respectively, XIC at 839 *m/z* in positive mode.**

using DART to verify the presence or absence of the API, etoricoxib. Intact tablets were analyzed, affording the diagnostic $[M + H]^+$ signal for etoricoxib at *m/z* 359, along with corresponding fragments at *m/z* 159 and 236 (Figure 7a). Analysis of the suspected counterfeit tablets did not result in signals at any of the corresponding etoricoxib *m/z* values, indicating the complete absence of the API (Figure 7b). A strong signal was observed at *m/z* 152, which corresponds to acetaminophen, a commonly used counterfeit ingredient. All analyses were completed within less than a minute, demonstrating the speed and ease with which DART can be used to identify counterfeit drug products.

Photodegradation Analysis. To determine the utility of DART for monitoring degradation of active pharmaceutical ingredients in formulated drug products, dry filled capsules containing a photosensitive API blend were exposed to 400 klux·h of ultraviolet light and analyzed for the presence of the known degradation product. Prior to UV exposure, powder blends were encapsulated in white, orange, or green gelatin capsules, which are known to provide increasing protection from the UV wavelength

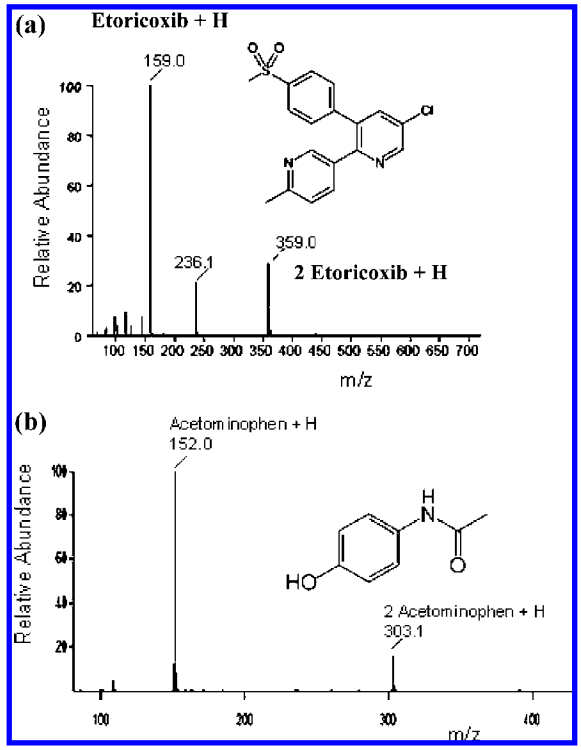

Figure 7. **Counterfeit analysis by DART. DART conditions: 350 °C, 3 L min^{-1} of helium, 4000 V discharge, electrodes 1 and 2 were set at 150 V and 300 V, respectively, scan range of 100−700 *m/z* in positive mode.**

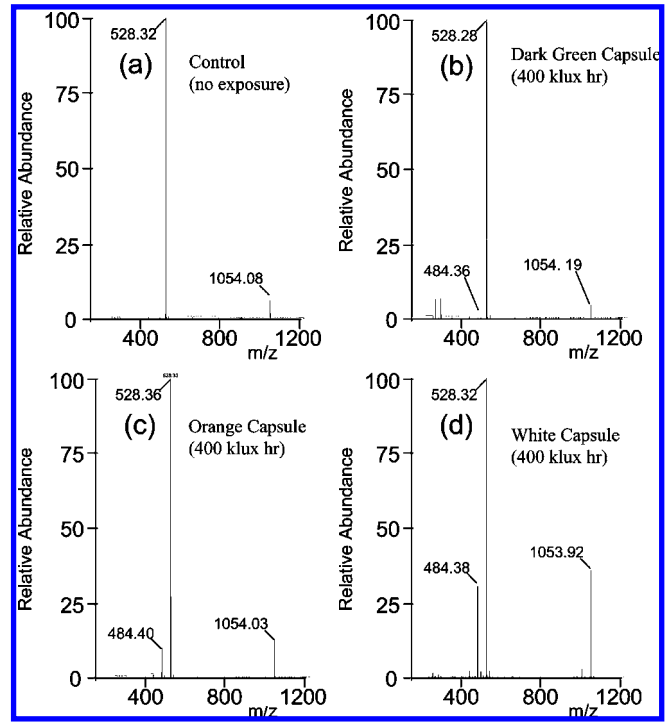

Figure 8. **Photodegradant analysis of formulated capsules by DART. DART conditions: 350 °C, 3 L min^{-1} of helium, 4000 V.**

Table 1. **Comparison of specific activity measured by LC-MS and DART**

API-isotope	SA via LC-MS (mCi mg^{-1})	SA via DART (mCi mg^{-1})
API-1 (^{3}H)	46.5	44.7
API-2 (^{14}C)	145.5	148.3
API-3 (^{3}H)	181.3	179.0
API-4 (^{14}C)	138.2	136.1

that causes photodegradation of the API. A control sample blend, not exposed to UV light, was also included in the study. Upon light exposure, each capsule shell was opened, and a glass capillary tube was dipped into the blend. Each glass capillary was then placed in the ionization region of the DART. In all cases, the parent API was observed at *m/z* of 528 $[M + H]^+$ (Figure 8). In addition to the parent API, an API dimer was also observed for each sample at *m/z* 1054. The control blend, which was not exposed to UV light, contained only these API-related peaks, whereas all other samples showed some formation of the degradant peak at *m/z* 484. On the basis of the relative amounts of this degradant peak in the various samples, it was concluded that white capsule shells afforded the poorest protection form UV light, with the green shells affording the greatest UV protection. If samples were solubilized and analyzed by traditional HPLC-UV technology, total preparation and analysis time would require several hours; however with DART, sample preparation time is virtually eliminated, and analysis time is significantly reduced, resulting in the ability to rank order the powder blends by degradant level formed within minutes.

Determination of Isotopic Abundance. The determination of specific activity (SA) is crucially important for the characterization of radiolabeled compounds prepared for drug metabolism studies (such as metabolite identification/pathways, and/or clinical studies). Since mass spectrometry can be used to directly measure isotopic abundance of the stable and radiolabeled molecules with good accuracy,[33] it was reasoned that relative abundance measurements using DART should allow for rapid and convenient specific activity measurements. The ability of DART to determine the isotopic abundance for compounds labeled with both ^{3}H and ^{14}C was assessed. Table 1 summarizes the SA results as determined by DART and HPLC-MS. In every case the DART could accurately report the specific activity for ^{3}H- and ^{14}C-labeled samples within 5% error. It is important to note that unlike the HPLC-MS assay which required a MS modifier to ionize the sample, the DART assay does not require any mobile phase modifier. Moreover, the DART assay for specific activity measurements was accurate and expeditious and generated almost no radioactive waste, an important environmental and economic concern for laboratories working with radiolabeled compounds.

Conclusion

Several examples which illustrate the utility of the DART for supporting preclinical pharmaceutical research have been provided. The DART has been shown to be an effective tool for analyzing samples that are difficult to analyze by ESI-MS. In addition, DART has been shown to provide rapid support for studies relating to production of isotopically labeled compounds, including measurement

of specific activity and analysis of preparative chromatography fractions. DART also proved useful for rapid analysis in support of formulation sciences, allowing the rapid determination of sample dose, monitoring of degradation studies, and identification of counterfeits, all without the need for sample preparation.

The use of DART for fast analytical support of high-throughput experimentation studies proved somewhat problematic. While the technique did allow for rapid reaction screening for an enzymatic oxidation reaction, attempts to use DART for more complex catalyst screening showed serious problems with interference caused by amine additives and other components in the sample matrix. The use of internal standards that were amenable to ionization by the DART offered some advantage, but only when plates containing comparable matrices were used.

In conclusion, DART has been shown to be a valuable tool for rapid analysis without the need for sample preparation. While the applicability of the technique is not universal for all analysis problems encountered in preclinical pharmaceutical development, it does offer a fast and effective alternative to more traditional analytical approaches, and promises to be a useful tool for future studies in this area.

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