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Characterization of Solid Counterfeit Drug Samples by Desorption Electrospray Ionization and Direct-analysis-in-real-time Coupled to Time-of-flight Mass Spectrometry

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The search for more versatile, sensitive, and robust ionization methods is a recurring theme in mass spectrometry (MS). Since the discovery of electrospray ionization (ESI)[1] and matrix-assisted laser desorption/ionization (MALDI),[2] many developments such as atmospheric pressure MALDI,[3] nanospray ionization,[4] Venturi-assisted electrospray,[5] and ion-funnel atmospheric pressure interfaces,[6] have paved the way to improved characterization of small molecules and biomolecules. One of the bottlenecks in achieving high sample throughput with both ESI and MALDI is the need to dissolve, extract, and/or filter the sample prior to analysis. Moreover, vacuum-incompatible materials cannot be easily investigated by MS without disturbing their innate structure. Recently, two novel methods for the direct ionization of solid samples under atmospheric pressure by MS were reported: desorption electrospray ionization (DESI)[7] and direct analysis in real time (DART).[8] More recently, McEwen et al. described a modified atmospheric pressure chemical ionization (APCI) technique for the direct analysis of solids which they named atmospheric pressure solids analysis probe (ASAP).[9] DESI makes use of a high-speed liquid spray directed at a sample held or deposited on a surface at atmospheric pressure. Ions generated during this process are sampled by a mass spectrometer. Several DESI applications such as the mapping of analytes separated by thin-layer chromatography,[10] the detection of explosives,[11,12] and the screening of pharmaceutical tablets[13–15] and illicit drugs[16,17] quickly followed the proof-of-principle description of the method.

DART involves an ionizing beam of metastable He atoms ($^3S_1$, 19.8 eV) generated by a corona discharge. The DART ionization mechanism is still not completely understood. In negative ion mode, the metastable He atoms generate electrons that produce negatively charged oxygen–water clusters, which then form the corresponding adducts. In positive ion mode, metastable He atoms generate protonated gaseous water clusters by Penning ionization.[8] Then, by proton exchange, these clusters form $[M+H]^+$ ions, which are generally the predominant species. DART's high throughput coupled with the high mass accuracy now attainable with modern time-of-flight mass (TOF) analyzers and accurate isotopic abundance measurements make it especially suitable for the rapid identification of unknown species in solid materials. One particularly relevant example is counterfeit drug samples. Counterfeit drugs are defined as those that are "deliberately and fraudulently mislabeled with respect to identity and/or source".[18] They may include products with the "wrong" ingredient(s), without active ingredient(s), or with an insufficient amount of active ingredient(s).

In recent years, a particularly alarming case of drug counterfeiting has been reported by field researchers[19,20] who have detected counterfeit products that mimic the vital antimalarial, artesunate.[21] The consumption of fake antimalarials has resulted in the death of many patients.[22–24] Evidence suggests that the production of counterfeit artesunate tablets is on an industrial scale. For example, one health care organization in southeast Asia unwittingly purchased 100,000 artesunate tablets which were later shown to be counterfeit.[25] Classic hyphenated analysis methods, such as liquid chromatography–mass spectrometry (LC–MS), lack the required sample throughput to survey such large numbers of samples in a reasonable amount of time.[26]

Figure 1a shows a schematic of the DART TOF MS setup used to screen 52 representative samples of a database containing more than 400 artesunate-based antimalarial tablets. Figure 1b and 1c show the negative ion mode DART TOF MS data of genuine and counterfeit artesunate (*M*) tablets, respectively.

The spectrum shown in Figure 1b has signals corresponding to the diagnostic $[M-H]^-$ artesunate anion (experimental $m/z = 383.1702$, calculated $m/z = 383.1711$) and palmitic acid, a ubiquitous contaminant. Artesunate fragment ions due to dissociation of the highly labile artesunate carboxylic acid side

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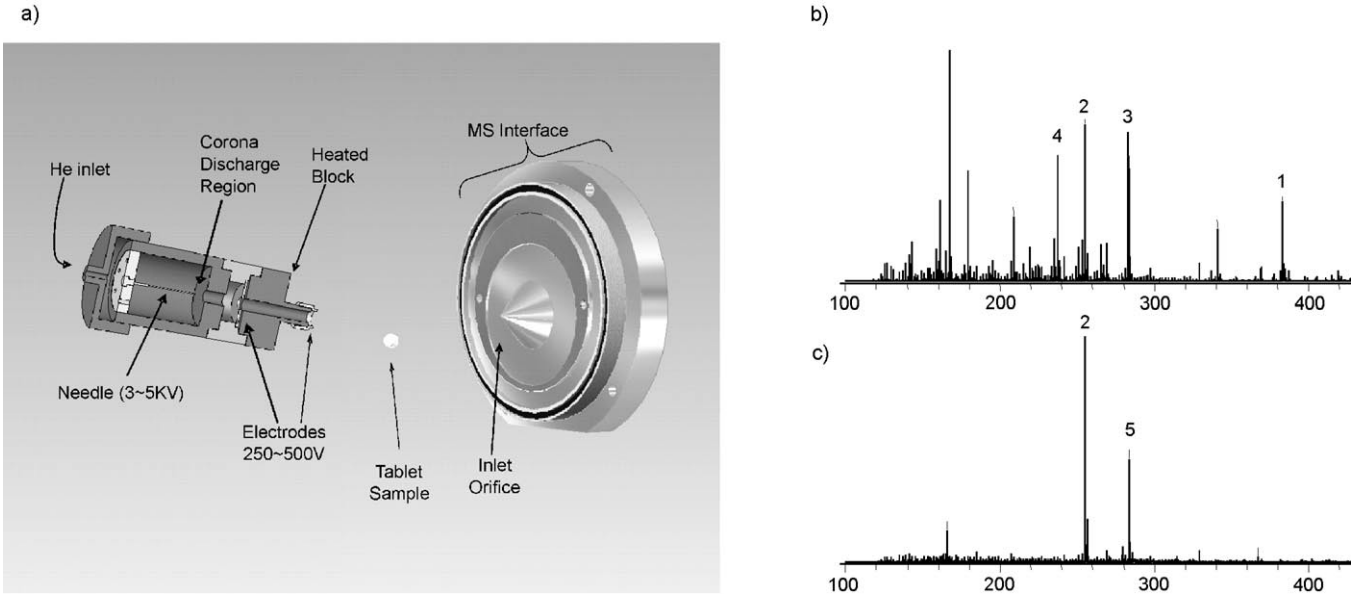

Figure 1. a) DART TOF MS setup; b) MS data for genuine artesunate tablet: 1 = $[M-H]^-$, 2 = [palmitic acid−H]$^-$, 3 = $[M-C_4H_4O_3-H]^-$, 4 = $[M-C_4H_6O_4-CO-H]^-$; c) MS data for counterfeit tablet: 5 = [stearic acid−H]$^-$.

chain were also detected in this spectrum. The spectrum shown in Figure 1 c, corresponds to a sample that had been classified as counterfeit by the fast red TR dye test,[27] and is completely devoid of any artesunate species, thus confirming the field result. Only palmitate and stearate anions were detected (calcium stearate is a common pharmaceutical excipient).

According to Figure 1 b and 1 c, negative ion mode DART TOF MS allows the rapid differentiation of counterfeit tablets from genuine artesunate tablets. However, the high specificity of this DART ion mode prevents the detection of analytes that are not easily deprotonated. Not only is the absence of artesunate in these tablets of concern, but also the potential presence of other physiologically active ingredients. These "wrong active ingredients"[28] are sometimes added to counterfeit pharmaceuticals to simulate either the organoleptic characteristics or the symptom-relief effects of the real product, or as waste powders left over from the manufacture of other pharmaceuticals. For this reason, an ionization mode that allows screening for artesunate and other common drugs is highly desirable.

Positive ion mode DART TOF MS was performed, but only a few artesunate fragment ions were detected. However, when the sample was exposed to ammonia vapors during DART ionization, the artesunate $[M+NH_4]^+$ ion was readily observed. Although the formation mechanism of this ion has yet to be studied, this DART approach involving a dopant proved extremely useful for analytical purposes. This method is conceptually similar to the reactive DESI experiment performed by Takats et al.[7] Figure 2 shows spectra of a genuine artesunate tablet obtained using DART ionization with NH_3 as a dopant (Figure 2 a) and DESI(Figure 2 b).

A total of 43 out of 52 (83%) of the samples studied by DART contained no detectable artesunate, in agreement with colorimetric test and LC results. Analysis of the packaging confirmed that these samples were counterfeit. Ammonia-doped DART not only produced a diagnostic $[M+NH_4]^+$ ion, but also

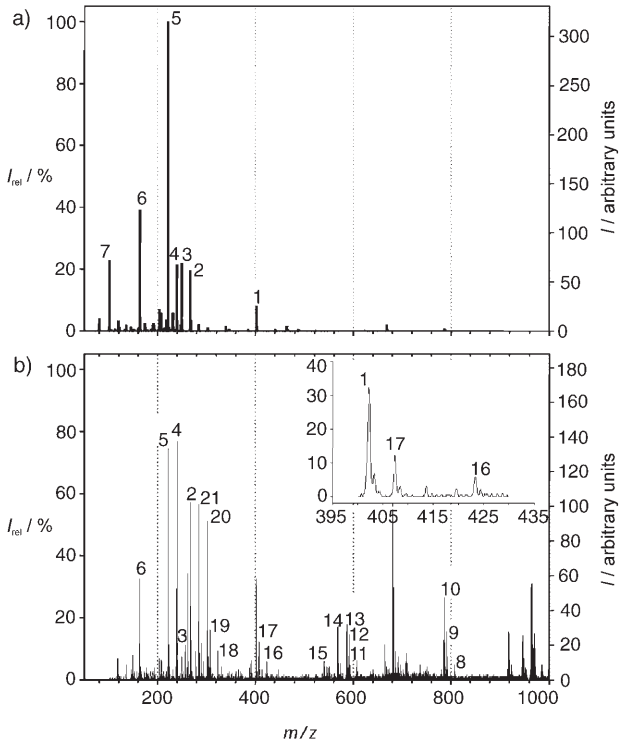

Figure 2. Screening of a genuine artesunate (*M*) tablet by a) ammonia-doped DART TOF MS (A = $C_4H_6O_4$, B = $C_4H_4O_3$): 1 = $[M+NH_4]^+$, 2 = $[M-A+H]^+$, 3 = $[M-A-H_2O+H]^+$, 4 = $[M-A-CO+H]^+$, 5 = $[M-A-CO-H_2O+H]^+$, 6 = $[M-A-C_4H_7O_3+H]^+$, 7 = $[A-H_2O+H]^+$; and b) DESI MS: 8 = $[2M+K]^+$, 9 = $[2M+Na]^+$, 10 = $[2M+NH_4]^+$, 11 = $[2M-A-B+K]^+$, 12 = $[2M-A-B+Na]^+$, 13 = $[2M-A-B+NH_4]^+$, 14 = $[2M-A-B-H_2O+NH_4]^+$, 15 = $[2M-A-B-H_2O-CO+NH_4]^+$, 16 = $[M+K]^+$, 17 = $[M+Na]^+$, 18 = $[M-B+K]^+$, 19 = $[M-B+Na]^+$ 20 = $[M-B+NH_4]^+$, 21 = $[M-A+NH_4]^+$.

fingerprint fragment ions that could be assigned with an average error of 2.2 mmu. Together the $[M+NH_4]^+$ and fragment ions accumulated 13.5 identification points, enabling the positive identification of the target analyte in accordance with new EU guidelines.[29,30]

Both DESI and DART produced spectra with good signal-to-noise ratios in five seconds, demonstrating an improvement of over two orders of magnitude in terms of sample throughput in comparison with LC–MS. DESI produced several adduct ions (Figure 2b, insert), including $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$, as well as ammoniated, sodiated, and potassiated fragment and cluster ions. However, lower DESI jet flow rates could change the relative abundances of these. Despite the low amount of internal energy believed to be deposited into the analyte ions during DESI, fragmentation was also observed; this is an indication of the lability of the $[M+H]^+$ ion itself. A fragmentation pattern similar to that shown in Figure 2b was also observed for ESI of artesunate dissolved in the DESI spray solvent, regardless of the capillary–skimmer potential difference. This similarity in behavior between ESI and DESI suggests that for this particular analyte, DESI possibly proceeds via a previously described dissolution-mediated "splashing mechanism".[7]

Figure 3 shows the DART TOF MS spectra of counterfeit artesunate samples of increasing sophistication. The sample shown in Figure 3a was collected in June 2003 in Savannakhet, Laos, and a type 7 fake hologram[31] was affixed to the blisterpack. Interestingly, this sample had $[M+H]^+$ and $[M+NH_4]^+$ protonated molecular ions consistent with artemisinin, the naturally occurring chemical found in *Artemisia annua* and the precursor for artesunate synthesis. Artemisinin may have been added incidentally as a powder available to the counterfeiters in a poor-quality production process or in an attempt to confer some antimalarial properties to the fake concoction.

Figure 3b shows the DART spectrum of a counterfeit sample collected in September 2004 in Luang Nam Tha, Laos with a well-crafted type 6 fake hologram affixed to the blisterpack; the presence of chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$, $\Delta_{mass}=1.4$ mmu) and metronidazole ($C_6H_9N_3O_3$, $\Delta_{mass}=1.4$ mmu) is apparent.

Figure 3c corresponds to a counterfeit artesunate sample collected at the Burma–Thai border in March 2005. This sample had a sophisticated (but fake) type 9 hologram affixed to the blisterpack, and it produced a negative result in the colorimetric fast red TR test. DART TOF MS revealed not only the presence of acetaminophen in this sample, but also low-intensity (5%) signals corresponding to artesunate fragment ions. This alarming finding was further confirmed by LC, which showed that this tablet contained 10 mg of artesunate per tablet, or 20% of the expected amount of active ingredient in the genuine product. We hypothesize that this is the latest "innovation" introduced by the counterfeiters in an attempt to deceive the colorimetric authentication tests currently in use in the field. The implications of this finding are far-reaching, as the continuous administration of low doses of artesunate could genetically select artesunate-resistant parasite strains, rendering this last-resource antimalarial medicine ineffective. Other compounds detected in counterfeit artesunate samples included the antimalarial combination pyrimethamine/sulfadoxine, which is also ineffective in southeast Asia, metamizole, an analgesic suspected of causing serious bone marrow disorders,[32] and chloroquine, an early-generation antimalarial that is now ineffective against multidrug-resistant *Plasmodium falciparum* parasites.[33]

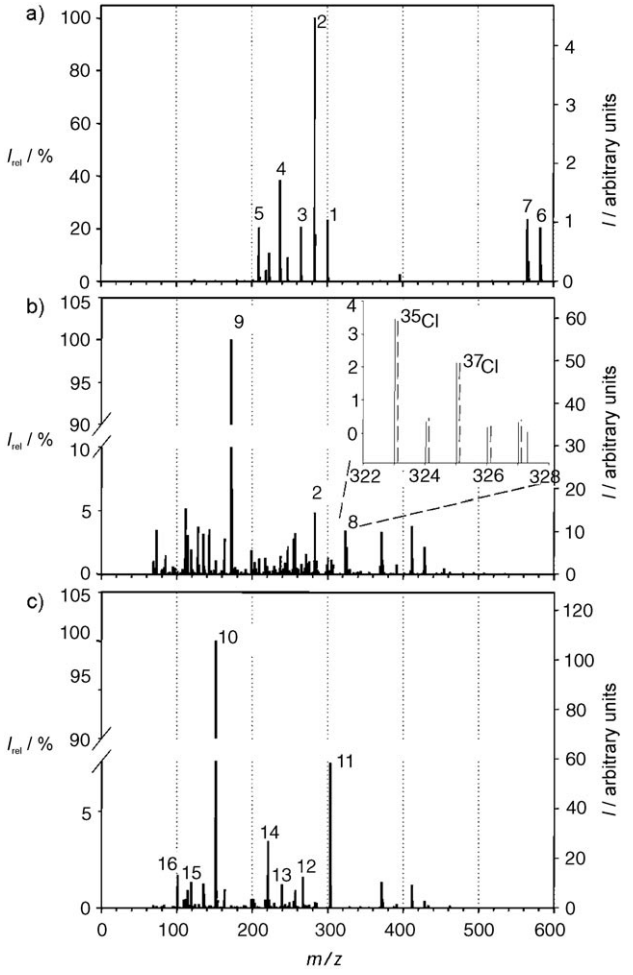

Figure 3. DART TOF MS spectra of sophisticated counterfeit artesunate samples (M = artesunate, N = artemisinin, A = $C_4H_6O_4$): 1 = $[N+NH_4]^+$, 2 = $[N+H]^+$, 3 = $[N-H_2O+H]^+$, 4 = $[N-H_2O-CO+H]^+$, 5 = $[N-H_2O-2CO+H]^+$, 6 = $[2N+NH_4]^+$, 7 = $[2N+H]^+$, 8 = [chloramphenicol+H]$^+$, 9 = [metronidazole+H]$^+$, 10 = [acetaminophen+H]$^+$, 11 = [2 xacetaminophen+H]$^+$, 12 = $[M-A+H]^+$, 13 = $[M-A-CO+H]^+$, 14 = $[M-A-CO-H_2O+H]^+$, 15 = $[A+H]^+$, 16 = $[A-H_2O+H]^+$.

In conclusion, our findings not only demonstrate the usefulness of DART for the rapid screening of counterfeit drugs, but have unprecedented implications for malaria control as well. We foresee that both DART and DESI will have a tremendous impact on a variety of scientific fields, ranging from drug quality control, screening, and discovery, to biological applications such as metabonomics and proteomics. In the absence of chromatography and as sample complexity increases, we expect that accurate mass and isotopic cluster intensity measurements could be complemented with MS–MS experiments for an even more confident identification of unknowns.

Experimental Section

Counterfeit and genuine artesunate tablets were collected in a wide area of southeast Asia encompassing Laos, Myanmar (Burma), and Thailand, and were kept refrigerated (4 °C) until analysis. Ammonium hydroxide (28% in water, Sigma–Aldrich) was used for ammonia-doped DART experiments. The DART ion source was coupled to an AccuTOF orthogonal time-of-flight mass spectrometer, operated as previously described.[8] Orifice 1 of the atmospheric pressure interface was set to 30 eV. Helium was used as the corona discharge gas at a flow rate of 2.8 L min^{-1} and was heated at 350 °C. The exit electrode was held at 300 V, and the corona discharge voltage was 3000 V. Tablets were manually held with a pair of tweezers between the DART source and the TOF MS inlet for 5 s. No memory effects were observed as long as care was taken not to contact the inlet orifice of the mass spectrometer with the tablet. The TOF inlet–sample distance was ~1 cm. Internal calibration and temperature-drift correction were performed using PEG 600 (Sigma). DESI was performed using a solution of MeOH/H_2O (50:50) as the desorption spray at a flow rate of 15 μL min^{-1}. The nebulizer gas pressure was set to 40 psi. Detection was performed with an LTQ linear ion trap mass spectrometer (Thermo Finnigan) tuned for optimum detection of the $[M+NH_4]^+$ ion. Tablets were exposed to the DESI jet for 10 s. DART data were acquired in centroid mode and DESI data, in profile mode. Both DESI and DART spectra were background corrected. LC analysis was performed using a C_{18} column (150×4.6 mm) with a mobile phase consisting of 50% acetonitrile and 50% 0.05 M perchlorate buffer (pH 2.5). Analyte detection was carried out with UV absorbance at $\lambda = 220$ nm. DART mass spectrometry data were exported as ASCII JEOL-DX centroided spectra and automatically searched against an in-house library of potential $[M+H]^+$ ions derived from 238 common drugs found in the List of Essential Drugs supplied by the World Health Organization (WHO).[34] A match was labeled positive if the difference between the experimental and theoretical masses was less than 5 mmu and the isotopic cluster pattern could be matched.

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