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**TECHNICAL NOTE**

**CRIMINALISTICS**

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# Validation of Thin Layer Chromatography with AccuTOF-DART™ Detection for Forensic Drug Analysis*

**ABSTRACT:** Thin layer chromatography (TLC) is a technique that is commonly employed in the forensic drug analysis of pharmaceutical preparations. Detection is typically accomplished using various visualization spray reagents. Conventional gas chromatography–mass spectrometry (GC-MS) analysis is typically performed to confirm the TLC results. Depending on the drugs tested and the instrument conditions required, this confirmation can take up to an hour to complete. Direct analysis in real time (DART™) is an ionization source, coupled to an accurate-mass time-of-flight mass spectrometer that has the capability to ionize materials under ambient conditions. To streamline analysis, the combination of TLC with DART™ detection is proposed to screen and subsequently identify drug compounds, all from the same TLC plate. DART™ confirmations of TLC analyses take <10 min to complete and compare favorably to GC-MS in sensitivity and selectivity. This study validates the use of TLC-DART in the forensic identification of the components of several pharmaceutical preparations.

**KEYWORDS:** forensic science, controlled substances, thin layer chromatography, direct analysis in real time, mass spectrometry, time of flight

Thin layer chromatography (TLC) is a technique used in forensic drug chemistry, which separates components of a drug mixture (1–10). TLC is one of the standard procedures used in many forensic drug laboratories when examining unknown drugs or mixtures. The separation of mixtures is dependent upon the pH and polarity of both the mixture and the solvent bath in conjunction with the thin layer stationary phase used. While the unknown drugs and mixtures are always run against standards, multiple compounds can share the same retention factor or produce similar chromophores when sprayed with detection reagents. This results in a low level of specificity, which can be raised through use of other detectors (1–10).

Currently, the Virginia Department of Forensic Science (VDFS) uses visualization spray reagents as detectors for the component spots on the plate (10). Commonly used sprays include potassium permanganate ($KMnO_4$), iodoplatinate, and ceric sulfate. All of the detection sprays used for TLC indicate class-specific chromophores. For identification, a drug-specific detection type is needed.

The AccuTOF-DART™ (JEOL USA, Peabody, MA and IonSense, Saugus, MA) employs an ambient ionization source in conjunction with an accurate-mass time-of-flight mass spectrometer (10–58). With a resolution of >6000, the AccuTOF™ allows for exact mass measurements to millidalton (mDa) accuracy. Little to no sample preparation is required before analysis is performed, whether the sample is in solid, liquid, or gas phase. The sample is held in the heated gas stream (typically helium) of the DART™ (direct analysis in real time) and is ionized. In-source collision-induced dissociation is used to promote fragmentation of protonated molecules to increase specificity. This procedure is controlled by varying the voltage applied to the first orifice (orifice 1) of the AccuTOF™. At low orifice 1 V, protonated molecules ($[M+H]^+$), formed from the interaction of ambient water molecules with sample molecules, pass into the AccuTOF™ unchanged. Depending upon the stability of the molecule, some fragment ions may also be formed. Higher orifice 1 V cause a higher degree of fragmentation. As described earlier by Steiner and Larson (58), "function switching" can be used to vary the orifice 1 V every fraction of a second throughout the data file. Compared with quadrupole gas chromatography–mass spectrometry (GC-MS), the AccuTOF™, used in conjunction with DART™ ionization, allows for richer data (spectra of the protonated molecule and fragmentation via function switching) to be collected, resulting in more specificity of the identity of the molecule (10–58). Currently at VDFS, the AccuTOF-DART™ can be used for screening of samples with confirmation of drug substances made via traditional means (GC-MS or Fourier transform infrared spectroscopy) (58).

Minimum standards for drug testing and reporting in the forensic community are recommended by the Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG). In Part III B, Section 2 of the SWGDRUG Recommendations document, methods of analyses are sorted into three categories based on specificity. The most highly specific methods are characterized as Category A. Category A includes such methods as infrared spectroscopy and mass spectrometry. Less specific methods of identification are characterized as Category B, including GC, liquid chromatography, pharmaceutical identifiers, and TLC. The least specific methods are characterized as Category C, which includes color tests and others (59).

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In order for a drug identification to be confirmed to SWGDRUG specifications, specific combinations of tests must be used. Positive results from a Category A test may be used in combination with a positive result from any other category test to confirm identification. If no Category A test is used, three separate tests must receive positive results, including at least two tests from Category B (59).

SWGDRUG guidelines call for validation before a technique can be used for identification in a forensic drug laboratory. These guidelines include determination of the lower limit of detection (LLOD), a measurement of selectivity of the test, and evaluation of the new technique against established analytical techniques to determine reproducibility (59). To validate TLC-DART for use in identifying the components of pharmaceutical preparations, standards were examined with the TLC-DART technique. The results of TLC-DART were compared to the results of the same standards from the currently accepted confirmation technique, GC-MS.

Standard procedure in VDFS laboratories used to examine pharmaceuticals begins with the physical examination of the markings on the tablets followed by TLC. The sample is then confirmed using GC-MS against the most likely drug standards (as anticipated from the physical examination and TLC screenings) (1–10). Many samples require extensive preparation prior to injection into the GC-MS system, with some requiring derivatization. Each injection into GC-MS takes an average of 5 min to run. For VDFS, a minimum of three injections are required for each unknown sample: the known standard(s) for each expected component of the sample, a blank, and the sample. This results in a total run time of about 30 min, including the time required between and before each run for instrument equilibration and sample introduction.

Modifying the analytical scheme for questioned pharmaceuticals using TLC (Category B) for separation and identifying the developed spots on the TLC plate directly with AccuTOF-DART™ (Category A) will streamline the process by eliminating the need for GC-MS, including the possible derivatization step. Additionally, all of the standards, blanks, and sample can be analyzed in one data file, with a new calibration in the same file. A TLC confirmation using the AccuTOF-DART™, including data reduction, takes no more than 10 min after the plate has been developed. As per Part III, Section 3.4 of the SWGDRUG Recommendations document, this procedure would meet the necessary requirements for confirmatory testing. It also maintains the minimal preparation before analysis that makes DART™ so appealing for forensic analyses.

Several studies have discussed the possibility of combining TLC with detection by AccuTOF-DART™. Previous research regarding the use of TLC plates in the gas stream of the DART™ ionization source shows that the technique is possible, but has not been attempted for the forensic analysis of drugs (42,55–57). The three pharmaceutical preparations (oxycodone with acetaminophen, hydrocodone with acetaminophen, and codeine with acetaminophen) that were successfully analyzed in this experiment show that the TLC-DART method of forensic drug analysis is both feasible and practical.

## Materials and Methods

TLC solvent tanks were obtained from the VDFS Controlled Substances Section. They consisted of rectangular glass containers, with tops, into which a developing solvent mix was placed. Based on previously established methodologies, the solvent systems chosen were the 9:1 (chloroform:methanol), 18:1 (ammonia-washed chloroform:methanol), and T-1 (100:1.5 methanol:ammonium hydroxide) bath systems. All solvents were HPLC grade with chloroform from EMD (Gibbstown, NJ), methanol from Fisher (Fairlawn, NJ), and ammonium hydroxide from VWR (Batavia, IL). The TLC detection sprays were $KMnO_4$; prepared by dissolving 1 g $KMnO_4$ (Mallinckrodt, St. Louis, MO) in 100 mL deionized water, acidified iodoplatinate; prepared by mixing 5 mL of 10% platinic chloride (Sigma Aldrich, St. Louis, MO) with 10 g potassium iodide (Mallinckrodt) in deionized water (the plate is oversprayed with 6 N hydrochloric acid [Fisher] to acidify) and ceric sulfate; prepared by dissolving 5 g ceric sulfate (Matheson, Coleman Bell, East Rutherford, NJ) in 500 mL of deionized water and 14 mL of sulfuric acid (VWR Scientific Products, West Chester, PA). The Analtech TLC plates (Newark, DE) were glass-backed, 10 × 20 cm, with silica gel GHLF layers of 250 μm. The silica gel layer contained a fluorescent dye for visualization under short-wave ultraviolet light. A SmartCut glass plate cutter (Camag, Wilmington, NC) was employed to create narrow plates, suitable for use in the AccuTOF-DART™ source. Pharmaceutical drug preparations (codeine/acetaminophen, hydrocodone/acetaminophen, and oxycodone/acetaminophen) and heterocodeine, neopine, and pseudocodeine were obtained from the standards collection of the VDFS Controlled Substances Section. Oxycodone HCl standard was obtained from Alltech, Inc. (State College, PA), and the hydrocodone bitartrate and codeine phosphate standards were obtained from Mallinckrodt.

The DART™ source employed helium as the ionization gas, and all measurements were taken in positive ionization mode. All measurements were taken with the AccuTOF™ ion guide peak voltage set at 600 V, the reflectron voltage at 990 V, orifice 1 V switching between 30 and 90 V, orifice 2 V at 5 V, ring lens voltage at 10 V, and an orifice 1 temperature of 80°C. The mass range was 101–600 daltons (Da). The DART™ ion source was used for all specimens with the helium gas flow rate set at 2.5 L/min, discharge needle set at 4000 V, discharge electrode set at 150 V, and grid electrode set at 250 V. Internal mass calibration was achieved using polyethylene glycol (PEG) 600 (Chem. Service, West Chester, PA), while drift compensation was set with the use of a cocaine lock mass solution (cocaine HCl from USP, Rockville, MD) within each DART™ data file. Instrument calibration was achieved daily by sampling methyl stearate (Eastman Organic Chemicals, Rochester, NY). Calibration was deemed acceptable if the measured mass of methyl stearate was within ±3 mDa of the calculated ($[M+H]^+$ of 299.2950 Da) mass. The AccuTOF™ was operated by JEOL MassCenter™ software (version 1.3.4m). These parameters represent the optimized settings for typical laboratory use of this instrument.

Glycerin (J. T. Baker Chemical Company, Phillipsburg, NJ) was used to aid in the desorption of the drug compounds from the silica gel plates (56). Several dilutions of glycerin solutions were prepared in methanol and sprayed on TLC plates. The plates were then subjected to DART™ analysis.

To determine the LLOD, oxycodone HCl, hydrocodone bitartrate, and codeine phosphate stock solutions were prepared in screw top glass tubes at 1 mg/mL of the basic drug. Dilutions in methanol were prepared at 0.7, 0.5, 0.3, 0.2, and 0.1 mg/mL concentrations. Sampling for all drugs was initially performed by the ''wanding'' method, dipping a glass melting point tube (Kimble Glass Company, Vineland, NJ) into each respective solution and holding it in the gas stream of the DART™. The dilutions were analyzed on the AccuTOF-DART™ using the function switching method, with centroided, background-subtracted spectra produced. An acceptance criterion was established such that the difference between the measured mass and the calculated mass of the protonated molecule was required to be within ±5 mDa after internal mass calibration and drift compensation. To test desorption and

ionization from the TLC plates, 5 μL of each of the prepared dilutions was spotted in a vertical array onto each TLC plate with one spot for each dilution. Eight TLC plate replicates were used for each drug dilution series, a total of 24 plates. Chromatography was not performed on these plates. The spots were visualized under short-wave UV light and marked for location with a no. 2 lead pencil. Using the plate cutter, the plates were vertically incised and broken, such that the spots were bisected. This yielded plates that were *c*. 12 mm in width. The incised plates were then sprayed with a 1:25 solution of glycerin in methanol and allowed to dry. This resulted in 16 half-plates being available for each drug. Each half-plate was carefully held in the edge of the DART™ gas stream such that the drugs in the marked areas would desorb off the plate, ionize, and be drawn into the mass spectrometer. No special holder or other modifications to the instrument were needed. The gas heater temperature was manually changed such that at least three half-plates, representing each drug, were examined at each of five temperatures of 275, 300, 325, 350, and 375°C.

An additional series of dilutions was then spotted onto another 24 TLC plates. Eight TLC plates were used for each drug dilution series. The plates were prepared as before. After preparation, each plate was held in the DART™ gas stream at the chosen temperature, and each spot was analyzed to establish the LLOD of the DART™ at the concentration just above where the acceptance criterion was no longer met.

Methanol solutions of the empirical formula isomers of codeine and hydrocodone (heterocodeine, neopine, and pseudocodeine) were sampled both by the "wanding" method as well as by the previously described TLC-DART sampling method to test the selectivity of the technique. The spectra at each voltage for each of the drugs were compared against the spectra obtained from the hydrocodone and codeine standards.

The pharmaceutical preparations were analyzed with the new TLC-DART methodology with the amount of tablet and solvent used determined by the composition of the tablets and the determination of the individual drug's LLOD. Each codeine/acetaminophen tablet contained 30 mg of codeine and 500 mg of acetaminophen. Based on the results of the LLOD determination, *c*. 1/32 of a tablet (0.94 mg codeine) was placed in 3 mL of ammonia-saturated chloroform to achieve the minimum concentration (0.3 mg/mL). A 1/32 of the codeine/acetaminophen tablet was measured by weight. Each hydrocodone/acetaminophen tablet contained 5 mg of hydrocodone and 500 mg of acetaminophen. Approximately 1/4 of a tablet (1.25 mg hydrocodone) was placed in 1.78 mL of ammonia-saturated chloroform to achieve the minimum concentration (0.7 mg/mL). Each oxycodone/acetaminophen tablet contained 5 mg of oxycodone and 325 mg of acetaminophen. Approximately 1/4 of a tablet (1.25 mg oxycodone) was placed in 2.5 mL of ammonia-saturated chloroform to achieve the minimum concentration required (0.5 mg/mL). Each tablet fragment was crushed with a mortar and pestle, for more thorough dissolution, and placed in a 5-mL vial before adding the solvent. A 5-μL glass capillary pipette was used to spot each solution onto six TLC plates. Two plates were placed in each of the three TLC baths. Once the solvent front neared the top of each plate, the plate was removed, dried, and then examined under short-wave UV. Any spots seen under UV were marked for location with a no. 2 lead pencil. One plate from each bath was then sprayed with $KMnO_4$ to visualize the acetaminophen; acidified iodoplatinate was sprayed to visualize the codeine, oxycodone, and hydrocodone with ceric sulfate spray to enhance any faint chromophores. The second plate from each bath was vertically incised, such that all of the component spots were bisected. The incised plates were then sprayed with the 1:25 solution of glycerin in methanol and allowed to dry. Each plate was held in the DART™ gas stream after the standard calibration of the run was completed by use of PEG-600 and the cocaine lock mass solution. Each spot on the plate was analyzed in the gas stream such that all separated compounds on the plate were examined.

For the reproducibility study, codeine/acetaminophen was prepared with a concentration about 0.3 mg/mL of codeine, oxycodone/acetaminophen with a concentration about 0.5 mg/mL of oxycodone, and hydrocodone/acetaminophen with a concentration about 0.7 mg/mL of hydrocodone. A reproducibility criterion was established such that the ionization seen for the $[M+H]^+$ for each drug, at the orifice 1 30 V setting, produced peaks that fell within ±5 mDa of the expected protonated molecule. Reproducibility of the fragmentation was checked with the orifice 1 90 V spectra. Replicate runs of 10 TLC plates each were conducted on three separate days for each of the three pharmaceutical preparations, for a total of 30 plates for each preparation, 90 total plates. The plates were spotted, and TLC was performed using the 9:1 TLC bath. Plate preparation was then performed as previously described. The left half-plate from each incised pair was held in the DART™ stream and analyzed, under the previously described parameters.

A time comparison of the TLC-DART analytical scheme was made against the conventional analysis method. As per VDFS procedure (10), the acetaminophen standard, codeine standard, a blank, and the 0.3 mg/mL codeine/acetaminophen sample were analyzed in separate runs on the Agilent (Little Falls, DE) 6890-5973N GC-MS instrument with parameters set at a 1-μL injection, 9-min run time, and temperature ramping from 50 to 290°C at 30° per minute on an HP-5ms 15 m × 0.25 mm × 0.25 μm column (J&W Scientific, Santa Clara, CA), with helium as the carrier gas with a flow rate of 1.8 mL/min. The injection port and transfer line temperatures were held at 290°C. The mass spectrometer was scanned over a range of 400–14 Da.

## Results and Discussion

To determine the ideal amount of glycerin needed to allow the drugs to desorb from the TLC plates, several concentrations of glycerin in methanol solutions were prepared: 1:100, 1:75, 1:50, and 1:25. These concentrations were tested for efficacy at several DART™ gas temperatures. A glycerin concentration of 1:25 and temperature of 325°C were chosen based on the ability to desorb the drugs from the TLC plate without causing excessive loss of the protonated molecule with these parameters and to meet the acceptance criterion where the protonated molecule was required to be within ±5 mDa of the calculated mass. This glycerin concentration and temperature setting was the best combination for all of the drugs tested.

The LLOD acceptance criterion, for the drug standards placed on the TLC plates and analyzed on the AccuTOF-DART™, failed at 0.2 mg/mL for codeine and 0.3 mg/mL for oxycodone and hydrocodone. The acceptance criterion was met at 0.3 mg/mL for codeine and at 0.5 mg/mL for oxycodone and hydrocodone; therefore, the LLOD for the AccuTOF-DART™ instrument was set at 0.3 mg/mL for oxycodone and at 0.5 mg/mL for codeine and hydrocodone.

When examining the empirical formula isomers for selectivity, pseudocodeine, codeine, and hydrocodone reacted with the iodoplatinate reagent to form purple chromophores, while neopine and heterocodeine reacted with iodoplatinate spray to form blue chromophores. The addition of ceric sulfate caused the purple chromophores to turn brown and the blue chromophores to darken. The co-elutions of the standards are depicted in Table 1. As indicated,

TABLE 1—*Thin layer chromatography co-elutions of the empirical formula isomers of codeine in the three baths.*

| | $CHCl_3$:MeOH 9:1 | $NH_4$-saturated $CHCl_3$:MeOH 18:1 | MeOH:$NH_4OH$ (100:1.5) T-1 |
|---|---|---|---|
| Codeine | Heterocodeine | Hydrocodone, Heterocodeine | Neopine, Heterocodeine |
| Hydrocodone | Neopine | Codeine, Heterocodeine | None |
| Neopine | Hydrocodone | None | Codeine, Heterocodeine |
| Pseudocodeine | None | None | None |
| Heterocodeine | Codeine | Codeine, Hydrocodone | Codeine, Neopine |

the codeine and heterocodeine isomers are indistinguishable based solely on TLC retention. When combined with DART™, all of the isomers have clear differences. At a 90 V orifice 1 value and 325°C gas stream on the DART™, a peak was seen for the protonated molecule within ±5 mDa of the expected $[M+H]^+$ of 300.1600 Da as well as fragmentation peaks consistent and unique to each standard. The peaks seen with the TLC-DART analysis were identical to the peaks seen with the ''wanding'' method of DART™ analysis. Figure 1 shows the orifice 1 90 V spectra of the five isomers.

Although there were co-elution issues, overlap of spots, and similarities in retention factors, each of the drugs was differentiable owing to the unique combination of characteristics and spectra produced. Because two TLC baths are typically used to increase specificity, only heterocodeine and codeine could be confused based solely on retention factor. Once the visualization spray reagents are applied, the difference in coloration seen with the iodoplatinate spray allows for immediate distinction of the two drugs despite their co-elution. It is also important to note that a search of available literature showed no pharmaceutical preparations currently

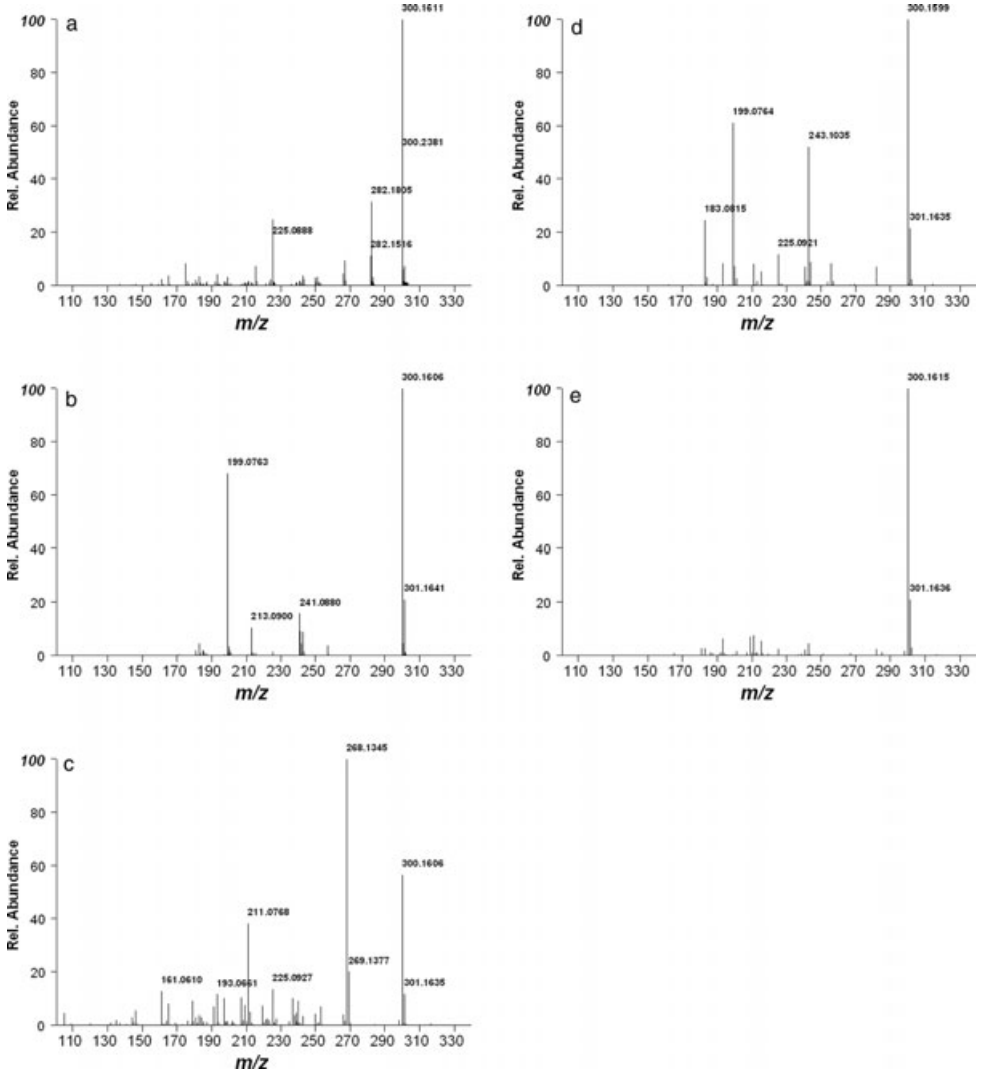


FIG. 1—*(a) Codeine, DART™ spectrum at orifice 1 90 V. (b) Hydrocodone, DART™ spectrum at orifice 1 90 V. (c) Heterocodeine, DART™ spectrum at orifice 1 90 V. (d) Neopine, DART™ spectrum at orifice 1 90 V. (e) Pseudocodeine, DART™ spectrum at orifice 1 90 V.*

marketed in the U.S. that contain any of the studied isomers of codeine and hydrocodone (60,61).

The components of the pharmaceutical preparations, at concentrations equivalent to the LLOD, were separated using two TLC plates for each drug in each of the three TLC baths, six total plates. Minimal to no separation of any of the pharmaceutical preparations was seen with either the T1 or 18:1 baths, with the sprays being able to detect only the acetaminophen on the plates from the T1 bath. The 9:1 bath gave the best separation of all three preparations; therefore, it was chosen as the bath to be used for the TLC-DART analysis.

Some difficulty was encountered in the desorption of the hydrocodone from the pharmaceutical preparation after TLC. When one of the plates that had been spotted with the 0.5 mg/mL hydrocodone/acetaminophen preparation solution was sprayed, the acetaminophen was at a detectable level but no hydrocodone was detected by the visualization spray reagents. When analyzing the plate at orifice 1 30 V, acceptable spectral results were obtained for both the acetaminophen and hydrocodone. However, the abundance of the $[M+H]^+$ for hydrocodone was so weak that the orifice 1 90 V spectrum yielded limited fragmentation ions for identification. These results would be unacceptable for the identification of the hydrocodone. The hydrocodone concentration was increased to 0.7 mg/mL, and two additional plates were run in the 9:1 (chloroform:methanol) TLC bath. With this concentration, visible chromophores for both the acetaminophen and hydrocodone were seen, and the orifice 1 90 V spectra yielded consistent fragmentation between runs.

One plate for each of the other pharmaceutical preparations was then sprayed with the visualization spray reagents. The $KMnO_4$ spray gave a visible yellow chromophore for the acetaminophen spot on both of the sprayed plates. The plates were then sprayed with acidified iodoplatinate that caused the yellow chromophore to fade and the codeine and oxycodone spots to give visible purple chromophores. The addition of ceric sulfate spray caused the purple chromophores to turn brown. These chromophores appeared as expected and indicated enough of the drug was present for preliminary screening to be successful. The LLOD for TLC-DART was reset at 0.7 mg/mL for hydrocodone and remained at the DART™ instrument LLOD settings of 0.3 mg/mL for oxycodone and 0.5 mg/mL for codeine.

Each of the unsprayed plates was analyzed using TLC-DART. At orifice 1 30 V, all of the plates showed the expected $[M+H]^+$ peak within ±5 mDa of 300.1600 Da for hydrocodone and codeine and within ±5 mDa of 316.1549 Da for oxycodone. The expected acetaminophen peak was also within ±5 mDa of 152.0711 Da. At orifice 1 90 V, the protonated molecule peak was still present for codeine and hydrocodone, with consistent fragmentation unique to each drug. The oxycodone $[M+H]^+$ peak was gone, but consistent fragmentation was present. The $[M+H]^+$ peak for acetaminophen was also gone, but the expected 110.0605-Da fragment was present. Figure 2 compares the orifice 1 90 V spectra of the three preparations after TLC. Acetaminophen peaks were seen in the spectra of all three of the pharmaceuticals owing to tailing of the acetaminophen; however, none of the other drugs were detected in the acetaminophen spots. The tailing of the acetaminophen was because of the high concentration (17–100 times the concentration of the drugs) present in the spotted samples, despite efforts to reduce the amount of acetaminophen extracted from the tablet material with the use of ammonia-saturated chloroform.

TLC-DART samples were tested for reproducibility over the course of a week. The results for all three 10-plate runs of the

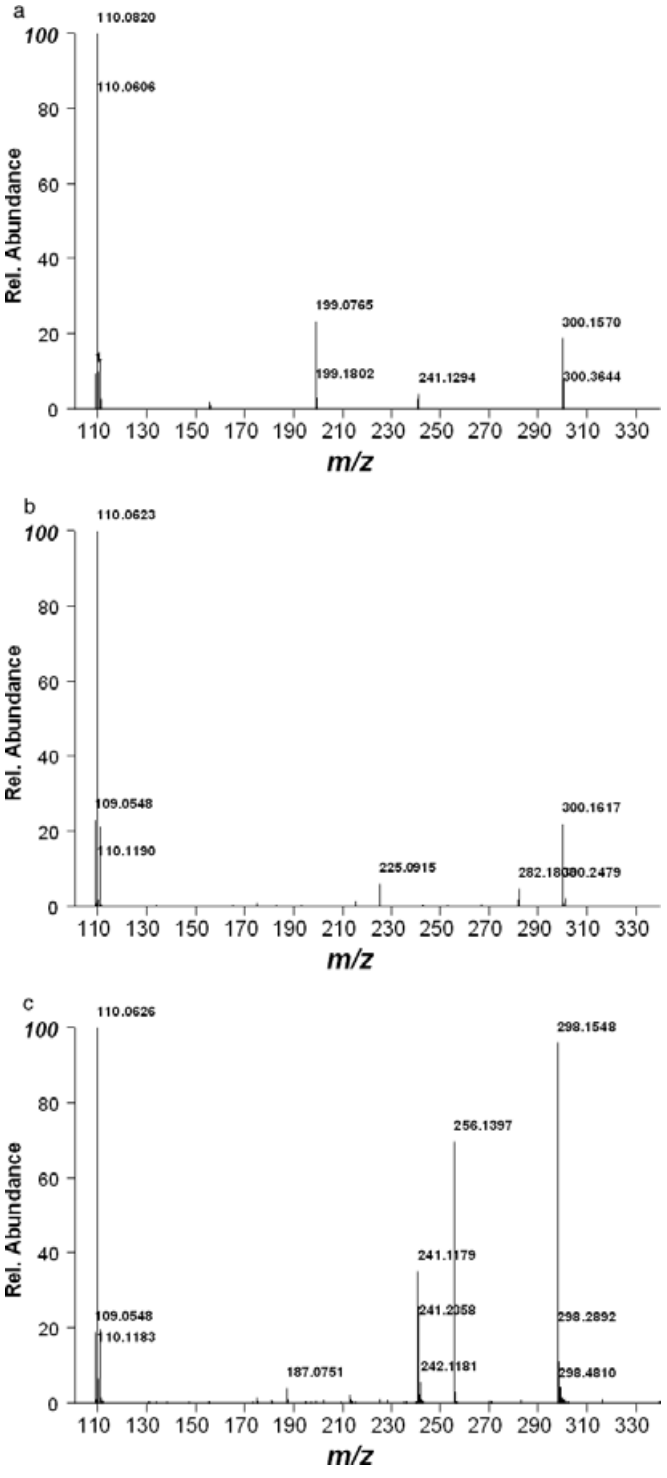


FIG. 2—*(a) Hydrocodone/acetaminophen, DART™ spectrum at orifice 1 90 V. (b) Codeine/acetaminophen, DART™ spectrum at orifice 1 90 V. (c) Oxycodone/acetaminophen, DART™ spectrum at orifice 1 90 V.*

reproducibility test were consistent with the results seen in the other validation tests. All peaks were within ±5 mDa of the major expected peak(s) for each component. The consistent results indicate reproducibility of the entire analytical scheme over the course of several days.

The amount of time needed for the identification of a pharmaceutical preparation was also examined. The TLC-DART analysis took <10 min to complete once TLC separation was completed.

The GC-MS analysis took 66 min to complete once the analysis was begun.

The use of TLC-DART eliminates the need for GC prior to structural identification, which saves a significant amount of time. The minimum concentration for each pharmaceutical preparation was used for all replicates to show that the results at the LLOD are accurate and reproducible. TLC was found to be the limiting factor for the further analysis via either DART™ or GC-MS. If the compounds were detected on the TLC plate, then, in this study, the compounds were easily detected with the confirmation techniques. The technique is simple to perform and extremely fast, compared to conventional confirmation methods. SWGDRUG guidelines state that only one other positive result is needed in addition to a positive Category A test. This analytical scheme utilized pharmaceutical identifiers (Category B), TLC (Category B), and AccuTOF-DART™ (Category A), thus exceeding the SWGDRUG guidelines. Future work in this direction would include validating this analytical scheme for identifying other pharmaceutical preparations and developing a better chromatographic system to ensure the full separation of the components prior to identification by AccuTOF-DART™.

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