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TECHNICAL NOTE

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Validation of the Direct Analysis in Real Time Source for Use in Forensic Drug Screening

ABSTRACT: The Direct Analysis in Real Time (DART) ion source is a relatively new mass spectrometry technique that is seeing widespread use in chemical analyses world-wide. DART studies include such diverse topics as analysis of flavors and fragrances, melamine in contaminated dog food, differentiation of writing inks, characterization of solid counterfeit drugs, and as a detector for planar chromatography. Validation of this new technique for the rapid screening of forensic evidence for drugs of abuse, utilizing the DART source coupled to an accurate mass time-of-flight mass spectrometer, was conducted. The study consisted of the determination of the lower limit of detection for the method, determination of selectivity and a comparison of this technique to established analytical protocols. Examples of DART spectra are included. The results of this study have allowed the Virginia Department of Forensic Science to incorporate this new technique into their analysis scheme for the screening of solid dosage forms of drugs of abuse.

KEYWORDS: forensic science, controlled substances, Direct Analysis in Real Time, mass spectrometry, validation, drugs of abuse

Direct Analysis in Real Time (DART) is a relatively new atmospheric pressure ionization technique, developed by IonSense, Inc. (Saugus, MA), that is beginning to see widespread application in many chemical analysis settings, including forensic analysis (1–9). The DART source can analyze solids, liquids, and gases merely by placing the desired test material into a heated gas flowing through the sampling area. Ionization occurs on the surface of the sampling medium. Coupling of this ion source to an accurate mass time-of-flight mass spectrometer gives quick and simple analyses with little to no sample preparation. While ionization can be done in both positive and negative mode, the large majority of drugs of abuse give usable spectra in positive ion mode. Ionization in positive ion mode is accomplished by charging a heated helium gas stream, forming metastable helium ions which react with ambient water vapor, producing hydronium ions which subsequently react with the sample molecules to induce ionization. The mechanisms of positive and negative ion production with the DART were previously discussed by Cody et al. (10).

In February 2007, the Virginia Department of Forensic Science put a DART source, coupled with a JEOL, Inc. (Peabody, MA) AccuTOF™ accurate mass time-of-flight mass spectrometer, on line for development as a screening and confirmation tool in the analysis of drugs of abuse. Screening and confirmation of solid dosage forms of drugs, by the Department, is done by color tests, thin layer chromatography, and time-consuming temperature-programmed runs on a gas chromatograph-mass spectrometer. Because the DART ionization technique was relatively new and untested in a forensic setting, a validation study needed to be carried out to determine the efficacy of the technique, with respect to these established analytical protocols. While the DART source produces spectra similar to other atmospheric pressure ionization techniques, especially electrospray ionization, differences would be expected due to the manner in which ions are produced.

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In general, DART ionization produces spectra with a characteristic peak at the protonated or deprotonated molecule. These ions are measured at their exact mass in the AccuTOF™ mass spectrometer. Elemental composition calculations, based on empirical formulas, can be performed on these ions to determine whether they fall within a specified error, usually measured in millimass units (mmu), of a known compound.

While accurate-mass spectra have an inherent specificity, confirmation is difficult if the possibility of an isomer exists. In order to be used as a confirmation step in a drug analysis scheme, it is important that the DART spectra have characteristic peaks that can be used to confirm the presence of the suspected drug compounds. By varying the voltage on the orifice 1 of the AccuTOF™, spectra with extensive fragmentation can be produced by in-source collision-induced dissociation (CID). Simultaneous collection of data at different orifice 1 voltages can be accomplished by utilizing the "function switching" mode of the AccuTOF™ operating software. Higher orifice 1 voltages generally result in more fragmentation and therefore more characteristic ions being produced. The combination of accurate mass measurement of the protonated molecule and characteristic CID fragmentation allows for the production of spectra that can be used as part of an identification scheme for drugs of abuse.

According to the Scientific Working Group for Drug Analysis (SWGDrug), there are three steps to the validation of a new technique for use in nonquantitative drug analysis schemes. These include determination of the lower limit of detection (LLOD) for the instrument, measurement of selectivity of the test and an evaluation of the new technique against established analytical techniques to determine reproducibility (11). The present study determined a LLOD using seven drug compounds representing different classes of drugs. Selectivity was determined by examining AccuTOF-DART spectra of compounds having the same empirical formula. The study then employed the use of previously confirmed drug case samples, subsequently run on the AccuTOF-DART system, to determine the reproducibility of this new technique as a screening and/or confirmatory step in a drug analysis scheme. Over 550 drug

case samples were blind tested on the AccuTOF-DART system and the results compared with those obtained by the traditional drug analysis scheme (color tests, thin-layer chromatography and gas chromatography-mass spectrometry [GCMS]) employed in this laboratory. We present here the results of these studies as they were used to justify the incorporation of the AccuTOF-DART system into the drug analysis scheme in the Virginia Department of Forensic Science.

Materials and Methods

Experiments were carried out using the DART ion source coupled to a JEOL AccuTOF™ mass spectrometer (JMS-100LC) operated in positive-ion mode. This system was controlled by "Mass Center" software (version 1.3.4 m; JEOL, Inc.). The AccuTOF™ was tuned by infusion of reserpine (Sigma-Aldrich, Inc., St. Louis, MO) through an electrospray ion source to meet the manufacturer's recommendations for resolution (>6000 FWHM). These tune settings were then utilized for all AccuTOF-DART analyses. Daily calibration was checked by sampling a methanol solution of methyl stearate (Eastman Chemicals, Rochester, NY) (2 mg/mL). In order to pass daily calibration, the measured mass of the $[M+H]^+$ of methyl stearate was required to be within ±3.0 mmu of the calculated mass for this ion (299.2950 Da). All measurements were taken with the ion guide peak voltage at 600 V, reflectron voltage of 910 V, orifice 1 voltage variable, orifice 2 voltage 5 V, ring lens voltage 6 V, and an orifice 1 temperature of 80°C. The mass range was 66–600 Da. The DART ion source was used for all experiments with the helium gas flow rate at 4.0 L/min, gas heater temperature of 275°C, discharge electrode needle at 4000 V, electrode 1 at 150 V, and electrode 2 at 250 V. These settings were chosen based on instrument tune values and to provide the optimum response for drugs of abuse. Internal mass calibration was achieved using a dilute solution of polyethylene glycol (PEG) 600 (Chem. Service, West Chester, PA) in methanol sampled within each data file. The mass calibration generated was applied to all data, corresponding to each specimen sampled within that data file. Data files typically contained the data from several specimens.

The AccuTOF™ was operated in function switching mode for all experiments. In this mode, tune files were established where the only difference was in the orifice 1 voltage. Orifice 1 voltages chosen were 20, 30, 60, and 90 V. The function switching method was set up to switch from each orifice 1 voltage, consecutively, every 0.25 sec. This created four "functions" of data which could then be examined separately.

Drug standards for use in the LLOD and selectivity experiments were alprazolam, lysergic acid diethylamide (LSD), lysergic acid methylpropylamide (LAMPA), psilocin, and heroin from Alltech, Inc. (State College, PA); hydromorphone from Bilhuber-Knoll Corp. (Orange, NJ); bufotenine from Cerilliant (Round Rock, TX); methamphetamine from K & K Labs (Jamaica, NY); morphine from Mallinckrodt (St. Louis, MO); butalbital, cocaine, scopolamine, phentermine, and trazodone from Sigma-Aldrich, Inc.; and testosterone propionate from USP (Rockville, MD). All solvents used were HPLC grade (Fisher Scientific, Fair Lawn, NJ).

All standards and specimens were dissolved in an appropriate solvent. Sampling was done by dipping the closed end of cleaned glass melting point tubes (Kimble Glass Company, Vineland, NJ) into the specimen vials and holding the tube in the DART gas stream, a process we refer to as "wanding." Each individual specimen or standard in a respective data file was "wanded" two times with the more intense signal being used for data analysis. Analysis of data was accomplished by creating averaged, background subtracted, centroided spectra that were subsequently calibrated to a PEG + H mass reference table. Comparison of measured $[M+H]^+$ and CID spectra to calculated masses and CID library spectra for each specimen was done in the SEARCHFROMLIST software in MSTools (ChemSW, Inc., Fairfield, CA).

Limit of Detection Study

The LLOD for the AccuTOF-DART system was determined by sampling seven drugs, representing different drug classes. The drugs used were alprazolam, butalbital, cocaine, heroin, methamphetamine, testosterone propionate, and trazodone. All drugs were primary standards. Stock solutions were prepared at 1 mg/mL for each standard. Dilutions in methanol of each standard were prepared at 0.5, 0.1, 0.05, 0.03, and 0.01 mg/mL. These were run on the AccuTOF-DART in function switching mode. Spectra at orifice 1 voltage of 20 V were examined for each drug at each dilution. An acceptance criterion was established where the measured mass of the $[M+H]^+$ for each drug was required to fall within the instrument manufacturer's specification of ±5.0 mmu of the calculated $[M+H]^+$ mass for that drug. The LLOD was determined to be the dilution level just above where the acceptance criterion failed. Ten subsequent AccuTOF-DART analyses of each drug at the LLOD were done to demonstrate repeatability.

Selectivity Study

Several combinations of drugs were chosen, based on their having the same empirical formula, and therefore the same calculated $[M+H]^+$, to determine whether the AccuTOF-DART could differentiate them. These drug combinations included methamphetamine/phentermine, cocaine/scopolamine, hydromorphone/morphine, psilocin/bufotenine, and LSD/LAMPA. One milligram per milliliter (methanol) standards were prepared and sampled using the AccuTOF-DART in function switching mode and their spectra at various orifice 1 voltages compared.

Comparison of AccuTOF-DART with GCMS

Sample specimens were collected from examiners after they had completed their normal drug analyses, which included confirmation via GCMS. All specimens were submitted for AccuTOF-DART analysis in autosampler vials, in various solvents including methanol, ammonia saturated chloroform and hexane. The autosampler vials were labeled with barcodes which allowed reference back to the data collected on the GCMS. No other information about the contents of the autosampler vials was submitted prior to AccuTOF-DART analysis, allowing all specimens to be run "blind." Run times were generally 3.5 min with typically eight specimens sampled within one data file. The orifice 1 20 V, PEG600-calibrated spectra were prepared and searched against a table of neutral masses of over 480 drugs, using the SEARCHFROMLIST program, based on the $[M+H]^+$ for all of the mass peaks found. Drugs were considered "identified" (or positively screened) if a mass peak was found within ±5.0 mmu of the calculated mass for that drug. From the list of drugs found, the drug with the highest Controlled Substances Act (CSA) Schedule was determined. GCMS data was analyzed for each specimen to determine what drugs were found by that technique, especially the drug of highest CSA Schedule. The names of all drugs found with the AccuTOF-DART and GCMS were entered into a database for comparison.

Results and Discussion

LLOD Study

The acceptance criterion began to fail at the 0.03 mg/mL level, where the measured mass of the protonated molecule for two of the seven drugs (heroin and alprazolam) fell outside the ±5.0 mmu range. At 0.01 mg/mL, five of the seven drugs (heroin, alprazolam, cocaine, testosterone propionate, and trazodone) failed. This placed the LLOD for the AccuTOF-DART system using function switching at 0.05 mg/mL. Standard deviations of the differences between calculated and measured mass for the 10 individual runs of each drug at 0.05 mg/mL ranged from 0.8 to 1.2 mmu. These were well within the ±5.0 mmu criterion. It is important to note that, while the function switching method gives rise to a tremendous amount of data for interpretation, sensitivity is somewhat sacrificed by "splitting" the ionization between the four different functions. Reducing the number of functions scanned would certainly lower the detection limit.

Selectivity Study

Figures 1–7 show spectra for each of the drug combinations at various orifice 1 voltages. In most instances, demonstrable differences in the spectra can be seen which would enable a chemist to differentiate the compounds from one another. In the case of psilocin/bufotenine and LSD/LAMPA, however, differences in the AccuTOF-DART spectra could not be seen at the orifice 1 voltages used in this study. Even at high orifice 1 voltages, little to no difference is seen in the spectra of these pairs of compounds. With no chromatographic method preceding the ion source, differentiating these compounds is impossible under the conditions employed in this study. This drawback of the technology hinders its usefulness as a confirmatory tool. When used for screening purposes, though, the AccuTOF-DART would provide valuable information about the constituents present in an unknown sample thereby narrowing the scope of further analysis. As with other techniques employed by forensic drug chemists, further confirmation by other analytical techniques would be required.

A preliminary analysis of LSD and LAMPA shows promise that at higher orifice 1 voltages differences can be seen in the spectra of these two compounds. While the screening method reported here

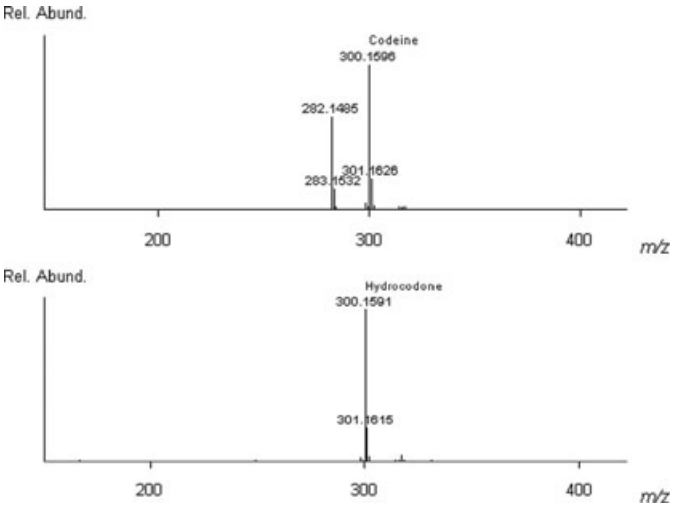

FIG. 2—*Codeine and hydrocodone spectra at orifice 1 30 V.*

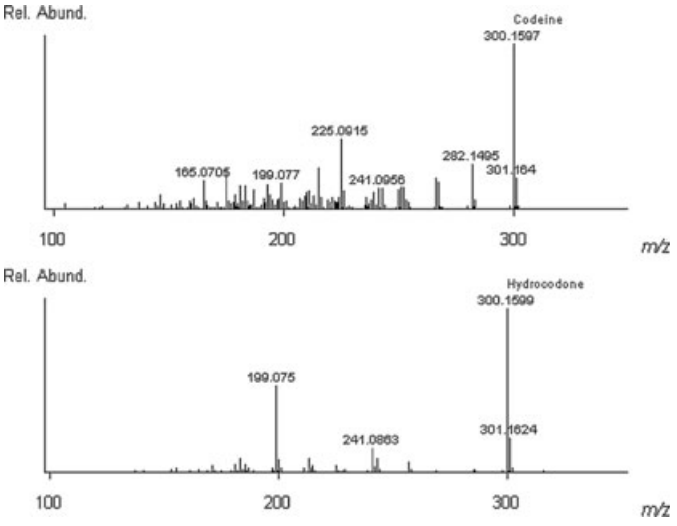

FIG. 3—*Codeine and hydrocodone spectra at orifice 1 90 V.*

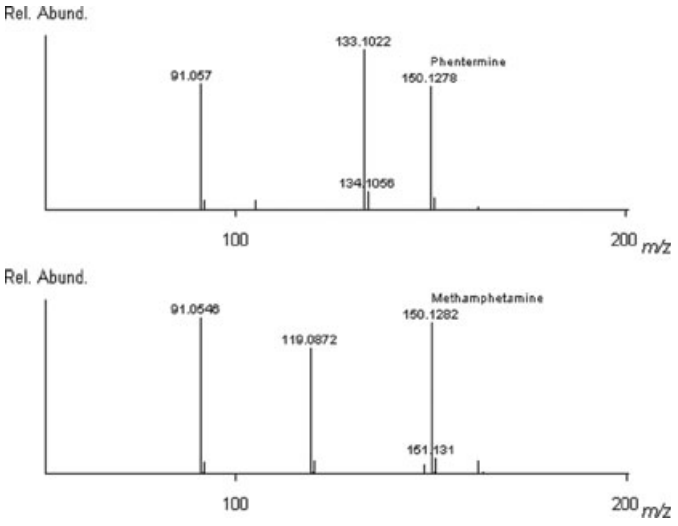

FIG. 1—*Phentermine and methamphetamine spectra at orifice 1 30 V.*

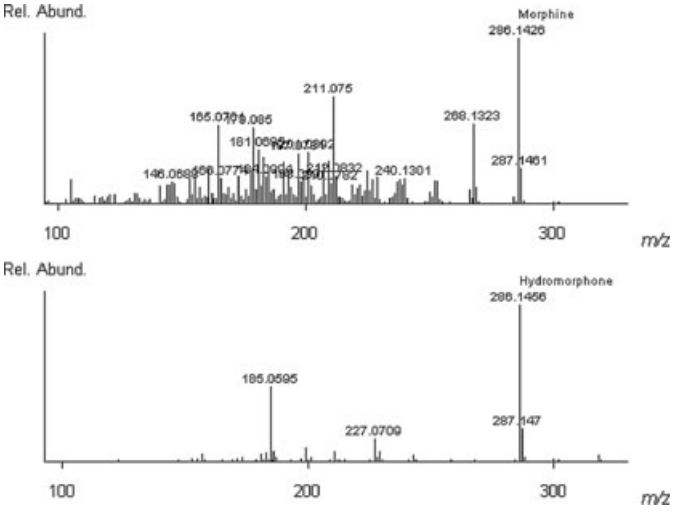

FIG. 4—*Morphine and hydromorphone spectra at orifice 1 90 V.*

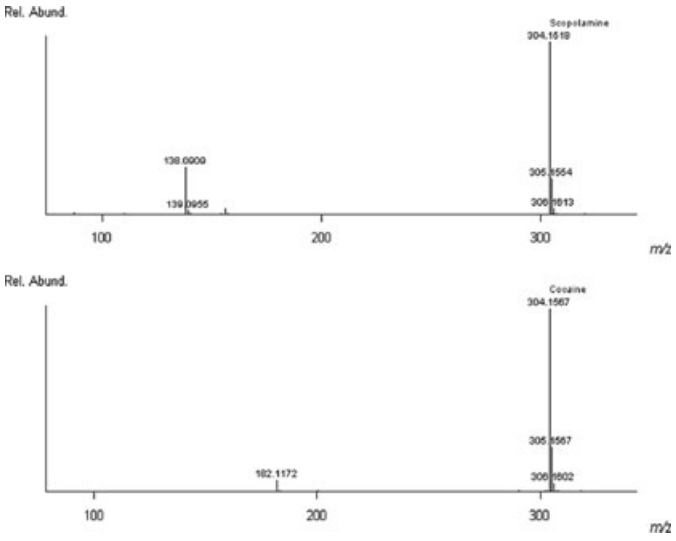

FIG. 5—*Scopolamine and cocaine spectra at orifice 1 30 V.*

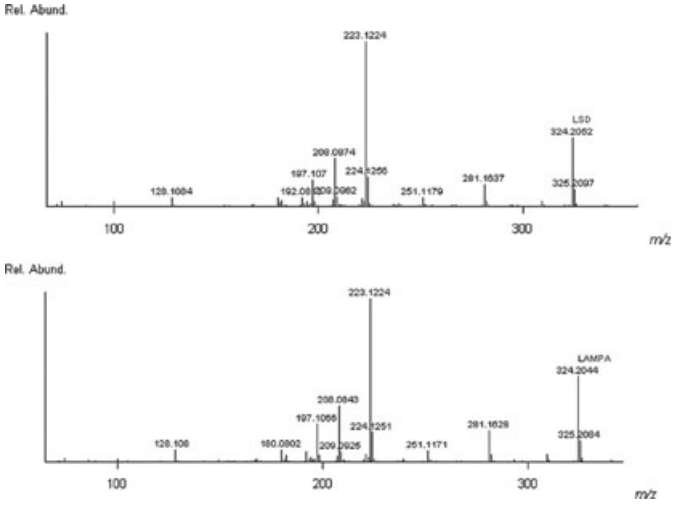

FIG. 6—*Lysergic acid diethylamide (LSD) and lysergic acid methylpropylamide (LAMPA) spectra at orifice 1 90 V.*

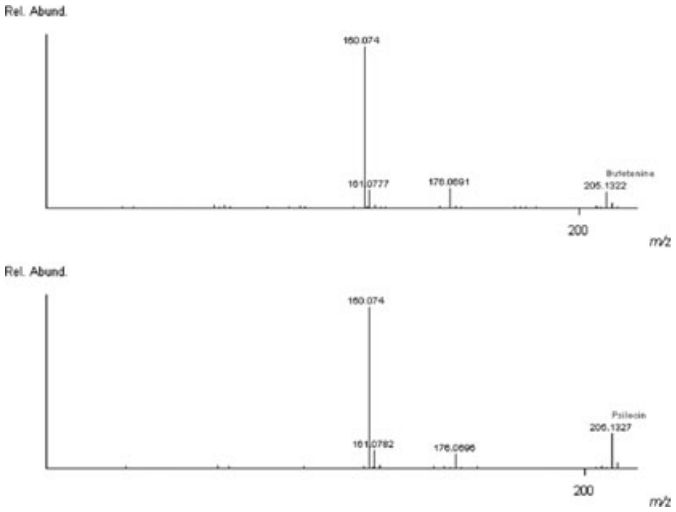

FIG. 7—*Bufotenine and psilocin spectra at orifice 1 60 V.*

would only be able to narrow the identity of an unknown to these two drugs, employing another DART method with a higher orifice 1 voltage could serve to distinguish them, thereby allowing a more definitive identification of the drug present. Further study will need to be done to determine the optimum orifice 1 voltage that will allow this differentiation and to determine the reproducibility of the fragmentation at this higher orifice 1 voltage.

Comparison with GCMS Data

Five-hundred and fifty-three case specimens were run on the AccuTOF-DART. The detected drugs of highest CSA Schedule, as determined by AccuTOF-DART and GCMS, were compared. With the exception of one specimen, the AccuTOF-DART and GCMS results agreed on the highest Scheduled drug for all 553 specimens.

Since there is no prior chromatography, AccuTOF-DART spectra show peaks that represent all the detectable drugs and ionized diluents present in the specimen. Figure 8 depicts a typical mixture spectrum obtained from the AccuTOF-DART, showing peaks for heroin, cocaine, procaine, caffeine, and 6-monoacetylmorphine. Note that the masses labeled by the peak search algorithm as "fluoxetine" (310.1429 Da) and "apomorphine" (268.1335 Da), in Fig. 8, are actually fragment ions of heroin, $[M\text{-}C_2H_4O_2]^+$ and $[M - (C_2H_4O_2 + C_2H_2O)]^+$, respectively.

The one exception noted above occurred with a specimen that contained heroin and, along with several other components, the unusual cutting material yohimbine (Fig. 9*a*). While yohimbine gives a $[M+H]^+$ at 355.2001 Da, an additional (unidentified)

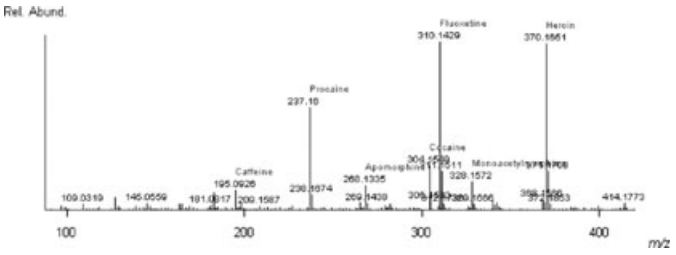

FIG. 8—*Typical AccuTOF-DART mixture spectrum.*

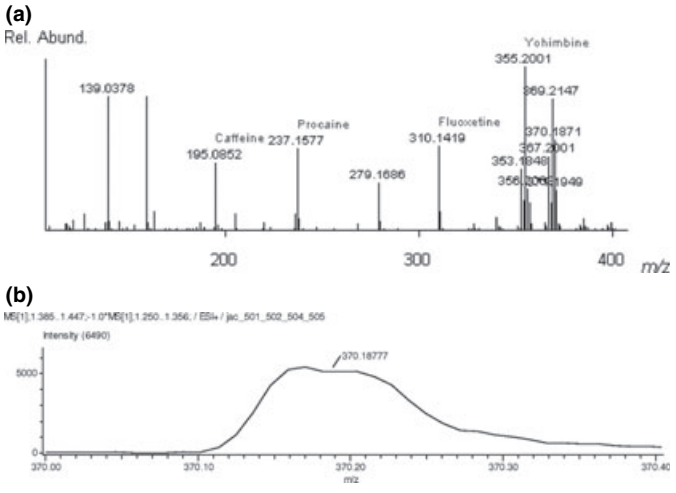

FIG. 9—*(a) AccuTOF-DART spectrum of heroin-containing sample with interference at mass 370 Da. (b) Profile spectrum at mass 370 Da showing doublet of two overlapping mass peaks.*

compound in this sample gave a large $[M+H]^+$ ion at 369.2147 Da. The ^{13}C isotope for this component peak appears at a mass of 370.21 Da. Heroin has its $[M+H]^+$ at 370.1654 Da. With the unknown component's isotope ion appearing so close to the heroin $[M+H]^+$, the MASS CENTER software was unable to distinguish two separate masses in the centroided peak that was labeled 370.1871 Da in Fig. 9*a*. As can be seen in Fig. 9*b*, when looking at the profile (continuous data collection) spectrum at this mass, a "doublet" of peaks can be seen, the more intense of which is centered at the heroin $[M+H]^+$ of 369.1654 Da, leading to the indication that heroin is indeed present in the specimen. The peak at 310.1419 Da further confirms the presence of heroin in this sample.

With respect to using the AccuTOF-DART as a screening tool, the spectra generated with this instrument were much richer in detail than the information typically obtained from the GCMS instruments. The AccuTOF-DART was able to simultaneously detect many more compounds than the GCMS since the DART ionization is of the entire mixture and is not encumbered by the limitations caused by temperature and time constraints on the GCMS instrument runs. GCMS runs are typically limited to specific oven temperature ranges in order to reduce the amount of time per run and thereby increase sample through-put. Because of this, some minor compounds detected on the AccuTOF-DART were not seen at all in the GCMS data.

While in general the AccuTOF-DART gives easily interpretable spectra, the heroin case example (Fig. 9*a*) serves as a warning that data obtained from this instrument needs to be examined very carefully. Single component samples are straightforward to interpret but, when dealing with multicomponent mixtures, the spectra produced can become extremely difficult to interpret, especially at higher orifice 1 voltages. Generation of single component library spectra allows for easier interpretation although differences in spectra can arise when mixtures of compounds with widely varying proton affinities are ionized. Because of this, compounds that give rise to DART fragment ions at low orifice 1 voltages give spectra with differing fragment ion intensities when combined with other drugs.

An example of the effect of proton affinities on DART ionization can be seen when mixing codeine with acetaminophen, as found in many pharmaceutical preparations. In Fig. 10, the large fragment ion ($[M-OH]^+$) produced by codeine at 282.1470 Da, by itself, becomes much smaller as the ratio of acetaminophen is increased in the mixture. The amount of energy available for fragmentation of the protonated codeine molecule is diminished as some of this energy is used to ionize the acetaminophen molecules. This is a classic example of a soft ionization process and demonstrates one of the difficulties in interpreting DART spectra.

The present study validates the use of the AccuTOF-DART for use as a screening tool in the general scheme of drug analysis. Due to some of the drawbacks pointed out here, more work will need to be done to move this instrumental technique towards routine use as a confirmation tool. Investigating further the ability of this system to differentiate isomeric compounds is on-going in this laboratory. It is envisioned that when used in conjunction with other characterizing techniques, such as the physical identification of pharmaceutical tablet and capsule markings, the AccuTOF-DART will soon be employed as a confirmation tool for drugs of abuse.

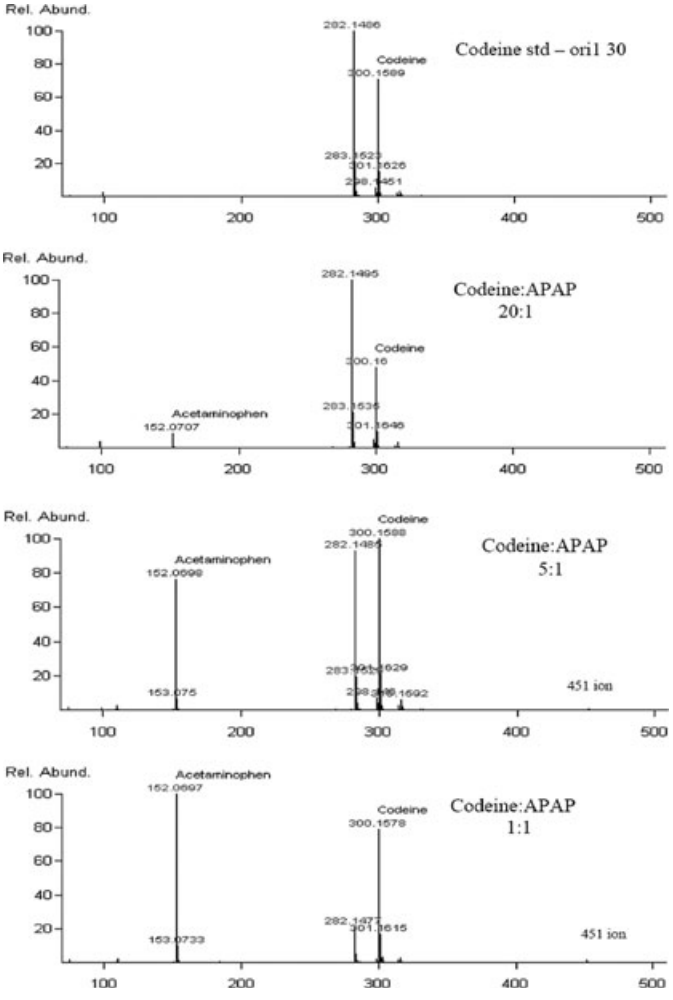

FIG. 10—*Addition of acetaminophen (APAP) to codeine with subsequent diminishing of the relative intensity of the codeine fragment ion at 282.1470 Da.*

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