Open Access Atmospheric Pressure Chemical Ionization Mass Spectrometry for Routine Sample Analysis

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We report the introduction and use of an atmospheric pressure chemical ionization liquid chromatography–mass spectrometry instrument that has been designed specifically for use by the synthetic chemist on an open access, walk-in basis. This instrument has been configured with an easy-to-use sample log-in terminal that requires the user to provide only a sample identification number and a user name. Sample analysis takes approximately 4 min and provides the synthetic and medicinal chemist with rapid and reliable mass spectrometry analysis. Since installation of the system, it has analyzed an average of about 80 samples per day and has the capacity to run over 100 samples per day without the intervention of a specialist operator. This capability has eliminated the need for an operator to analyze routine samples and allows the mass spectroscopist more time to deal with problem solving. (*J Am Soc Mass Spectrom* 1995, 6, 387–393)

Mass spectrometry plays a key role in the structural elucidation of synthetic compounds. The assignment of chemical structure for a particular compound is frequently made on the basis of molecular weight as determined by mass spectrometry and the interpretation of proton and/or carbon nuclear magnetic resonance (NMR) spectra. The combination of data from these two techniques can provide rapid structural identification. The acquisition of chemical structural data from NMR experiments has been carried out routinely by medicinal and synthetic chemists for many years. This process has been facilitated by the availability of sophisticated instrumentation that can be used by the nonspecialist. In contrast, however, synthetic chemists have relied on dedicated specialists for routine mass spectrometry analysis. Generally this has isolated the chemist from the data acquisition process and made mass spectrometry a less accessible technique than NMR. The need for more efficient sample analysis combined with increased numbers of samples for analysis has made one or two day analysis turnaround times a rate determining factor in structural characterization. Because most syntheses are multistep processes, rapid mass spectrometric analysis has a direct impact on the efficiency of synthesis. As a result we have evaluated a number of options for automated mass spectrometry analysis, particularly those that allow the chemists to acquire their own data. There have been several reported examples of automated instruments that incorporate various levels of automation and different instrument configurations. Several studies have used a filament-type desorption probe [1–3] or heated probe [4], as well as thermospray- [5, 6] and particle beam ionization-based instruments (Frank Pullen, Pfizers Pharmaceuticals UK, private communication). Kiplinger [7] reported the use of hands-on instrumentation that utilizes particle beam chemical ionization (CI) and thermospray that has produced molecular weight information for 75–90% of target samples. The use of electrospray ionization (ESI) on a Sciex API III liquid chromatography–mass spectrometry (LC-MS) (Sciex, Thornhill, Ontario) instrument has been described for automated batch sample analysis where the individual chemist submits a predetermined amount of dry sample attached to a sample submission form [8].

The introduction of atmospheric pressure ionization, particularly atmospheric pressure chemical ionization (APCI) combined with LC-MS has expanded dramatically the role of mass spectrometry in the pharmaceutical industry in the area of drug development. The robustness and reliability of APCI LC-MS has made the technique more suited to the analysis of large batch samples and facilitated the detection and identification of drugs and drug-related metabolites in

biological matrices such as plasma [9–13], urine [14], and bile [15]. The sensitivity of APCI combined with the selectivity and specificity of LC-MS and liquid chromatography–tandem mass spectrometry (LC-MS/MS) techniques frequently makes it the method of choice for the quantification of drugs and drug metabolites. The APCI ion source is capable of operation with minimal tuning and optimization, particularly for the analysis of synthetic samples in which sensitivity is rarely a limiting factor. Although it may be possible to increase sample signal for a particular compound or compound type, the relatively high sample concentrations that inevitably are used by the chemist mean there is little or no benefit to be gained by optimization. The essence of the analysis is to obtain rapid sample molecular weight information. Thus instrumental parameters are kept identical for all samples without any obvious loss of mass spectral quality. The instrumentation described in this work has been used by synthetic chemists involved in the drug discovery process, but is equally suited to other areas that require the analysis of synthetic compounds. However, the choice of a particular ionization technique will depend on the general class of compounds under analysis.

Experimental

The open access LC-MS system comprises a benchtop quadrupole mass spectrometer equipped with an APCI LC-MS interface (VG Platform, Fisons Instruments, Altrincham, UK), a high-performance liquid chromatography (HPLC) pump, and an autosampler fitted with an external carousel (HP 1050, Hewlett-Packard, Wilmington, DE). The mass spectrometer, HPLC, and autosampler are controlled from a common Microsoft Windows-based software system (VG OpenLynx, Fisons Instruments, Altrincham, UK) that runs as two separate programs distributed between two 486i 66-MHz personal computers (Digital Equipment Corp., Maynard, MA). The system PC and the log-in PC are connected onto a Microsoft Windows for Workgroups network. The system PC automatically controls the operation of the HPLC, autosampler, and the mass spectrometer, including data acquisition and post-acquisition processing. The system PC and all operational parameters of the HPLC and mass spectrometer (i.e., mass spectrometer tuning, solvent flow rate) are not accessible from the log-in terminal; hence they cannot be changed by the chemist.

The log-in PC provides a simple interface to the system for the user because it has limited functionality and serves to isolate the user from the LC-MS analysis. The log-in PC allows the user to enter details of samples to be analyzed. Post-acquisition, processed spectra are retrieved automatically by the log-in PC, over the network from the system PC, and sent to the laser printer. The user is prevented from mass spectra manipulation.

The source temperature is set at 120 °C and the APCI probe is operated at a temperature of 420 °C with a gas nebulizer gas pressure of 60 lb in.$^{-2}$ (4.13 × 10^5 Pa) and source cone voltage at 20 V. The electron multiplier is operated with a low gain (set at 450 V for positive ion mode and 650 V for negative ion mode).

The mass spectrometry experiment is initiated by the user from the dedicated log-in terminal that consists of an open access window that requires entry of a sample notebook number and a user name. Once this information has been entered, the "Load Sample" command can be given by a carriage return or by clicking the mouse pointer. The default ionization mode is set to positive APCI; however, the user can specify the negative ion mode by selecting the corresponding mode option. After the Load Sample is initiated, a window is displayed that tells the user to place the sample vial into a specific carousel position and displays the data file number for that sample. Once an analysis is initiated, the sample vial is selected by the autosampler and an automatic injection is made. Because there is no HPLC column, the sample enters the APCI source approximately 20 s after injection and is generally detected for a total of 20 to 30 s dependent on the sample solvent. Data acquisition occurs for a total of 1.2 min, after which time the sample spectra acquired between 20 and 40 s are combined, background subtracted, smoothed, peak centered, and finally printed. An example of the total ion chromatogram for sample analysis is shown in Figure 1. The total cycle time for each sample analysis is about 4 min 15 s, although any number of samples can be queued by using the log-in terminal subject to the capacity of the sample carousel.

Sample Preparation

Samples for analysis (1–100 μg) are placed in a 2-mL sample vial and 1 mL of solvent is added. Commonly
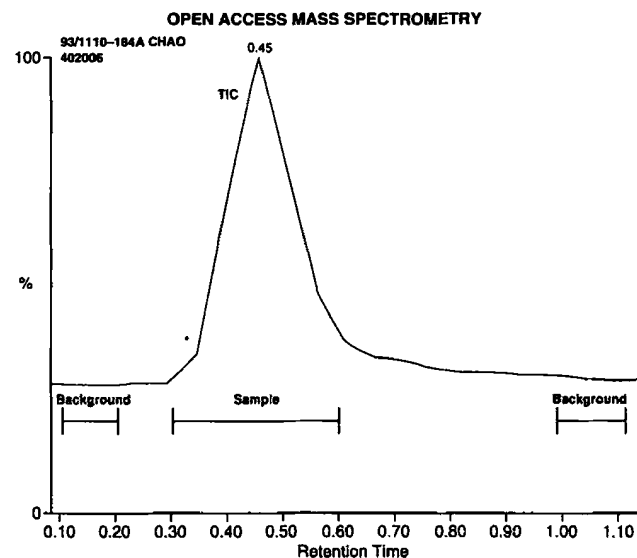

Figure 1. Total ion current trace for a typical sample analysis.

used solvents are methanol, water, and acetonitrile, although, if necessary, solvents such as methylene chloride, dimethyl sulfoxide, and chloroform can be used. The solvent delivery system is operated with a mobile phase of methanol at a flow rate of 0.5 mL min^{-1}. The sample injection volume is 5 μL per sample.

Results

During the evaluation of ionization techniques for an open access system, approximately 60 representative samples were analyzed by thermospray, electrospray ionization (ESI), and APCI. The majority of compounds selected had molecular weights in the mass range 200–600 u and included both neutral molecules as well as those containing precharged functional groups. This comparison showed that APCI generated more intense molecular ions, less fragmentation, and better signal-to-noise ratio than thermospray for the selected compounds. Moreover, APCI gave molecular ions for all the samples analyzed with the exception of three peptides (molecular weight of approximately 1000 u). In contrast, about 30% of the more thermally labile samples did not give molecular ions in the thermospray ionization mode. ESI proved to be a softer ionization technique than APCI and produced molecular weight information for the synthetic peptides. However, the ESI spectra were characterized by multiply charged ions and higher chemical background than the corresponding APCI spectra. Because the great majority of synthetic samples produced in our laboratories give good molecular ions by APCI, we have elected to use predominantly this ionization mode. A recent independent comparison of 75 compounds by electrospray, fast-atom bombardment, matrix-assisted laser desorption, and APCI also concluded that APCI afforded the highest success rate for obtaining molecular weight information on the largest variety of compounds of interest [16].

An example of an APCI mass spectrum is shown for a reaction by-product from the synthesis of an amino acid derivative (Figure 2). Note the protonated molecule at m/z 649 with the corresponding sodium ion adduct 22 u higher at m/z 671. The presence of the MNa$^+$ species is commonly observed in these APCI mass spectra and presumably indicates there is some ion formation from the liquid phase (cf. MH$^+$ from gas-phase ionization). Figure 2 also shows some diagnostic fragmentation at values of m/z 365, 323, and 204. Frequently, however, the APCI spectra of many compound classes show only abundant molecular ion species with little or no significant fragmentation. The ability to generate intense molecular ions also is shown for a trichloro-substituted pyrimidine compound (Figure 3), with the diagnostic molecular isotope distribution shown by the ions at m/z 484, 486, and 488. The lack of fragmentation has not been a problem for the
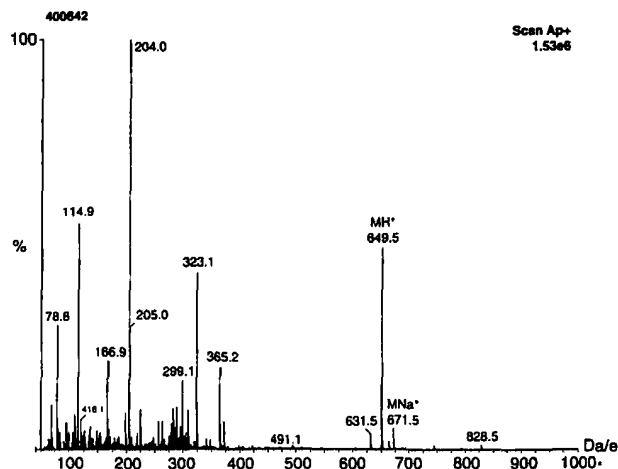

Figure 2. Positive ion APCI mass spectrum of a reaction by-product from the synthesis of an amino acid derivative.

chemist, whose general requirement is for distinct and unambiguous molecular weight information.

Since installation of this instrument, an average of 76 samples per day have been analyzed over a six-month period, that is, a total of approximately 8000 samples. This compares with 20 to 30 samples per day prior to the introduction of the system. In addition to providing analysis of synthetic intermediates and target compounds, the sensitivity of this instrument in the APCI mode is ideally suited to the following types of analysis.

Reaction Monitoring

Because samples can be analyzed in less than 5 min, it is possible to carry out reaction monitoring experiments. An example of this is the radical bromination of an acid compound (Figure 4a). This oxidation reaction was carried out by using N-bromosuccinimide in the presence of a free radical initiator (azo-bis-isobutyronitrile) to give the intermediate bromine-substituted compound that spontaneously eliminated hydrogen bromide to form the product with a molecular weight 2 u less than the starting material. The acquired negative ion APCI mass spectrum of the starting material shows the deprotonated molecular ion at m/z 225 with the major fragment at m/z 181 due to the neutral loss of carbon dioxide. Figure 4b shows the mass spectrum acquired about 30 min after the start of the reaction by taking two drops of the crude reaction mixture from the reaction vessel, putting it in the sample vial, and adding 1 mL of methanol. The sample was then logged in and analyzed in the usual manner. The resulting mass spectrum indicated the formation of the desired product at m/z 223 (with the corresponding loss of carbon dioxide to give m/z 179), as well as the starting material two mass units higher. On the basis of this mass spectrum the reaction was allowed to continue for about another 30 min, until completion.
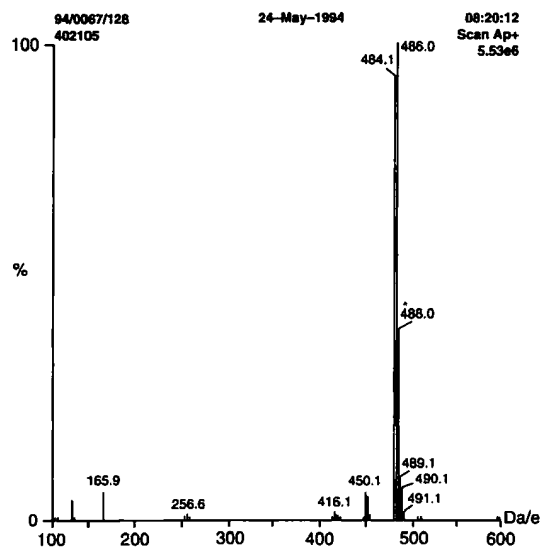
Figure 3. Positive ion APCI mass spectrum of a trichloro-substituted pyrimidine compound.

Analysis of Preparative High-Performance Liquid Chromatography Fractions

HPLC is often used to isolate synthetic compounds. Consequently it is convenient to collect individual fractions and directly carry out mass spectrometric analysis. Figure 5 shows an example for an unknown reaction by-product that appeared in the preparative HPLC analysis of a benzothiazepine derivative. The acquired mass spectrum showed an intense MH$^+$ ion at m/z 691, with a characteristic sodium adduct species at m/z 713. Typical quadrupole resolution is shown in Figure 5 (inset). Interpretation of this spectrum, based on the chemistry, indicated the formation of a dialkylborinic acid ester. The spectrum also shows distinct fragmentation, which is diagnostic for the R$_1$, R$_2$, and R$_3$ groups.

A second example is typified by the analysis of two overlapping peaks from a preparative HPLC. An initial synthesis gave two diastereomeric amine alcohols (benzothiazepine derivatives; Figure 6a). Analysis of a subsequent repeat synthesis indicated two overlapping HPLC peaks (Figure 6b). To determine whether an additional component had caused the loss of peak separation, three individual fractions were collected and analyzed by mass spectrometry. The APCI spectra of the two amine alcohols were identical and gave MH$^+$ ions at m/z 456 (Figure 7a). Analysis of the overlapping peak fraction showed an additional molecular ion species at m/z 454, that is, 2 u less than the amine (Figure 7b). In view of the synthetic reaction conditions, this species was assigned as the nonreduced imine compound.

Analysis of Thin-Layer Chromatography Plates

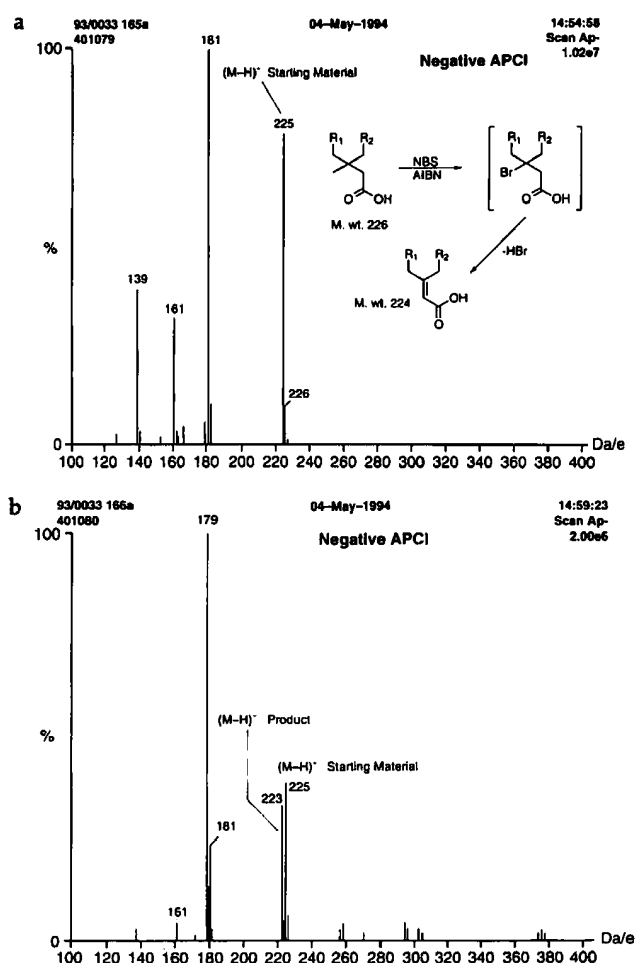Open access mass spectrometry allows the chemist to analyze spots on thin-layer chromatography (TLC)

Figure 4. (a) Negative ion APCI mass spectrum of starting material that shows the [M − H]$^-$ ion at m/z 225. The reaction was carried out with N-bromosuccinimide (NBS) in the presence of a free radical initiator (AIBN, azo-bis-isobutyronitrile). (b) Negative ion APCI mass spectrum of crude reaction mixture after 30 min that shows the presence of the reduced product [M − H]$^-$ at m/z 223.
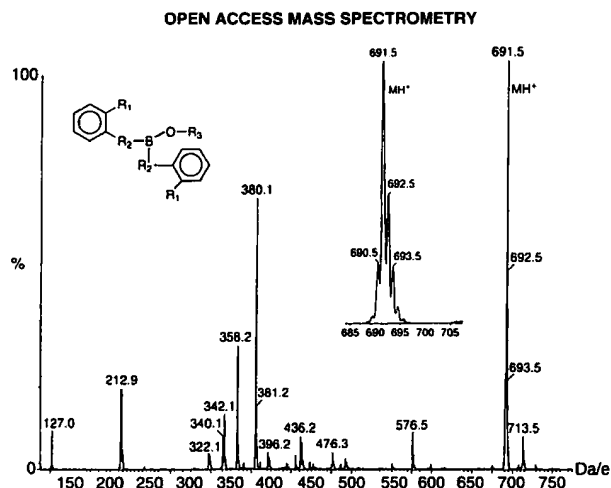

Figure 5. Positive APCI mass spectrum of a dialkylborinic acid ester that was formed as a reaction by-product. Expansion of the molecular ion region (inset) demonstrates the typical resolution used for analysis.
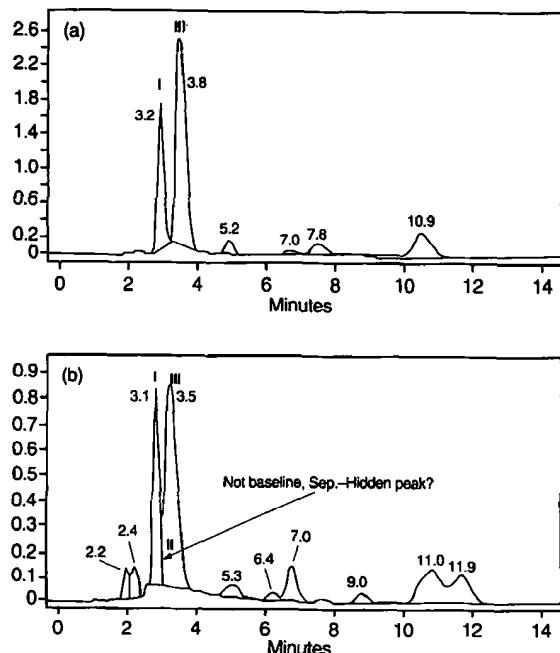
Figure 6. (a) HPLC UV analysis of two benzothiazepine diastereomers formed as synthesis products (I and III). (b) HPLC UV analysis of a repeat synthesis of the benzothiazepine amino alcohols that shows lack of peak separation due to an additional reaction by-product (II).

Figure 8. Positive APCI mass spectrum of the upper band that has been scraped from a TLC plate. The inset shows the corresponding HPLC UV analysis of the TLC band that shows the two diastereomers (MH$^+$ at m/z 488).

plates and rapidly carry out structural identification. A particular spot can be scraped off the plate, solvent extracted, and the resulting solution analyzed. An example is given for a TLC plate that gave two bands (designated 08B and 08C). Mass spectrometry of 08B indicated a protonated molecular ion of m/z 488,
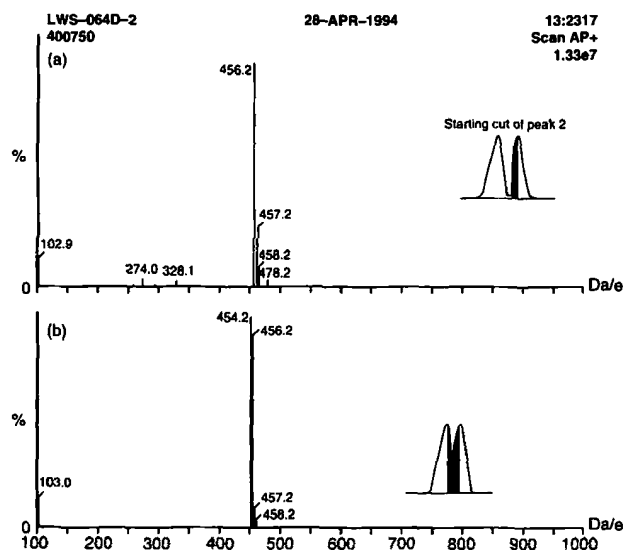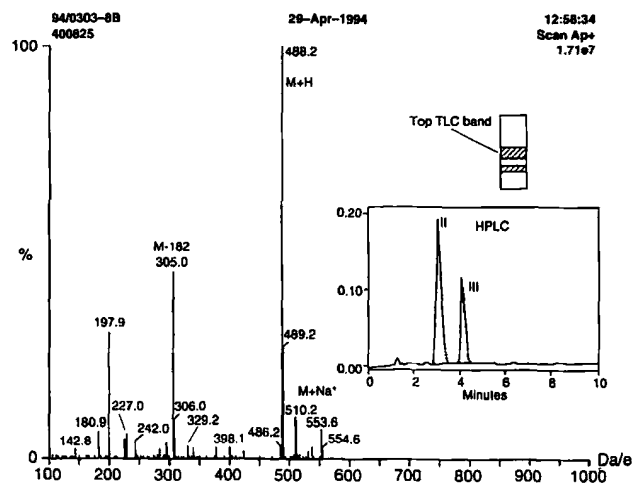
consistent with the target compound (Figure 8). HPLC analysis of this band showed two separate peaks due to two diastereomers [Figure 8 (inset)]. Analysis of band 08C indicated two compounds with protonated molecular ions of m/z 472 (M$_1$) and 531 (M$_2$) (Figure 9). The presence of the sodium adducts at m/z 494 and 553 was further confirmation of the molecular mass assignments. The higher molecular weight compound was consistent with formation of an impurity, and the HPLC analysis of band 08C showed a peak with a minor shoulder that reflects the presence of a second component (inset, Figure 9). Inspection of the HPLC UV chromatogram in Figure 9 reveals a number of minor peaks, and the APCI spectrum is consistent with the presence of additional molecular species, for example, M$_3$H$^+$ at m/z 516 and M$_3$Na$^+$ at m/z 538.

Figure 7. (a) Positive APCI mass spectrum of benzothiazepine amino alcohols [HPLC fraction (III) in Figure 6a] MH$^+$ m/z 456. (b) Positive APCI mass spectrum of benzothiazepine amino alcohols [HPLC fraction (II) in Figure 6b]. Note the MH$^+$ species at m/z 454 due to formation of the imine alcohol derivative.
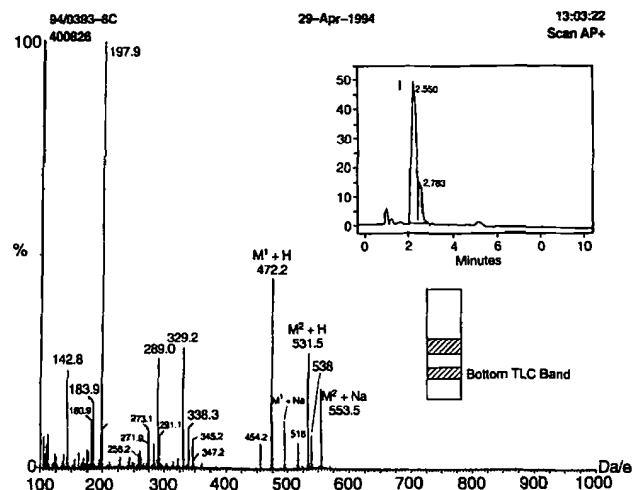

Figure 9. Positive APCI mass spectrum of the lower TLC band that shows two MH$^+$ species at m/z 472 and 531. The HPLC UV analysis (inset) shows a major component (peak I) with a minor shoulder due to an impurity (m/z at 531).

Discussion

The choice of APCI as the ionization technique for open access mass spectrometry was based on its ease of automation as well as the suitability of this ionization mode for the analysis of the majority of organic compounds synthesized in our laboratories. We note, for example, that synthetic peptides constitute a minority of the samples analyzed and that laboratories heavily devoted to peptide synthesis would find ESI a more appropriate approach to open access instrumentation. Because it is a prerequisite that individual users generate and analyze their own data, it is important that the mass spectra be readily interpretable by the synthetic chemist. Thus, although ESI may be considered a softer ionization and give mass spectra for a wider range of compound types than APCI, the interpretation of spectra that contain multiply charged ions, particularly for mixtures, is generally more complicated. Significant multimer formation has not been observed in the APCI spectra, although we have not inspected the nearly 8000 spectra generated since its installation. Instrument users have adapted quickly to the sample quantities required for optimum analysis and peak saturation has not been a problem. The multiplier is operated with a low gain setting because high sensitivity is not a requirement. Moreover the post-acquisition processing software flags any saturated peaks in the spectrum and indicates that mass assignments may be inaccurate. In such cases the chemist simply dilutes the sample and repeats the analysis. It is understood that not all compounds will be amenable to APCI mass spectrometry; however, the aim of an open access mass spectrometry system is to successfully generate mass spectra for a wide range of target compounds. The vast majority of polar, low molecular weight synthetic compounds (molecular weight less than 1000 u) synthesized in our laboratories give detectable molecular ions, particularly (but not exclusively) those that contain a heteroatom. There are some specific compound classes that generally do not give molecular ions by APCI, for example, synthetic peptides with more than 3 or 4 amino acid residues, bis-quaternary ammonium compounds, and bis-acids. However these types of compounds can be analyzed by using other ionization techniques, for example, ESI. Our Platform instrument can be operated in the open access ESI mode by using the appropriate electrospray probe and solvent delivery conditions. Indeed, the original goal was to apportion operation between APCI and ESI on a daily basis; however, the general success of APCI combined with the expectation of immediate analysis has necessitated a single mode of operation. We have not observed any significant sample memory effects during the operation of the system, which is an important consideration for an instrument with multiple users who are analyzing different compounds with varying ionization efficiencies. The cycle time between sample analyses is approximately 4 min during which time the mobile phase flows continuously at 0.5 mL min^{-1}. This provides a self-cleaning effect and results in no signal carryover from one sample to the next.

The introduction of an open access mass spectrometer to directly support medicinal and synthetic chemistry has led to an increased use of mass spectrometry for structural elucidation. The ability to rapidly and reliably generate structural information has led to an increased level of confidence in mass spectrometry and a greater appreciation of the analytical requirements for generation of interpretable mass spectra. Short analysis times make mass spectrometry the first option for sample characterization. Also, the synthetic chemist has been provided with a convenient means of optimization of reaction conditions, following the progress of multistep syntheses, and characterization of reaction products. The present instrument configuration allows the analysis of about 112 samples per 8-h day and eliminates the need for an instrument operator to analyze routine samples. This change affords the mass spectroscopist more time to be involved in the more complex structural elucidation problems that inevitably arise on a regular basis.

Acknowledgments

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