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ARTICLE *in* ANALYTICAL CHEMISTRY · APRIL 2003

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Attenuated Total Internal Reflectance Infrared Microspectroscopy as a Detection Technique for High-Performance Liquid Chromatography

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The use of single reflection attenuated total internal reflection infrared microspectroscopy as a detector for high-performance liquid chromatography (HPLC) is demonstrated. The terminus of the HPLC column is placed at the focus of an ATR infrared microscope, allowing several advantages over other detection techniques. These include the following: (1) the reduction or elimination, or both, of detection cell dead volume, (2) the ability to interrogate a nearly pure aliquot of analyte, and (3) the ability to signal average spectra of the same aliquot (depending on its size) thereby improving the signal-to-noise ratio of the measurement and, concomitantly, the analytical characteristics of the method. Static measurements of succinylcholine chloride in water have shown a detection limit of 0.7 parts per thousand (ppt). Two-microliter injections of succinylcholine chloride in a 5-cm × 1-mm C-18 column with a flow rate of 60 μL/min have shown a detection limit of 1.9 ppt. This analytical concentration corresponds to a mass of 3.8 μg in the injection loop and a mass of 350 fg in the sampling volume. The potential of this detector for HPLC is demonstrated, and future improvements are also discussed.

Infrared microspectroscopy has gained wide acceptance in the analytical community as both a qualitative and a quantitative research tool, due to the fact that it provides direct molecular identification of the materials being studied. In recent years, sampling methodologies normally associated with the analysis of macrosized samples (e.g., reflection−absorption, attenuated total internal reflection, diffuse reflectance, grazing angle reflectance) have been adapted for microscopy, thereby allowing microsamples to be investigated. One method, which has experienced tremendous growth over the past several years, is ATR microspectroscopy.[1−5] This method has several advantages which include limited sample preparation, an easily controlled optical path length, and, for microanalysis, a magnification factor equal to the refractive index of the internal reflection element. From a microscopic standpoint, this latter benefit translates into an increased spatial and volumetric resolution.

Several methods have been previously developed in which infrared spectroscopy is employed as a chromatographic detector. Two general approaches include methods that eliminate the solvent, followed by postcolumn detection and those in which the detection is conducted on line (in situ). Solvent elimination techniques, such as thermospray and ultrasonic nebulization, have been described by Griffiths and others.[6−10] While these methods provide excellent mass detection limits and analytical concentration detection limits, their implementation requires complex interfaces and the elimination of the solvent. The in situ methods have generally employed CIRCLE (cylindrical internal reflectance) flow cells. The CIRCLE[11−14] cell, designed for in situ monitoring of the analyte, is simpler to implement, but both the standard cell volume (24 μL) and the ultramicro-CIRCLE cell with a volume of 1.75 μL[15,16] are too large for capillary HPLC or CE.

More recently, Harris et al.[17] and Niemczyk[18] investigated the application of thin sol−gel films deposited directly onto the internal reflection element (IRE). The sol−gels can be modified to selectively concentrate the analyte in the thin film, thereby increasing the number of molecular interactions per unit time, with the infrared beam. Detection limits in the part per billion regime have been demonstrated. However, due to the cell design, the volume and consequently the number of photons interacting with the sample is not optimal.

Over the past several years, new developments in the area of ATR microspectroscopy have taken place. These developments

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10.1021/ac0205917 CCC: $25.00
Published on Web 02/04/2003

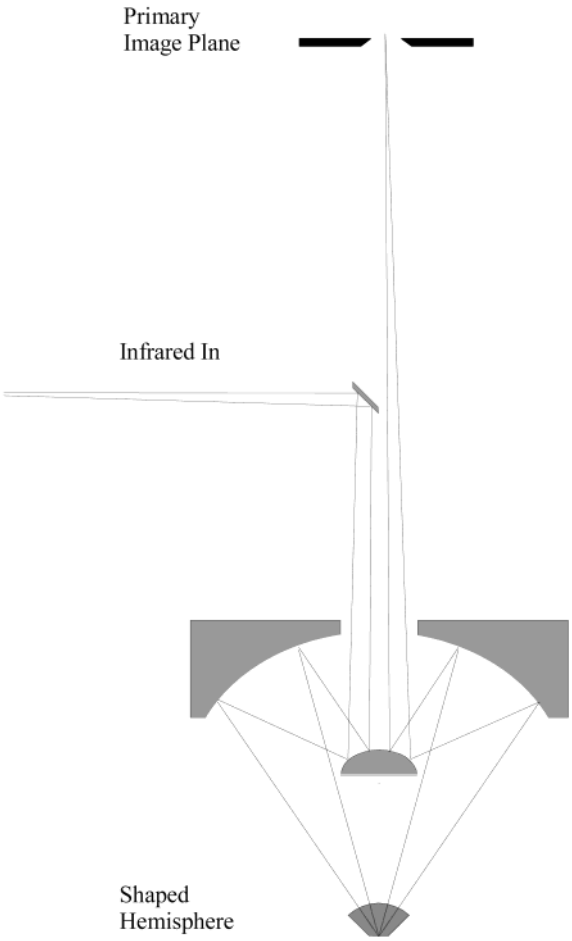

Figure 1. Optical diagram of Perkin-Elmer AutoIMAGE, showing aperture splitting beam splitter and germanium ATR crystal.

have centered on the use of a shaped germanium hemispherical internal reflection element, which unlike the CIRCLE cell design permits only one reflection to take place as the light traverses through the crystal. As illustrated in Figure 1, half of a microscope objective is employed to introduce light into the IRE and the remaining half is employed to collect the exiting light. The diameter of the infrared beam at the IRE/solution interface can be approximated from diffraction theory by

$$d = 1.22\lambda(n_c \sin \theta)^{-1} \quad (1)$$

where: λ is the wavelength of light, n_c is the refractive index of the internal reflection element, and θ is the half-angle acceptance of the microscope objective. For a germanium IRE ($n_c = 4.0$) and common microscope optics ($\sin \theta = 0.3$), the beam diameter is equal to the wavelength of light being employed. In effect, the wavelength is reduced by the refractive index of the internal reflection element. To guarantee that this beam diameter is achieved, a confocal aperture is placed at the primary image plane of the microscope. It should be noted that the IRE provides a magnification factor equal to its refractive index. This factor allows either smaller beam diameters to be achieved (i.e., the wavelength of light is reduced by a factor equal to the refractive index of the IRE) or equal beam diameters. The aperture of the microscope can be opened to larger dimensions enabling a higher signal throughput to be achieved compared to a normal transmission measurement.

From a volumetric consideration, the optical path length, which is more commonly known as the penetration depth, is given by

$$d_p = \frac{\lambda_c}{2\pi[\sin^2 \theta - (\eta_s/\eta_c)^2]^{1/2}} \quad (2)$$

where $\lambda_c = \lambda/n_c$ and n_s is the refractive index of the sample. Since the infrared light exponentially decays into the solution, the sampling volume can be approximated as a cone. The base of the cone is the diameter of the infrared light at the IRE/sample interface, and its height is equal to the penetration depth (d_p). The sampling volume can be diffraction limited or can be increased by changing the size of the microscope aperture, the IRE refractive index, or both.

We have undertaken a preliminary investigation into the use of attenuated total internal reflection (ATR) infrared microspectroscopy as an alternative detection method for capillary column high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). The main benefit of this methodology over current detection schemes is that it provides direct in situ molecular identification of the solutes being separated. The purpose of this study is to investigate the use of ATR microspectroscopy as a detector for microbore and eventually capillary HPLC. Three dynamic experiments and one static experiment were conducted. Initially, a calibration set was developed for flow injection (FI). Second, the same experiment was repeated, but with a standard HPLC C-18 column in place. Third, a chromatographic separation was performed with succinylcholine chloride and methyl 3-hydroxybenzoate. Finally, a drop of aqueous succinylcholine chloride was placed on a glass slide, and a calibration curve was developed using static measurements. The first three experiments were conducted to address the feasibility of the methods under conditions relevant to a chromatographic separation. The final experiment was conducted to determine the analytical capabilities under stopped-flow conditions where time considerations can be relaxed.

EXPERIMENTAL SECTION

All spectra were collected with a Perkin-Elmer AutoIMAGE microscope interfaced to a Spectrum 2000 FT-IR. The microscope was equipped with a liquid nitrogen-cooled, wide band 250 × 250 μm MCT detector and the standard Ge ATR microsampling accessory. The aperture at the primary image plane was set to 100 × 100 μm, which produced a 180-fL sampling volume. Data collection and processing were performed with Perkin-Elmer Spectrum Time Base (Version 2.0) on a Dell PC running Windows 95. No modifications were made to the instrument or computer. All spectra were collected at 8-cm^{-1} resolution. Single-scan backgrounds of flowing mobile phase were collected prior to each FI or HPLC run. The average time to collect a spectrum was 1.6 s. The entire optical path of the instrument was purged with boil-off from a liquid nitrogen tank.

The FI and HPLC instruments are described as follows: The solutions were circulated with a SSI 222C HPLC pump into a SSI LP-21 pulse dampener and a Rheodyne 7125 injector. The column

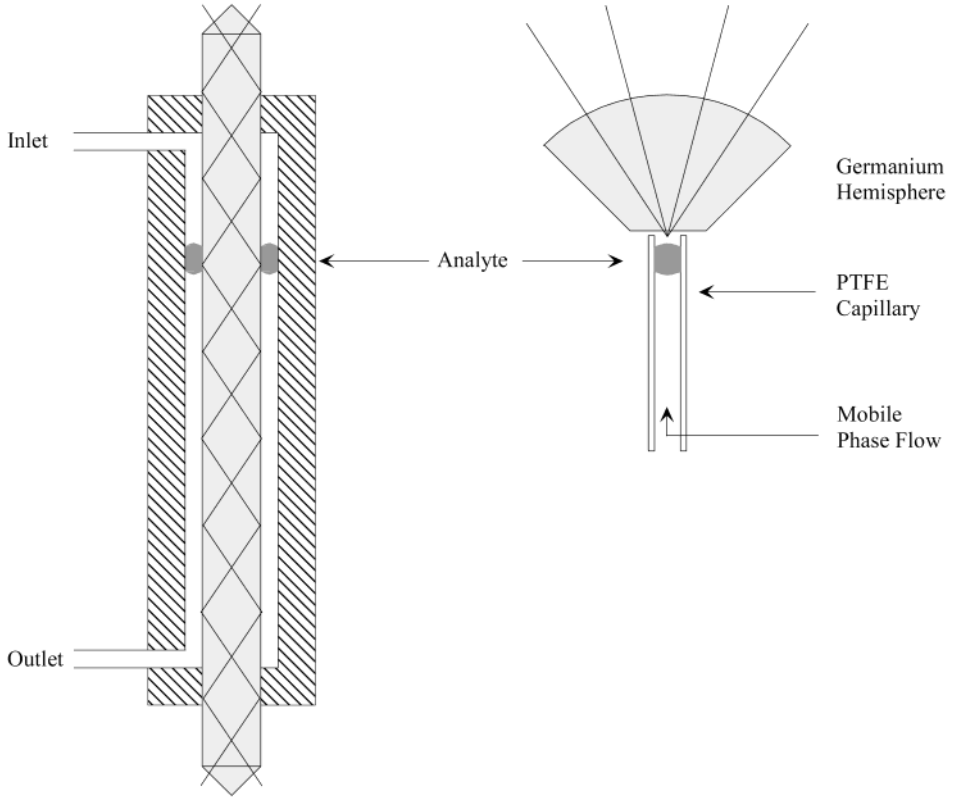

Figure 2. Comparison of the ultramicro-CIRCLE cell (left) and the germanium micro-ATR interface (right). See text for details.

(LC Packings), 5 cm long with an inside diameter of 1 mm, was packed with 3-μm, Inertsil ODS-3 (C18) particles. Solution injection volumes of either 20 or 2 μL were passed through an in-line filter using flow rates of 60 and 80 μL/min. Standards were made of succinylcholine chloride (Sigma, St. Louis, MO) in water ranging from 8 to 328 ppt. The preservative methyl 3-hydroxybenzoate was also purchased from Sigma. The water used was distilled, deionized, and degassed with helium.

The column was coupled to the ATR microscope with a section of poly(tetrafluoroethylene) (PTFE) (Cole-Palmer Instrument Co.) capillary with an inside diameter of 0.002 $\pm$ 0.001 in. (51 $\pm$ 25 μm). The inside diameter measured with the AutoIMAGE microscope averaged 70 μm. This capillary was held in place by mounting it into a stainless steel HPLC male−male Swagelok union with a drilled-out center. A piece of polypropylene tubing served as a sleeve to hold the PTFE capillary in the union. One end of the union, in conjunction with a pair of washers and an end cap, held the tube fixed on a metal slide. The terminus of the capillary, with the other union end cap in place, was cut flush with the end cap using a razor blade. The end cap was subsequently removed, and as long as the ferrule was not touched, the capillary remained in place. Removal of the end cap was necessary to allow the eluent to drain away from the crystal. With the end cap in place, surface tension would cause the solution to bead up the sides of the IRE, which produced a modulated baseline in the chromatogram.

The metal slide with the column end was mounted onto the AutoIMAGE stage and brought into focus. A visual image was made to track the position of the crystal in relation to the end of the tubing. The tip of the IRE was lowered, and a background was taken. At a point midway between the inside edge and the outside edge of the tubing, the column was raised 1 μm at a time until the PTFE absorption peaks were just visible. The capillary was then lowered 1 μm. In effect, this procedure put the IRE 1−2 μm from the end of the capillary. The capillary was then slowly moved on center with the IRE. The distance between the IRE and the capillary must be minimized to reduce the effect of "postcolumn broadening" as the droplet exits the capillary. This affects detection limit, reproducibility, and band shape. Water was then circulated through the system for ~1 h at 60 or 80 μL/min to allow the system to equilibrate. All calibration curve data points are based on the integrated absorbance of the C−O−C stretch of succinylcholine over the range of 1133−1193 cm^{-1}.

For the FI measurements, the mobile phase was run for about 2−3 min before the first injection was made to determine baseline noise. Subsequent sample injections were made when the previous peak was detected. The sample of succinylcholine chloride and methyl 3-hydroxybenzoate was injected at time zero on the chromatogram. Noise was calculated as 0.2 times the peak-to-peak noise over the range 2100−1900 cm^{-1}. The detection limit was calculated by taking three times the noise of the blank and dividing by the slope of the calibration plot. Each experimental condition produced its own detection limit. Chromatographic peak asymmetry values were calculated using the peak width at 10% peak height.[19]

For the static measurements, a few drops of the solution were placed on a dimpled glass slide, which was then slowly raised until the IRE tip just made contact with the solution. Spectra were then obtained by coadding 64 individual scans, and pure water was used for the generation of a background spectrum. Each

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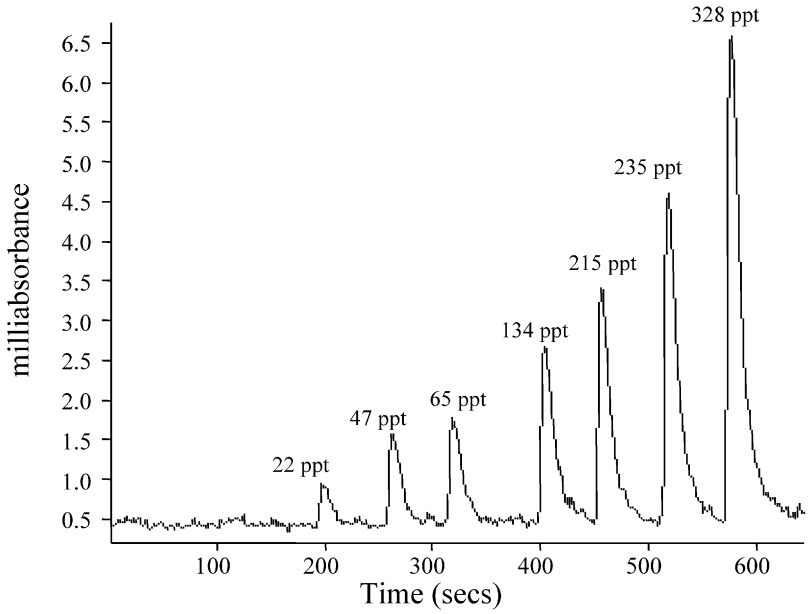

Figure 3. Microbore HPLC peaks of succinylcholine chloride using a 60 μL/min water flow rate and a 2-μL injection. Each data point is integrated absorbance from 2200 to 1100 cm^{-1} from each spectrum.

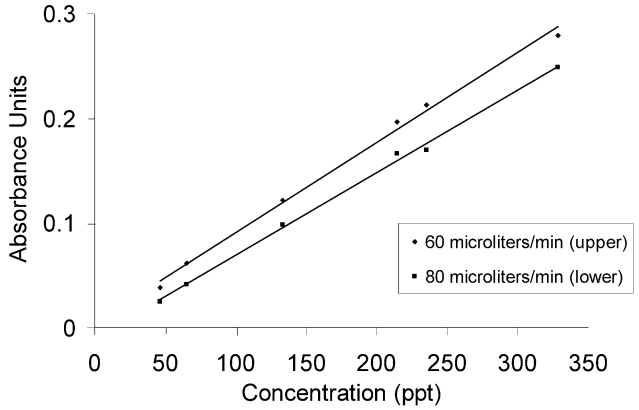

Figure 4. Calibration plot of FI (see Figure 3) at 60 and 80 μL/min flow rates. Each data point is the average of the five center spectra of each FI peak using integrated absorbance from 1193 to 1163 cm^{-1}. Each spectrum required 1.6 s to collect.

Table 1. Detection Limits Based on Flow Rate and Injection Volume[a]

flow rate (μL/min)	static	80	80	60
injection volume (μL)	n/a[b]	20	2	2
Without Column				
concentration detection limit (ppt)	0.7	1.0	1.3	1.9
injection mass detection limit (μg)	n/a	20.6	2.7	3.8
detection volume detection limit (fg)	126	185	240	346
With Column				
concentration detection limit (ppt)	n/a	n/a	0.7	0.7
injection mass detection limit (μg)	n/a	n/a	1.4	1.5
detection volume detection limit (fg)	n/a	n/a	126	131

[a] Injection mass detection limit represents the mass in the sample loop when injected, and the detection volume detection limit represents the mass in the evanescent wave. [b] n/a, not available.

measurement was made in triplicate, and a new background was collected for each data point. The crystal was cleaned between each static measurement with a 50% methanol/water solution on a Kimwipe. A single-scan background preceded all FI and HPLC measurements. Water continued to flow between each FI and HPLC measurement, which automatically cleaned the crystal. The background appears to return to baseline levels after each analyte.

RESULTS AND DISCUSSION

In Figure 2, the outer bore of the ultramicro-CIRCLE cell is much larger than the penetration depth. This characteristic increases the dead volume of the ultramicro-CIRCLE cell and CIRCLE or tunnel cells in general, which affects not only the spectroscopic sensitivity (decreasing effective concentration) but also the chromatographic efficiency through band broadening. The ultramicro-CIRCLE cell has a total volume of 1.75 μL. Considering that the length and radius of the wetted IRE are 1.1 and 0.063 cm, respectively, and that the penetration depth is 5.67×10^{-5} cm, the corresponding sampling volume is 0.025 μL.[15] Therefore, only ~1.4% of the total cell volume is actually interrogated. In reality the situation is much worse since these considerations assume that the IRE is uniformly illuminated over its entire length. In addition, light traversing through a large multibounce IRE is prone to transmission losses, which ultimately decrease the signal in a given measurement. The wetted length of a ZnSe crystal in an ultramicro-CIRCLE cell is 1.1 cm;[15] therefore, the path length of the light through the crystal is ~1.25 cm. In contrast, these transmission losses are minimized in a micro ATR configuration whose path length is 3 mm. Second, the single reflection reduces the absorbance associated with the solvent, which in a multi-reflection geometry can lead to blind spectral regions where the solvent is totally absorbing and solvent compensation is poor. As shown in Figure 2, this configuration will offer several major benefits with respect to capillary liquid chromatography. First, the hemisphere geometry of the IRE focuses the light into a very small volume element, which at its maximum will be one pL (assuming a 250 $\times$ 250 μm aperture). This volume element is 6 orders of magnitude smaller than the ultramicro-CIRCLE cell. One could argue that the optical path length (OPL) in the microscope configuration is reduced (OPL = 3.4 μm versus OPL = 1.1 μm),

Table 2. Comparison of a Single Reflection Crystal (Present Work) to Multibounce CIRCLE Cells

study	cell	cell vol	sampling vol	ratio cell vol to sampling vol	optical path length (μm)	flow rate (mL/min)	injection vol (μL)	scans (background)	detection limit[a] (ppt)
Miller[13]	micro-CIRCLE cell	25 μL	0.22 μL	113	6.0	0.40	200	500	0.2
McKittrick[15]	ultramicro-CIRCLE cell	1.75 μL	0.025 μL	70	3.43	0.40	200	50	2
present	germanium hemisphere	4.23 pL	0.18 pL	24	1.1	0.060	2	1	1.9

[a] Miller calculated detection limit as (S/N = 2) but did not mention whether by peak height or area. McKittrick said that the detection limit was calculated by peak area and was the same for 20-, 50-, or 200-μL injection. Our detection limit was calculated as (S/N = 3) peak area from 1133 to 1193 cm^{-1}.

which would yield a lower absorbance and signal, but the opposite is true. In the microconfiguration, the sample and sampling volumes have been optimized allowing more photon/sample interactions to take place over a given time element. As a result, the effective concentration of the detected analyte is not compromised as in the CIRCLE cell designs. One final advantage of the method is that the interface is simplified compared to previous configurations. All that is needed is a mechanism to bring the end of the capillary into the focus of the microscope.

Figure 3 illustrates the instrument response curves for 2-μL injections ranging in concentration from 22 to 328 ppt using a flow rate of 60 μL/min. Based on the injection volume and flow rate, the full width at half-maximum (fwhm) for each response should be 2 s, assuming no band broadening. As can be seen from the figure, the average fwhm is on the order of 22 s and the peak asymmetry is 1.45 ± 0.10. Figure 4 illustrates the calibration curve generated from the instrument responses of Figure 3. Five spectra in the center of each response curve were used to obtain the integrated band intensity of the C−O−C stretch located near 1165 cm^{-1}. A similar calibration curve is also shown for a flow rate of 80 μL/min. Each curve demonstrates good linearity over a concentration range of 22−328 ppt. Sensitivity was ∼0.9 milliabsorbance unit per ppt, with intercepts of less than 5 milliabsorbance units. The R^2 values were greater than 0.995 for all measurements. The response of the detector is higher at lower flow rate due to signal averaging near the peak maximum. Data presented in the curve were generated without a chromatographic column in place. In an effort to gain information under chromatographically relevant conditions, a 1-mm-diameter packed column was added to the system. Results obtained under these conditions showed response curves with similar fwhm's; however, the band asymmetry increased to 3.20 ± 0.9. The analytical concentration detection limits were calculated to range from 0.7 to 1.9 ppt depending upon conditions using three times the noise divided by the slope of the calibration curve (Table 1). Static experiments were conducted to determine the quantitative capacity of the method in cases where integration times could be relaxed such as in stop-flow measurements. Data collected using 64 coadded scans produced a marginal improvement with an analytical concentration detection limit of 0.7 ppt.

In previous FIA determinations of succinylcholine chloride using the CIRCLE cell by Miller et al.,[13] and later using the ultramicro-CIRCLE cell by McKittrick et al.,[15] the reported concentration detection limits were 0.2 and 2 ppt, respectively.

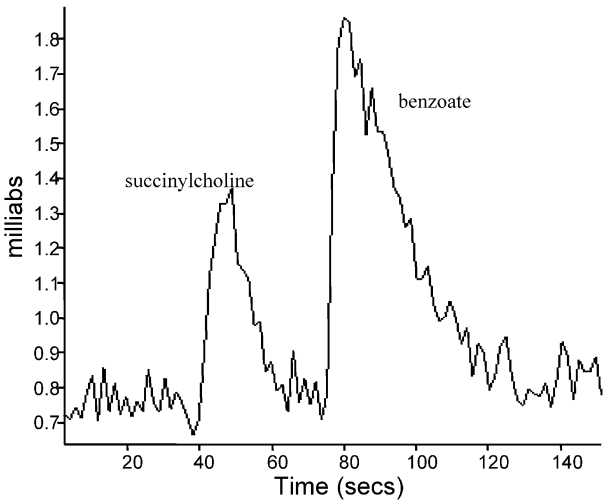

Figure 5. HPLC separation of 100 ppt succinylcholine and 90 ppt methyl 3-hydroxybenzoate with 2-μL injection, 60 μL/min flow rate, and a mobile phase of 60/40 methanol/water.

Characteristics pertinent to each investigation are summarized in Table 2. A detailed comparison of the methods includes the cell volume, optical path length, and noise characteristics for the measurement conditions.

All three investigations employed injection volumes that were significantly larger than the cell volume. As such, any argument based on the ratio of sampling volume to the actual cell volume is moot. Spectroscopically, all configurations should yield the same signal if the path length and detection characteristics are the same. From the standpoint of optical path length, the CIRCLE cells would be favored by roughly a factor of 5. The work conducted by McKittrick et al. employed a Perkin-Elmer 1800 FT-IR interfaced to a Spectra-Tech IR PLAN microscope. The signal-to-noise ratio for instruments of that era was 1000:1. The current Perkin-Elmer AutoIMAGE microscope has a 5000:1 signal-to-noise ratio for similar conditions, thereby favoring the current method by a factor of 5. The scan conditions of McKittrick et al. were also slightly different in that the ratios of 50 background scans to 5 sample scans were taken. In the present method, the ratio of five background scans to five sample scans was determined. Independent tests show signal-to-noise values reported by McKittrick et al. would be favored by a factor of 1.4×. Taking all these considerations into account, one would expect comparable results reported between the two methods.

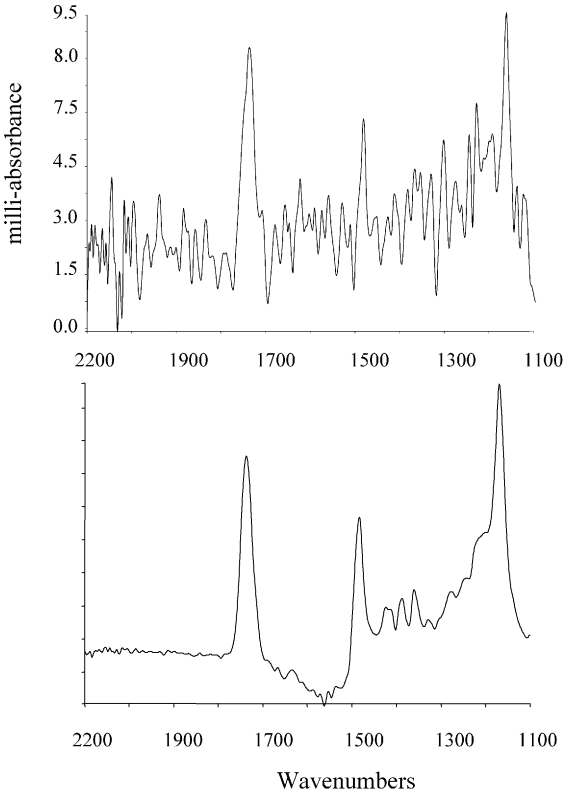

Figure 6. Spectra of succinylcholine chloride (100 ppt) of the first LC peak in Figure 5 (top) and stationary solution (bottom).

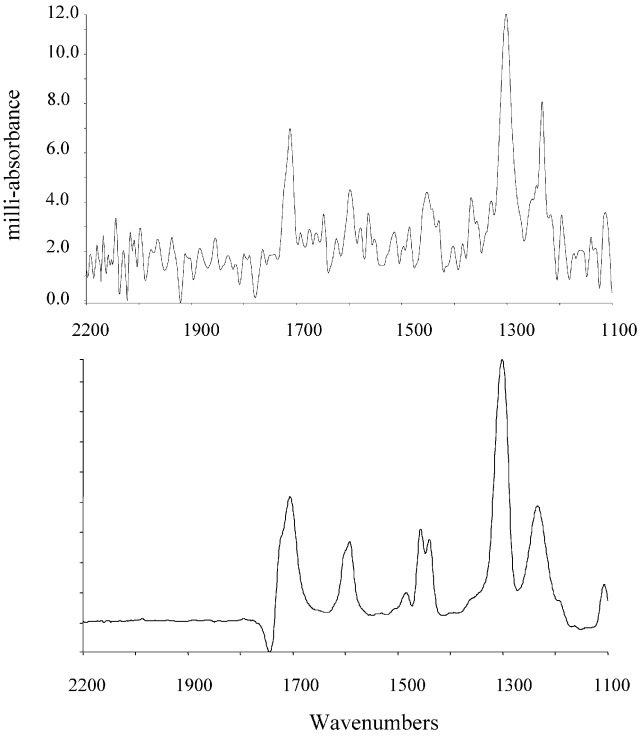

Figure 7. Spectra of methyl 3-hydroxybenzoate (90 ppt) of the second LC peak in Figure 5 (top) and stationary solution (bottom).

To demonstrate the viability of the method on a pharmaceutically relevant system, a separation of succinylcholine and methyl paraben (methyl 3-hydroxybenzoate) was conducted. Succinylcholine is often found with the preservative methyl paraben in pharmaceutical solutions. It was possible to separate these two analytes with baseline resolution as shown in Figure 5. A single-scan spectrum of each of the compounds at their chromatographic peak is also shown in Figures 6 and 7 (top). These spectra represent the limit of identification of approximately three times the background noise level.[12] Spectra representative of 64 coadded scans for each sample are provided for comparison. The major bands of succinylcholine chloride as identified by Miller[13] were the C═O stretch (1737 cm^{-1}), the CH_2 and CH_3 deformation bands (1480−1490 cm^{-1}), and the C−O−C stretch (1165 cm^{-1}). The methyl 3-hydroxybenzoate bands are the C═O stretch at 1705 cm^{-1}, the C═C stretch at 1591 cm^{-1}, the aromatic ring stretch at 1457 cm^{-1}, and a CH_3 asymmetric stretch at 1440 cm^{-1}, and esters conjugated to a C═C show up at two bands, 1302 and 1233 cm^{-1}.

CONCLUSIONS

The results presented in this report clearly demonstrate the future potential of single bounce attenuated total internal reflection infrared microspectroscopy as an improved chromatographic detection method when molecular information is sought. Analytical concentration detection limits comparable to those reported previously have been obtained using 1 order of magnitude less injected sample volume. By a judicious choice of the internal reflection element and remote aperture size, the sampling volume can be controlled. The sampling volume in the present investigation was 180 fL. However, a diffraction-limited sampling volume of 35 fL should be obtainable. Taking into account the sampling volume and the analytical concentration limit, the mass of material being detected is on the order of 180 fg. As such, the method offers excellent mass detection limits. A detailed optical and volumetric analysis shows that the method can be vastly improved. Table 2 shows the ratio of the total cell volume to that of the sampling volume is 24 and has been minimized, but sample can still pass around the evanescent cone without being detected. The solution to this problem is to make the diameter of the column half the diffraction-limited diameter of the infrared beam at the IRE/solution interface. For the present flow cell configuration and considering the analytical wavelength, the diameter of the column should be 5.5 μm. Making the column diameter smaller will also improve the chromatographic characteristics. One quickly realizes that the method based on this diameter is more suited for CE detection than capillary HPLC and would provide many benefits in situations where a limited quantity of material is available. CE detection is typically on column, but CE peaks are on the order of 30−120 s, which allows scan averaging.[20] Single bounce ATR has been recently demonstrated to be useful for the detection of proteins in water; this system could be easily adapted for chromatography.[21]

Two other areas of improvement include reducing the size of the infrared detector and applying a selective film on the IRE. A smaller detector will aid in lowering the detection limit by reducing the noise in the measurement. A 25 × 25 μm instead of 250 × 250 μm MCT detector will reduce the noise equivalent power (NEP ∼ $A^{1/2}D^{*-1}$) by a factor of 10.[22] Major gains, possibly by 6

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orders of magnitude, for improving the analytical detection limit are anticipated by placing a suitable sol−gel coating on the surface of the IRE. FI work conducted by Niemecyck and Harris[17,18] has already demonstrated ppb detection limits on multibounce systems. Finally, attenuated total internal reflection microspectroscopy need not be relegated to the infrared region. The same principles and benefits apply for visible and ultraviolet absorption spectroscopy. In these regions, the extinction coefficients are much larger and the wavelengths are much smaller. This latter fact means that the sampling volumes are now in the attoliter (10^{-18}) regime. The largest hurdle to realize these benefits will be the design and engineering of the appropriate cells.

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ACKNOWLEDGMENT

The authors thank the Perkin-Elmer Corp. and the Miami University Research Challenge Program.

Received for review September 25, 2002. Accepted December 31, 2002.

AC0205917