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Investigation of a Unique Adjunct to Enzyme Immunoassay in Order to Rapidly Perform First-Pass Screening for Drugs and Poisons in Postmortem Toxicology Cases

R&D Award 2003-IJ-CX-K007 Final Report

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Abstract

The analysis of blood and urine samples collected at autopsy for the identification and quantitation of drugs that may have contributed to the cause of death is a time consuming and labor intensive process. This project was designed to investigate the potential use of a liquid chromatograph/mass spectrometer/mass spectrometer (LC/MS/MS) as an adjunct to conventional enzyme based immunoassay testing following a simple one hour acetone based extraction process to streamline this analysis. The instrument selected for the research was the Applied Biosystems Model 2000 QTRAP because of its linear ion trap capabilities. Drug standards were injected directly into the mass spectrometer system to determine the optimal parameters for detection of each drug. Elution of drugs from the LC column was performed using a linear gradient from 95% water/5% methanol to 5% water/95% methanol. The optimal run times for detection and identification of drugs present in a sample was determined to be 20 minutes to allow identification of up to 3 different drugs which co-eluted with the same retention time from the column. Quantitative analysis of samples can be completed in as little as 8 minutes per sample. Concordance studies were conducted with over 100 non-probative cases that had been previously analyzed using conventional liquid/liquid or solid phase extraction methods followed by gas chromatography/mass spectrometry. The results of the concordance studies demonstrated the LC/MS/MS was capable of detecting and identifying all drugs previously identified with 4 notable exceptions – morphine, hydromorphone, nalorphine and THCA. Studies to determine the effectiveness of the instrument to perform quantitative analysis were also conducted. These studies determined that quantitation of drugs was possible using the same extraction procedure and that the variance was similar to that obtained from more conventionally used methods. In summary, the research conducted on this project demonstrated that a linear ion trap LC/MS/MS is capable of the detection, identification and quantitation of over 100 different drugs. By incorporating this type of analysis into routine casework a time and labor savings of up to 20-50% could be realized in the analysis of post-mortem samples while still meeting all of the requirements of an accreditation standard.

Introduction

Analysis of biological samples for the presence of various drugs that may have contributed to cause of death or driving under the influence (DUI) is the realm of the forensic toxicologist. Historically this has been accomplished by a rapid but non-specific screening for different drug classes (amphetamines, opiates, cocaine, barbiturates, benzodiazepines and THC) using enzyme immunoassay (EIA) (1-4). The EIA screening is limited by the number of commercially available reagents, the cost of those reagents and the fact that many drugs do not provide a positive response when tested. Examples of commonly encountered drugs that do not produce a positive response in these six screening tests are methadone, fentanyl, and amitriptyline. Samples that screen positive are then subjected to more intensive analysis using gas chromatography/mass spectrometry (GC/MS) to identify and quantitate the drugs present in the sample. There are two significant disadvantages to this approach: 1) many modern drugs don't react with the commercially available EIA reagents that are commonly used in forensic laboratories; 2) time consuming and labor intensive extraction procedures are often required to prepare the samples and/or derivatize the drugs prior to injection into the GC/MS. The method developed in this award 2003-IJ-CX-K007 (5) essentially eliminates these two issues by utilizing the same simple acetone based extraction process used for the EIA analysis, followed by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) to identify and quantitate the drugs present in a sample. This project resulted in the development and implementation of an operational method capable of identifying over 100 different drugs and quantitating a substantial number of those drugs. The new method was not intended to and does not replace screening via immunoassay, but acts as a powerful addition by quickly providing specific information as to the drug content of a sample without the need for additional sample consumption and extraction.

The GBI-DOFS pioneered work to adapt existing immunoassay procedures to alternative biological specimens (1-4). The acetone precipitation method developed by Lewellen and McCurdy has gained widespread acceptance and is a standard procedure used for the presumptive identification of drugs in blood and other biological samples by EIA. Herrin, McCurdy and Wall developed a comprehensive drug screen by LC/MS/MS utilizing the same extraction technique used for the EIA analysis (5). This method removes a 100 µL aliquot of the EIA extract and analyzes it by positive mode electrospray ionization on an Applied Biosystems QTrap tandem mass spectrometer. The instrument software scans the signal from each sample for over 110 different drugs using multiple reaction monitoring (MRM) coupled with information dependent acquisition (IDA) of an enhanced product ion (EPI) scan. The MRM method uses a list of compound specific parent to daughter ion transitions to scan for drugs. At the end of every MRM scan, the instrument will automatically trigger the EPI scan for a specific drug if a preset condition for the MRM intensity is met. The EPI selectively collects a complete MS fragmentation pattern that can then be compared to a library of known standards. The MRM transition information obtained from the EIA extraction can also be used for quantitation of a wide-range of commonly encountered drugs.

The method described above is effective as a general screen for the vast majority of drugs routinely encountered in casework. Incorporation of the LC/MS/MS analysis into normal casework should be considered as an adjunct or supplement to the EIA testing already conducted. This is because, like all general screens, detection of some important drugs and drug classes were not optimized. Both methods produce data that is valuable to the analysis of forensic toxicology cases. Morphine, hydromorphone and carboxy-THC, acidic drugs (e.g. valproic acid), and most barbiturates did not prove amenable to analysis in the general method and require further method optimization. Investigation of alternate sample preparation, chromatographic or ionization modes targeted to those compounds may assist in creating and optimizing a set of methods and procedures utilizing LC/MS/MS to handle the real world case load facing forensic toxicology laboratories. This is a desirable outcome because the sample preparation time required for the LC analysis is significantly reduced when compared to that required for GC/MS analysis.

General screening and analyte specific methods that include opioids and cannabinoids have been developed for LC/MS/MS. (6-12, 14) The general unknown screen for 238 drugs using MRM scanning developed by Gergov et al.(7) and extended by Mueller et al. (6) to 301 drugs with IDA triggered collection of EPI spectra includes opiates and cannabinoids. Gergov's reported limits of detection for morphine and tetrahydrocannibinol at 0.100 mg/L and 0.050 mg/L respectively are not useful for human performance toxicology. These and other general unknown screens (9) use either liquid-liquid (LLE) or solid phase (SPE) extraction to produce a clean extract and concentrate the drugs. Additionally these use acetonitrile as the primary organic mobile phase. Several ESI-LC/MS/MS methods have been developed specifically to quantitate opioids (10-12) and cannabinoids (8) in biological fluids. Slawson et.al. report a limit of quantitation of 0.500 mcg/L for morphine. Maralikova and Weinmann developed methods for the quantitation of THC, THC-OH, and THC-COOH with limits of quantitation of 0.8 ng/mL, 0.8 ng/mL and 4.3 ng/mL in plasma. Like the general screens these methods use SPE and acetonitrile as the primary organic mobile phase. LLE and SPE extractions of biological matrixes have been shown to produce less ion suppression of the analyte signal compared to protein precipitation methods. (13, 15) Concentration of the sample generally leads to a concentration of the suppressing agents as well. In our protein precipitation method morphine and hydromorphone elute during the greatest period of ion suppression effectively eliminating them from the method. An addition of a simple clean up step and/or modification of chromatographic conditions warrant investigation.

Development of screening methods for other classes of drugs such as barbiturates, acidic drugs, and quaternary amines using the protein precipitation method have not been thoroughly investigated. Analysis of quaternary amines and other drugs by positive mode ESI-LC/MS/MS have been previously reported using solid and liquid phase extractions. (9,18) Barbiturate analysis has been performed using negative mode electrospray ionization.(16) However in that work, serum samples were extracted using super-critical fluid extraction, a technique not in wide spread use in forensic toxicology laboratories. Valproic acid has been analyzed by negative mode ionization using SPE. (17) Development of a general screening method for those drugs best suited for analysis by negative mode ionization, requiring only an additional injection of the same EIA extract,

would be a valuable tool when the high cost of initial screening for drugs like barbiturates is weighed against the relatively low number of positive cases.

In cases where the cause of death is undetermined from the autopsy results, analysis for the presence of a wide range of drugs that could have contributed to the death is often required. Such analyses are very time consuming since they reveal only limited information at each stage. These analyses also may require several different extraction procedures from the samples to identify different classes of drugs, potentially consuming large amounts of the original sample. Development of a method that provides all of the qualitative and quantitative information obtained from the conventional EIA and GC/MS methods, but requiring significantly less labor, time and sample is therefore a desirable goal. This report summarizes the results of studies designed to accomplish this goal through the use of a liquid chromatograph/mass spectrometer/mass spectrometer (LC/MS/MS) for analysis of the extract used for the EIA procedure.

Materials and Methods

Drug Standards – All standard drug compounds were of pharmaceutical purity obtained from various pharmaceutical companies and/or vendors. Standard solutions of each drug (1 mg/mL) were prepared for dilutions and use in subsequent studies.

Instrumentation – The instrument selected for this research was the Applied Biosystems, Inc. QTRAP™ 2000 with an attached Perkin Elmer Series 200 binary HPLC system equipped with an autosampler, solvent degasser, and column heater. A PEAK gas generator was used to supply the curtain, source, and exhaust gases for the mass spectrometer system. The instrument software used for data collection and analysis was Analyst version 1.4 or 1.4.1. The QTRAP™ 2000 has four quadrupoles – Q0, Q1, Q2 and Q3, each of which has independent data acquisition parameters.

HPLC Column and Mobile Phase - The column used for the majority of the studies was a MetaSil Basic RP (3 micron, 50 X 2.0mm). Mobile Phase A – 0.1% formic acid, 1 g/L (~15mM) ammonium formate in HPLC grade water; Mobile Phase B – 0.1% formic acid, 1 g/L (~15mM) ammonium formate in HPLC grade methanol. All mobile phases were degassed prior to use.

Biological Samples – All blood samples used in these studies were whole blood purchased from the Red Cross that had been screened negative for the presence of any drugs (excepting nicotine and caffeine) or samples from previously analyzed cases that were scheduled for destruction.

Enzyme Immunoassay – The EIA procedure is described fully in (Lewellen and McCurdy, 1988) but the basic procedure discussed there is as follows. Add 2.5 mL acetone to 1 mL whole blood, remove the supernatant to a fresh tube and evaporate to dryness. The residue is resuspended in 0.5 mL of methanol/pH 7.0 phosphate buffer (50:50). For these studies 50 µL of a 3 µg/mL mepivacaine solution was added to each sample for use as an internal standard. Mepivacaine was selected as the internal standard because it was already in use in the laboratory for this purpose and it is extremely rare that it is found in casework samples. Additionally, studies were conducted to verify that the presence of the mepivacaine in the EIA extract did not interfere with the results of the immunoassay analysis.

Instrument Parameters and Mass Spectral Library – Diluted drug standards (1 µg/mL approximate) were directly infused into the mass spectrometer at a rate of 10 µL/min to determine optimal parameters for each drug. The parameters were:

- CE – Collision Energy (applied to Quadrupole 2)
- DP – Declustering Potential (applied to the orifice plate)
- EP – Entrance Potential (applied to Quadrupole 0)
- CEP – Collision Entrance Potential (applied to the collision cell entrance lens
- CXP – Collision Exit Potential (applied to the collision cell exit lens)

In addition to these parameters, multiple reaction monitoring (MRM) transitions for detection/identification of each compound were determined. The Information Dependent Acquisition (IDA) criteria was set to conduct 2 experiments, one for MRM and the other using the Q-Trap linear ion trap (LIT mode) for a high sensitivity product ion scanning (Enhanced Product Ion or EPI). This mode has the advantage in that a concomitant product ion spectrum is produced for MRM transition ions. The scan rate for the LIT mode was set at 1000 amu per sec. The IDA criteria for the EPI was set to have a maximum rolling collision energy of 80V and all target ions were excluded for a period of 15 sec after the second occurrence.

Results

Determination of Instrument Parameters

The initial objective was to determine the initial instrument settings and ion transitions for each drug compound that will be analyzed using the LC/MS/MS in order to obtain the maximum detection sensitivity. Each drug was infused directly into the mass spectrometer at a rate of 10 µL per minute and at a concentration of approximately 1 µg/mL, although some drugs were found to require considerably less concentration. The instrument was set to allow passage of the M+1 ion through the Q1 filter then Q3 was scanned for ion fragments. The collision energy (CE), declustering potential (DP), entrance potential (EP), collision entrance potential (CEP) and collision exit potential (CXP) parameters that were determined through this procedure were used in subsequent studies. See Appendix A, Table 1 for a listing of the drugs and parameters.

Selection of Dwell Time Settings

The dwell time setting on the instrument determines how long the mass spectrometer looks for any particular ion transition and is thus directly linked to the detection sensitivity. Dwell times for the detection of ions produced from the drugs studied were determined empirically to be 10 msec for most drugs from the initial studies. However, thirty-nine drugs were identified that required increased or decreased sensitivity. The minimum detectable concentrations were established based on the accepted therapeutic blood levels. To determine the optimal dwell times for these 39 drugs, a series of injections at various concentration levels and different dwell settings was made. For some of the drugs, increasing the default dwell time to 25 msec was sufficient to increase the detection sensitivity to necessary levels. The dwell times for small number of compounds such as acetaminophen, caffeine, and carbamazepine were set to 1 msec in order to significantly reduce detection sensitivity of these drugs. By selecting the appropriate dwell times for each drug, the total scan time, including the EPI scan was finalized at approximately 2.8 seconds.

A supplemental study was performed to determine the impact on detection sensitivity of changing the dwell time in the range 0.04 msec to 500 msec. Two groups of drugs were prepared to complete this study. Group 1 drugs (haloperidol, sertraline, and hydrocodone) were used to study the effects of raising the dwell times to increase sensitivity. Group 2 drugs (meprobamate, trazodone, and methadone) were used to study the effects of lowering the dwell times to decrease sensitivity. The concentration of drugs in Group 1 was 0.05 mg/L and Group 2 was 1 mg/L. For this study, the only six MRM transitions used were those for the six drugs. Eight instrument methods were created beginning with the established method as the model, changing only the dwell time for each method. The peak area responses from each drug were plotted versus dwell times. Examples from each drug group are shown in Figure 1A, B. Area counts for each Group 1 drug went up as the dwell times were increased, however, beginning about at 50 ms the benefit began to become less pronounced. Raising the dwell from 10 to 50 produced an average of 36% gain in sensitivity. Increasing the dwell from 50 to 500 only produced an average gain of 9%, but would severely affect the scan time for the run. Lowering the dwell times from 10 to 0.04 had mixed results. Decreases in

sensitivity were found with trazodone (-17%) and meprobamate (-52%), but an increase in sensitivity was found for methadone using dwells below 1ms. Consultations with the instrument manufacturer determined this result is due to sampling error when ultra-short dwell times are used. See Figures 1A, B in Appendix A.

Although the results show a sensitivity gain for the Group I drugs at dwell times up to 50 msec, the maximum dwell time used for sample analysis was set to 25 msec in order to achieve a balance between sensitivity and the time interval required for each scan. By minimizing the scan interval time, the overall accuracy in peak area determination is improved and the greater number of scans across a peak allows for a greater probability of detecting multiple drugs that overlap chromatographically.

Liquid Chromatography Methods and Optimization

The HPLC solutions in Materials and Methods were used for all studies. Several different parameters were examined to determine the most effective set of conditions for separation of drugs and to ensure that the maximum number of drugs could be detected from the EIA extract. Samples placed into the HPLC autosampler consisted of either 100 µL of the EIA extract or dilutions of the drug standards. An injection volume of 10 µL of each sample was determined to be the optimal volume to ensure complete filling of the sample loop.

HPLC Gradient Profile – In order to determine the best balance between drug detection and instrument throughput, three different HPLC gradient profiles were examined using a mixture of 25 drugs each present at 0.1 mg/L. The three profiles are shown in the Table 2 of Appendix A. Results from this study determined that the 20 minute profile provides the best combination of throughput with detection capabilities. (Appendix A, Figure 1A-C). In the 10 minute profile, the retention time of several drugs overlapped, making the initial screening process somewhat more problematic by increasing the probability that a drug could be not detected. In the 20 minute profile, only one of the 25 drugs, clonazepam, was not detected. The 30 minute profile did not significantly improve the separation of the drugs on the column, but cut the potential throughput by an additional 33% and therefore was not further studied.

Drug Test Mix – In order to be able to evaluate the condition of the column and suitability of the mobile phases for analysis, a test mix of six drugs was developed. The six drugs selected were oxycodone, mepivacaine, piroxicam, imipramine, amitriptyline and methadone. The selections of these drugs was based on the need to have drugs that eluted at various times during the gradient profile and at least two drugs that elute with very close retention times. When the column is performing as expected the piroxicam peak will separate from the imipramine, amitriptyline peak and the methadone peak will show almost baseline resolution. See Figure 3A in Appendix A.

HPLC Column – The initial column selected for the research was the MetaSil Basic RP (3 micron, 50 X 2.0mm). During the course of the study four drugs were could not be analyzed using this column. The four drugs in question were morphine, hydromorphone, nalorphine and THCA. Three additional columns were purchased and investigated through the injection of the drug test mix to see if better chromatography results could be

achieved. As shown in Figures 3A-D in Appendix A, there was no significant improvement of results with any of the columns when compared to the MetaSil Basic RP, so the Metasil column was selected for all additional studies.

Drug Retention Times and Limits of Identification – The initial step in the identification of the drugs observed in a sample is the calculation of the retention time of the unknown peak relative to the retention time of the internal standard mepivacaine. Table 3 in Appendix A provides a listing of the relative retention times for each of the drugs included in this study. One of the goals of this research was to ensure that the sensitivity of the LC/MS/MS instrument using the extract obtained from the EIA procedure was sufficient to identify each of the drugs studied to at least therapeutic levels or lower. The limit of identification (LOI) for each drug was determined by preparing blood samples at the following final concentrations of each drug - 0.005, 0.01, 0.02, 0.03, 0.05, and 0.07 mg/L. 1 mL of each sample was processed using the EIA procedure and 100 µL removed for the LC/MS/MS analysis. For those drugs that have therapeutic levels above 0.07 mg/L, supplementary samples were tested at concentrations up to 0.7 mg/L. The LOI was empirically determined as the lowest concentration at which an enhanced product ion (EPI) spectrum could be obtained with sufficient detail to unambiguously identify the drug.

Most of the drugs produced satisfactory results when compared to conventional GC/MS analysis. The exceptions were morphine, hydromorphone, and nalorphine that were not detectable at any therapeutic or toxic level. Fentanyl and buspirone were not detectable at the lower therapeutic levels. Clonidine was detectable but at levels approximately 3 times therapeutic. It is interesting to note that all of the drugs for which the sensitivity levels do not meet the desired target level elute from the column relatively early. It is possible that the sensitivity for these drugs is impacted by residue from the extraction process which is eluting through the column at approximately the same time.

Sample Carryover – The potential for carryover of results from one sample to the next must be minimized and eliminated if possible when using an autosampler to perform analytical testing. There are two potential sources of carryover on this instrument: 1) Pre-column carryover – results from some of the analyte from a previous injection lodging in the equipment or supplies before the column as relates to the flow of sample. The result is demonstrated as an analytical peak; 2) Column carryover – results from some of the analyte from previous injection lodging in or after the column. The result is demonstrated as background signal.

To investigate the possibility of these two types of carryover from one sample to the next, two studies were conducted. The first involved injection of samples extracted from blood containing cocaine, benzoylecgonine, and diphenhydramine at concentrations of 20 mg/L followed by two blank samples. The addition of an extra 2 minute column rinse step with 95% methanol at the end of each sample 20 minute run decreased, but did not eliminate, the appearance of carryover of both types in the blank samples. A subsequent experiment determined that simply rinsing the autosampler needle twice with 700 µL of buffer eliminated the pre-column carryover peaks and substantially reduced the column

carryover to the point where it did not interfere with interpretation (data not shown). The extra 2 minute methanol rinse was found to be unnecessary.

Reproducibility – The ability of the instrument to reproduce a chromatographic spectrum was investigated by injecting a twenty replicates of the drug mixture – benzoylecgonine, cocaethylene, cocaine, hydrocodone, and lidocaine where each of the drugs was present at 1mg/mL concentration. Each sample also contained the mepivacaine internal standard. The results of this are shown in Table 4, Appendix A. The data for Table 4 was collected over a period of six days from three runs of 20 injections each. The %CV of the absolute retention time during this set of experiments ranged from 0.28 – 1.08 with the later eluting drugs generating the lowest variance. The peak height remained relatively stable over this six day period, but for actual analysis ratios to the mepivacaine internal standard peak should also be reviewed.

Column Equilibration – In order to investigate the possibility that the 20 minute gradient profile time could be reduced by decreasing time allotted for step 1 of the gradient, four new profiles (Table 5, Appendix A) were created that reduce the equilibration step at the beginning of each sample and then increased the time at the end. The linear gradient step for the mobile phases was maintained at a constant 16.9 minutes. Replicate samples containing cocaine, mepivacaine, and hydromorphone were injected. Reducing the equilibration times immediately following at the beginning of the injection did not significantly impact cocaine which elutes after the internal standard mepivacaine, but the RRT for hydrocodone increased by 58% from 0.29 to 0.46. Based on this result, the EQ0 conditions were maintained.

Drug masking – The possibility exists when analyzing unknown samples that one or the EPI scan may not be triggered if two or more drugs are present with similar retention times. To examine this possibility a series of seven samples was prepared that contained up to seven drugs that had similar retention times. Each sample contained 0.02 mg/L of hydroxyzine. Samples 1-7 also contained 0.2 mg/L amitriptyline. Benztropine was added to samples 2-7, nortriptyline to samples 3-7, paroxetine to samples 4-7, maprotiline to samples 5-7, flunitrazepam to samples 6-7 and clonazepam to sample 7. The total volume of each sample was adjusted to 5 mL. The samples were injected and analyzed using the 20 minute gradient profile. From the results obtained, it was determined that the 20 minute gradient minimized the possibility of peak masking due to similar retention times. The primary caveat to this conclusion was if one of the overlapping drugs was present at much lower concentrations.

A second study was conducted to determine if drugs with similar mass and retention times produced any problems for interpretation. Two pairs of samples were prepared that met this criteria. In the first sample of each pair, drug 1 was present at 0.1 mg/L and drug 2 at 1.0 mg/L. In the second sample of each pair the drug concentrations were reversed. Table 6 in Appendix A shows the two drug pairs, their compound mass and the transition ion that is detected, and the RRT. Both drugs were detected at the concentrations tested in each of the samples; however the resultant EPI spectrum is a varying combination of both drugs, the contributed ions dependent on the individual drug concentrations. An example of this type of mixed spectra is shown in Figure 5, Appendix A. There are no

drugs with the same molecular weight that were examined in this study that when present prevented detection of the other.

Several preliminary studies to investigate quantitation capabilities were performed using calibration standards containing 75 drugs at concentrations of 0.125, 0.25, 0.50, 1.0, 1.5 and 2.0 mg/L in blood. There were several drugs that did not trigger an EPI scan in these preliminary experiments. Careful examination of the analytical data produced from these studies revealed that the detection failures were primarily due to two reasons:

1. The dwell times for drugs with overlapping retention times were too long. While one drug was being detected, a second drug that produces a lower response could elute and the instrument software would ignore the second drug because it was still focused on the response from the first drug. This led to the dwell time studies discussed earlier.
2. The transition responses from commonly encountered and consumed substances like acetaminophen and caffeine were observed throughout the entire chromatogram because these compounds are present in such high concentration compared to the drugs of interest. 'Detuning' by setting the dwell time to 1 msec for these drugs caused the instrument to essentially ignore the signal from these drugs and eliminated this issue.

In addition to the reduced dwell times, it was decided to alter the quantitative analysis method to not generate an EPI spectrum. This further shortened the time required for each instrument scan. With all of the changes incorporated, the scan time for a quantitative analysis is 2.1 seconds vs. 2.65 seconds for the qualitative analysis method. This reduction of only 0.5 seconds enabled an increase in the precision of the quantitative results obtained from an average of 33% to approximately 20%. The latter value is comparable to the precision obtained from more conventional liquid/liquid extractions followed by GC/MS analysis.

Quantitation

The LC/MS/MS was demonstrated to adequately detect and identify 111 of the 114 drugs studied during this research in the experiments outlined above. The next stage was to determine if the instrument could be used to perform quantitative analysis on those same drugs using the same EIA extraction method. To investigate this potential the following studies were performed.

A series of six bloods were extracted using the EIA method. Each of the bloods contained drugs at concentrations of 0.125, 0.25, 0.50, 1.0, 1.5 and 2.0 mg/L. 7.5 µL of the extract was injected using the 20 minute gradient profile. The correlation coefficients of the calibration curves produced were greater than 0.97 in most instances. The curve for cocaine is shown in Figure 6A, Appendix A. Dilutions (1:11) of these samples were then re-injected to see if additional drugs were detected and also to determine the impact on the linearity and intercept. No additional drugs were detected, but the intercept did pass closer to the origin (Figure 6B). In those instances where the correlation coefficient was <0.97, the value increased to greater than 0.98 using the diluted samples. This study demonstrated that the LC/MS/MS was capable of generating a linear calibration curve

over the range of 0 – 0.5 mg/L in undiluted samples and up to 5 mg/L for samples that were diluted.

Technically the instrument can produce a qualitative result and a quantitation from a single sampling and injection as demonstrated above. However, from an operational and quality assurance standpoint for a forensic laboratory, it is preferable to have a replicate test. Since a second 20 minute injection of each sample to attain the quantitative data would cut the instrument throughput potential by 50%, experiments were conducted to determine if a shorter gradient time and a decreased amount of blood could be used to obtain the quantitative result.

Early experiments had shown the potential to conduct a 10 minute gradient profile with only a small loss of resolution. The initial quantitation experiments included MRM detection, followed by generation of an EPI spectrum for ions of interest. Dwell times had not been optimized at the time of those studies, resulting in potential detection failures in the case of samples containing multiple drugs. Taking advantage of the refined dwell times and the a priori knowledge of what drugs are present in each sample after the qualitative analysis, it was possible to reduce the overall gradient profile to 8 minutes for the quantitative analysis.

The second component of this study was to determine if a smaller quantity of blood could be used for the EIA extraction. Since the aliquot removed for the original EIA extraction for qualitative analysis was 1 ml, but only 100 µL of the 500 µL final solution was used for the LC/MS/MS analysis, a volume of 200 µL of blood was selected and proven to produce reliable results. To verify that the quantitative values obtained from the new procedure were equivalent to existing GC/MS procedures in place, fifteen drugs were spiked into fifteen replicate pairs of blood samples at a concentration of 0.3 mg/L each. One of each replicate pair of was extracted using the EIA procedure followed by the 8 minute gradient LC/MS/MS analysis, with the other sample extracted using routine liquid/liquid methods followed by GC/MS analysis. The results are shown in Table 7, Appendix A. These results demonstrate that the LC/MS/MS analysis produces quantitative results at least as accurate as the GC/MS method. In addition to these fifteen drugs, a total of 34 different drugs have been demonstrated to produce suitable calibration curves and precision. See Table 8, Appendix A for a listing of the drugs that have been confirmed to produce satisfactory quantitation results.

A secondary goal was to determine if a calibration curve could be generated, stored and used for subsequent analysis without having to repeatedly extract and analyze known calibrator samples. The results of this study were not promising (data not shown) because the variance of the ratio of the analyte peak area to that of the internal standard was too high on a day to day basis. This type of stored curve is suitable for a rough estimate of concentration during the qualitative analysis so that in cases where drugs are present in extremely high concentrations, dilutions can be made prior to the quantitative analysis to obtain responses that remain on the linear calibration curve.

Concordance Studies

The ultimate test of any new procedure is how it compares with existing methodologies. Are the results of the new procedure at least equivalent to the existing procedure and do they improve on the existing procedure in any way? Two separate concordance studies were undertaken during this project to examine these questions.

The first concordance study involved the reanalysis of samples from 168 previously analyzed cases that were scheduled for sample destruction. One important note regarding the GC/MS analysis is that the liquid/liquid (LL) or solid phase extraction (SPE) extractions consume 3-5 mL of blood per drug class as compared to the 1 ml or less required for the LC/MS/MS analysis. Sample consumption is an important distinction when considering the overall utility of a method. These samples were extracted using the EIA procedure and the resultant extract injected onto the LC/MS/MS. Results from this study were compiled into three categories: concordant, minor discrepancies and not concordant. Initially there were nine cases that appeared to produce non concordant results, but subsequent re-analysis using both GC/MS and LC/MS/MS determined that the initial results were most likely due to drug degradation during sample storage. One drug identified through GC/MS analysis (midazolam) was not identified in the LC/MS/MS analysis. Conversely there were several drugs that were identified through the LC/MS/MS analysis (none of which were material to the conclusions reached in the cases), that were not identified in the GC/MS analysis.

The second concordance study involved parallel testing of samples for both qualitative and quantitative information. 50 cases were tested using both existing methods and the newly developed LC/MS/MS methods. A total of 87 drugs were identified, but the initial quantitations from the LC/MS/MS method were significantly different in 8 cases from the first two batches of samples tested. No conclusive determination of the root cause of the variations was discovered, other than potential differences in sample preparation by different individuals. There were no significant differences observed in the third batch of samples and this issue has not been observed subsequent to that experiment.

To demonstrate the practical utility of this method, a group of 123 cases was analyzed using both existing GC/MS methods and the LC/MS/MS method. From this group of 123 cases, there were 27 cases that screened negative via the six immunoassay panel of cocaine, THC, opiates, barbiturates, benzodiazepines, and amphetamines, yet were found to contain drugs in the LC/MS/MS analysis. An additional 18 cases had drugs present that are not detected by the immunoassay in conjunction with drugs that do screen positive. In those cases, the additional drugs would not normally have been detected until after the first GC/MS analysis. By using the LC/MS/MS data, the analyst was more fully aware of the necessary controls and procedures necessary to complete those analyses. In a third subset of 11 cases, drugs were detected via the LC/MS/MS that were not identified by GC/MS because of insufficient sensitivity. The final subset of these 123 cases in which the LC/MS/MS played a significant role in streamlining the analysis was the 28 cases in which a general total base drug screen was necessary. Those 28 cases all screened negative with the six immunoassay classes and the LC/MS/MS. By obtaining this information at the beginning of the case analysis, no additional work was necessary on those cases. The remainder of the 123 cases would have been equivalent regardless of the method chosen. As a result of using the LC/MS/MS procedure the analysis of a total of 84 cases (68%) was enhanced.

Drugs Not Amenable to Current Procedure

As discussed earlier, there were four drugs that did not produce results from the LC/MS/MS equivalent to those that can be obtained from current GC/MS methods.

These drugs were morphine, hydromorphone, nalorphine and THCA. In the case of the first three, the drug appears to elute from the column at such an early retention time that the signal is masked by ion suppression from components present in the EIA extract buffer. Additional studies are still underway to determine if different gradient profiles or other instrumental parameters could improve the detection of these drugs. Until a more satisfactory analysis on the LC/MS/MS is possible, these drugs will continue to be analyzed using current methods, i.e. immunoassay followed by GC/MS for confirmation. THCA is detected on the LC/MS/MS, but the sensitivity of the instrument wasn't sufficient to detect the presence of the compound in the EIA extract. Instead the extract had to be further concentrated or a different extraction method employed that either used more sample or further concentrated the drug.

Discussion

This project was designed to determine if the simple extraction process used in the analysis of samples via enzyme immunoassay could be utilized to prepare samples for analysis of samples via LC/MS/MS. Although several other groups have used LC/MS/MS for analysis of toxicology samples (6-12, 14), the preparative steps were more complex than those involved in the method described in this project. The studies conducted in this project have definitely proven that the EIA extract is suitable for both the qualitative and quantitative analysis of the majority of drugs encountered during routine toxicology analysis. There were a few drugs that were not amenable to the analysis, but in developing this procedure, it was decided that the first step would be a general method that worked for the majority of drugs.

The extraction procedure is relatively quick and inexpensive when compared to liquid/liquid or solid phase column methods, requiring only about 60-90 minutes to complete for a batch of 30-40 samples. One of the primary benefits of utilizing this method vs. SPE is that this extraction process is already required for the immunoassay procedure, thus no additional processing other than placing an aliquot into the LC/MS/MS vial is required to obtain a full detail screen of the drugs present in the sample. Furthermore, this extraction process is simple to perform and can be completed by a technician, allowing more highly trained scientists to focus their time on data analysis and interpretation.

The LC/MS/MS provides confirmatory information about the drugs present in the sample. In the method described, the immunoassay is still considered 'presumptive'. The first LC/MS/MS analysis provides a definitive determination of the drugs present in the sample with the exceptions noted previously. The presence of the drug is confirmed through the relative retention time, the specific mass transition and the mass spectrum from that parent ion. A second extraction and injection into the LC/MS/MS can then be used to replicate the initial results and/or obtain quantitative information. From a qualititative standpoint, the information obtained from the LC/MS/MS is at least equivalent to that obtained from more routinely used methods with the exception of the drugs discussed above. In some respects, the information from the new procedure poses a problem to the practicing forensic toxicologist, because drugs are often detected at levels that have no impact on the conclusions that will be reached in the case. Therefore, the laboratory needs to evaluate the results to establish operational guidelines of when the presence of the drug will trigger further quantitative analysis.

The quantitative analysis obtained from the LC/MS/MS is equivalent to that obtained from GC/MS in both values obtained and the level of variance observed. There are some significant advantages to conducting the quantitative analysis on the LC/MS/MS however. First, the amount of sample required is only 200 µL as compared to the 3-5 mL required for most liquid/liquid or solid phase preparations. This is an important advantage since it preserves sample for additional testing if needed. Secondly, no derivatization steps are required to prepare the drugs for volatilization in the GC. Third, the time and labor involved in the extraction process is only approximately 20% of that required for GC/MS analysis. In cases where multiple drug classes are observed that would normally require several extractions to obtain all necessary analytical information, up to 80% of the time and labor involved in extractions could be saved by obtaining

equivalent information after only a single simple extraction and LC/MS/MS injection. The data analysis and interpretation of the information obtained from the LC/MS/MS appears to take approximately the same amount of time as that needed for GC/MS analysis.

The purchase of an LC/MS/MS instrument is a significant investment for a forensic laboratory. To justify such a large investment, the laboratory must understand the alternatives and the benefits that would be realized. One alternative is to purchase and validate commercial immunoassay reagents in an effort to produce a more complete presumptive data set on what drugs are present in the system. This could minimize the number of drugs that are only detected after the first GC-MS analysis. Unfortunately, the immunoassay tests are still only presumptive in nature and do not provide the structural elucidation necessary to confirm the presence of a specific drug compound. GC-MS analysis is still required to obtain the structural information. Although GC-MS analysis is an extremely valuable technique, it does have limitations in that many drugs require derivatization in order to make them sufficiently volatile to inject onto the instrument and other drugs are so thermally labile that they are destroyed in the process of injecting them onto the GC. Both of these problems are eliminated in the LC/MS/MS process described here.

In summary, the LC/MS/MS instrument can be successfully used to identify and quantitate a wide variety of drugs routinely encountered in forensic toxicology using a simple, inexpensive extraction process. The implementation of this type of instrumentation has the potential to save significant amounts of time and labor involved in sample preparation steps, especially in cases where there is no suspected cause of death and general screening procedures must be employed or in cases where multiple drug classes have been identified. Just in the first few months of operational use (in parallel with GC/MS analysis) in our laboratory, the results from the procedures developed during this project have demonstrated that the operational effectiveness of the toxicology group will be significantly improved.

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Appendix A

Table 1

Mass Spectrometer Parameters

Compound	M+1	Fragment	CE	DP	EP	CEP	CXP	Dwell
Acebutolol	337.2	116	29	51	10.5	20.27	4	10
Acetaminophen	152	65	39 (12)	46 (70)	9.0 (4)	14.0	4	1
Acethylmorphine-6	328.1	165	55	61	9.5	19.92	4	25
Alprazolam	309.1	205	53	46	10.0	19.23	4	25
Amitriptyline	278.2	233	27	36	9.5	17.99	4	10
Amoxapine	314.1	271	29	41	10.5	19.38	4	10
Amphetamine	136.1	91	23	26	5.0	12.00	4	10
Atomoxetine	256.2	148	13	21	4.5	20.00	4	10
Benzoylecgonine	290.1	168	27	36	9.0	18.49	4	10
Benztropine	308.2	167	41	51	10.5	19.23	4	10
Brompheniramine	319.1	274	23	21	8.0	19.57	4	25
Bupivacaine	289.2	140	27	41	10.5	20.00	4	1
Bupropion	240.1	184	17	31	10.0	16.51	4	10
Buspirone	386.2	122	43	61	9.5	22.20	4	10
Caffeine*	195.1	110	31(13)	51(25)	10.5(4)	18.00	4	1
Carbamazepine	237.1	194	31	61	10.0	16.44	4	1
Carbinoxamine	291.1	167	49	26	6.5	18.49	4	1
Carisoprodol	261.2	176	13	31	9.0	17.33	4	1
Chlordiazepoxide	300.1	227	37	46	10.5	24.00	4	10
Chlorpheniramine	275.1	230	29	26	6.5	17.87	4	25
Chlorpromazine	319.1	58	57	36	9.0	19.57	2	25
Chlorprothixene	316.1	231	41	51	10.0	19.45	4	10
Citalopram	325.2	109	41	51	10.0	19.92	4	10
Clomipramine	315.2	58	59	36	8.5	19.45	2	10
Clonazepam	316.0	270	45	61	9.0	19.45	4	25
Clonidine	230.0	124	55	61	9.0	16.12	4	25
Clozapine	327.1	270	39	46	9.5	19.92	4	10
Cocaethylene	318.2	196	25	41	9.5	19.57	4	10
Cocaine	304.1	182	27	36	9.5	19.07	4	10
Codeine	300.2	152	85	56	10.5	18.87	4	25
Cyclobenzaprine	276.2	215	49	36	10.5	17.91	4	10
Desipramine	267.2	72	25	56	11.0	17.56	4	25
Dextromethorphan	272.2	128	75	56	11.0	17.76	4	1
Diazepam	285.1	193	45	56	10.5	26.00	4	25
Diltiazem	415.2	178	33	41	10.0	23.29	4	25
Diphenhydramine	256.2	167	21	16	4.5	17.14	4	10
Doxepin	280.2	107	33	36	10.5	18.10	4	25
Doxylamine	271.2	167	49	21	5.0	17.76	4	10
Estazolam	295.1	205	53	51	10.0	18.64	4	10
Fenfluramine	232.1	159	33	46	10.5	16.20	4	10
Fentanyl	337.2	188	29	41	9.5	20.27	4	25
Flecainide	415.1	301	35	56	10.5	23.29	4	10

Flunitrazepam	314.1	268	27	61	11.0	19.38	4	25
Fluoxetine	310	44	31	26	5.0	28	4	10
Flurazepam	388.2	315	27	41	10.5	22.24	6	10
Haloperidol	376.1	123	55	46	10.5	21.77	4	25
Hydrocodone	300.2	199	41	61	10.0	18.87	4	25
Hydromorphone	286.1	185	41	71	10.5	22.00	4	25
Hydroxyzine	375.2	201	25	26	8.5	21.77	4	10
Imipramine	281.2	86	23	36	4.5	18.10	2	10
Ketamine	238.1	125	39	36	10.5	16.44	4	10
Lamotrigine	256.0	211	33	61	9.5	17.14	4	10
Lidocaine	235.2	86	25	31	10.5	16.32	4	1
Loratadine	383.1	337	41	56	8.0	22.05	6	10
Lorazepam	321.0	275	29	36	7.5	19.64	4	25
Loxapine	328.1	271	27	46	10.0	19.92	4	25
Maprotiline	278.2	191	47	51	10.5	17.99	4	10
MDA	180.1	105	29	21	4.5	14.00	4	10
MDMA	194.1	163	15	36	9.5	14.00	4	10
Meclizine	391.2	201	25	26	10.0	22.36	4	10
Meperidine	248.2	220	33	46	9.0	16.83	4	1
Mepivacaine	247.2	98	27	41	10.5	16.83	4	10
Meprobamate	219.1	158	13	31	10.5	16.00	4	1
Mesoridazine	387.1	98	55	56	10.5	22.20	4	10
Metaxalone	222.1	161	15	31	9.5	15.78	4	10
Methadone	310.2	265	19	26	5.0	19.23	4	10
Methamphetamine	150.1	91	23	31	4.5	14.00	4	10
Methylphenidate	234.1	84	29	31	4.5	16.28	4	25
Metoclopramide	300.1	227	21	36	6.5	18.87	4	10
Metoprolol	268.2	56	43	36	9.0	17.6	2	10
Midazolam	326.1	223	49	56	9.0	19.92	4	10
Mirtazapine	266.2	195	35	36	8.5	20.65	4	10
Morphine	286.1	152	57	56	10.5	26.00	4	25
Morphine-6-glucuronide	462.2	286	47	66	9.0	24.48	4	25
Nalorphine	312.2	152	81	66	10.5	19.38	4	10
Nefazodone	470.2	274	33	66	10.5	25.41	4	10
Nifedipine	347.1	254	25	41	9.0	20.65	4	10
Nordiazepam	271.1	140	39	56	10.5	20.00	4	10
Normeperidine	234.3	160	19	31	3.5	22	4	25
Norpropoxyphene	326.2	252	13	11	4.5	19.92	4	10
Nortriptyline	264.2	233	17	46	10.5	17.45	4	10
Olanzapine	313.1	256	33	41	10.0	19.38	4	25
Orphenadrine	270.2	181	17	21	9.5	22.00	4	1
Oxazepam	287.1	241	35	41	10.0	18.33	4	1
Oxycodone	316.1	241	43	51	8.5	19.45	4	25
Paroxetine	330.1	70	45	56	8.5	20.00	4	10
Pentazocine	286.2	218	23	46	8.5	18.33	4	10
Perphenazine	404.1	171	29	56	9.0	18.00	4	10
Phencyclidine	244.2	159	17	26	7.5	16.67	4	25
Phentermine	150.1	91	27	26	8.5	12.00	4	10
Piroxicam	332.1	95	41	46	9.5	20.07	4	1
Promethazine	285.1	198	37	26	8.0	24.00	4	10

Propoxyphene	340.2	58	31	11	4.5	20.39	2	10
Propranolol	260.2	116	23	36	9.5	17.33	4	10
Pseudoephedrine	166.1	115	35	26	9.5	12.00	4	10
Quazepam	387.0	258	57	66	10.5	22.20	4	1
Quetiapine	384.2	253	27	41	9.5	22.09	4	10
Quinine	325.2	172	47	51	10.0	19.92	4	1
Ranitidine	315.1	176	21	31	6.0	19.45	4	10
Scopolamine	304.1	138	33	46	10.5	19.07	4	25
Sertraline	306.1	275	17	21	5.0	19.07	12	25
Strychnine	335.2	184	53	86	10.5	20.19	4	10
Temazepam	301.1	255	27	36	9.0	18.87	4	1
Terbutaline	226.1	152	21	46	10.5	15.97	4	10
Theophylline	181.1	124	25	51	10.5	14.00	4	1
Thioridazine	371.2	98	51	46	10.5	21.62	4	10
Tramadol	264.2	58	39	26	6.5	17.45	4	10
Trazodone	372.2	176	33	51	10.5	21.62	4	1
Triazolam	343.0	239	57	56	10.5	20.49	4	10
Trifluoperazine	408.2	113	41	56	9.5	23.02	4	25
Trihexyphenidyl	302.2	98	35	31	10.0	18.92	4	10
Venlafaxine	278.2	58	33	26	7.5	17.99	2	10
Verapamil	455.3	165	41	56	9.5	24.84	4	1
Zolpidem	308.2	235	49	66	9.5	19.23	4	10

Figure 1A

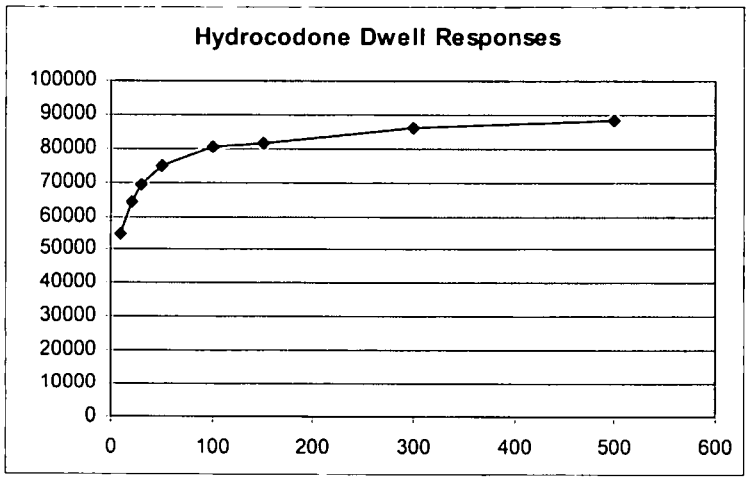

Figure 1B

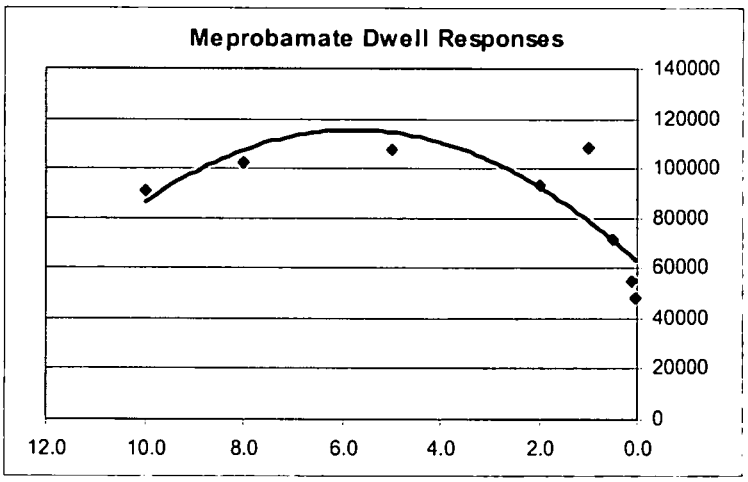

Figure 1A: A typical graph of the effect of increasing the dwell time and measuring an analyte response, hydrocodone in this case. It shows that up to a point an increased dwell yields significant increase in response and beyond that the benefit is diminished. Higher dwell times increase the total analytical scan time, a negative attribute. 1B: Lowering the dwell time reduces the drug response. The lowest practical dwell used in a method is 1 ms.

Table 2

HPLC Gradient Profiles

10 minute run	20 minute run	30 minute run
0 – 1 min, 95% A:5% B	0 – 1 min, 95% A:5% B	0 – 1 min, 95% A:5% B
1 – 8.9 min, 95% A:5% B → 95% B:5% A	1 – 17.9 min, 95% A:5% B → 95% B:5% A	1 – 27.9 min, 95% A:5% B → 95% B:5% A
8.9 – 9 min, 95% B:5% B → 95% B:5% A	17.9 – 18 min, 95% B:5% B → 95% B:5% A	27.9 – 28 min, 95% B:5% B → 95% B:5% A
9 – 10 min, 95% A:5% B	18 – 20 min, 95% A:5% B	28 – 30 min, 95% A:5% B

Mobile Phase A – 0.1% formic acid, 1 g/L (~15mM) ammonium formate in HPLC grade water; Mobile Phase B – 0.1% formic acid, 1 g/L (~15mM) ammonium formate in HPLC grade methanol.

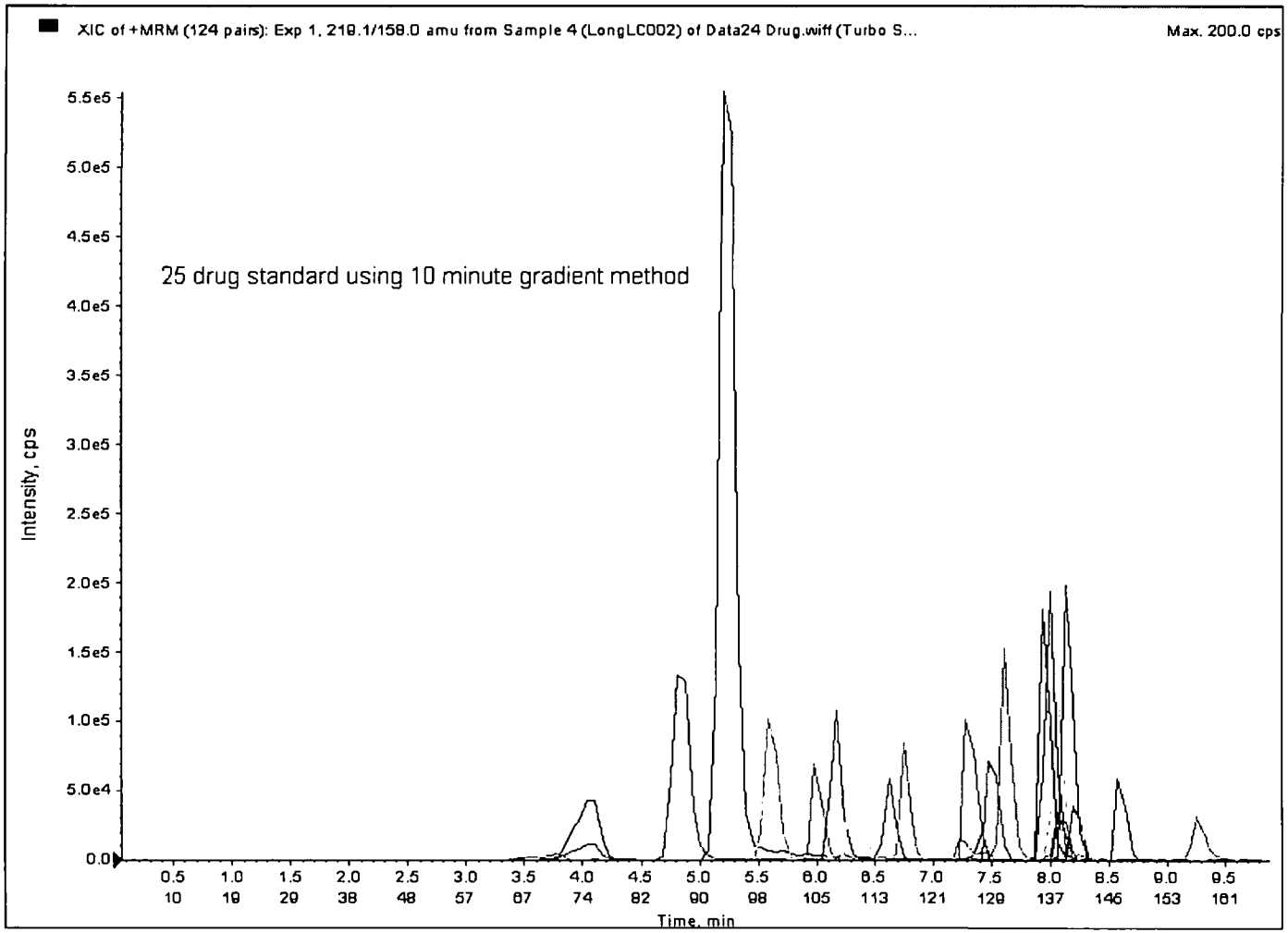

Figure 2A – 10 minute HPLC gradient profile of 25 drug mixture.

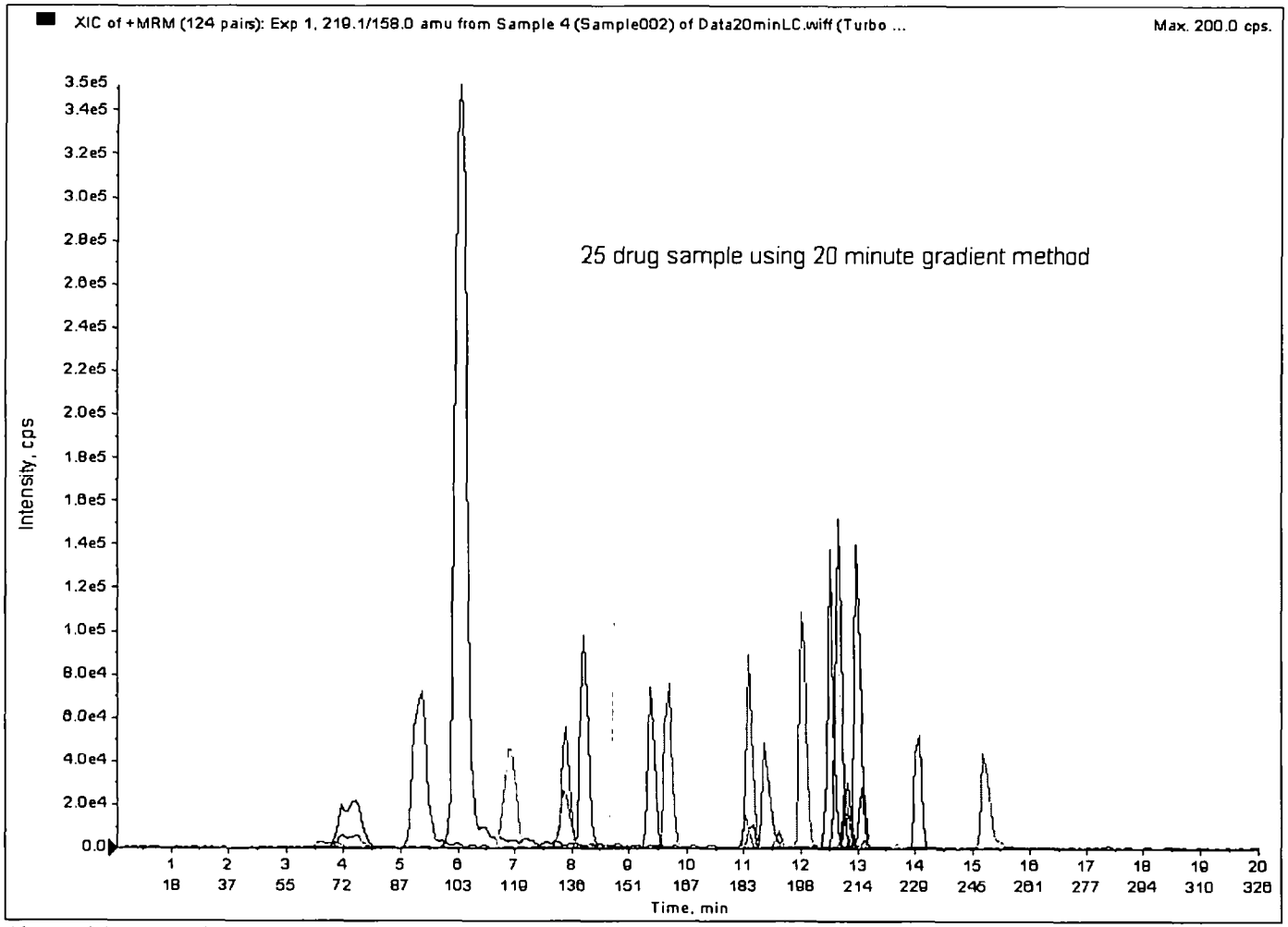

Figure 2B – 20 minute HPLC gradient profile of 25 drug mixture.

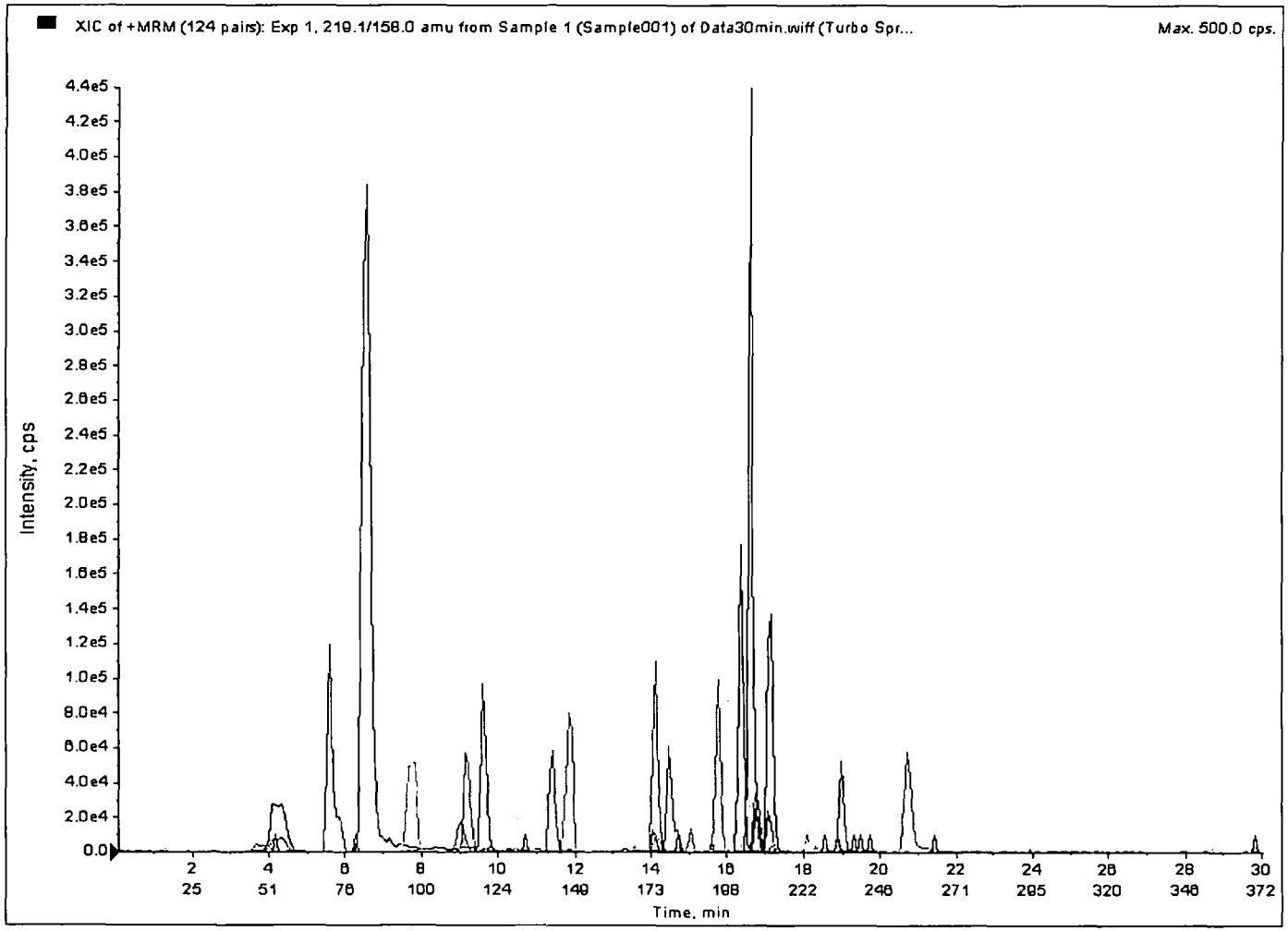

Figure 2C – 30 minute HPLC gradient profile of 25 drug mixture.

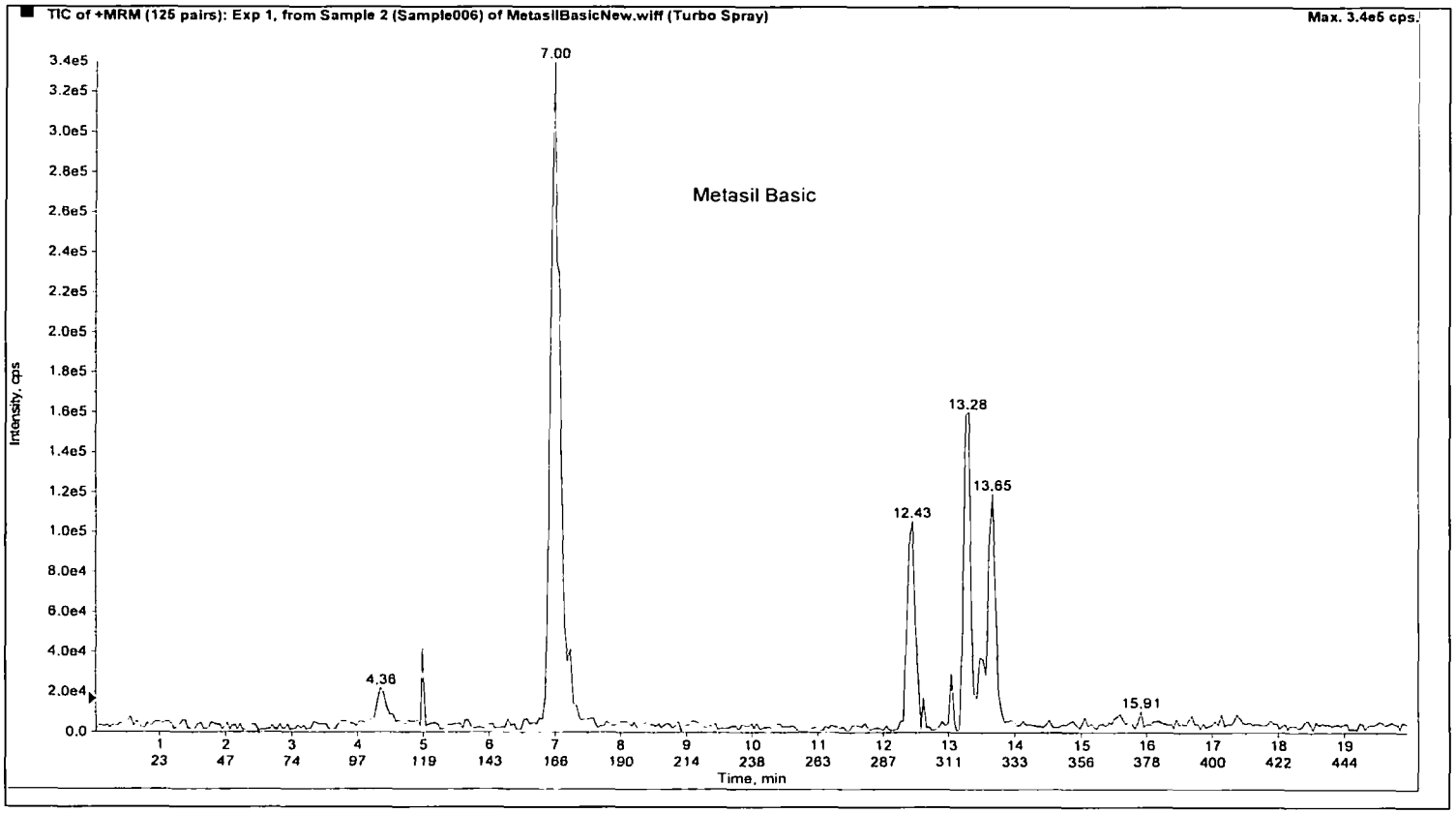

Figure 3A – Chromatography results of a six drug test mix using Metasil Basic column and 20 min HPLC gradient profile.

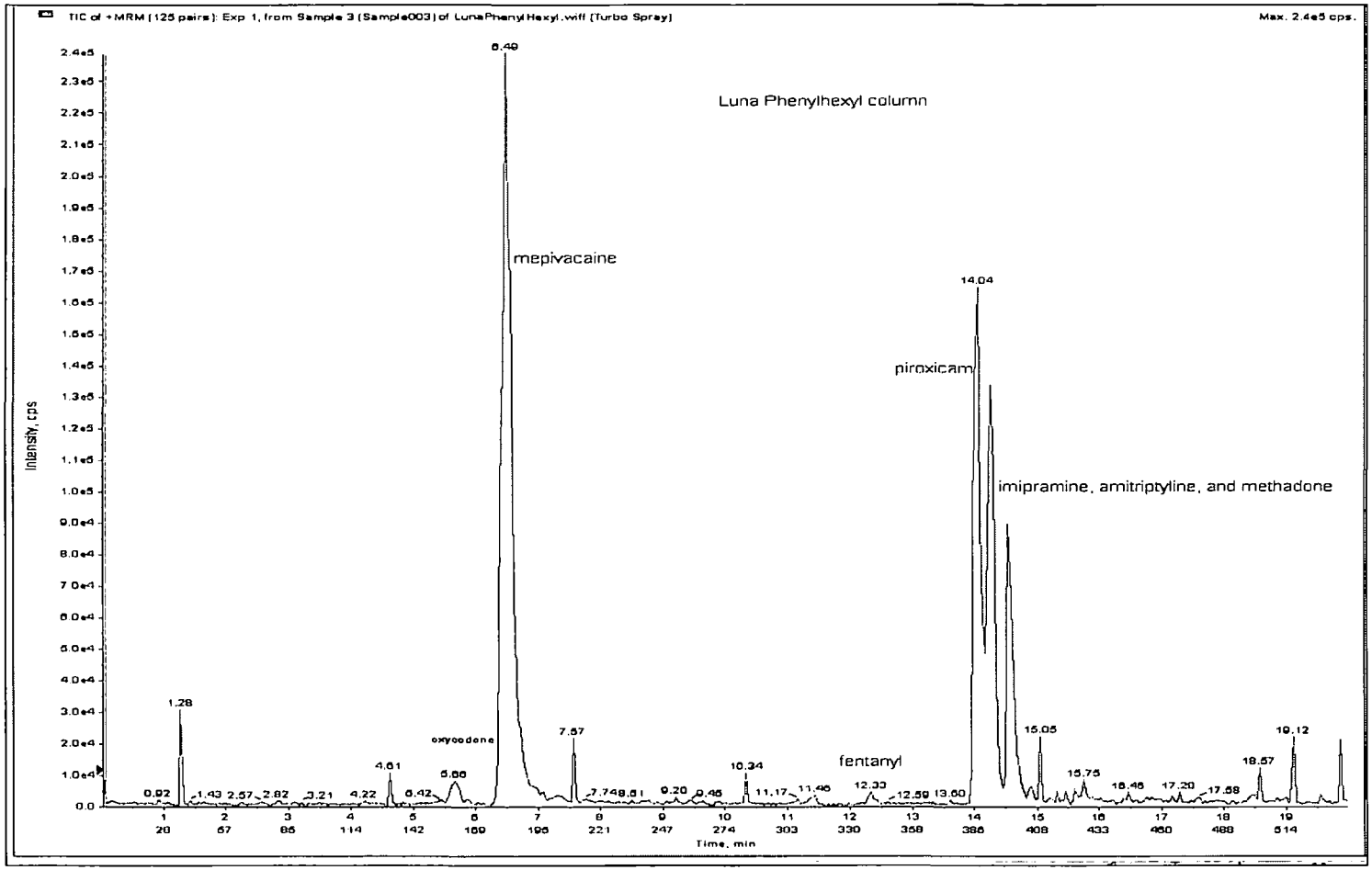

Figure 3B – Chromatography results of a six drug test mix using Phenomenex Luna 3u Phenyl - Hexyl column and 20 min HPLC gradient profile.

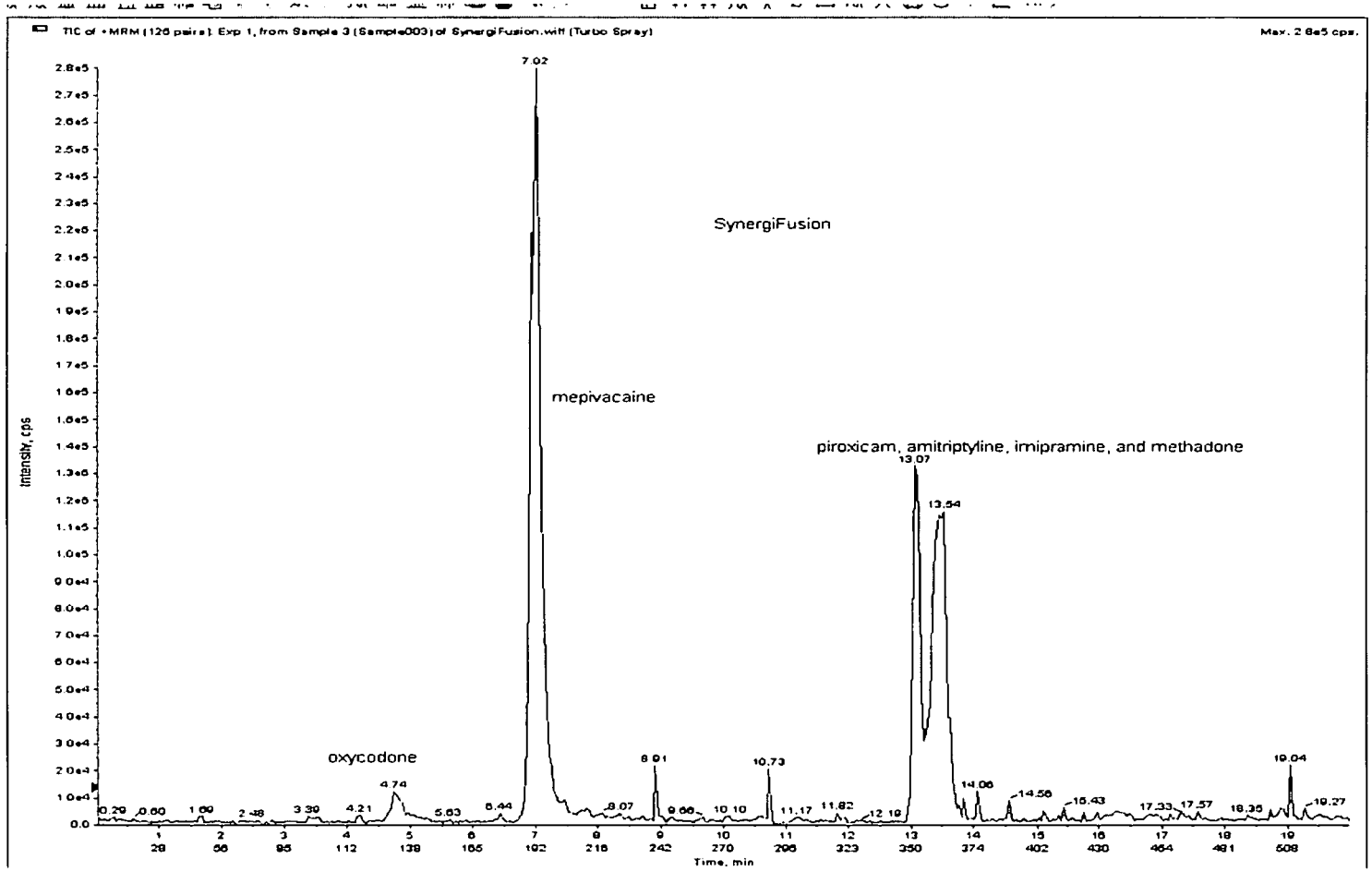

Figure 3C – Chromatography results of a six drug test mix using Phenomenex Synergi 4u Fusion - RP 80A column and 20 min HPLC gradient profile.

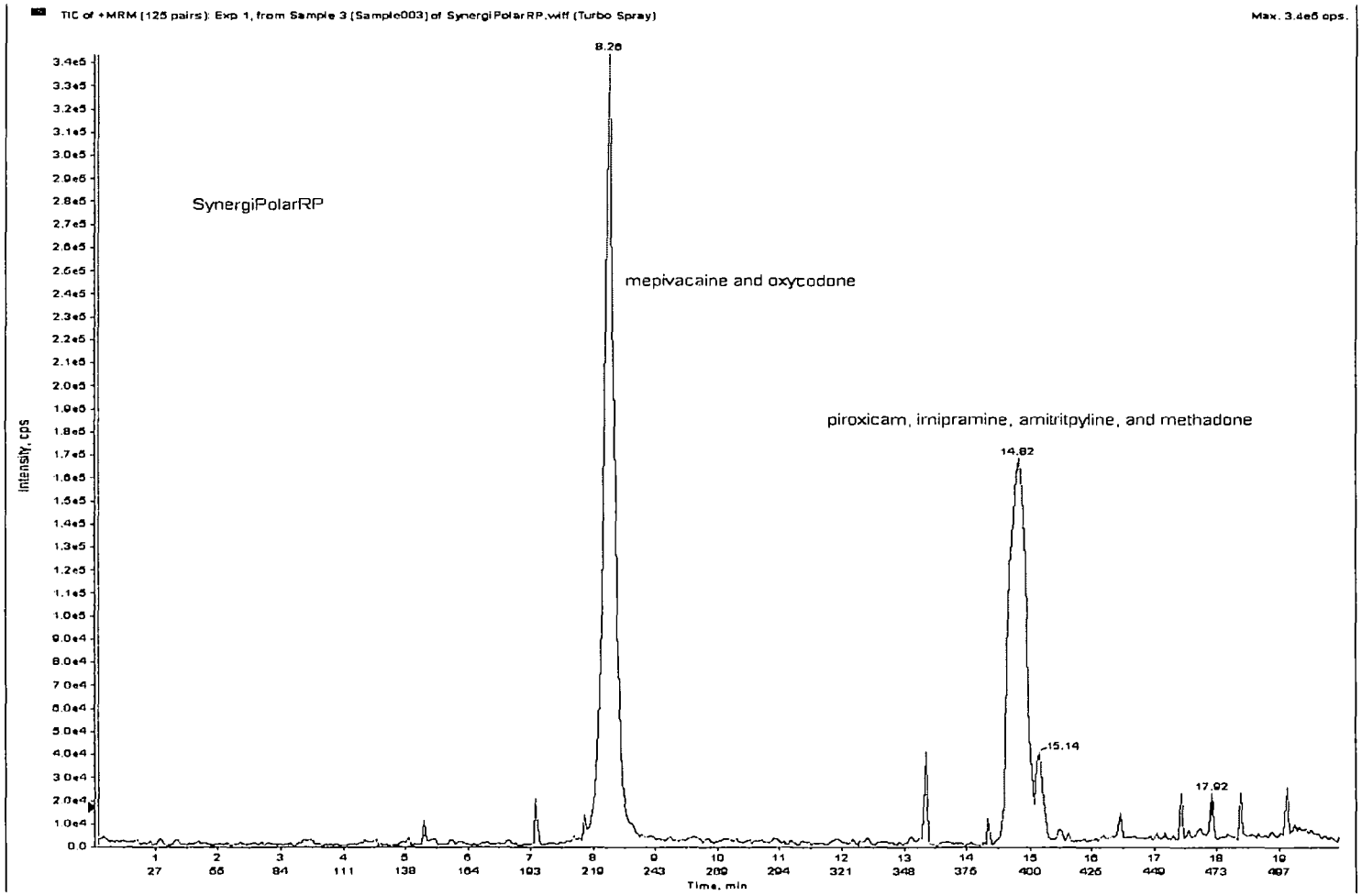

Figure 3D – Chromatography results of a six drug test mix using Phenomenex Synergi 4u Polar - RP 80A column and 20 min HPLC gradient profile.

Table 3

Relative Retention Times and Limits of Identification

RRT	drug	M+1	Fragment	LOI (mg/mL)	Desired Target Level (mg/L)
0.14	Morphine-6-glucuronide	462.2	286	ND	
0.26	Terbutaline	226.1	152	0.05	0.006
0.27	Hydromorphone	286.1	185	>0.3	0.02
0.45	Ranitidine	315.1	176	ND	
0.51	Morphine	286.1	152	>0.3	0.1
0.58	Codeine	300.2	152	0.1	0.1
0.58	Pseudoephedrine	166.1	115	0.02	0.08
0.6	Acetaminophen	152	65	5	10
0.6	Oxycodone	316.1	241	0.05	0.03
0.62	Scopolamine	304.1	138	0.01	0.001
0.65	Clonidine	230	124	0.07	0.001
0.68	Hydrocodone	300.2	199	0.02	0.03
0.69	Amphetamine	136.1	91	0.05	0.1
0.71	Methamphetamine	150.1	91	0.03	0.05
0.72	MDA	180.1	105	0.1	0.3
0.72	Theophylline	181.1	124	1.5	10
0.73	Olanzapine	313.1	256	0.1	0.02
0.78	Acethylmorphine, 6-	328.1	165	ND	
0.78	MDMA	194.1	163	0.02	0.1
0.89	Lidocaine	235.2	86	0.03	1.5
0.94	Phentermine	150.1	91	0.05	0.1
0.95	Caffeine	195.1	138	5	10
0.97	Doxylamine	271.2	167	0.01	0.1
0.97	Strychnine	335.2	184	0.03	0.1
1	Mepivacaine	247.2	98	ND	
1.12	Ketamine	238.1	125	0.005	1
1.13	Metoclopramide	300.1	227	0.005	0.05
1.14	Benzoylecgonine	290.1	168	0.02	0.3
1.21	Tramadol	264.2	58	0.005	0.3
1.3	Metoprolol	268.2	56	0.02	0.07
1.31	Cocaine	304.1	182	0.005	0.3
1.33	Methylphenidate	234.1	84	0.02	0.03
1.34	Acebutolol	337.2	116	0.005	0.8
1.36	Mirtazapine	266.2	195	0.005	0.03
1.39	Lamotrigine	256	211	0.02	2
1.4	Quinine	325.2	172	0.02	3
1.43	Meperidine	248.2	220	0.005	0.2
1.45	Zolpidem	308.2	235	0.005	0.06
1.52	Bupropion	240.1	184	0.02	0.1
1.55	Cocaethylene	318.2	196	0.005	

1.6	Pentazocine	286.2	218	0.01	0.2
1.6	Trazodone	372.2	176	0.005	1
1.63	Carbinoxamine	291.1	167	0.05	
1.63	Chlorpheniramine	275.1	230	0.02	0.05
1.64	Buspirone	386.2	122	0.005	0.001
1.65	Phencyclidine	244.2	159	0.05	0.08
1.67	Fenfluramine	232.1	159	0.01	0.04
1.68	Venlafaxine	278.2	58	0.02	0.1
1.7	Bupivacaine	289.2	140	0.3	0.9
1.72	Brompheniramine	319.1	274	0.03	0.05
1.72	Meprobamate	219.1	158	0.45	5
1.79	Fentanyl	337.2	188	0.005	0.001
1.8	Dextromethorphan	272.2	128	0.005	0.3
1.8	Propranolol	260.2	116	0.02	0.01
1.83	Diphenhydramine	256.2	167	0.005	0.08
1.83	Midazolam	326.1	223	0.005	0.08
1.85	Citalopram	325.2	109	0.01	0.1
1.85	Clozapine	327.1	270	0.02	0.2
1.85	Flurazepam	388.2	315	0.005	0.03
1.87	Doxepin	280.2	107	0.005	0.1
1.88	Chlordiazepoxide	300.1	227	0.01	0.7
1.89	Flecainide	415.1	301	0.01	0.2
1.9	Quetiapine	384.2	253	0.005	0.3
1.92	Diltiazem	415.2	178	0.005	0.1
1.93	Mesoridazine	387.1	98	0.02	1
1.95	Piroxicam	332.1	95	0.25	2
2	Orphenadrine	270.2	181	0.005	0.2
2	Verapamil	455.3	165	0.005	0.2
2.02	Haloperidol	376.1	123	0.005	0.006
2.03	Amoxapine	314.1	271	0.02	0.05
2.03	Propoxyphene	340.2	58	0.3	0.2
2.05	Carbamazepine	237.1	194	0.01	11
2.05	Loxapine	328.1	271	0.01	0.01
2.05	Oxazepam	287.1	241	0.03	1
2.07	Cyclobenzaprine	276.2	215	0.005	0.02
2.07	Promethazine	285.1	198	0.005	0.03
2.08	Imipramine	281.2	86	0.03	0.05
2.08	Methadone	310.2	265	0.005	0.1
2.08	Norpropoxyphene	326.2	252	0.5	
2.09	Trihexyphenidyl	302.2	98	0.005	0.03
2.1	Desipramine	267.2	72	0.01	0.05
2.1	Metaxalone	222.1	161	0.02	
2.12	Amitriptyline	278.2	233	0.01	0.1
2.13	Benztropine	308.2	167	0.005	0.08
2.14	Nortriptyline	264.2	233	0.005	0.05
2.15	Hydroxyzine	375.2	201	0.005	0.07
2.15	Maprotiline	278.2	191	0.005	0.1

2.15	Paroxetine	330.1	70	0.005	0.6
2.17	Clonazepam	316	270	0.07	0.02
2.17	Flunitrazepam	314.1	268	0.005	0.008
2.18	Atomoxetine	256.2	148	ND	
2.19	Estazolam	295.1	205	0.01	0.05
2.2	Carisoprodol	261.2	176	0.45	10
2.23	Alprazolam	309.1	205	0.005	0.03
2.24	Triazolam	343	239	0.005	0.03
2.25	Lorazepam	321	275	0.01	0.02
2.25	Temazepam	301.1	255	0.01	0.4
2.26	Nalorphine	312.2	152	>0.3	
2.28	Fluoxetine	310	44		0.1
2.28	Nefazodone	470.2	274	0.005	0.1
2.29	Clomipramine	315.2	58	0.01	0.1
2.3	Chlorprothixene	316.1	231	0.03	0.04
2.31	Sertraline	306.1	275	0.005	0.06
2.32	Chlorpromazine	319.1	58	0.01	0.02
2.33	Thioridazine	371.2	98	0.1	0.2
2.34	Diazepam	285.1	193	0.005	0.1
2.37	Nordiazepam	271.1	140	0.01	0.1
2.39	Nifedipine	347.1	254	0.07	0.04
2.47	Perphenazine	404.1	171	ND	0.02
2.49	Meclizine	391.2	201	0.01	
2.49	Trifluoperazine	408.2	113	0.01	0.001
2.59	Quazepam	387	258	0.2	11
2.74	Loratadine	383.1	337	0.005	0.01

Table 4

Reproducibility

		Retention Time (in seconds) N=58			Peak Area N=60		
1 mg/ml	RRT	Mean	Std. Dev	%CV	Mean	Std. Dev.	%CV
Benzoylecgonine	1.14	695.43	2.264	0.33	4550147	523029.9	11.49
Cocaethylene	1.55	930.26	2.646	0.28	1745482	169416.6	9.71
Cocaine	1.31	779.26	2.173	0.28	361300	44884.1	12.42
Hydrocodone	0.68	393.86	4.249	1.08	1473218	121365.7	8.24
Lidocaine	0.89	496.07	4.573	0.92	4657577	509962	10.95

Table 5A

Equilibration Time Gradient Profiles

Samples	95A:5B	95A: 5B ->5A:95B	95B:5A wash	95A:5B final equilibrium (total run time)
EQ0	0-1.0 min	1.0-17.9 min	17.9-18.0 min	20.0 min
EQ1	0-0.8 min	0.8-17.7 min	17.7-17.8 min	19.3 min
EQ2	0-0.7 min	0.7-17.6 min	17.6-17.7 min	18.7 min
EQ3	0-0.6 min	0.6-17.5 min	17.5-17.6 min	18.1 min
EQ4	0-0.5 min	0.5-17.4 min	17.4-17.4 min	17.5 min

Table 5B

Effect on RRT of Changing Equilibration Steps in Gradient Profile

	Cocaine	Hydromorphone
EQ0	1.29	0.29
EQ1	1.30	0.355
EQ2	1.295	0.425
EQ3	1.30	0.45
EQ4	1.305	0.46

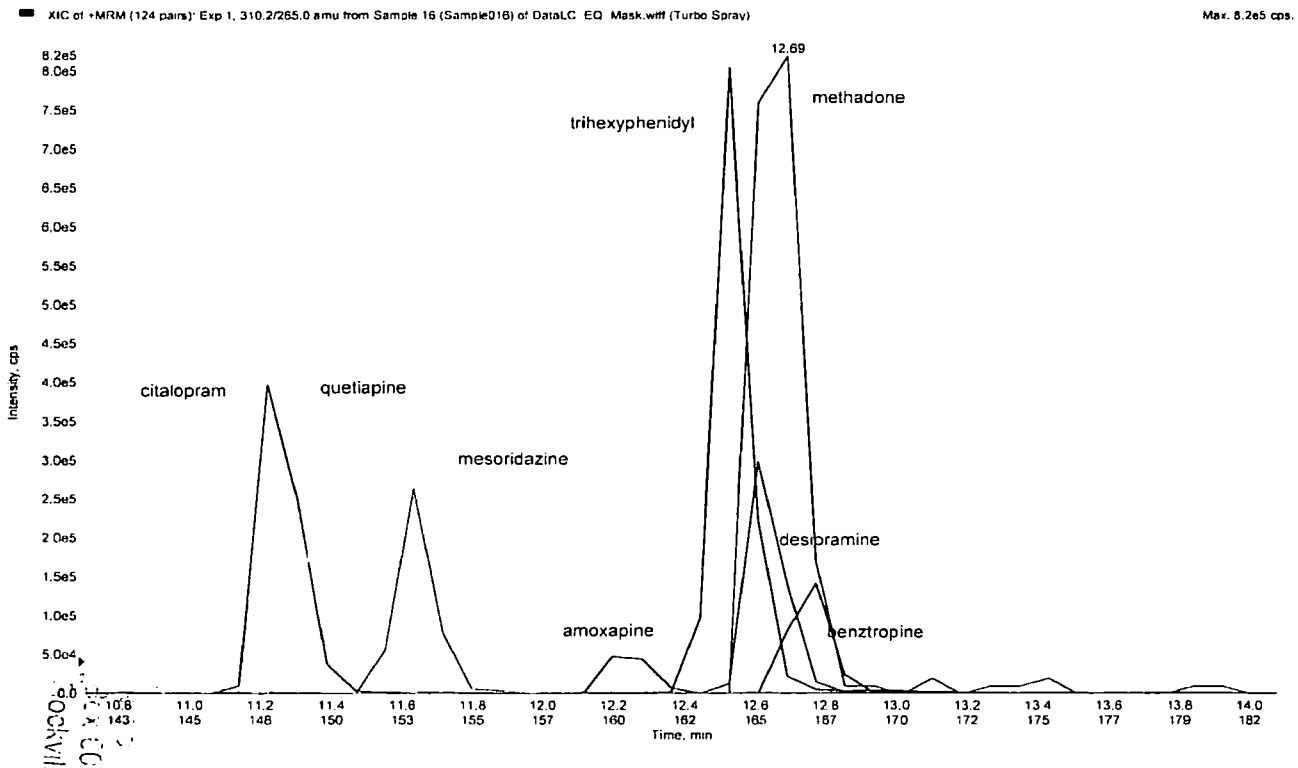

Figure 4: Chromatogram of 7 drug mixture using drugs that similar retention times.

Rockville, MD 20849-6000

Table 6

Drugs with same mass and similar RRT

Drug Pair	MRM	RRT
flecainide	415-301	1.89
diltiazem	415-178	1.92
maprotilene	278-191	2.15
amitriptyline	278-233	2.12

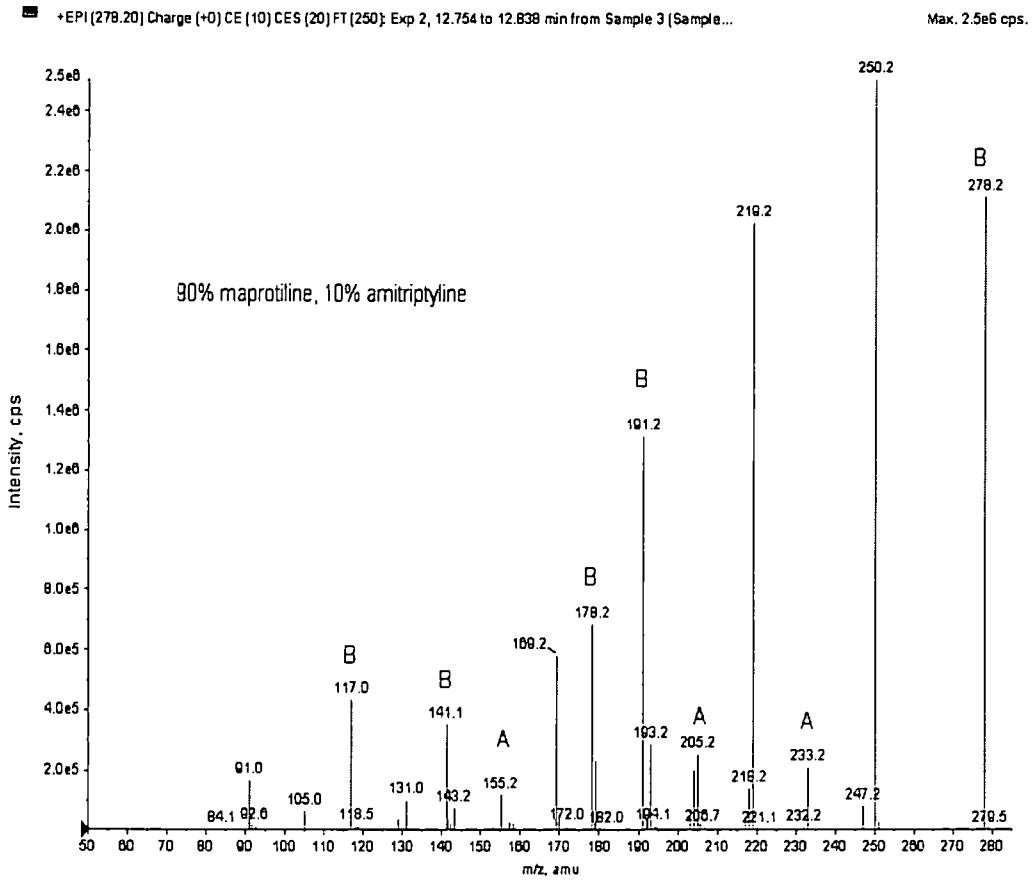

Figure 5: EPI spectrum from mixture containing 1 mg/L maprotiline and 0.1 mg/L amitriptyline. The peaks label 'A' are derived from amitriptyline, 'B' from maprotiline.

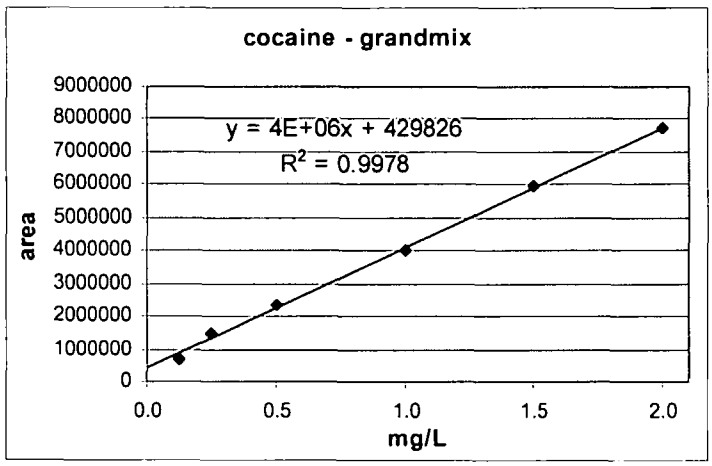

Figure 6A

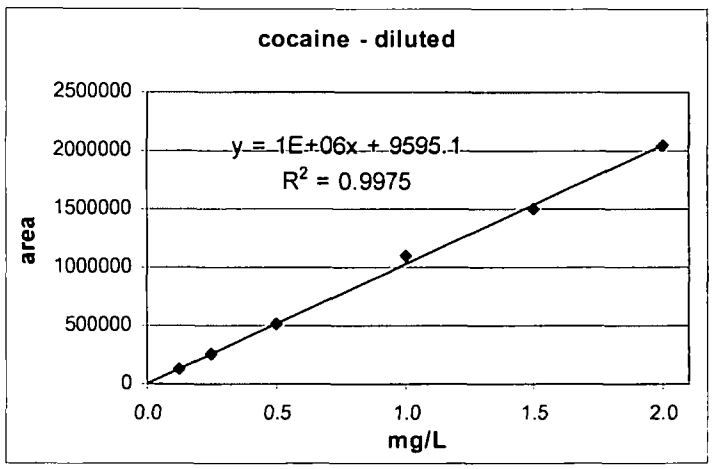

Figure 6B

Figure 6A: Calibration curve for cocaine extracted using EIA procedure and injected using 20 minute gradient profile. 6B: Calibration curve generated following injection of 1:11 dilution of the samples.

Table 7

	LCMS				GCMS			
n=15	Mean value	Accuracy	Std. Dev.	%CV	Mean value	Accuracy	Std. Dev.	%CV
fluoxetine	0.28946	3.51	0.07673	26.51	0.258	14.07	0.099	38.58
tramadol	0.34715	15.72	0.07401	21.32	0.287	4.22	0.054	18.72
methadone	0.29631	1.23	0.08034	6.54	0.255	14.89	0.089	34.84
amitriptyline	0.35302	17.67	0.08508	0.48	0.259	13.56	0.096	36.83
nortriptyline	0.28678	4.41	0.09455	2.15	0.256	14.67	0.076	29.67
citalopram	0.29735	0.88	0.08965	30.15	0.283	5.56	0.079	27.72
oxycodone	0.22223	25.92	0.06797	30.58	0.266	11.19	0.084	31.46
verapamil	0.25851	13.83	0.06592	25.50	0.279	6.89	0.121	43.22
propoxyphene	0.35124	17.08	0.05060	14.41	0.267	10.89	0.088	33.02
promethazine	0.36143	20.48	0.10892	30.14	0.291	3.11	0.089	30.73
sertraline	0.34350	14.50	0.10440	30.39	0.256	14.67	0.109	42.56
norpropoxyphene	0.35321	17.74	0.06592	18.66	0.233	22.44	0.072	30.89
paroxetine	0.26672	11.09	0.05888	22.07	0.251	16.22	0.082	32.67
zolpidem	0.32160	7.20	0.07293	22.68	0.336	12.00	0.065	19.38
trazodone	0.26738	10.87	0.03814	14.26	0.347	15.56	0.110	31.74
AVERAGE		**12.14**		**19.72**		**11.995**		**32.134**

Table 7: Results of quantitative analysis using 200 uL EIA extraction or liquid/liquid method followed by 8 min gradient profile on LC/MS/MS or GC/MS analysis. Accuracy = | Mean – 0.3| ÷ 0.3 x 100.

Table 8

amitriptyline	fluoxetine	oxycodone	zolpidem
benztropine	haloperidol	paroxetine	
carbamazepine	hydroxzine	pentazocine	
chlorpromazine	imipramine	promethazine	
citalopram	lidocaine	propopoxyphene	
clozapine	metaxalone	propranolol	
cyclobenzaprine	methadone	sertraline	
desipramine	mirtazapine	tramadol	
dextromethorphan	norpropoxyphene	trazodone	
diphenhydramine	nortriptyline	venlafaxine	
doxylamine	olanzaprine	verapamil	

Table 8: Drugs confirmed to produce satisfactory quantitative analysis using the LC/MS/MS method.

Table 9

Description	EIA + GC/MS	EIA + LC/MS/MS
Retrieve 35 samples from storage	0.5 hr	0.5 hr
Remove aliquot for immunoassay	1.0 hr	1.0 hr
Perform immunoassay (EIA) extraction	2.0 hrs	2.0 hrs
Remove aliquot for LC/MS/MS	N/A	0.5 hr
Perform immunoassay analysis	3.0 hrs	3.0 hrs
Qualitative LC/MS/MS injections	N/A	15.0 hrs*
EIA data analysis	2.0 hrs	2.0 hrs
LC/MS/MS data analysis (qualitative)	N/A	7.5 hrs
Retrieve 35 samples from storage	0.5 hr	0.5 hr
Remove aliquot for extraction	1.0 hr	1.0 hr
Total base extraction to identify drugs	6 hrs	N/A
GC/MS injections	15.0 hrs	N/A
GC/MS data analysis	7.5 hrs	N/A
Benzodiazepine extraction (quantitate)	6.0 hrs	N/A
GC/MS injections	15.0 hrs	N/A
GC/MS data analysis	7.5 hrs	N/A
Retrieve 25 samples from storage	0.5 hr	0.5 hr
Remove aliquot for extraction	1.0 hr	1.0 hr
Strong base extraction (quantitate)	6.0 hrs	N/A
GC/MS injections	15.0 hrs	N/A
GC/MS data analysis	7.5 hrs	N/A
Retrieve 25 samples from storage	0.5 hr	N/A
Remove aliquot for extraction	1.0 hr	N/A
Opiate extraction (quantitate)	6.0 hrs	6.0 hrs
GC/MS injections	15.0 hrs	15.0 hrs
GC/MS data analysis	7.5 hrs	7.5 hrs
EIA extraction for quantitation	N/A	2.0 hrs
Quantitative LC/MS/MS injections	N/A	6.0 hrs*
LC/MS/MS data analysis (quantitative)	N/A	7.5 hrs
Report preparation	7.5 hrs	7.5 hrs
Report & data administrative review	7.5 hrs	7.5 hrs
Report & data technical review	7.5 hrs	7.5 hrs
TOTAL (including overnight instrument runs)	149.5 hrs	101.0 hrs
TOTAL (excluding overnight instrument runs)	89.5 hrs	59.0 hrs
TOTAL (excluding equivalent tasks & overnight instrument runs)	42.0 hrs	17.0 hrs

Table 9 represents optimal analysis timelines for a theoretical batch of 35 samples plus controls to be analyzed. Ten of the samples test negative via both immunoassay and total

base extraction methods. The remainder of the samples require up to three different extractions to complete the identification and quantitate the drugs present via GC/MS.
* - Instrumental analysis is conducted overnight via automated sampling.