NOVEL METHODS FOR THE QUANTITATION OF CENTRAL NERVOUS SYSTEM AGENTS

by

LEAH NICOLE WILLIAMSON

(Under the Direction of Michael G. Bartlett)

ABSTRACT

Humans can be exposed to central nervous system agents environmentally and/or therapeutically. Chronic exposure to these agents can cause damage to the central nervous system. In order to study the neurological effects of these agents, sensitive and selective analytical methods must be developed and validated in biological matrices, such as brain tissue, plasma, and whole blood. Chlorpyrifos (CPF) is an organophosphate pesticide that inhibits acetylcholinesterase, which is an enzyme that is necessary for normal function of the nervous system. CPF is metabolized by cytochrome P450 into chlorpyrifos-oxon (CPF-O) and TCP. CPF-O is about 3,000 times more potent than CPF in its inhibition of acetylcholinesterase activity, while TCP is non-toxic and is eliminated by the kidneys. Therapeutically, chronic exposure to antipsychotic drugs can result in abnormalities in motor function. Additionally, it is important to determine the extent to which a correlation exists between plasma and brain levels of these agents and cognitive function. Chapter 1 is the introduction and literature review that describes the layout of the dissertation and reviews the literature for analytical methods using liquid chromatography with time-of-flight mass spectrometry (TOF-MS) for quantitation. Chapter 2 reviews the literature for analytical methods using gas chromatography and TOF-MS

for quantitation. Chapters 3 and 4 present analytical methods for the quantitation of CPF and its metabolites in rat brain tissue and blood using liquid chromatography and tandem mass spectrometry (LC-MS/MS). A TOF-MS analytical method for the quantitation of five antipsychotic drugs in rat plasma is presented in chapter 5.

INDEX WORDS: High performance liquid chromatography, tandem mass spectrometry, time-of-flight mass spectrometry, quantitation, chlorpyrifos, antipsychotics

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DEDICATION

To Mom and Dad.

For encouraging me, believing in me, and loving me unconditionally.

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Chapter 1

INTRODUCTION AND LITERATURE REVIEW

Tandem mass spectrometry (MS/MS) is widely used for the quantitation of small molecules in biological matrices. Time-of flight mass spectrometry (TOF-MS) is usually used for accurate mass determination because of the high resolving power. However, over the last 20 years, TOF-MS has been used for quantitation. This chapter reviews the literature for methods using liquid chromatography (LC) and TOF-MS for quantitation. Chapter 2 reviews the literature for methods using gas chromatography (GC) and TOF-MS for quantitation. LC and GC/TOF-MS have been used for quantitation of molecules in different matrices such as foodstuffs and pharmaceuticals. Unlike scanning MS/MS instruments, TOF-MS instruments acquire mass data over a wide mass range at rapid acquisition rates. This allows for the acquisition of quantitative and accurate mass data simultaneously. TOF-MS methods have been developed and validated and have shown similar results for accuracy and precision when compared to MS/MS instruments.

Chlorpyrifos (CPF) is an organophosphate pesticide that inhibits acetylcholinesterase, which is necessary for normal nervous system function. CPF is metabolized by cytochrome P450 into chlorpyrifos-oxon (CPF-O) and TCP. TCP is non-toxic and eliminated by the kidneys, while CPF-O is 3,000 times more potent than CPF in inhibiting acetylcholinesterase. It is important to determine the extent to which there is a correlation between plasma and brain levels of CPF, CPF-O, and TCP and cognitive function. These levels are in the ng/ml range and require analytical methods that are highly specific and sensitive. Chapter 3 presents a method to

quantitate CPF and its metabolites in rat brain tissue using coupled-column liquid chromatography/tandem mass spectrometry. Mass spectrometry methods require clean-up steps to reduce endogenous compounds from interfering with the analyte response. Typical clean-up methods such as solid phase extraction, can be time consuming and expensive. Coupled-column LC is an online sample clean-up method that can reduce the sample preparation time, as well as expense. Chapter 4 presents a method to quantitate CPF and its metabolites in rat blood using LC-MS/MS which uses a simple liquid/liquid extraction for sample clean-up.

In chapter 5, a method has been validated to quantitate antipsychotic drugs in rat plasma using TOF-MS. The goal of the study was to evaluate TOF-MS as a quantitation tool and to compare it with a MS/MS method that was previously published for antipsychotic drugs in rat plasma. Normally, TOF-MS instruments are limited for quantitation because of their narrow dynamic range. Software and hardware improvements have been made to overcome this. It was important to determine if the quantitative and qualitative data was affected by the software generated data.

The methods presented in the following chapters were validated for specificity, linearity, accuracy, precision, recovery, and matrix effects. The results were acceptable according to the current FDA bioanalytical validation requirements. The methods were applied to samples from rats that were exposed to CPF or antipsychotic drugs.

Over the past 20 years time-of-flight mass spectrometers (TOF-MS) instruments have been used qualitatively for identification. Unlike quadrupole scanning instruments, TOF instruments have the capability of collecting spectra over a short period of time, typically milliseconds. Instead of scanning the m/z of selected ions, all of the ions are pulsed down a field-free flight tube. Packets of ions are pushed at the same time, but as they travel in the field-

free region, the smaller ions will travel faster than the larger ions. Therefore, all of the ions will reach the detector at different times. Because the velocity of the ions is proportional to the mass, the mass-to-charge ratio (*m/z*) can be calculated by knowing the time that an ion reaches the detector. Also because no scanning is involved, all ions reach the detector, giving the instrument a theoretically limitless mass range.

Due to the fact that all of the ions reach the detector, saturation can occur. This saturation results in a mass shift, as well as a small dynamic range. Poor mass accuracy can lead to questionable or false data. Because a small linear dynamic range is typical of TOF instruments, they are considered less attractive for quantitation purposes. To overcome the small dynamic range, a voltage is applied to the Z-focus lens. This decreases the intensity of the ions entering the flight tube. At higher concentrations, fewer ions therefore reach the detector and saturation is less likely to occur. Using this feature, the dynamic range can be as high as four orders of magnitude.

TOF instruments are also well known for their high resolution capability and are typically used in LC-MS. Most instruments have a resolution of at least 10,000 at full width half maxima (FWHM) peak height. The resolution has further been improved by adding a reference spray, or lock-spray, function. This allows the instrument to continuously sample a reference compound during each run which internally calibrates the instrument. Mass errors are typically 3 ppm or less when using the lock-spray function.

Using TOF instruments for quantitation has recently gained in popularity in fields ranging from pharmaceuticals to the environment (see Table 2.1). Precision and accuracy of the methods were found to be comparable to data obtained on quadrupole instruments. Ion chromatograms can also be extracted using a narrow mass window. This increases the

probability that the chromatographic peaks are free from background interferences. In some cases, method development using TOF instruments may be shorter when compared to triple quadrupole instruments because precursor-to-product ion transitions do not have to be determined and optimized. Due to these recent instrumental improvements, TOF instruments are now being used more frequently for quantitation, in addition to analyte verification.

Quantitation in biological matrices

Several papers have been published to quantitate various small molecules in biological matrices. Donepezil hydrochloride has been quantified in human plasma using loratadine as an internal standard (Lu*, et al.*, 2004). A Mariner 5140 TOF mass spectrometer was used to detect the compounds that were separated on a Kromasil-ODS column using mobile phases of methanol and acetate buffer. The compounds were extracted from the plasma by liquid-liquid extraction using a mixture of isopropyl alcohol and hexane. Calibration curves were plotted as the peak area ratio of drug to internal standard and showed good linearity (r^2=0.9998) from 0.1-15 ng/ml. The LOQ was 0.1 ng/ml and the precision was less than 15% over three days for the QC concentrations of 0.2, 5.0, and 10.0 ng/ml. The mean recovery of the donepezil from the plasma was 99.4±6.3%. The stability was tested over two freeze-thaw cycles and donepezil was found to be stable in plasma. The method was applied to healthy subjects to obtain pharmacokinetic parameters after the administration of a single 5-mg oral dose.

The quantitation of five pharmaceutical drugs in human plasma has been determined using atmospheric pressure ionization with time-of-flight mass spectrometry (Zhang*, et al.*, 2000a). Doxepin, desipramine, imipramine, amitriptyline, and trimipramine (internal standard) were extracted from human plasma by liquid-liquid extraction using hexane. The drugs were separated using a C18 column with mobile phases of ammonium acetate and acetonitrile. A PE

Biosystems API-TOF operated in positive ion mode was used for detection. Specificity, linearity, lower limit of quantitation, recovery, precision and accuracy were assessed. No chromatographic interferences were observed in an extract of blank human plasma. The linear range was 2-100 ng/ml for desipramine and 1-50 ng/ml for the other four tricyclic amines. The correlation coefficients ranged from 0.990-0.999. At higher concentrations, detection saturation was observed and was believed to be caused by the 'dead time' associated with the time-to-digital converter electronics. A software upgrade was used to correct for this and the dynamic range increased 200-fold. The LOQ for desipramine was 2 ng/ml while the LOQs for the other drugs were 1 ng/ml. The precision was within 16% and the accuracy was within 14%. The acquisition rate was also investigated. It was found that a faster acquisition rate resulted in a lower S/N. The average recovery of desipramine at three QC concentrations (5, 40, and 80 ng/ml) was 75%, while the average recovery for the other drugs (2.5, 20, and 40 ng/ml) was greater than 92%. The precision values for the four tricyclic amines were within 11.1% while the accuracy values were within 8%. The precision and accuracy values for desipramine were higher (17.7% and 14.5%, respectively). The poor results were believed to be the result of desipramine having a lower extraction recovery. The accurate masses of the protonated molecules were measured by infusing a mass reference standard (Jeffamine D230) post-column. The mass accuracy was determined in spiked plasma samples and reference standards. The mass error values of all five drugs at the three QC concentrations were less than 10 ppm.

Unlabeled and deuterium-labeled α-tocopherol have been quantitated in human blood components (Hall*, et al.*, 2003) using HPLC and time-of-flight mass spectrometry. Human plasma, erythrocytes, platelets, and lymphocytes were isolated from human blood, diluted with phosphate buffered saline (PBS), and then extracted using hexane. The tocopherols were

separated on a reverse-phase Waters Symmetry® column using 100% methanol. A Micromass LCT™ mass spectrometer was operated in negative ion mode. The response of the standards was linear from 0-1.5 μM (0-150 pmol injected). Concentrations above 150 pmol resulted in saturation of the detector. However, the response of the labeled tocopherols from the various blood components was found to be non-linear and required the used or a polynomial fit with an r^2>0.995. The LOQ values were 50, 100, and 50 fmol injected in plasma, RBC's and platelets, respectively. The quantitative accuracy was assessed by comparing the TOF-MS method with HPLC/ECD. Plasma samples were obtained from volunteers that were taking part in an α-tocopherol supplementation study. It was found that the TOF-MS method correlated closely with the ECD method (r^2=0.9399). Precision was also assessed and within-day and between-day CVs were no greater than 10% in all three matrices. The method was applied to blood components taken from humans after ingesting 150 mg of d_6-labeled RRR-α-tocopherol acetate. The values α-tocopherol in the blood components were similar to published values.

Time-of-flight mass spectrometry was compared to selected reaction monitoring (SRM) mass spectrometry for the quantitative determination of idoxifene in human plasma (Zhang and Henion, 2001). Human plasma samples were treated by liquid-liquid extraction using hexane. Deuterated idoxifene was used as the internal standard. The compounds were separated on a Luna CN column with mobile phase compositions of water, ammonium acetate, acetonitrile, and formic acid. The mass spectrometer used was a PE Biosystems API-TOF operated in positive ion mode. The internal standard was used a reference standard to conduct a one-point mass calibration during the run time. This resulted in a mass accuracy of 0.8 ppm. The method was validated by assessing selectivity, LOQ, linearity, precision, and accuracy. Blank plasma and plasma spiked at the LOQ were used to verify the selectivity of the method. The LOQ was 5

ng/ml, which was 10 times higher than the LOQ for the SRM method. The precision and accuracy for the method were 5.1 and -5.7%, respectively. The response was linear from 5-2000 ng/ml with a correlation coefficient of 0.9992. The linear range for the SRM method was chosen to be 0.5-1000 ng/ml (r^2=0.999). There was significant carry over when the concentration was above 1000 ng/ml. Precision and accuracy were assessed at 3 concentrations (15, 1000, and 1500 ng/ml). The precision RSD values were less than 8% and the accuracy values were less than 2% (percent deviation).

Four aconitine alkaloids have been quantitated in human plasma and *Acontium* tubers using TOF-MS (Kaneko*, et al.*, 2006). Plasma samples were treated by solid phase extraction, while the tuber samples were treated by liquid-liquid extraction. The alkaloids were separated using a Shiseido C18 column with mobile phases containing ammonium acetate and acetonitrile. An AccuTOF mass spectrometer was used for the analysis in positive ion mode. The method was specific because there were no interfering peaks in blank plasma samples. The linear range was 10-300 ng/ml with correlation coefficients greater than 0.9977 for all four alkaloids. The LODs were 0.5 ng/ml for jesaconitine, and 0.2 ng/ml for mesaconitine, hypaconitine, and aconitine. The accurate mass error for all four alkaloids was less than 10 ppm. Accuracy and precision were assessed at 2 QC concentrations (50 and 200 ng/ml). The intra-day precision was less than 14%, while the day-to-day precision was less than 25%. The intra-day accuracy was less than 15% while the day-to-day accuracy was less than 16%. The method was applied to real tuber samples to determine the alkaloid content. Three of the four alkaloids were detected in the three tuber samples.

Capillary chromatography was coupled to time-of-flight mass spectrometry for salcatonin determination in human plasma and urine (Aguiar*, et al.*, 2005). Urine was centrifuged and the

supernatant was directly injected onto the μLCMS system, while plasma was treated by protein precipitation. The salcatonin and the ^{13}C-labeled internal standard were separated on a Zorbax C18 column with a mobile phase consisting of TFA in water and TFA in acetonitrile. A QSTAR hybrid tandem time-of-flight was operated in TOF-MS mode for the detection (positive ion mode). Specificity, linearity, LOQ, precision, and accuracy of the method were assessed. There were no observed interfering peaks in blank lots of human urine and plasma. The LOQ was 10 ng/ml for urine and 25 ng/ml for plasma. Since the analyte and internal standard co-eluted, the potential differences in ionization efficiency were minimized. The linear range was 10-1000 ng/ml for urine (r^2=1.0000) and 25-2500 ng/ml for plasma (r^2=0.9998). For the 3 QC concentrations in each matrix, the accuracy (% bias) ranged from 100.5 to 106.4% for urine and 76.3 to 100.1% for plasma. The precision (%CV) was less than 9.5% in both matrices.

High-throughput bioanalysis of rat plasma has recently been investigated using a UPLC system with TOF-MS (O'Connor and Mortishire-Smith, 2006). The use of TOF-MS as a quantitation technique was compared to using a tandem MS system. TOF-MS data was acquired on a Micromass QToF2 mass spectrometer. The compounds were separated on an Acquity BEH C18 column with 0.25 mM ammonium formate and acetonitrile. Plasma samples were treated by protein precipitation. Further clean-up was achieved by diverting the first minute to waste using a column switching valve. The linear range for the TOF-MS method was 6.4-2000 ng/ml, however, the precision was 42% at 6.4 ng/ml. The precision was <15% for concentrations above 32 ng/ml. Accuracy was between 87% and 111%. The precision and accuracy was also assessed for 3 QC concentrations (5, 50, and 500 ng/ml). The precision and accuracy was poor for the low point (48% and 54% respectively). However, the precision and accuracy were acceptable for the medium and high points (<5% and >99% respectively). The LOQ of the TOF method

had a higher LOQ than the tandem MS method (30 ng/ml vs. 5 ng/ml). The method was applied to rats that were orally dosed with 4 mg/kg of a clinical agent. All samples had concentrations above the LOD and pharmacokinetic parameters were determined. Seven metabolites were identified and the accurate masses were determined. All metabolites had a mass error of less than 5 mDa.

UPLC and TOF-MS have also been used to determine histamine and its metabolites in mouse hair (Kawanishi*, et al.*, 2006). A Micromass LCT Premiere operated in positive and negative ion modes was used for the detection. The compounds were separated on an Acquity BEH C18 column using ammonium formate and acetonitrile. The compounds were extracted from dried mouse hairs by using methanol, hydrochloric acid, and ultrasonication. The linear range of the method was 1.0-25 pmol/mg hair (r^2=0.998). The precision and recovery was assessed at 25 pmol (3.8% and 98.3% respectively). The method was applied to mice without any treatment. The concentration of histamine and 3 metabolites in the hair shafts was the first to be reported.

Barcelo-Barrachina, *et al.* (2004) have investigated the determination of heterocyclic amines in meat extract by HPLC/TOF-MS. A Micromass Q-TOF-2 mass spectrometer was operated in positive ion mode. The data was acquired using single MS mode as well as MS-MS mode. The compounds were separated by reverse phase HPLC using a Symmetry C8 column and mobile phases consisting of acetonitrile and acetic acid/ammonium acetate buffer. Meat extracts were treated by solid-phase extraction. The method was evaluated for LOD, LOQ, linearity, and short-term precision. The LODs of the compounds ranged from 0.2 to 2.9 ng/g in meat extract. The response of the analytes was linear up to 1.5 μg/g with correlation coefficients greater than 0.999. For 2 QC concentrations (0.07 and 0.4 μg/g), the RSDs were less than 8%.

Accurate mass measurements of the compounds were also observed and the percent errors were less than 2 mDa. The method was also applied to real samples. The method of standard addition was used for quantitation. The results were found to be consistent with results obtained in the same lab using different LC-MS methods.

A screening method has been validated to determine perfluoroalkylated substances (PFAS) in biota (Berger and Haukas, 2005). A Micromass LCT mass spectrometer was operated in negative ion mode. The PFAS's were separated on an Ace 3 C18 reversed phase column using ammonium acetate in methanol and water. Polar cod and glaucous gull livers were homogenized and treated by liquid-liquid extraction. The method was assessed for recovery, instrument and method detection limits, linearity, specificity, matrix effects, precision, and accuracy. The instrument LODs for the PFAS's were all less than 8 pg injected. The specificity of the method was determined by extracting a mass chromatogram for each PFAS. Extracting a high resolution mass chromatogram resolved the compounds from all of the matrix components. The recoveries ranged from 60 to 115%. Matrix effects were quantified based on the response of the analyte in the matrix and the response of the standard solution. The matrix effects ranged from 0.05 to 2.58 in polar cod liver and 0.08 to 2.51 in glaucous gull liver. The method detection limit ranged from 0.04 to 1.3 ng/g wet weight. The RSDs were 35% or less. Because there was a lack of certified reference materials, accuracy was determined by comparing the results to an ion pair extraction (IPE) method. The mean values between the two methods were found to be in excellent agreement. The screening method was successfully applied to liver samples from 12 different species, as well as brain samples of fish and birds (data was not shown).

Atmospheric pressure ionization TOF-MS has been used to rapidly identify and quantitate 4 drugs in rat plasma (Zhang, *et al.*, 2000b). A Micromass LCT was used and was

operated in positive ion mode. The compounds were separated isocratically on a packed column with C18 material using 50% methanol and 50% 2 mM ammonium acetate buffer. The rat plasma samples were treated by a semiautomated liquid-liquid extraction using ethyl acetate. The recovery ranged from 45 to 60%. The LOQs ranged from 5 to 25 ng/ml. Calibration curves were obtained from the LOQ to 2000 ng/ml and the correlation coefficients were greater than 0.9944. Precision and accuracy were assessed at 3 QC points (50, 500, and 1000 ng/ml). The precision was not greater than 6.4% and the accuracy ranged from 96 to 111%. The linearity, precision, accuracy, and LOQs were compared to those obtained using a triple quadrupole instrument in MRM mode. It was found that there was a 5-10 fold improvement in the detection limit using the triple quadrupole instrument. The TOF instrument had a similar dynamic range with almost identical precision and accuracy. Quantitation and selectivity using the TOF instrument were also improved by narrowing the mass window of the extracted ion chromatograms. Metabolites were also identified by extracting accurate mass chromatograms. If the mass agreed with the theoretical mass within 10 ppm, then the identity of the unknown metabolite was confirmed.

Hydroxylated polychlorinated biphenyls (PCBs) and other halogenated phenolic compounds have been determined in eggs from birds of prey using LC TOF-MS (Berger*, et al.*, 2004). A Micromass LCT operated in negative ion mode was used for the detection. Gas chromatography/high resolution mass spectrometry was used for comparison. HPLC separation was carried out on a C18 column with 1 mM ammonium acetate in methanol and water mobile phases. Hen's eggs were used for the method development and validation. The content of whole eggs were homogenized and mixed with acidified sodium sulfate. The compounds were extracted using a glass column and a cyclohexane/acetone mixture. The samples were further

cleaned by using gel permeation chromatography. The LOQs for the four PCBs ranged from 15 to 100 pg/g, however, the correlation coefficients were not reported. Recoveries ranged from 56 to 98%. The RSDs were less than 10% for a spiked sample at 1 ng/g. The linear range was from the lower limit to 100 pg injected (~400 pg/g). Both LC and GC methods were applied to real egg samples from four different prey species. Pentachlorophenol was found in all four samples. Tetrabromobisphenol A was also found in all four samples but only by using the GC method. Twenty-six PCBs were identified and quantified using the TOF-MS method. The values reported from the LC and GC methods were similar.

LC-TOF-MS has been used to determine Benzodiazepines in urine samples (ElSohly*, et al.*, 2006). An Agilent 1100 SL mass spectrometer was operated in positive ion mode. Chromatographic separations were achieved on a Phenomenex Luna C8 column using gradient elution with water and acetonitrile, both containing 0.1% acetic acid. The human urine samples were treated with potassium phosphate buffer and chloroform/IPA for the extraction. Calibration curves were constructed in the range of 10-500 ng/ml for 22 benzodiazepines. The correlation coefficients were greater than 0.9994. The LOQs ranged from 2 to10 ng/ml and the LODs ranged from 0.5 to 3 ng/ml. Recoveries were assessed at 25 and 50 ng/ml and ranged from 84 to 98%. Precision and accuracy were assessed at 50 and 100 ng/ml. The day-to-day CVs ranged from 0.46 to 5.37%. Accurate mass data was acquired and the mass error was 3 ppm or less. The method was applied to human urine samples to determine the amount of benzodiazepines.

Tian *et al.* (2006) have used LC-TOF-MS to determine puerarin (PU) and its metabolite in human urine. A Waters CapLC-MS system was operated in negative ion mode. The compounds were separated on a Micro-Tech Scientific ODS column using mobile phases of 10 mmol/L ammonium acetate in water and 10 mmol/L ammonium acetate in acetonitrile. The

urine samples were treated by protein precipitation using acetonitrile. Puerarin was linear in the range of 0.1 to 25 nmol/ml while the metabolite, daidzein (DA) was linear from 0.05 to 10 nmol/ml (r^2>0.998). The LOQs were 0.1 and 0.05 nmol/ml for PU and DA respectively. Recovery for both analytes ranged from 79.4 and 98.5%. Three concentrations were assessed for each analyte for accuracy and precision over 3 days. The intraday precision ranged from 2.6 to 12.2% and the intraday accuracy was between 87.3 to 108.4%. The interday precision ranged from 6.0 to 8.5% and the interday accuracy ranged from 88.5 to 105.8%. The method was applied to human urine samples.

Quantitation in foodstuffs

Tolonen and Uusitalo (2004) have developed a fast screening method to analyze total flavonoid content in plants and foodstuffs using TOF-MS. A LCT TOF mass spectrometer was used and operated in both positive and negative ion modes using polarity switching. The separation took place on a SymmetryShield RP8 column and a Luna C18 pre-column using 0.1% formic acid and methanol mobile phases. Plant and green tea samples were extracted using methanol, while the wine and orange juice samples were centrifuged and diluted with methanol. Calibration curves were constructed from 0.04 to 10 μg/ml. Accurate mass data was also acquired and all samples were found to be accurate within 4 mDa. The linear ranges were 0.2-10 μg/ml for one of the compounds, and 0.2-4 μg/ml for the others. The responses for all the compounds were linear with r^2 values greater than 0.980. Precision values were less than 14% while the accuracy ranged from 80-120%. The response was also measured using a PDA detector in line with the TOF-MS detector. It was found that the TOF-MS detector resulted in lower detection limits, but the PDA detector increased the response over the concentration range tenfold. Event though the sensitivity was increased with the PDA detector, specificity was lost

with the wine and juice samples. The TOF-MS had greater specificity because the molecular ion chromatogram was extracted and therefore resolved from the background components. Plants, red wines, orange juices, and green teas were sampled and the flavonoid content was in agreement with the values reported in the literature.

Herbicides in olive oil have been analyzed using TOF-MS (Garcia-Reyes*, et al.*, 2006). The mass spectrometer used was an Agilent MSD TOFMS operated in positive ion mode. The separation took place on a Zorbax Eclipse XDB-C8 column using acetonitrile and 0.1% formic acid. The herbicides were extracted from the olive oil using a preliminary liquid-liquid extraction followed by matrix solid-phase dispersion (MSPD). The mass accuracy of the protonated molecule and some of the fragments was also determined. All mass errors were less than 0.6 mDa. Linearity was assessed by spiking blank olive oil samples at concentrations ranging from 0.005 to 0.5 mg/kg. All herbicides had a r^2 greater than 0.994 and RSDs of 4% or less. Matrix effects were also studied by comparing the slopes of the calibration curves in olive oil with those in neat solutions. The signal was suppressed by as much as 36%, therefore matrix-matched standards were used for quantitation. The selectivity of the TOF-MS instrument was investigated by extracting the ion chromatograms using different mass range windows. It was found that when using a narrow 0.05 Da window, interferences were eliminated which resulted in an improved signal-to-noise ratio. Eight real olive oil samples were analyzed using the reported method. Only one of the samples had traces of herbicide residues at a concentration near the LOQ. However, other labs found traces of a herbicide using GC methods. When this LC method was used, a peak was observed at a similar retention time using a wide mass window. The accurate mass spectrum confirmed that the peak observed was not from the compound of

interest. This verified that other labs using analytical techniques with low resolution were reporting false positives.

Multi-residue pesticide analysis in fruits and vegetables has also been investigated by Ferrer, *et al.* (2005a). An Agilent MSD TOF instrument was operated in positive ion mode for detection. The pesticides were separated using a Zorbax Eclipse XDB-C8 column with acetonitrile and 0.1% formic acid mobile phases. Fruit and vegetable homogenates were treated with ethyl acetate to extract the pesticides. Accurate mass measurements were acquired and the errors were less than 2 ppm. Linearity was assessed using 6 points ranging from 0.01 to 0.5 mg/kg. The correlation coefficients were greater than 0.992. The method was also found to be precise (run-to-run RSDs between 0.8 and 7% and day-to-day RSDs between 2 and 10%). The matrix effects were also studied by comparing the slope of the standards spiked in the matrix and the neat standards. The LODs ranged from 0.5 to 50 µg/kg in three of the matrices. The method was also applied to real market samples. The results were significantly close to the results obtained using a quadrupole instrument in SIM mode. Ferrer, *et al.* (2005b) reported a method to quantitate pesticides in vegetables using the same instruments and extractions techniques. The mass accuracy errors were less than 2 ppm for the molecular ions. Linearity was assessed and the response was linear from 0.005 to 1 mg/kg (r^2>0.991). Intra-day precision RSDs ranged from 2-3% while the inter-day precision RSDs ranged from 4-5%. The matrix effects were assessed by comparing the calibration slopes of the spiked standards in the matrix to the neat standards. The variation in the slopes ranged from 87 to 110%. The method was applied to real vegetable samples. The values obtained using the LC/TOF-MS method were significantly similar to the values obtained using a triple quadrupole instrument.

A LC/TOF-MS method has been developed to simultaneously detect trichothecenes, zearalenone, and aflatoxins in foodstuffs (Tanaka*, et al.*, 2006). Mass spectrometry was performed using an Agilent MSD TOF instrument operated in APCI positive ion mode. A Zorbax Eclipse XDB C18 column was used with 10 mM ammonium acetate and methanol mobile phases. The mycotoxins were extracted from corn, wheat, cornflasks, and biscuits using solid phase extraction. Mass accuracy data was acquired and the mass error was less than 2.5 ppm. Matrix effects were assessed in terms of signal intensity and mass accuracy. It was found that the matrix had little effect on the mass error (±5 ppm) and no enhancement or reduction of the response was observed. Calibration curves were prepared in standard solutions at concentrations of 0.2 to 20 ng/ml. The response was found to be linear over this range with correlation coefficients greater than 0.999. The recoveries were found to range from 71 to 133%. The precision RSDs ranged from 2.1 to 7.1%. Accurate mass of the protonated molecule was used to confirm identity. The method was applied to determine the amount of mycotoxins in wheat.

Quantitation in environmental samples

TOF-MS has been used for quantitation of compounds in the environment such as river water, wastewater, and atmospheric aerosols. An on-line LC/TOF-MS method has been developed to analyze pesticides in river water (Sasaki*, et al.*, 2006). The mass spectrometry was performed on a Waters LCT-Premier mass spectrometer operated in positive ion mode. The SPE pre-column was an Oasis HLB extraction column and the analytical column was an Atlantis dC18 column. Mobile phases of water, acetonitrile, and 2% formic acid were used for sample clean-up, loading, and elution. The response was linear from 1 to 100 ppb (r^2>0.999). The LODs ranged from 0.0034 to 3.3 ppb. The recoveries ranged from 67 to 126%. The

reproducibility (%CV) ranged from 1.1 to 11%. The %RSDs were determined for standards, "clean" water, and "dirty" water samples.

Núñez, *et al.* (2004) have compared TOF-MS to triple quadrupole MS in the analysis of quaternary ammonium herbicides in drinking water. The mass spectrometer used was a Q-TOF Ultima operated in positive ion mode. Data was acquired in single MS acquisition mode. The separation was carried out on a Kromasil C8 column with Heptafluorobutyric acid solution in formic acid/ammonium formate buffer and acetonitrile mobile phases. The water samples were treated by automated solid phase extraction. Calibration curves were acquired at concentrations between 0.05 and 20 µg/L (r^2>0.990). The LODs were in the range of 0.2-9.0 µg/L, which were similar to the LODs using the triple quadrupole instrument in SIM mode. The run-to-run reproducibility RSDs were less than 9%. The recoveries ranged from 50 to 90%. The method was successfully applied to spiked tap water, mineral water, and groundwater samples.

Organic microcontaminants in surface water have been identified using on-line solid-phase extraction liquid chromatography with TOF-MS (Hogenboom*, et al.*, 1999). The mass spectrometer used in the study was a Micromass LCT TOF mass spectrometer operated in positive ion and negative ion modes. A precolumn packed with PLRP-S material was used for trace enrichment and a Zorbax SB-C18 column was used for the separation. Ammonium acetate and methanol were used as the mobile phases. Using this on-line setup, the sample preparation time was 11 minutes, while the total analysis time was 32 minutes. Accurate mass data was acquired and the mass accuracies were between 1.4 and 17.3 ppm. The response of the 10 pesticides was linear from 0.1 to 10 µg/L (r^2>0.9922). The LODs ranged from 0.1 to 0.2 µg/L. The repeatability over two consecutive days had RSDs less than 23%. Real water samples were

collected to develop the method, but the method was not applied to any real samples in this work.

Microcystins have been determined in lake water using integrated solid-phase microextraction with microbore LC/TOF-MS (Zhao*, et al.*, 2006). An Applied Biosystems/MDS QSTAR Pulsar hybrid QTOF-MS was operated in full-scan MS mode (positive ion mode). The microcystins were separated on a BetaBasic-18 microcolumn with mobile phases of methanol and water both containing 0.01% formic acid. A carbowax templated resin (CW-TPR) SPME fiber was found to have the best extraction efficiency. The pH, salt concentration, addition of methanol, and extraction time were all studied and optimized. Calibration curves were obtained at concentrations of 1 to 250 µg/L. The response was found to be linear with correlation coefficients greater that 0.994. The LODs were 0.3 and 0.8 µg/L for the two microcystins studied. No microcystins were detected using a real lake water sample. The lake water sample was spiked at two concentrations to determine the recovery, accuracy, and precision of the method. The recoveries were greater than 71% and the RSDs were less than 10%.

Twenty nine pharmaceuticals have been analyzed in wastewater using a UPLC/TOF-MS system (Petrovic*, et al.*, 2006). A Water's QToF-Micro mass spectrometer was operated in positive and negative ion modes. Quantitation data was acquired in TOF mode. The chromatography was performed on a Waters Acquity C18 column with ammonium acetate/acetic acid and acetonitrile/methanol mobile phases. River and wastewater samples were filtered and then preconcentrated using solid-phase extraction. Accurate mass data was acquired for the protonated and deprotonated molecules with errors between 0.7 and 4.4 ppm. The run-to-run RSDs were 0.5 to 5.3%, while the day-to-day RSDs were 2.1 to 9.1%. The method detection limits (MDLs) ranged from 10 to 500 ng/L, which were found to be approximately one order of

magnitude higher than using a triple quadrupole instrument in MRM mode. Matrix effects were studied. The majority of the compounds had less than 30% suppression. The method was applied to 5 wastewater samples and 10 river water samples. A majority of the compounds were detected in the wastewater, however, none of them were detected in the river water samples. This was because of the higher MDLs for the river water.

Pól, *et al.* (2006b) have investigated two-dimensional liquid chromatography TOF-MS in the analysis of acidic compounds in atmospheric aerosols. The mass spectrometer used was a Bruker Micro TOF-LC operated in negative ion mode. The stationary phase for the first dimension was SCX with a 10 µm pore size. The second dimension consisted of a C18 XBridge column. The mobile phases were acetonitrile and 17.5 mM acetic acid. Samples were collected on quartz filters and extracted three times with methanol by static sonication-assisted solvent extraction (SAE). Calibration curves were constructed (2-50 µg/ml) and were found to be linear (r^2>0.9823). The limits of detection ranged from 0.01 to 1.14 ng. The method was applied to real aerosol samples from two different locations. An external mass calibration was used prior to every analysis and the mass error was less than 4 ppm for most analytes. The amounts of the acids (ng/m^3) were reported for an urban sample and a rural sample.

Quantitation in plant extracts

The appetite suppressant P57 found in *Hoodia gordonii* has been determined in plant extracts using TOF-MS (Avula, *et al.*, 2006). An Agilent Series 1100 SL mass spectrometer was used and was operated in positive ion mode. UV data was also collected for comparison. A Phenomenex Gemini C18 column was used for the separation with mobile phases of water and acetonitrile, both with 0.1% acetic acid. Dry samples were sonicated in methanol, centrifuged, and filtered before analysis. The recovery ranged from 98.5 to 103.5%. The response was linear

from 25 to 1000 ng/ml (r^2=0.99997). The LOQ of TOF method (25 ng/ml) was much lower than the LOQ of the UV method (500 ng/ml). Intra-day and inter-day precision RSDs were less than 3.8% over 3 days. The method was applied to plant samples and dietary supplements. Out of the five tested commercially available capsules, only 2 had measurable quantities of P57. Five batches of one capsule were tested and there was no detectable P57.

The low calorie sweetener *Stevia Rebaudiana* has been characterized using two-dimensional liquid chromatography TOF-MS by Pól, *et al.* (2006a). A Bruker MircoTOF-LC mass spectrometer was operated in negative ion mode for the detection. Different column combinations were tested. The NH_2 and C18 columns resulted in the best separation of the *Stevia* glycosides. Gradient elution was used with acetonitrile and water mobile phases. *Stevia* leaves were extracted by pressurized hot water extraction. The extract was filtered upon cooling and directly injected into the liquid chromatography system. The molecular peaks were identified and were within 10 ppm accuracy. A calibration curve was constructed for one of the glycosides. The LOD was 43.4 ng/g. A correlation coefficient of 0.9935 was reported, however, the linear range was not. Two kinds of leaves from different origin were used to apply the method. Only stevioside was quantified because it was the only commercially available standard.

Comprehensive two-dimensional LC has been used to analyze Lamiaceae herbs for their antioxidant phenolic acid content (Kivilompolo and Hyotylainen, 2007). A Bruker Daltonics MicroTOF mass spectrometer was operated in negative ion mode. A Waters Atlantis C18 column was used for the first-dimension and a Beckman Ultrasphere CH cyano column was used for the second dimension. The compounds were separated on the first dimension using gradient elution and on the second dimension isocratically using 15% acetonitrile and 0.5% acetic acid in

water. Different extraction methods were tested and it was found that dynamic sonication assisted extraction (DSAE) with methanol was superior. Calibration curves were constructed from 0.5 to 5 mg/L (r^2>0.9828). The LODs ranged from 18 to 90 ng/ml. The optimized LC×LC system was applied to real herb extracts.

A highly sensitive LC-TOF-MS has been developed to quantitate nicotianamine in young tobacco leaf and rice seed (Wada*, et al.*, 2007). A Jeol JSM-T100LC AccuTOF was operated in positive ion mode. A Phenomenex Synergi Hydro RP column was used for the chromatographic separation. Gradient elution was used using 0.5% formic acid, water, and acetonitrile. Nicotic acid (NA) was derivatized using 9-fluroenyl methoxycarboxyl chloride so that it will retain in the column. Rice grain samples were suspended in deionized water, centrifuged, and concentrated in a micro vacuum. Calibration curves were obtained from 0.25 to 4 nmol (r^2=0.99). The method was applied to determine the nicotic acid content in real rice grain extracts and young tobacco leaves.

Quantitation of explosive materials

Quantitation and identification of explosive residues has become increasingly important in crime scene investigation and home land security especially since the 9/11 terrorists attacks. Kinghorn, *et al.* (2006) have used LC/TOF-MS to quantitate and identify 22 explosive materials. An Agilent MSD TOF mass spectrometer was used for the detection. Five different detection methods were studied (UV, ESI+, ESI-, APCI+, and APCI-) and APCI- was found to give the best response. A Zorbax Extended-C18 column was used for the chromatographic separation with methanol and water mobile phases. Accurate mass data was also acquired and the mass error was less than 3 ppm for all analytes. The LOQs ranged from 0.5 to 5000 µg/L. Mass accuracy data was also acquired with varying concentrations (1-100,000 µg/L). It was found that

at high concentrations (100,000 µgL) the mass accuracy was greater than 10 ppm in most cases. The peak area repeatability was also observed at the same concentrations. For concentrations above the LOQ, the RSDs were less than 10%. Ten explosives were evaluated for linearity. It was found that some of them displayed excellent linearity across four orders of magnitude. Most had a correlation coefficient of 0.998 or greater. Blank soil samples were also spiked to assess the recovery. The recovery ranged from 75 to 120%. The method was applied to two blind samples obtained from crime evidence. The chromatogram retention time resulted in two possibilities; however, the TOF accurate mass data confirmed the identity of the unknown.

TOF versus Quadrupoles

TOF instruments have several advantages over quadrupole instruments. One advantage is the high resolution capability. Typically quadrupole instruments have unit mass resolution, whereas TOF instruments have resolution of at least 10,000 FWHM. This allows for resolution of chromatographic peaks from background interferences, or from other compounds with the same unit mass. This also makes it an excellent tool for screening to verify the presence of a compound or compounds of interest. The newer TOF instruments which can correct for peak saturation have improved dynamic ranges and linearity can be achieved for up to four orders of magnitude. Unlike using a quadrupole instrument in SIM mode which only monitors a selected ion, TOF instruments are capable of acquiring chromatograms for all ions in the acquisition range. Because there is no scanning involved using TOF-MS, the sensitivity is higher when compared to using a quadrupole instrument in SIM mode. However, it does appear that MRM methods using triple quads remain about 3-5 times more sensitive.

The precision and accuracy have also been shown to be similar between TOF and quadrupole instruments. Because of this, TOF instruments have recently been utilized more

frequently for quantitation and mass confirmation simultaneously. With TOF instruments, method development times can also be shorter. This is because precursor-to-product ion transitions do not have to be determined or optimized. In the future, it appears that more methods will be developed using TOF instruments as opposed to quadrupole instruments for these reasons.

Conclusions

Because of recent improvements in the design of TOF instruments, the linear dynamic range has significantly improved. Precision and accuracy of the TOF methods are comparable to quadrupole methods (SIM and MRM). The high resolution capability of the TOF instruments also allows accurate mass data to be acquired simultaneously. Because of these advantages, more scientists are now using TOF instruments for quantitative analyses. Almost half of the papers in this review were from 2006. TOF-MS has been used as a quantitative tool in different fields from environmental chemistry to food science. This diversity shows the increasing popularity and reliability of TOF instruments as quantitation tools and demonstrates the high probability of its use in future applications.

Table 2.1. LC/TOF-MS methods for quantitation

Author(s)	Analyte	Matrix	LOD or LOQ	Linearity (r^2)
Hogenboom *et al.* (1999)	Polar microcontaminants	Surface water	0.1-0.2 µg/L (LOD)	0.1-10 µg/mL (>0.9922)
Zhang *et al.* (2000a)	5 pharmaceuticals	Human plasma	1-2 ng/ml (LOQ)	LOQ-100 ng/ml (>0.990)
Zhang *et al.* (2000b)	4 pharmaceuticals	Rat plasma	5-25 ng/ml (LOQ)	LOQ-2000 ng/ml (>0..9944)
Zhang *et al.* (2001)	Idoxifene	Human plasma	5 ng/ml (LOQ)	5-2000 ng/ml (0.9992)
Hall *et al.* (2003)	Tocopherols	Human blood components	50-100 fmol injected (LOQ)	LOQ-15 pmol injected (>0.995)
Barceló-Barrachina *et al.* (2004)	20 heterocyclic amines	Beef extract	0.4-3.6 ng/g (LOD)	LOQ-1.5 µg/g (>0.999)
Berger *et al.* (2004)	Polychlorinated biphenyls	Preditory bird's eggs	15-100 pg/g (LOQ)	LOQ-400 pg/g (NR)
Lu *et al.* (2004)	Donepezil hydrochloride	Human plasma	0.1 ng/mL (LOQ)	0.1-15 ng/mL (0.9998)
Nunez *et al.* (2004)	Quaternary ammonium herbicide	Drinking water	0.2-9.0 µg/L (LOD)	15-800 µg/L (>0.991)
Tolonen *et al.* (2004)	Flavonoids	Foodstuffs	0.04-0.1 µg/mL (LOD)	0.2-10 µg/ml (>0.980)
Aguiar *et al.* (2005)	Salcatonin	Human urine	10 ng/mL (LOQ)	1-1000 ng/mL (1.0000)
		Human plasma	25 ng/mL (LOQ)	25-2500 ng/mL (0.9998)
Berger *et al.* (2005)	Perfluoroalkylated substances	Polar cod and glucous gull live	0.2-8 pg injected (LOD)	NR
Ferrer *et al.* (2005a)	15 pesticides	Fruits and vegetables	0.3-50 µg/kg (LOD)	LOQ-0.5 mg/kg (>0.992)
Ferrer *et al.* (2005b)	3 pesticides	Vegetables	0.001-0.01 mg/kg (LOD)	LOQ-1 mg/kg (>0.991)
Avula *et al.* (2006)	P57	Hoodia gordonii	25 ng/mL (LOQ)	25-1000 ng/mL (>0.999)
Garcia-Reyes *et al.* (2006)	4 herbicides	Olive oil	1-5 µg/kg (LOD)	0.005-0.5 mg/kg (>0.9948)
Kaneko *et al.* (2006)	Aconitum alkaloids	Human plasma	0.2-0.5 ng/mL (LOD)	10-300 ng/mL (>0.9977)
Kawanishi *et al.* (2006)	Histamine and its metabolites	Mice hair	<1 pmol (LOD)	1.0-25 pmol (0.998)
Kinghorn *et al.* (2006)	Explosives	Soil	0.5-5000 µg/L (LOQ)	LOQ-10,000 µg/L (>0.998)
O'Connor and Mortishire-Smith (200	Compound 1 and its metabolites	Rat plasma	30 ng/mL (LOQ)	30-2000 ng/mL (>0.99)
Petrovic *et al.* (2006)	29 pharmaceuticals	Wastewater	10-500 ng/mL (MDL)	10 ng/L-5 mg/L (>0.99)
Pol *et al.* (2006a)	Stevia glycosides	Stevia leaves	43.4 ng/g (LOD)	NR (0.9935)
Pol *et al.* (2006b)	Acidic compounds	Atmospheric aerosols	0.01-1.14 ng (LOD)	2-50 µg/mL (>0.9823)
Sasaki *et al.* (2006)	Pesticides	River water	0.0034-3.3 ppb (LOD)	1-100 ppb (>0.999)
Tanaka *et al.* (2006)	Mycotoxins	Foodstuffs	0.1-6.1 ng/g (LOD)	0.2-200 ng/mL (>0.999)
Zhao *et al.* (2006)	Microcystins	Lake water	0.3-0.8 µg/L (LOD)	1-250 µg/L (>0.9943)

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Chapter 2

QUANTITATIVE GAS CHROMATOGRAPHY/TIME-OF-FLIGHT MASS SPECTROMETRY: A REVIEW[1]

[1] Williamson, L.N. and Bartlett, M.G. *Biomedical Chromatography*. In Press. Reprinted here with permission of publisher.

Abstract

Time-of-flight (TOF) instruments have recently gained popularity in quantitative analyses. Normally, TOF mass spectrometers are used for accurate mass measurements for empirical formula verification. However, over the past decade, they are being used quantitatively as well. Because of the fast separations and narrow peaks that result from gas chromatography separations, scanning mass spectrometers are not ideal detectors. TOF mass spectrometers, however, have the ability to collect spectra at a faster rate. Two-dimensional gas chromatography has also been introduced to further resolve peaks from complex matrices. Two-dimensional gas chromatography results in a faster separation as well as narrower peaks. This paper reviews the methods currently in the literature for the quantitation of compounds using one and two-dimensional gas chromatography and TOF mass spectrometry detection.

Keywords: Gas chromatography, time-of-flight mass spectrometry, quantitation, review

Introduction

Time-of-flight mass spectrometers (TOF-MS) are advantageous over quadrupole mass spectrometers because of their fast acquisition rates and high resolution capabilities. Gas chromatograms have narrow peaks and require a fast scanning detector. TOF-MS, unlike scanning instruments, have the ability to acquire chromatograms in microseconds, depending on the acceleration potential. During TOF-MS acquisitions, ions are pulsed down a field-free flight tube in packets. The packets leave the source at the same time and initially have the same velocity. As they travel down the field-free flight tube, the smaller ions will travel faster than the larger ions. The smaller ions will therefore reach the detector before the larger ions. Because the velocity of the ions is proportional to the mass, the mass-to-charge ratio (*m/z*) can be calculated by knowing the time that it takes to reach the detector. TOF mass spectrometers have high resolution of at least 10,000 at full width half maxima (FWHM) peak height. This allows for peaks to be resolved from closely related interfering matrix components. Also, accurate mass data can be obtained during each run for empirical formula verification.

One-dimensional gas chromatography often does not provide efficient separation of complex mixtures. Two-dimensional gas chromatography (GC × GC) further resolves chromatographic peaks by sampling peaks from the first dimension, and further separating them in the second dimension. A typical GC × GC system consists of two GC columns with different retention mechanisms connected in series. During the separation on the first column, fractions of the eluate are retained and focused on the entrance of the second column. A cryogenic modulator is used to retain and focus the peaks from the first dimension. The second column is usually shorter and narrower than the first column, which results in a fast separation. The data is

usually presented in a contour plot, where the axes represent the retention times in the first and second dimension, and the shading represents the intensity of the peak.

Over the past decade, one and two-dimensional GC has been coupled with TOF-MS for quantitative analyses (see Table 3.1). Several papers in this review compare quantitative data with detection methods such as ultraviolet (UV), single quadrupole MS, nitrogen-phosphorus (NP), and flame ionization detection (FID). The precision and accuracy data from TOF-MS detection were found to be comparable to other detection methods.

Quantitation in biological matrices

Bromazepam has been quantitated in human blood using GC-TOFMS (Aebi*, et al.*). An Agilent GC 6890 and a Pegasus II TOFMS were used for the detection. A DB5-MS column with 0.18 µm film thickness was used for the separation. Human whole blood was spiked and treated by liquid-liquid extraction using *n*-butyl acetate. A five-point calibration curve was constructed (50-300 ng/ml) and the correlation coefficients were 0.99793 and 0.99729 for ion 1 (*m/z* 236) and ion 2 (*m/z* 288), respectively. Based on a signal to noise ration of 5:1, the absolute amount of bromazepam that could be detected was 0.6 ng for ion 1 and 1.1 ng for ion 2. This was found to be much lower than the amounts for quadrupole GC-MS (9 and 13 ng). This GC-TOFMS method was used for quantitation and confirmation. The mass spectra of bromazepam gave match factors of 90% or better with the available libraries. The method was successfully applied to real human blood samples.

Human serum and milk samples have also been analyzed for the determination of polybrominated diphenyl ethers, polybrominated and polychlorinated biphenyls, and organochlorine pesticides (Focant*, et al.*, 2004a). The compounds were separated in two dimensions. The columns were DB-1 dimethylpolysiloxane and HT-8 (8% Phenyl-

polycarboranesiloxane) columns for the first and second dimension, respectively. A LECO Pegasus 4D instrument was used for the detection. The serum samples were treated by solid phase extraction, while matrix solid-phase dispersion was used to treat the milk samples. Ten-point calibration curves were constructed for 59 compounds and were linear from 0.5-2000 pg/µl. The method detection limits (MDLs) in serum and milk ranged between 1 and 15 pg/µl. The two-dimensional gas chromatography with isotope-dilution time-of-flight mass spectrometry (GC × GC-IDTOFMS) method was compared to a validated GC-Isotope dilution high-resolution mass spectrometry method. RSDs for both methods were less than 12% and PCB levels measured using both methods were highly correlated. For 3 compounds analyzed, the coefficients of variance (CVs) were 0.8, 2.5, and 10.8% using TOF detection. Several human milk samples were analyzed using both methods.

Gas chromatography-isotope dilution time-of-flight mass spectrometry was also used to analyze human serum for selected polychlorinated biphenyls (PCBs) (Focant*, et al.*, 2004b). A Leco Pegasus III TOF instrument was equipped with an Agilent 6890 GC. A DB-XLB capillary column was used to separate the compounds. The samples were cleaned up using an automated system from Fluid Management Systems which is based on multilayer silica, basic alumina, and carbon absorbents prepacked in Teflon columns. Calibration curves were constructed for 38 PCBs ranging from 0.5 to 1000 pg/µl (r^2>0.99339). The limits of detection (LODs) were close to 20 pg/µl injected. Spiked bovine serum samples were used to compare the method to a GC-IDHRMS method. Quantification data was found to be similar for both methods except for one compound, CB138. This was due to the fact that the column used for the GC-IDHRMS method did not allow for the chromatographic separation of CB138 and CB158.

Kalinova, *et al*. (2006) have used two-dimensional GC with TOF-MS to identify insect pheromones. A LECO Pegasus 4D instrument was used for the analyses. A slightly polar DB-5 column was used for the first dimension, and a polar BPX-50 column was used for the second dimension. Pheromone glands were excised from larvae and the pheromones were extracted using hexane. Synthetic (Z9,E12)-tetradeca-9,12-dien-1-ol was diluted in hexane to determine the linear range of the method. A five-point calibration curve was constructed (10-2000 pg/µl, r^2=0.9893). The LOD was 10 pg/µl. The method was applied to determine the pheromone concentration in larvae of the persimmon bark borer *E. batangensis.*

Two-dimensional gas chromatography time-of-flight mass spectrometry was also used for drug screening and confirmation (Song*, et al.*, 2004). A LECO Pegasus III equipped with an Agilent HP6890 GC was used for the analyses. A HP-5MS column was used for the first dimension, while a BPX50 column was used for the second dimension. Drug standards were spiked in drug-free human whole blood to develop the method. Blood was treated with butyl chloride for extraction. Calibration curves were constructed ranging from 0.02 to 5.0 µg/ml for four of the standards. The correlation coefficients ranged from 0.9783 to 0.9988. Intra-day precision was assessed over 2 days with CVs no greater than 21.0%. The method was applied to four real blood samples. One of the samples was analyzed using GC-nitrogen-phosphorus detection for comparison. The results reported for both methods were the same.

Quantitation in foodstuffs

Fish oil in dietary supplements has been analyzed for mono- to octachlorobiphenyls using solid-phase microextraction (SPME) with TOF detection (Agustin*, et al.*, 2005). An Agilent 6890N gas chromatograph was used for the chromatographic separation, while a LECO Pegasus III TOF-MS system was used for detection. Adsorption chromatography was used to clean up

the fish oil samples. Polydimethylsiloxane-divinylbenzene (PDMS-DVM) and polydimethylsiloxane (PDMS) fibers were used to extract the compounds. Linearity was assessed over two levels (low and high). Coefficients of variation of 0.99 or better were observed. The RSDs reported were quite high (above 15%) but this was found to be common when using SPME methods. A certified reference (cod liver oil) was used to test the method. The results from the TOF method agreed with the certified data. One PCB value was reported higher because of co-eluting interferences in the matrix.

Dalluge, *et al.* (2002a) have investigated two-dimensional GC TOF-MS for the determination of pesticides in food extracts. A HP 6890 GC and a LECO Pegasus III time-of-flight mass spectrometer were used for the analysis. The first dimension consisted of a CP-SIL 5 CB low bleed/MS phase column. A BPX-50 phase column was used for the second dimension. Chopped food samples (carrots and celeriac) were treated with sodium sulfate and ethyl acetate for extraction. Six pesticides were used to determine the linearity and detection limits of the method. Calibration curves were linear from 0.01 to 3 ng/µl (r^2>0.998). The LODs were between 3 and 23 pg/µl. The RSDs of the peak areas at 100 pg/µl were no greater than 11%.

Difficult matrix introduction-gas chromatography-time-of-flight mass spectrometry (DMI-GF-TOF-MS) has also been used to determine pesticides in grapes and pineapples (de Koning*, et al.*, 2003). An Agilent 6890 GC was used for the chromatographic separation, while a LECO Pegasus III was used for detection. To reduce the sample clean up time, direct thermal desorption (DTD) was used online with the gas chromatograph. The DMI setup consisted of a DTD liner holding a disposable Atlas MPI-µ-vial. A FOCUS XYZ sample preparation robot was used for sample handling and cleanup. The linear range was studied for 13 pesticides (10-200 ng/ml). The calibration curves in both pesticide-free pineapple and grapes had a correlation

coefficient of at least 0.990. The LODs were between 1 and 10 ng/g. One of the pesticides (Tolyfluanid) in pineapple had a deconvolution problem, and the linearity was not determined. The RSDs were reasonably low (<6%) except for dichlofluanid, iprodion, and tolylfluanid, which tend to be sensitive to thermal degradation.

Acetonitrile extraction/partitioning was compared to dispersive solid-phase extraction in the determination of herbicide residues in barley samples (Diez*, et al.*, 2006). An Agilent 6890 GC with a LECO Pegasus III TOF MS was used for the analysis. Three different extraction methods were tested: QuEChERS (Anastassiades*, et al.*, 2003) (quick, easy, cheap, effective, rugged, safe), acetone, and ethyl acetate. QuEChERS extraction was designed for samples with more than 75% moisture and uses anhydrous magnesium sulfate and sodium chloride to define the separation phases. Linearity was assessed by using linearity coefficients. A coefficient of 95 or greater was considered linearly fitted for chromatography. For the range of 2.5 to 100 µg/kg, the compounds showed good linearity with coefficients greater than 95. The LODs ranged from 1.0 to 2.3 µg/kg. The recoveries ranged from 70 to 110% except for the nitrile herbicides, which were not well-deconvoluted.

Large volume-difficult matrix introduction-gas chromatography-time-of-flight mass spectrometry (DMI-GC-TOF-MS) has been used to analyze pesticide residues in lettuce (Patel*, et al.*, 2003). The DMI-GC-TOF-MS system consisted of an ATAS programmable injector, FOCUS automated sample processor with a Direct Thermal Desorption device, an Agilent 6890 GC, and a LECO Pegasus III time-of-flight mass spectrometer. Organic lettuce was treated with ethyl acetate, anhydrous sodium sulfate, and sodium hydrogen carbonate. Calibration curves were constructed from 0.0025 to 0.5 µg/ml with correlation coefficients greater than 0.990. The recoveries ranged from 73 to 118% with RSDs up to 13%.

Patel, *et al.* (2004) have used the same DMI-GC-TOF-MS system to determine pesticides in baby food. Organic baby food was treated in the same manner with ethyl acetate, anhydrous sodium sulfate, and sodium hydrogen carbonate. Calibration curves for 98 pesticides were linear from 0.005 to 0.1 mg/kg. Correlation coefficients were greater that 0.980. Recoveries were higher than 69%.

Ryan, *et al.* (2005) have investigated solid phase micro extraction (SPME) with isotope dilution and two-dimensional gas chromatography in the analysis of methoxypyrazines in wine. An Agilent 6890 GC was used along with a LECO Pegasus III TOF mass spectrometer. The first dimension of the GC × GC system consisted of a BPX5 (5% phenyl polysilphenylene-siloxane) column. The second dimension was a PB20 poly(ethylene glycol) column. A Polydimethylsiloxane/Divinylbenzene (PDMS/DVB) fiber was used to extract the methoxypyrazines. One of the methoxypyrazines was used to assess the linearity and LOD of the method. The LOD was 1.95 ng/L. The calibration curve was linear from 1.95 to 200 ng/L (r^2=0.994). The method was applied to a real wine sample. The results were compared to using a nitrogen-phosphorus detector and were in very good agreement.

Organic microcontaminants have been determined in water and tea using GC-TOF-MS (Vreuls*, et al.*, 1999). The GC-TOF-MS system consisted of an Agilent 6890 GC equipped with a LECO Pegasus II TOF-MS. The chromatographic separation took place on a CP-Sil 8 CB column. Solid phase extraction was used to treat the water samples, while in-vial liquid-liquid extraction was used for the tea samples. Sediment samples were also analyzed and were extracted by ultrasonic solvent-assisted extraction. Standard solutions containing 18 pesticides and herbicides were linear in the 0.01-5 ng/µl range with correlation coefficients between

0.99709 and 0.99995. The LODs ranged from 0.3 to 60 pg injected. The RSDs of the peak areas were between 4 and 11%. The method was applied to real river water and tea samples.

Two-dimensional gas-chromatography-time-of-flight mass spectrometry has been used to determine pesticide residues in fruit (Zrostlikova*, et al.*, 2003). A LECO Pegasus 4D instrument was used which consisted of an Agilent 6890 GC and a Pegasus III TOF-MS. An Agilent DB-XLB column and a DB-17 column were used for the first and second dimension, respectively. Homogenized peach and apple samples were treated with ethyl acetate and sodium sulfate. The samples were further purified with high-performance gel permeation chromatography. One-dimensional GC was also performed for comparison. The LODs for the two-dimensional GC ranged from 0.2 to 100 µg/kg of peach, which was lower by a factor of 1.5-50 versus one-dimensional GC. The RSDs of the peak areas ranged from 4.2 to 6.6%. Good linearity was achieved in the range of 5-500 ng/ml (r^2=0.9982-0.9996).

Quantitation in environmental samples

Fast gas chromatography-time-of-flight mass spectrometry has been used for the determination of polychlorinated biphenyls and other contaminants in sediment samples (Cochran, 2002). A LECO Pegasus III GC-TOFMS was used for the sample analysis. The samples were either introduced into a narrow bore column (0.10 mm) or a 0.53 mm column operated in vacuum-outlet mode. PCBs were extracted from sediment samples using an ultrasonic bath and hexane. The samples were then cleaned by solid phase extraction. Calibration curves were found to be linear from 25 to 1000 pg/µl, but correlation coefficients were not reported. The method was applied and the measured values were found to be generally close to the calculated values. Using the narrow bore column, the LODs were between 1.0 and 16 pg/µl.

Organic microcontaminants have been determined in wastewater using gas chromatography and TOF-MS (Dalluge*, et al.*, 2002b). Sample analyses were performed on an Agilent 6890 and a Micromass GCT TOF-MS. A DB-5 MS column was used to separate pesticides and polycyclic aromatic hydrocarbons (PAHs). A longer DB-5 column was used to separate the polychlorinated biphenyls (PCBs). A LECO Pegasus II TOF-MS was used for comparison. Wastewater samples were treated by an automated solid phase extraction (SPE) system. PCBs were extracted in eel samples based on total lipid extraction. The LODs were compared between the two instruments. The LODs for the Micromass instrument were between 1 and 4 pg (injected mass), while the LODs for the LECO instrument were between 0.5 and 6 pg. The response of the PAHs were linear, however, the calibration plots for the pesticides and PCBs were fit to a second-order polynomial. The linear range was between 3 and 1000 ng/µl and the correlation coefficients were between 0.9953 and 0.9998.

Focant *et al.,* (2004a) have used two-dimensional gas chromatography-isotope dilution time-of-flight mass spectrometry (GC × GC-IDTOF-MS) to measure polychlorinated dibenzo-p-dioxins (PCDD), polychlorinated dibenzofurans (PCDF), and coplanar polychlorinated biphenyls (cPCB) in ash, sediment, vegetation, and fish samples. The GC × GC-IDTOF-MS system consisted of an Agilent 6890 GC and a LECO Pegasus 4D TOF-MS. A Rtx-Dioxon 2 column was used for the first dimension, and a Rtx-500 was used as the second dimension. Dried soil and sediment samples were ground and homogenized. Fish samples were homogenized, extracted with hexane, and passed through a column containing anhydrous sodium sulfate. Ash samples were sonicated with hydrochloric acid. Soil, sediment, and ash samples were extracted with toluene and then subjected to an open column chromatographic procedure. Seventeen PCDDs and four PCBs were used to assess the linearity. The response was linear at a

concentration range of 0.5-2000 pg/µl. The correlation coefficients ranged from 0.99564 to 0.99999. The method was applied to real samples and the results were compared to GC-IDHRMS data. The values were found to be similar.

Zou *et al.* (2003) have investigated thermal desorption and fast GC-TOF-MS for the rapid analysis of polycyclic aromatic hydrocarbons (PAHs) in fly ash. An Agilent 6890 plus GC system was linked to a LECO Pegasus II time-of-flight mass spectrometer. A HP-5 capillary column was used to separate 16 PAHs. The fly ash samples were treated by thermal extraction. Calibration curves were linear in the range of 1.5 to 60 mg/g (correlation coefficients > 0.99). Recovery was assessed over three days and was greater than 83% (RSD <2.8). The LODs ranged from 0.35 to 1.43 µg/g. Two different fly ash samples were analyzed to apply the method, and compare thermal extraction with a solvent extraction method. The results for both extraction methods were comparable for all PAHs except naphthalene. The difference may have been due to the higher volatility of naphthalene.

TOF versus Quadrupoles

TOF-MS instruments have some advantages over quadrupole instruments when using them for quantitative analyses. During TOF acquisitions, spectra are generated for all of the ions as opposed to selected ions using a quadrupole instrument. This makes the TOF instrument ideal for screening applications where several analytes need to be detected at once. Because TOF mass spectrometers are not scanning instruments, the fast acquisition speed is also ideal for fast gas chromatographic separations. TOF instruments are also known for their high mass resolution (at least 10,000 at full width half maxima (FWHM)). Quadrupole instruments typically have only unit mass resolution. A TOF instrument can therefore be used to acquire accurate mass data for empirical formula confirmation along with quantitation data. Also, compounds can be

resolved from endogenous peaks from matrix components. These advantages make GC-TOF-MS an ideal tool for compound quantitation and identification.

Conclusions

Time-of-flight instruments have recently been used more frequently as a quantitative tool. A time-of-flight mass spectrometer has been shown to be an ideal detector for gas chromatography because of the fast separations and narrow peaks that result. Because of the high resolution capability of the TOF instruments, qualitative data can also be obtained along with quantitative data. Compounds have been resolved from complex matrices by using two-dimensional GC with TOF detection. Because of the high resolution of the TOF instruments, sample clean-up and method development time can be reduced. Coupling GC with TOF-MS yields promising qualitative and quantitative results which should continue to drive this combination of instruments.

Table 3.1. GC/TOF-MS methods for quantitation

Author(s)	Analyte	Matrix	LOD or LOQ	Linearity (r^2)
Aebi, *et al.* (2002)	Bromazepam	Human whole blood	0.6 ng injected (LOD)	50-300 ng/ml (0.997)
Augustin, *et al.* (2005)	PCBs	Fish oil	0.8-31 ng/g (LOD)	2-500 ng/ml (>0.9901)
Cochran, *et al.* (2002)	PCBs	Sediment	1.0-16 pg/µl (LOD)	25-1000 pg/µl (NR)
Dalluge, *et al.* (2002a)	Pesticides and PAHs	Waste water and eel	0.5-6 pg injected (LOD)	3-1000 ng/µl (>0.9953)
Dalluge, *et al.* (2002b)	Pesticides	Carrots and celeriac	3-23 pg/µl (LOD)	0.01-3 ng/µl (>0.998)
de Koning, *et al.* (2003)	Pesticides	Pineapple and grapes	1-10 ng/g (LOD)	10-200 ng/ml (>0.990)
Diez, *et al.* (2006)	Herbicides	Barley	1.0-2.3 µg/kg (LOD)	2.5-100 µg/L (>95[†])
Focant, *et al.* (2004a)	PCBs	Human serum	20 pg/µl injected (LOD)	0.5-1000 pg/µl (>0.99339)
Focant, et al. (2004b)	PCBS and pesticides	Human milk and serum	1-15 pg/-µl (LOD)	0.5-2000 pg/µl (>0.99339)
Kalinova, et al. (2006)	Pheremones	Larvae	10 pg/µl (LOD)	10-2000 pg/µl (0.9893)
Patel et al. (2003)	Pesticides	Lettuce	0.01-0.05 mg/kg (LOD)	0.0025-0.5 µg/ml (<0.990)
Patel et al. (2004)	Pesticides	Baby food	0.005-0.01 mg/kg (LOD)	0.005-0.1 mg/kg (>0.980)
Ryan et al. (2005)	Methoxypyrazines	Wine	1.95 ng/ml (LOD)	1.95-200 ng/L (0.994)
Song et al. (2004)	Drugs	Human whole blood	0.02-0.05 µg/ml (LOD)	0.02-5.0 µg/ml (>0.9783)
Vreuls et al. (1999)	Microcontaminants	Tea, water, and sediment	0.3-60 pg injected (LOD)	0.01-5 ng/µl (>0.99709)
Zou et al. (2003)	PAHs	Fly ash	0.35-1.43 µg/g (LOD)	1.5-60 mg/g (> 0.99)
Zrostlikova et al. (2003)	Pesticides	Peach and apple	0.2-100 µg/kg	5-500 ng/ml (>0.9982)

[†] linearity coefficient ($r = (1-s_b/b)\bullet 100$)

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Chapter 3

DETERMINATION OF CHLORPYRIFOS AND ITS METABOLITES IN RAT BRAIN TISSUE USING COUPLED-COLUMN LIQUID CHROMATOGRAPHY/ELECTROSPRAY TANDEM MASS SPECTROMETRY[1]

[1] Williamson, L.N., Terry Jr., A.V., and Bartlett, M.G. *Rapid Communications in Mass Spectrometry* 2006; **20**: 2689-95. Reprinted here with permission of publisher.

Abstract

A method has been developed to quantify chlorpyrifos (O,O-diethyl O-[3,5,6,-trichloro-2-pyridyl] phosphorothionate) and its metabolites chlorpyrifos-oxon (O,O-diethyl-O[3,5,6,trichloro-2-pyridinly] phosphate) and TCP (3,5,6,-trichloro-2-pyrinidol) in rat brain tissue by coupled-column liquid chromatography/electrospray ionization tandem mass spectrometry (LC/LC/ESI-MS/MS). Rat brains were homogenized and treated by protein precipitation using ice-cold acetonitrile. The supernatant was directly injected onto the coupled-column system. Sample clean-up was achieved on a Zorbax Extended-C_{18} column (2.1×50 mm, 5 μm) using a mobile phase of acetonitrile/water with 0.0025% formic acid (40:60, v/v). The compounds were separated isocratically on a Zorbax Eclipse XDB C_8 column (2.0×150 mm, 5μm) using a mobile phase of acetonitrile/water with 0.0025% formic acid (75:25, v/v). Chlorpyrifos and chlorpyrifos-oxon were detected in positive ion mode using multiple reaction monitoring (MRM). TCP was detected in negative ion mode using parent to parent transition monitoring.

The method was validated and the specificity, linearity, limit of quantitation (LOQ), precision, accuracy, stability, and recoveries were determined. Calibration curves for all three analytes yielded correlation coefficients of 0.993 or greater. The LOQ was 25.3 ng/g for chlorpyrifos and 6.3 ng/g for chlorpyrifos-oxon and TCP. All precision relative standard deviations (RSDs) were less than 16% for the LOQ and less than 11% for the other QC samples. This method was successfully applied to 6 rats that were injected subcutaneously with chlorpyrifos.

Introduction

Chlorpyrifos (O,O-diethyl O-[3,5,6,-trichloro-2-pyridyl] phosphorothionate) is an organophosphate insecticide that is used agriculturally (Lorsban ™) and commercially (Dursban ™). Chlorpyrifos is used because it inhibits acetylcholinesterase, an enzyme that is necessary for normal function of the nervous system. Chlorpyrifos is metabolized by Cytochrome P-450 into Chlorpyrifos-oxon (O,O-diethyl-O[3,5,6,trichloro-2-pyridinly] phosphate) and TCP (3,5,6,-trichloro-2-pyrinidol). Chlorpyrifos-oxon is about 3,000 times more potent than Chlorpyrifos in its inhibition of acetylcholinesterase , which leads to neurotoxicity.[1] TCP is nontoxic and is eliminated by the kidneys. The use of chlorpyrifos has been restricted by the Environmental Protection Agency, however, TCP has recently been detected in the urine of 96% of approximately 2,000 samples analyzed from subjects living in the United States.[2]

Analytical methods have been published to detect chlorpyrifos and its metabolites in biological matrices. Chlorpyrifos, chlorpyrifos-oxon, and TCP have been detected in plasma and urine using high performance liquid chromatography with ultraviolet detection.[3-5] Gas chromatography with mass spectrometry detection has also been used to detect chlorpyrifos and its metabolites in plasma, serum, and urine.[6-9] One disadvantage of using GC for quantitation is that the chlorpyrifos-oxon can hydrolyze to TCP because of the high temperature in the injection port. Therefore an internal standard must be used, as well as a derivatizing agent for the TCP.

One concern when using electrospray ionization mass spectrometry for detection is matrix effects, therefore, the sample clean-up is important. Sample clean-up methods such as solid phase extraction (SPE) and liquid-liquid extractions (LLE) can be very time consuming and can yield a low sample throughput. Online sample clean-up using a coupled-column system has recently gained popularity for analyzing biological samples.[10-16] Coupled-column systems

interfaced with tandem mass spectrometers have been used to analyze compounds in biological matrices.[12, 17-19] A coupled-column method has been published to detect chlorpyrifos and TCP in human serum and urine.[11] This method, however, did not detect the toxic chlorpyrifos-oxon metabolite, and there were two separate methods for the detection of the chlorpyrifos and the TCP.

Animal studies have indicated that repeated exposures of chlorpyrifos at sub-threshold levels can lead to persistent impairments in cognitive function.[20] It is important that a sensitive and accurate method be developed to detect the small amounts of chlorpyrifos and its metabolites in the brain to further explore the underlying neurological bases for the cognitive changes. This method detects chlorpyrifos, chlorpyrifos-oxon, and TCP in one injection and has a run time of 9 minutes. This method was found to be rapid and efficient and did not require the use of derivatizing agents.

EXPERIMENTAL

Chemicals and reagents

Chlorpyrifos (Fig. 4.1(a)), chlorpyrifos-oxon (Fig. 4.1(b)), and TCP (Fig. 4.1(c)) were purchased from Chem Service (West Chester, PA, USA). HPLC-grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized water was generated from a Continental Deionized Water System (Natick, MA, USA). Formic acid was purchased from J.T. Baker (Phillipsburg, NJ, USA).

Calibration standards and quality control standards

Standard stock solutions were made by dissolving chlorpyrifos, chlorpyrifos-oxon, and TCP in acetonitrile (1 mg/ml) and were stored at 4°C. The stock solutions were further diluted with acetonitrile to make working solutions with concentrations of 3.0 and 4.0 µg/ml. Standard

calibration samples were mixed then spiked in blank rat brain homogenate to yield final concentrations of 6.3, 25.3, 63.3, 126.5, 189.8, 253.0, 506.1, and 1,012.1 ng/g. Quality control (QC) samples were spiked in rat brain homogenate to yield final concentrations of 12.7 for TCP and chlorpyrifos-oxon, 50.6 for chlorpyrifos, and 151.8, and 759.1 ng/g for all three analytes.

Liquid chromatography

An Agilent 1100 Series HPLC system (Palo Alto, CA, USA) equipped with a degasser, binary pump, autosampler, and thermostated column compartment was used. A 6-port column switching valve was used to utilize the coupled-column system (Fig. 4.2). The clean-up column was an Agilent (Palo Alto, CA, USA) Zorbax Extended-C_{18} column (2.1×50 mm, 5 μm) equipped with a 4.0 × 2.0 mm Phenomenex (Torrance, CA, USA) Security Guard C_{18} guard column. The analytical column was an Agilent (Palo Alto, CA, USA) Zorbax Eclipse XDB C_8 column (2.0×150 mm, 5μm) equipped with a 4.0 × 2.0 mm Phenomenex (Torrance, CA, USA) Security Guard C_8 guard column. The stationary phase of the analytical column consisted of an extra-dense monolayer of C_8 bonded to an ultra-pure silica surface.

The mobile phase used for the clean-up column was acetonitrile/deionized water with 0.0025% formic acid (40:60, v/v). The mobile phase used for the analytical column was acetonitrile/deionized water with 0.0025% formic acid (75:25, v/v). The flow rate was 0.30 ml/min.

Mass Spectrometry

The coupled-column HPLC system was interfaced to a Quattro micro API tandem mass spectrometer equipped with a Z-spray ion source and syringe pump (Manchester, UK). Nitrogen gas was used as the desolvation gas and was set to a flow rate of 500 L/h with a temperature of 350°C. The cone gas flow was set to 50 L/h. Argon was the collision gas used and the collision

cell pressure was 2.2×10^{-3} mbar. TCP was detected in negative ion mode using a capillary voltage of 2.5 kV, a cone voltage of 20 V, and a collision energy of 2 eV. Chlorpyrifos and chlorpyrifos-oxon were detected in positive ion mode with a capillary voltage of 3.5 kV, a cone voltage of 20 V, and a collision energy of 20 eV. The polarity was switched from negative to positive after 4.0 minutes. The source temperature was 120°C. Samples were acquired in multiple reaction monitoring (MRM) mode and were processed using Masslynx 4.0 software.

Sample preparation

This method was applied to 6 (n=3 per group) male albino Wistar rats obtained from Harlan (Indianapolis, IN, USA). The rats were housed in pairs in a temperature controlled room (25°C) with a 12 hour light/dark cycle. The rats were injected with 18 mg/kg chlorpyrifos and sacrificed after 6 and 24 hours. The chlorpyrifos was dissolved in dimethyl sulfoxide (DMSO) and then added to peanut oil. The rats were then injected subcutaneously. On the day of sacrifice, the rats were anesthetized with ketamine (40 mg/kg) and xylazine (18 mg/kg). With the rat lying on its back, the chest wall was opened to expose the heart. The rat was infused intercardially with 50 ml of phosphate-buffered saline (PBS) into the left ventricle. The rat was then decapitated to remove the brain from the skull. Whole brains were then submerged in isopentane (-70°C). The brains were stored at -70°C until analysis.

For the validation of the method, whole rat brains were supplied from untreated rats. The brains were minced and homogenized in deionized water (1 ml/g) using a Brinkmann (Westburg, NY, USA) Polytron TP 1020 homogenizer . 70 µl of blank brain homogenate were spiked with 10 µl of each analyte. The samples were briefly vortexed and were prepared by protein precipitation by adding 400 µl of ice-cold acetonitrile. The samples were then vortexed for one

minute, and centrifuged for 10 minutes at 13,000 rpm. 10 µl of the supernatant were injected onto the coupled-column system.

Method validation

Calibration curves were acquired (8-point for chlorpyrifos and chlorpyrifos-oxon and 7-point for TCP) by plotting peak area against the analyte concentration. The curves were acquired daily for 3 days (n=5 for intra-day and n=15 for inter-day). Precision and accuracy were determined for the 3 QC points (12.7, 151.8, and 759.1 ng/g brain for TCP and chlorpyrifos-oxon and 50.6, 151.8, and 759.1 ng/g brain for chlorpyrifos) and the LOQ (6.3 ng/g for TCP and chlorpyrifos-oxon and 25.3 ng/g brain for chlorpyrifos). Precision was reported as percent relative standard deviation (RSD) (Equation 4.1) and accuracy was reported as percent error (Equation 4.2).

$$(4.1) \qquad \%RSD = 100 \times \frac{s\,\text{tan}dard\ deviation}{mean}$$

$$(4.2) \quad \%Error = 100 \times \left| \frac{\text{exp}erimental\ concentration - theoretical\ concentration}{\text{exp}erimental\ concentration} \right|$$

Recoveries were also determined. Absolute recovery was determined by dividing the response of the analyte in brain homogenate by the response of the standard solution in acetonitrile. Relative recovery was determined by dividing the response of the analyte in brain homogenate by the response of the analyte in brain homogenate that was spiked after the extraction process. The matrix effects were determined by a method reported by Matuszewski, et al. [21] and were calculated to be the response of the homogenate spiked after the extraction divided by the response of the standard solution in acetonitrile.

The stability of the stock solutions were determined at their storage conditions of 4°C. Other stability experiments were determined at concentrations of 12.7 and 759.1 ng/g for TCP

and chlorpyrifos-oxon and 50.6 and 759.1 ng/g for chlorpyrifos. Freeze/thaw stability was determined over 3 cycles. Autosampler stability was determined by preparing samples and injecting them at different time intervals over 24 hours. Bench top stability was determined by spiking blank brain homogenate and preparing the samples at different time intervals over 24 hours.

RESULTS AND DISCUSSION

Development of the analytical method

Full scan and product ion mass spectra were obtained by infusing 100 μg/ml of chlorpyrifos, chlorpyrifos-oxon, and TCP into the electrospray ionization (ESI) probe at a rate of 10 μl/min. TCP ionized in negative ion mode, while chlorpyrifos and chlorpyrifos-oxon ionized in positive ion mode. The collision-induced dissociation of TCP yielded an abundant ion at m/z 198, which corresponded to the $[M-H]^-$ of TCP. Since the abundance of the TCP ion was high relative to the chloride ion, the deprotonated molecular ion was used for quantitation. This same transition was used in a previous study of TCP.[11] The MRM transitions monitored were: m/z $[M-H]^-$ 198→198 for TCP, m/z $[M+H]^+$ 336→280 for chlorpyrifos-oxon, and m/z $[M+H]^+$ 352→200 for chlorpyrifos.

Column-switching optimization

The analytes first passed through the clean-up column before being separated on the analytical column. The biological matter was flushed to waste, and then the switching valve was activated to allow the analytes to enter the analytical column. The clean-up column was first directly connected to the mass spectrometry interface to determine the time to activate the switching valve. TCP was the first compound to elute, so the mobile phase was optimized to retain the TCP while allowing all of the biological matter to elute to waste. The formic acid

content of 0.0025% was chosen because good peak shape was obtained while maintaining a high signal for the acidic TCP, as shown in a previous study.[11] Chromatograms of blank brain homogenate and TCP standard eluting from the clean-up column can be seen in Figure 4.3. The switching valve was activated after 1 minute.

Method Validation

Specificity

From the MRM chromatograms of brain homogenate from an untreated rat (Fig. 4.4) it can be seen that there are no interfering peaks observed where the analytes elute. However, a region of enhancement was seen between 2 and 2.75 minutes in the TCP MRM chromatogram. Because this enhancement is seen in the MRM chromatograms of the standards as well, we believe that the baseline changes are a result of the pressure change that occurs after the column switching valve is activated. In comparing the MRM chromatograms of the spiked brain homogenate and the standard solutions (Fig. 5), this method was shown to be selective for chlorpyrifos, chlorpyrifos-oxon, and TCP.

Linearity

Standard solutions were made fresh each validation day and a calibration curve was generated for each analyte. The linear range was 25.3 to 1012.1 ng/g for chlorpyrifos and 6.3 to 1012.1 ng/g for chlorpyrifos-oxon and TCP. A weighting factor of $1/x^2$ was used for each curve. The coefficients of variation (r^2) for each compound were: 0.9985 ± 0.0009 for chlorpyrifos, 0.9945 ± 0.0042 for chlorpyrifos-oxon, and 0.9926 ± 0.0021 for TCP.

Accuracy and Precision

Precision and accuracy measurements were acquired for the 3 QC points and the LOQ for each compound. The accuracy and precision data can be seen in Table 4.1. Values for the intra-

day precision and accuracy ranged from 2.78-9.75% and 3.71-12.55%, respectively, for the QC samples and 3.89-16.35% and 2.58-12.56% for the LOQ samples. Inter-day precision and accuracy ranged from 5.57-9.75% and 6.56-13.62%, respectively for the QC samples and 8.13-13.46% and 5.89-9.83% for the LOQ samples.

Recovery and Matrix Effects

Absolute recovery, relative recovery, and matrix effects data are summarized in Table 4.2. Relative recoveries ranged from 81.56-92.47%, so protein precipitation was efficient for the extraction of the compounds. The brain homogenate matrix seemed to have minimal effect on the analyte signal (<12% suppression or enhancement), except for the case of chlorpyrifos at 759.1 ng/g (22.2% enhancement). This larger enhancement may be due to the fact that at the higher analyte concentration, there is less competition between the analyte and the biological matter.

Stability

The stock solutions were stable at the storage conditions (4°C) for 3 months. Spiked samples were found to be stable over 3 freeze/thaw cycles. Prepared samples were stable in the autosampler for up to 24 hours. Spiked samples were stable at room temperature for 24 hours as well. For all stability studies, there was no significant decrease in the peak areas for the analytes.

Application of the method

The concentration data for chlorpyrifos and its metabolites are reported in Table 4.3. Chlorpyrifos levels in the brain at 6 and 24 hours were similar showing that chlorpyrifos may persist in the brain for long periods relative to the blood. In an earlier study of chlorpyrifos following an oral dose of 15 mg/kg the blood levels of chlorpyrifos fell approximately 90% from 6 to 24 hours.[22] The levels of TCP were 3-10 fold lower than chlorpyrifos in the brain. This

finding is interesting because the levels of TCP were found to be 10-20 fold higher than chlorpyrifos in the blood.[22] Finally, we did not observe any of the chlorpyrifos-oxon metabolite in the brain samples. This finding is consistent with the physiological-based pharmacokinetic model for chlorpyrifos, which predicts 100-fold lower levels of the chlorpyrifos-oxon in the brain versus the blood.[23]

Conclusions

This method is the first validated method to quantitate chlorpyrifos and its two main metabolites; chlorpyrifos-oxon and TCP in rat brain tissue. The coupled-column system for online sample clean-up along with the short run time of the method resulted in a high throughput of samples. This online sample clean up method will save time and money over solid phase and liquid-liquid extraction. All analytes had high recoveries (81-92%) from the brain homogenate. The method was accurate and precise with %RSD and %Error values of <17% over three validation days.

Acknowledgements

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Table 4.1. Intra- and Inter-day precision (%RSD) and accuracy (%Error) of the method in rat brain homogenate

Analyte		Theoretical Concentration (ng/g)	Experimental Concentration (ng/g)	%RSD	%Error
Chlorpyrifos	(n=5)	25.3	25.26 ± 0.98	3.89	2.58
		50.6	48.60 ± 3.89	8.00	7.66
		151.8	161.85 ± 13.96	8.62	7.91
		759.1	825.00 ± 49.91	6.04	8.89
	(n=15)	25.3	25.28 ± 2.05	8.13	5.89
		50.6	52.85 ± 5.29	10.01	8.54
		151.8	170.17 ± 11.01	6.47	12.54
		759.1	806.71 ± 60.33	7.47	9.12
Chlorpyrifos-oxon	(n=5)	6.3	6.90 ± 1.02	14.88	12.56
		12.6	12.72 ± 0.89	7.00	5.20
		151.8	162.48 ± 11.62	7.15	7.57
		759.1	819.15 ± 27.30	3.33	7.91
	(n=15)	6.3	6.73 ± 0.71	10.53	9.17
		12.6	13.63 ± 0.94	6.93	9.62
		151.8	169.21 ± 10.49	6.20	11.65
		759.1	778.71 ± 46.72	5.99	5.79
TCP	(n=5)	6.3	6.37 ± 1.04	16.35	11.28
		12.6	14.18 ± 0.39	2.78	12.55
		151.8	165.60 ± 16.15	9.75	9.07
		759.1	761.17 ± 36.49	4.79	3.71
	(n=15)	6.3	6.42 ± 0.86	13.46	9.83
		12.6	13.11 ± 1.77	13.55	13.62
		151.8	170.75 ± 10.91	6.39	12.48
		759.1	800.22 ± 44.63	5.57	6.56

Table 4.2. Absolute recovery, relative recovery, and matrix effects (mean ± SD) of analytes in rat brain homogenate (n=5)

Analyte	Concentration (ng/g)	Absolute Recovery (%)	Relative Recovery (%)	Matrix Effect (%)	Type of Effect
Chlorpyrifos	50.6	91.51 ± 5.57	88.10 ± 6.33	104.04	4.04% enhancement
	151.8	88.68 ± 6.47	85.15 ± 5.59	104.13	4.13% enhancement
	759.1	106.61 ± 9.35	87.44 ± 8.98	122.20	22.20% enhancement
Chlorpyrifos-oxon	12.6	86.45 ± 7.55	92.47 ± 7.47	93.46	6.54% suppression
	151.8	76.07 ± 1.85	83.92 ± 3.88	90.78	9.22% suppression
	759.1	92.88 ± 3.28	91.22 ± 1.91	101.79	1.79% enhancement
TCP	12.6	87.17 ± 11.59	82.19 ± 6.87	105.94	5.94% enhancement
	151.8	90.37 ± 8.87	81.56 ± 9.93	111.93	11.93% enhancement
	759.1	80.06 ± 6.62	82.38 ± 5.97	97.13	2.86% suppression

Table 4.3. Concentrations of chlorpyrifos and TCP in rats dosed at 18 mg/kg after 6 hours (n=3) and 24 hours (n=3) of exposure reported as mean ± SD (%RSD)

Rat Group	Chlorpyrifos Concentration (ng/g)	TCP Concentration (ng/g)
6 hours	74.64 ± 4.69 (6.29)	23.29 ± 4.65 (19.96)
24 hours	90.15 ± 14.84 (16.47)	9.34 ± 0.52 (5.60)

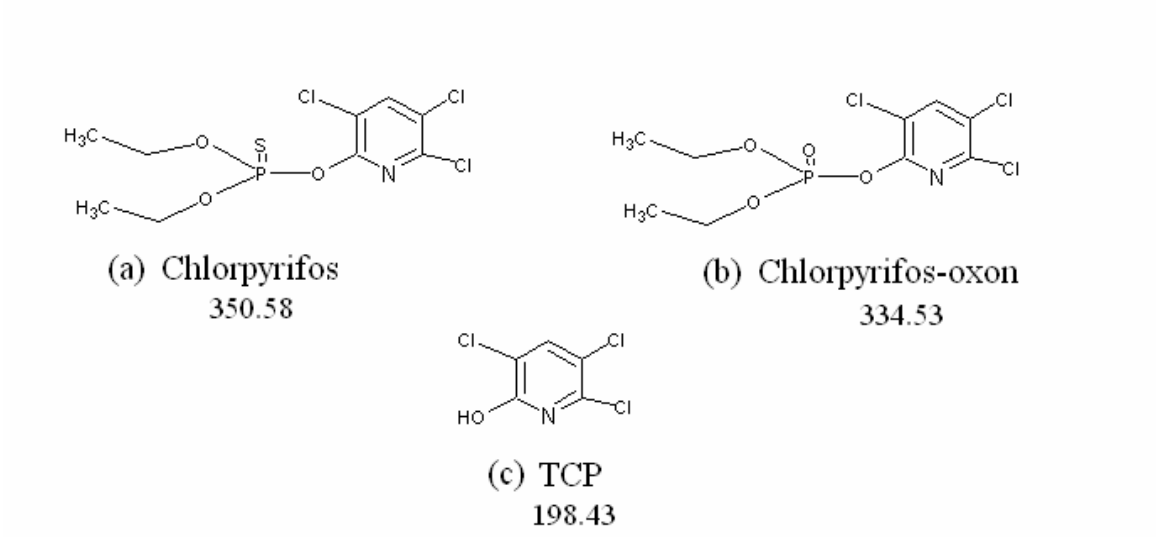

Figure 4.1. Chemical structures of (a) chlorpyrifos, (b) chlorpyrifos-oxon, (c) TCP, and their molecular weights.

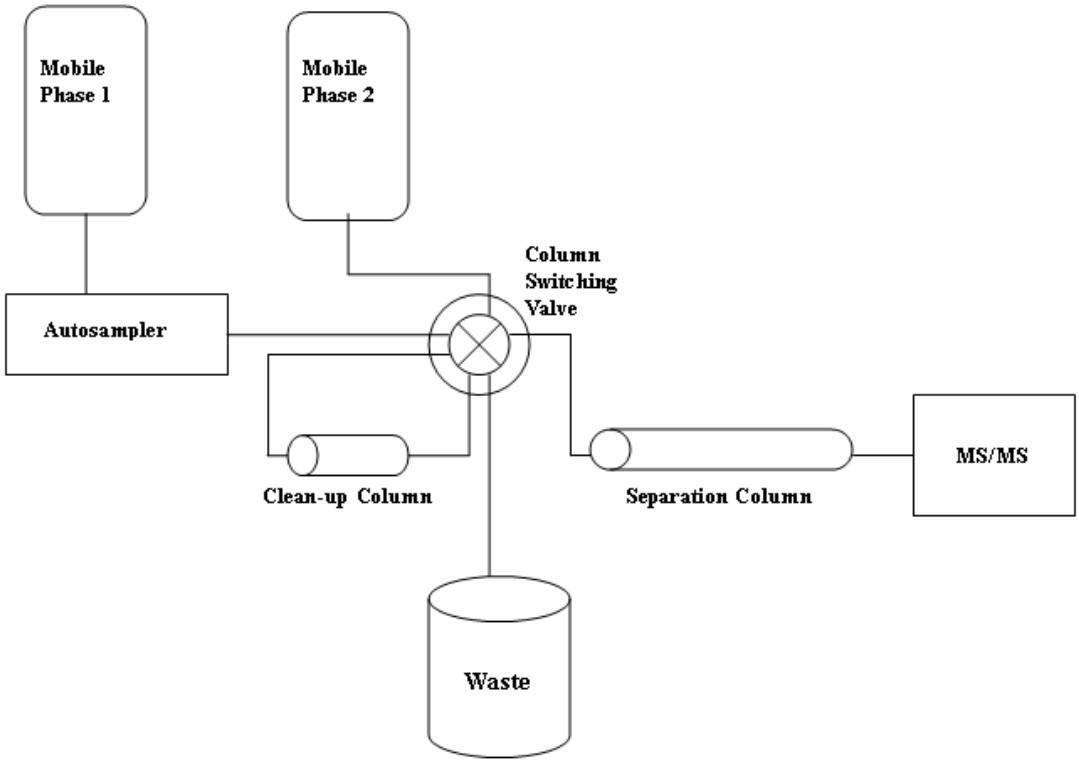

Figure 4.2. Schematic of coupled-column HPLC system.

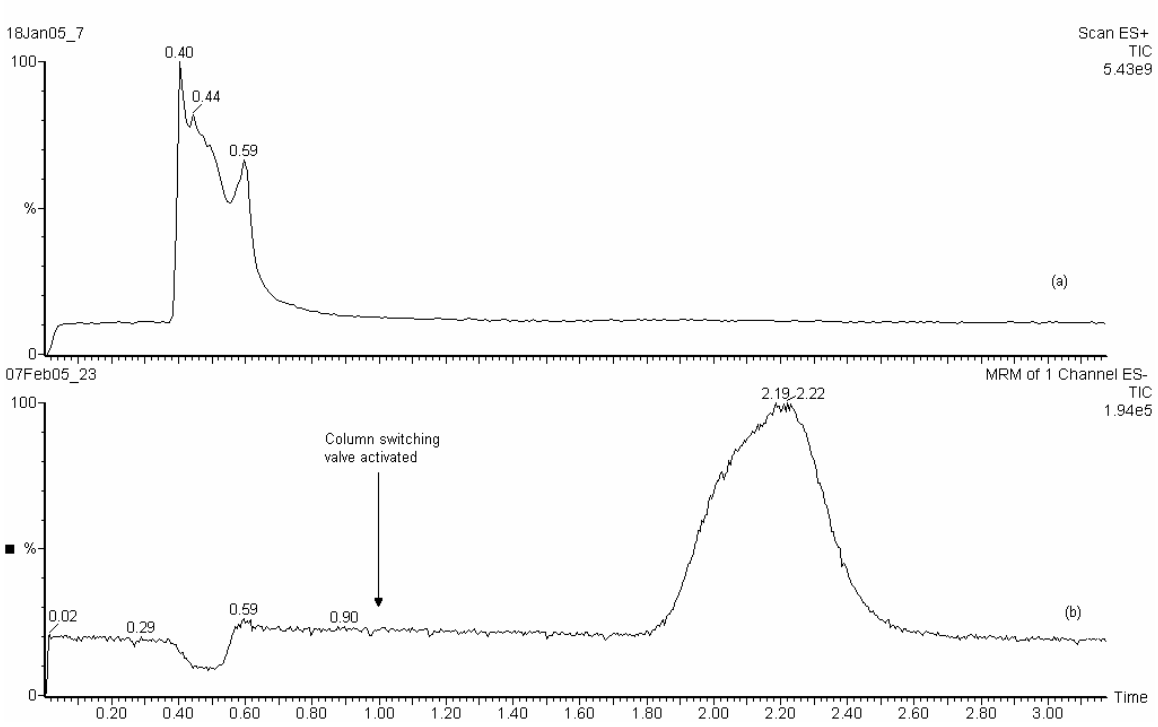

Figure 4.3. (a) Full scan chromatogram of blank brain homogenate and (b) MRM chromatogram of TCP standard eluting through the clean-up column.

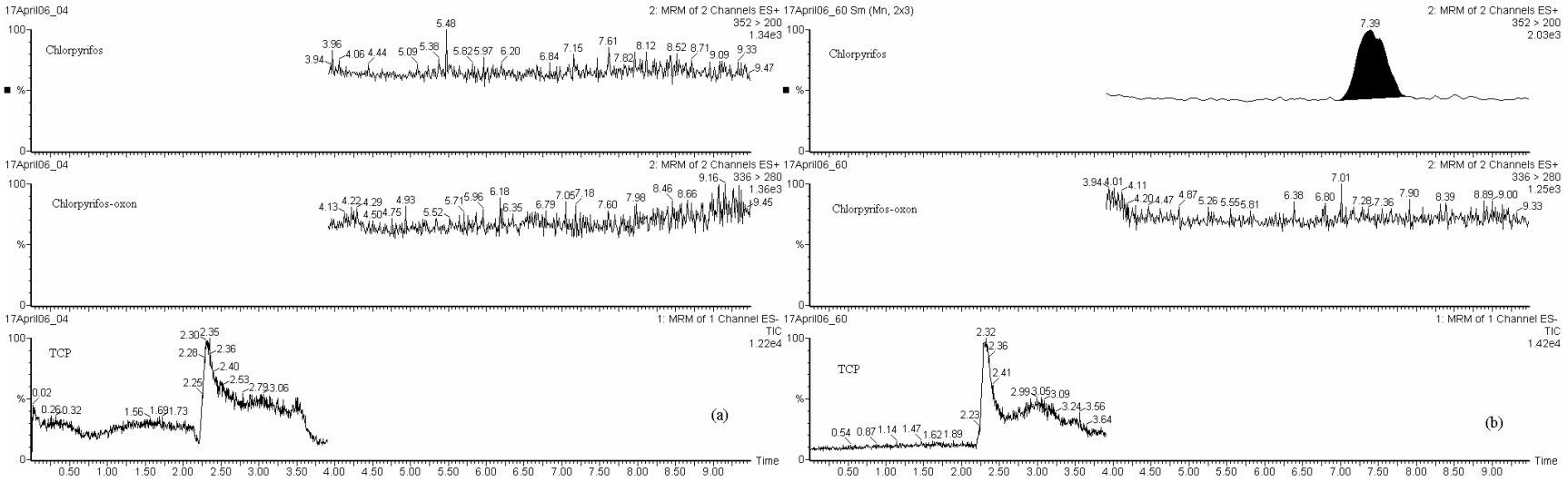

Figure 4.4. MRM chromatograms of brain homogenate from (a) an untreated rat and (b) a rat dosed with 18 mg/kg chlorpyrifos

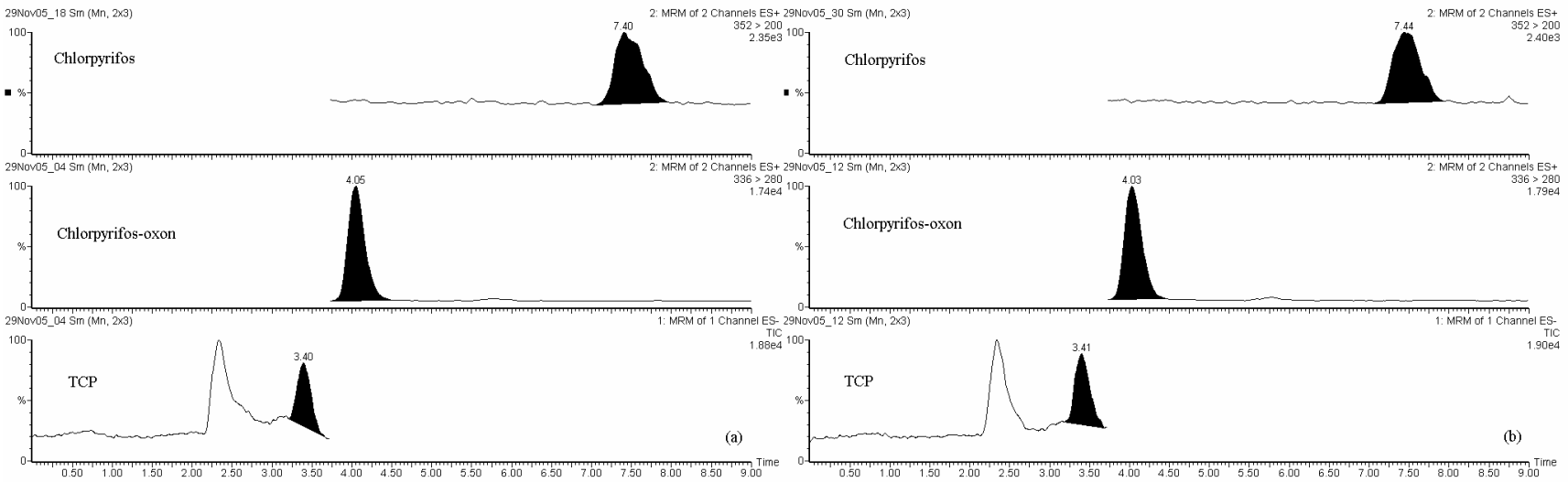

Figure 4.5. MRM chromatograms of (a) brain homogenate spiked with TCP (12.6 ng/g), chlorpyrifos-oxon (12.6 ng/g) and chlorpyrifos (50.6 ng/g) and (b) standard solutions at the same concentrations.

Chapter 4

DETERMINATION OF CHLORPYRIFOS AND ITS METABOLITES IN RAT BLOOD USING LIQUID CHROMATOGRAPHY/ELECTROSPRAY TANDEM MASS SPECTROMETRY[1]

[1] Williamson, L.N., Terry Jr., A.V., and Bartlett, M.G. *Journal of Liquid Chromatography & Related Technologies*, 2007; **30**: 273-285. Reprinted here with permission of publisher.

Abstract

A method has been developed to quantify chlorpyrifos (O,O-diethyl O-[3,5,6,-trichloro-2-pyridyl] phosphorothionate) and its metabolites chlorpyrifos-oxon (O,O-diethyl-O[3,5,6,trichloro-2-pyridyl] phosphate) and TCP (3,5,6,-trichloro-2-pyridinol) in rat blood by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). Rat blood was treated by liquid-liquid extraction and the analytes were separated by gradient elution on an Agilent Zorbax Extended-C_8 column (2.0×150 mm, 5μm). Chlorpyrifos and chlorpyrifos-oxon were detected in positive ion mode using multiple reaction monitoring (MRM). TCP was detected in negative ion mode using parent-to-parent transition monitoring.

This method was validated and the specificity, linearity, limit of quantititation (LOQ), precision, accuracy, stability, and recoveries were determined. The method was then applied to determine the level of chlorpyrifos and its metabolites from rats exposed to a subcutaneous injection of 10 and 18 mg/kg.

Introduction

Chlorpyrifos (O,O-diethyl O-[3,5,6,-trichloro-2-pyridyl] phosphorothionate) is an organophosphate insecticide that is widely used agriculturally (Lorsban ™). This insecticide is used because it inhibits acetylcholinesterase, an enzyme that is necessary for normal function of the nervous system. It is metabolized by Cytochrome P-450 into Chlorpyrifos-oxon (O,O-diethyl-O[3,5,6,trichloro-2-pyridinly] phosphate) and TCP (3,5,6,-trichloro-2-pyrinidol). TCP is also formed by the hydrolysis of chlorpyrifos-oxon. Chlorpyrifos-oxon is about 3,000 times more potent than Chlorpyrifos in its inhibition of acetylcholinesterase , which leads to neurotoxicity.[1] TCP is nontoxic and is eliminated by the kidneys. The use of chlorpyrifos has been restricted by the Environmental Protection Agency, however, TCP has recently been detected in the urine of 96% of approximately 2,000 samples analyzed from subjects living in the United States.[2]

Methods have been published to detect chlorpyrifos and its metabolites in blood, serum[3, 4], and brain tissue.[5] The method proposed by Brzak et al. [3] using gas chromatography/mass spectrometry requires isotope-labeled internal standards and derivatizing agents. Because of the high temperature at the GC inlet port, the chlorpyrifos and chlorpyrifos-oxon are hydrolyzed to TCP. However, the internal standards corrected for the low chlorpyrifos and chlorpyrifos-oxon and the high TCP concentrations. Two separate methods were used to quantitate the chlorpyrifos and chlorpyrifos-oxon and the TCP because the derivatizing agent for the TCP caused the chlorpyrifos-oxon to degrade. The method proposed by Sancho et al. [4] detected chlorpyrifos and TCP in serum, however, the chlorpyrifos-oxon metabolite was not detected. This may be due to the fact that the serum was not acidified to stop the chlorpyrifos-oxon hydrolysis.

Therefore, if using this method to detect chlorpyrifos and its metabolites in real serum samples, higher TCP concentrations may be reported.

Chlorpyrifos and its metabolites have been detected using high performance liquid chromatography with UV detection.[6-9] A method has also been published to detect chlorpyrifos in serum using liquid chromatography and atmospheric pressure chemical ionization mass spectrometry.[10] These methods reported limit of quantitations (LOQs) of 150-200 ng/ml. Because TCP levels in the urine are a marker for chlorpyrifos exposure, several methods have been published to measure TCP in urine.[4, 11-15]

Animal studies have indicated that repeated exposures of chlorpyrifos at sub-threshold levels can lead to persistent impairment in cognitive function.[16] It is important that a sensitive and accurate method be developed to detect the small amounts of chlorpyrifos and its metabolites in the blood to further explore the underlying neurological bases for these cognitive changes. The method we propose detects chlorpyrifos, chlorpyrifos-oxon, and TCP in one injection with a run time of 10 minutes. This method also did not require the use of derivatizing agents or internal standards.

EXPERIMENTAL

Chemicals and reagents

Chlorpyrifos (Fig. 5.1(a)), chlorpyrifos-oxon (Fig. 5.1(b)), and TCP (Fig. 5.1(c)) were purchased from Chem Service (West Chester, PA, USA). HPLC-grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized water was generated from a Continental Deionized Water System (Natick, MA, USA). Formic acid was purchased from J.T. Baker (Phillipsburg, NJ, USA). Trichloroacetic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Calibration standards and quality control standards

Standard stock solutions were made by dissolving chlorpyrifos, chlorpyrifos-oxon, and TCP in acetonitrile (1 mg/ml) and were stored at 4°C. The stock solutions were further diluted with acetonitrile to make working solutions with concentrations of 3.0 and 2.0 µg/ml. Standard calibration samples were spiked in blank rat blood from different lots to yield final concentrations of 1, 5, 15, 25, 50, 75, 100, and 200 ng/ml. Quality control (QC) samples were spiked in rat blood to yield final concentrations of 1, 2, 5, 10, 30, and 150 ng/ml.

Liquid chromatography

An Agilent 1100 Series HPLC system (Palo Alto, CA, USA) equipped with a degasser, binary pump, autosampler, and thermostated column compartment was used. The column used was an Agilent (Palo Alto, CA, USA) Zorbax Extended-C_8 column (2.0×150 mm, 5µm) equipped with a 4.0 × 2.0 mm Phenomenex (Torrance, CA, USA) Security Guard C_8 guard column. The compounds were separated by gradient elution with mobile phases of deionized water with 0.0025% formic acid (A) and acetonitrile (B) at a flow rate of 0.30 ml/min. The formic acid content of the mobile phase was chosen to be 0.025% because good peak shape and a high signal for the acidic TCP were maintained, as shown in a previous study.[4] The initial conditions were 40% A and 60% B. From 0 to 2 minutes, B was increased from 60% to 80% and held for 1 minute. From 3 to 4 minutes, B was decreased back to the starting conditions. The column then re-equilibrated from 4 to 10 minutes.

Mass Spectrometry

The HPLC system was interfaced to a Quattro micro API tandem mass spectrometer equipped with a Z-spray ion source and syringe pump (Manchester, UK). Nitrogen gas was used as the desolvation gas and was set to a flow rate of 500 L/h with a temperature of 350°C. The

cone gas flow was set to 50 L/h. Argon was the collision gas used and the collision cell pressure was 2.2×10^{-3} mbar. TCP was detected in negative ion mode using a capillary voltage of 2.5 kV, a cone voltage of 20 V, a dwell time of 200 ms and a collision energy of 2 eV. Chlorpyrifos and chlorpyrifos-oxon were detected in positive ion mode with a capillary voltage of 3.5 kV, a cone voltage of 20 V, a dwell time of 200 ms, and a collision energy of 20 eV. The source temperature was 120°C. Samples were acquired in multiple reaction monitoring (MRM) mode and were processed using Masslynx 4.0 software.

Sample preparation

This method was applied to 18 male albino Wistar rats obtained from Harlan (Indianapolis, IN, USA). All procedures were reviewed and approved by the Medical College of Georgia Committee on Animal Use for Research and the Veterans Affairs Medical Center Subcommittee on Animal Use and were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by US National Institute of Health. The rats were housed in pairs in a temperature controlled room (25°C) with a 12 hour light/dark cycle. The rats were injected with chlorpyrifos every other day for 30 days followed by a wash-out period of 14 days where no injections took place. The chlorpyrifos was dissolved in dimethyl sulfoxide (DMSO) and then added to peanut oil. The rats were injected subcutaneously at concentrations of 10 and 18 mg/kg. On the day of sacrifice, the rats were anesthetized with ketamine (40 mg/kg) and xylazine (18 mg/kg). The jugular vein was located and ~ 500 µl of blood was withdrawn. The blood was transferred to a Microtainer that contained the TCA solution (75 µl/ml blood) and briefly vortexed. The blood was stored at -70°C until analysis.

For the validation of the method, untreated rat blood was obtained from Harlan (Indianapolis, IN, USA). Trichloroacetic acid solution (1 g/ml) was added to decrease the pH of

the blood to ~4 (75 µl/ml blood). Acidifying the blood prevented the chlorpyrifos-oxon from enzymatically hydrolyzing to TCP. From a previous study, it was found that proteins with A-esterase activity for chlorpyrifos-oxon had maximum activity at pH 4.9-5.2.[17]

35 µl of the acidified rat blood was spiked with 5 µl of each analyte. The samples were briefly vortexed and were prepared by liquid-liquid extraction using 200 µl of ethyl acetate/acetonitrile (60:40, v/v). The samples were then vortexed for 5 minutes and centrifuged for 10 minutes at 13,000 rpm. The organic layer was extracted and dried under a gentle stream of nitrogen. The residue was reconstituted in 50 µl of acetonitrile. The samples were sonicated and centrifuged for 10 minutes at 13,000 rpm. 10 µl was used as the injection volume.

Method validation

Calibration curves were acquired (8-point for chlorpyrifos and chlorpyrifos-oxon and 7-point for TCP) by plotting peak area against the analyte concentration. The curves were acquired daily for 3 days (n=5 for intra-day and n=15 for inter-day). Precision and accuracy were determined for the 3 QC points (10, 30, and 150 ng/ml for TCP and 2, 30, and 150 ng/ml for chlorpyrifos and chlorpyrifos-oxon) and the LOQ (5 ng/ml for TCP 1 ng/ml for chlorpyrifos and chlorpyrifos-oxon). The LOQ values were determined by observing where precision and accuracy values did not exceed 20%. Precision was reported as percent relative standard deviation and accuracy was reported as percent error.

Recoveries were also determined. Absolute recovery was determined by dividing the response of the analyte in the blood by the response of the standard solution in acetonitrile. Relative recovery was determined by dividing the response of the analyte in the blood by the response of the analyte in blood that was spiked after the extraction process. The matrix effects were determined using the method reported by Matuszewski, et al. [18] and were calculated to be

the response of the homogenate spiked after the extraction divided by the response of the standard solution in acetonitrile.

The stability of the stock solutions were determined at their storage conditions of 4°C. Other stability experiments were determined at concentrations of 10 and 150 ng/ml for TCP and 2 and 150 ng/ml for chlorpyrifos and chlorpyrifos-oxon. Freeze/thaw stability was determined over 3 cycles. Autosampler stability was determined by preparing samples and injecting them at different time intervals over 24 hours. Bench top stability was determined by spiking blank blood and preparing the samples at different time intervals over 24 hours.

RESULTS AND DISCUSSION

Development of the analytical method

Full scan and product ion mass spectra were obtained by infusing 100 µg/ml of chlorpyrifos, chlorpyrifos-oxon, and TCP into the electrospray ionization (ESI) probe at a rate of 10 µl/min. TCP ionized in negative ion mode, while chlorpyrifos and chlorpyrifos-oxon ionized in positive ion mode. The collision-induced dissociation of TCP yielded an abundant ion at *m/z* 198, which corresponded to the $[M-H]^-$ of TCP, and *m/z* 35 for the chloride ion. Since the abundance of the TCP precursor ion was high relative to the chloride ion, the deprotonated molecular ion was used for quantitation. This same transition was used in an earlier study of TCP.[4] The MRM transitions monitored were: *m/z* 198→198 for TCP, *m/z* 336→280 for chlorpyrifos-oxon, and *m/z* 352→200 for chlorpyrifos. The MRM transitions monitored were selected based on the chromatograms that yielded the highest signal-to-noise ratio.

Method Validation

Specificity

From the MRM chromatograms of blank blood, it can be seen that there are no interfering peaks. In comparing the MRM chromatograms of the spiked blood and the standard solutions (Figure 5.2), this method was shown to be specific for chlorpyrifos, chlorpyrifos-oxon, and TCP.

Linearity

Standard solutions were made fresh each validation day and a linear calibration curve was generated for each analyte. The linear range was 1 to 200 ng/ml for chlorpyrifos and chlorpyrifos-oxon and 5 to 200 ng/ml for TCP. A weighting factor of $1/x^2$ was used for each curve. The coefficients of variation (r^2) for each compound were: 0.9910 ± 0.0028 for chlorpyrifos, 0.9922 ± 0.0010 for chlorpyrifos-oxon, and 0.9777 ± 0.0039 for TCP.

Accuracy and Precision

Precision and accuracy measurements were acquired for the 3 QC points and the LOQ for each compound. The accuracy and precision data can be seen in Table 5.1. Values for the intra-day precision and accuracy ranged from 3.23-14.16% and 2.68-10.75%, respectively, for the QC samples and 6.50-15.21% and 4.87-9.48% for the LOQ samples. Inter-day precision and accuracy ranged from 3.23-11.45% and 5.16-11.34%, respectively for the QC samples and 8.31-11.34% and 7.07-11.28% for the LOQ samples.

Recovery and Matrix Effects

Absolute recovery, relative recovery, and matrix effects data are summarized in Table 5.2. Relative recoveries ranged from 77.93-102.01%, so liquid-liquid extraction was a sufficient method for the extracting the compounds from the blood. The TCP signal was greatly suppressed (> 35%) at all three concentrations. This certainly contributed to the higher LOQ for

TCP. This suppression may be due to the fact that TCP was the first compound to elute. From this, we concluded that residual biological matter competes more effectively for the charge against TCP.

Stability

The stock solutions were stable at the storage conditions (4°C) for 3 months. Spiked samples were found to be stable over 3 freeze/thaw cycles. Prepared samples were stable in the autosampler for up to 24 hours. Spiked blood samples were found to have a change in the peak areas when left at room temperature. At 24 hours, the concentrations of the chlorpyrifos and chlorpyrifos-oxon decreased by as much as 49% while the concentrations for TCP increased by about 23%. This could be due to the fact that the hydrolysis of chlorpyrifos and chlorpyrifos-oxon could be temperature dependent as well as pH dependent. There was no change in the concentration of these samples after 4 hours at room temperature. Therefore, it is recommended that the blood samples be prepared directly upon thawing.

Application of the method

The concentration data for chlorpyrifos and its metabolites are reported in Table 5.3. The TCP concentrations were shown to reach a maximum value after the first week of injections, then continued to decrease until the end of the 30 days. The concentration of chlorpyrifos decreased after the 8^{th} injection and then increased after the 12^{th} injection. A MRM chromatogram from a rat dosed with 10 mg/kg of Chlorpyrifos after a 14-day wash-out period can be seen in Figure 5.3. We observed no chlorpyrifos-oxon in the blood. This is consistent with the findings that the chlorpyrifos-oxon levels in rat blood are 50-140 times lower than chlorpyrifos levels. [19] In an earlier study where an oral dose of chlorpyrifos was given at 15

mg/kg, the blood levels of TCP were 10-20 fold higher than chlorpyrifos levels.[20] This is consistent with our findings.

Conclusions

This validated method was efficient for the determination of chlorpyrifos, chlorpyrifos-oxon, and TCP in rat blood. The liquid-liquid extraction sufficiently recovered all three analytes from the blood. All analytes had high recoveries (>77%) from the blood. The method was accurate and precise with %RSD and %Error values of <16% over three validation days.

Acknowledgements

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Table 5.1. Intra- and Inter-day precision (%RSD) and accuracy (%Error) of the method in rat blood

Analyte		Theoretical Concentration (ng/ml)	Experimental Concentration (ng/ml)	%RSD	%Error
Chlorpyrifos	(n=5)	1	0.98 ± 0.12	12.74	8.06
		2	1.92 ± 0.17	8.84	7.08
		30	32.71 ± 2.86	8.74	10.07
		150	160.00 ± 9.40	5.86	7.64
	(n=15)	1	1.05 ± 0.12	11.34	9.63
		2	1.92 ± 0.14	7.52	5.59
		30	32.66 ± 2.01	6.16	9.50
		150	163.00 ± 9.80	6.01	8.93
Chlorpyrifos-oxon	(n=5)	1	1.03 ± 0.16	15.21	9.48
		2	1.92 ± 0.05	2.42	4.45
		30	33.22 ± 1.22	3.68	10.75
		150	151.35 ± 4.89	3.23	2.68
	(n=15)	1	1.09 ± 0.10	9.59	11.28
		2	2.05 ± 0.15	7.19	6.15
		30	33.10 ± 1.26	3.81	10.34
		150	150.15 ± 9.56	6.34	5.16
TCP	(n=5)	5	4.88 ± 0.32	6.50	4.87
		10	10.89 ± 1.54	14.16	9.18
		30	31.77 ± 2.11	6.65	7.57
		150	160.47 ± 17.94	11.18	7.16
	(n=15)	5	4.76 ± 0.39	8.31	7.07
		10	10.67 ± 1.22	11.45	11.34
		30	32.86 ± 2.06	6.26	10.09
		150	154.84 ± 13.74	8.88	6.28

Table 5.2. Absolute recovery, relative recovery, and matrix effects (mean ± SD) of analytes in rat blood (n=5)

Analyte	Concentration (ng/l)	Absolute Recovery (%)	Relative Recovery (%)	Matrix Effect (%)	Type of Effect
Chlorpyrifos	2	109.44 ± 6.01	96.73 ± 5.25	113.21	13.21% enhancement
	30	89.17 ± 3.45	77.98 ± 2.35	114.37	14.37% enhancement
	150	89.44 ± 3.70	80.22 ± 5.49	111.70	11.70% enhancement
Chlorpyrifos-oxon	2	107.90 ± 5.09	97.55 ± 5.97	110.70	10.7% enhancement
	30	89.29 ± 1.94	77.93 ± 2.83	114.63	14.63% enhancement
	150	90.03 ± 1.28	79.18 ± 7.93	114.30	14.30% enhancement
TCP	10	50.02 ± 3.69	102.01 ± 10.06	49.18	50.82% suppression
	30	45.08 ± 5.34	84.01 ± 20.30	54.88	45.12% suppression
	150	50.50 ± 3.10	79.18 ± 7.93	64.27	35.73% suppression

Table 5.3. Concentrations of chlorpyrifos and TCP in rat blood (n=6) after 5th dose (Day 9) of chlorpyrifos. Reported as mean ± S.D. (%RSD)

Dosage	Chlorpyrifos (ng/ml)	TCP (ng/ml)
10 mg/kg	2.20 ± 0.86 (39.16)	44.23 ± 13.32 (30.11)
18 mg/kg	5.06 ± 0.89 (17.50)	54.80 ± 17.64 (32.19)

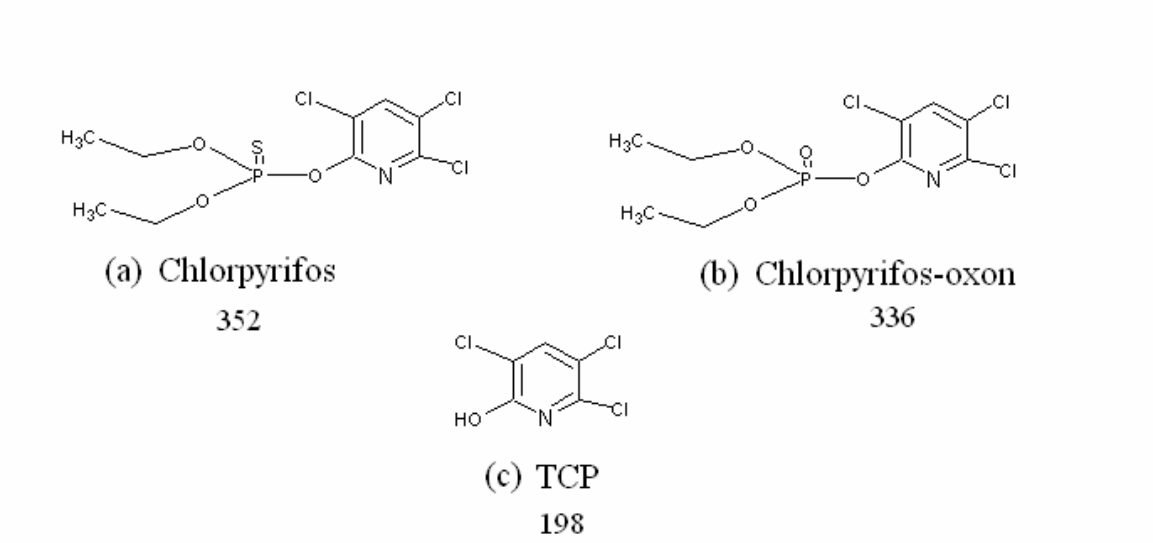

Figure 5.1. Chemical structures of (a) chlorpyrifos, (b) chlorpyrifos-oxon, and (c) TCP.

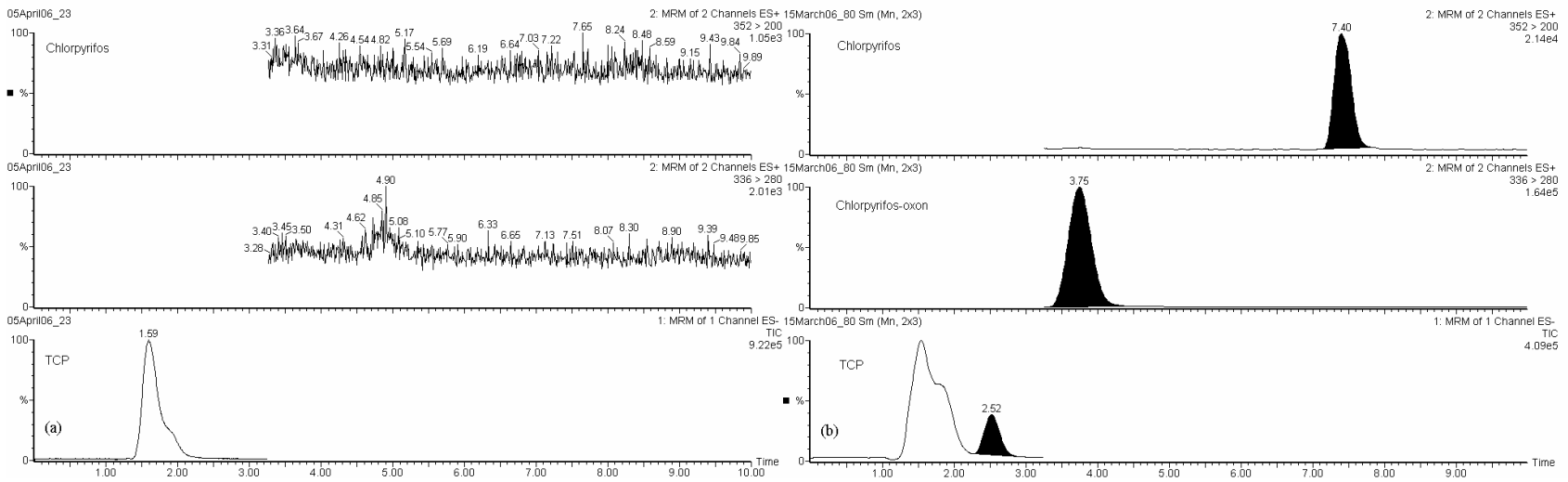

Figure 5.2. MRM chromatograms of (a) blank blood and (b) blood spiked at 15 ng/ml.

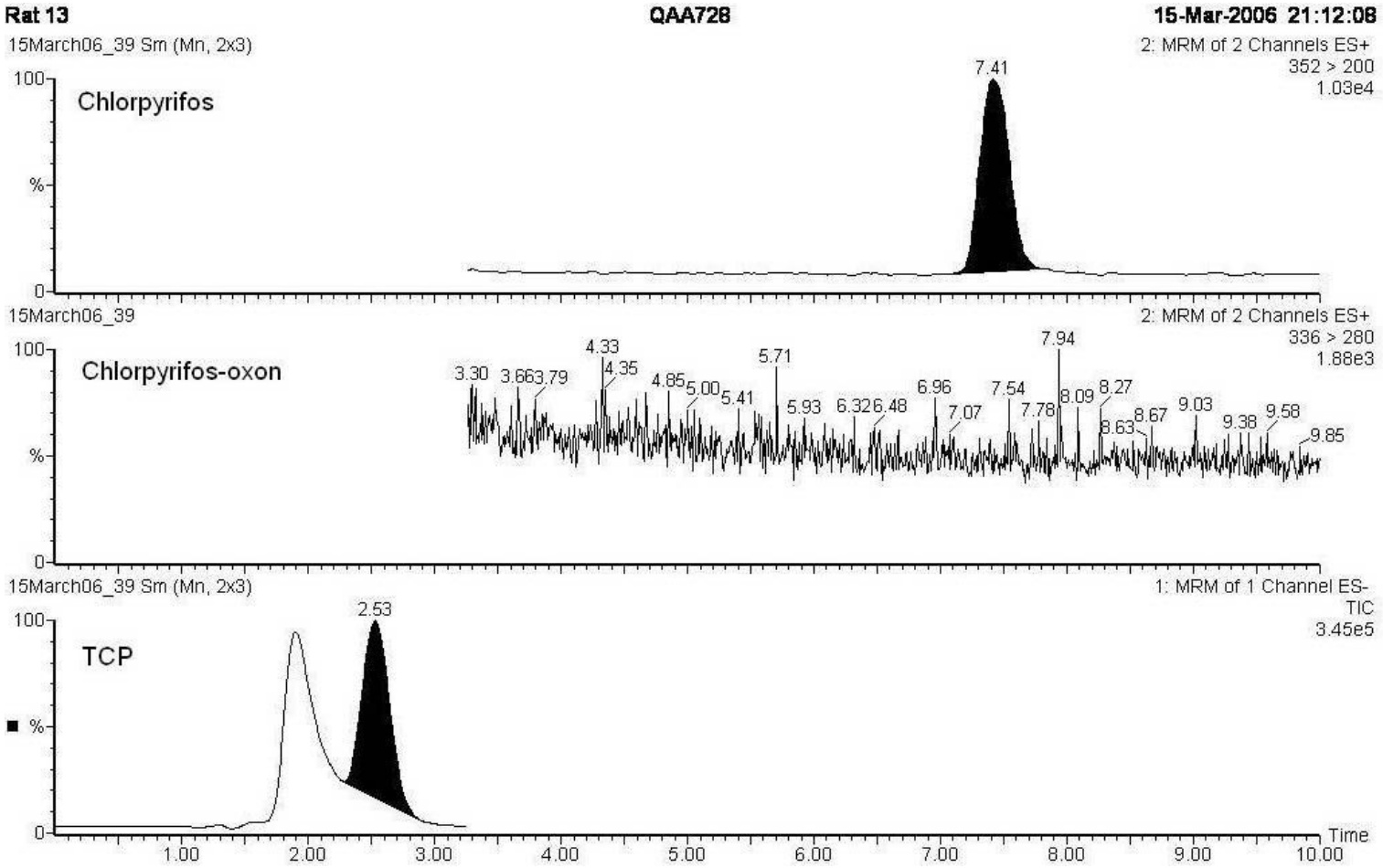

Figure 5.3. MRM chromatogram of blood from rat dosed with 10 mg/kg chlorpyrifos after 14-day wash-out period.

Chapter 5

USE OF LIQUID CHROMATOGRAPHY/TIME-OF-FLIGHT MASS SPECTROMETRY FOR QUANTITATIVE ANALYSIS: APPLICATION TO ANTIPSYCHOTICS[1]

[1] Williamson, L.N., Zhang, G., Terry Jr., A.V., and Bartlett, M.G. Submitted to *Journal of Mass Spectrometry*

Abstract

Tandem mass spectrometry (MS/MS) is frequently used for the detection of small molecules in biological samples because of the high specificity and sensitivity associated with these instruments. Time-of-flight mass spectrometers (TOF-MS) are typically used qualitatively for accurate mass determination; however, recently they have gained more attention for quantitation. A selective and sensitive TOF-MS method was validated to determine risperidone, its active metabolite paliperidol, haloperidol, clozapine, and olanzapine in rat plasma using midazolam as an internal standard (IS). Comparisons were made between the use of LC-TOF and LC-MS/MS using a triple quadrupole for these compounds. Rat plasma samples were treated by liquid-liquid extraction using isopropyl ether. A Waters Atlantis™ dC-18 column was used for chromatographic separation with acetonitrile and 5 mM ammonium acetate (pH 6.1) mobile phases. A Waters LCT Premier™ TOF-MS was operated in positive ion mode. For quantitation, ion chromatograms for each analyte were extracted from the total ion chromatograms using a mass window of 50 mDa. The method was validated for specificity, linearity, precision, accuracy, matrix effects, and recovery. Accurate mass data was also obtained for each analyte and was within 3.2 ppm accuracy. The linear range was 2 to 200 ng/ml. The precision and accuracy for both inter-day and intra-day did not exceed 13.6%. The matrix effects were less than 30% and the relative and absolute recoveries were greater than 50%. The method was successfully applied to plasma from rats that were chronically treated with antipsychotics.

Introduction

Quantitation of small molecules in biological matrices requires analytical methods with high selectivity and sensitivity. High performance liquid chromatography (HPLC) with ultraviolet detection (UV) is common for these analyses; however, limits of quantitation are often in the µg/ml range. HPLC used with mass spectrometry (MS) detection has more advantages over UV detection including, improved limits of quantitation, high selectivity, small sample volumes, and higher throughput.[1] Even though tandem mass spectrometers (MS/MS) are highly specific, accurate mass data cannot ordinarily be obtained from them. Time-of-flight mass spectrometers (TOF-MS), however, have the capability to resolve compounds with a resolving power of up to 10,000 at full width half maxima (FWHM) peak height and make accurate mass measurements of better than 5 ppm.

Over the past several years, TOF-MS has been used more frequently for quantitation.[2, 3] Because TOF mass spectrometers are not scanning instruments, a wide mass range can be acquired at a rapid pace without losing significant sensitivity. Methods using TOF-MS have been reported to have similar results for accuracy and precision when using MS/MS.[4-6] These methods also reported a limits of quantitation (LOQs) 5-10 times higher when using TOF-MS versus MS/MS. The linear range for TOF-MS is usually narrow because of the detector becoming saturated at high concentrations. Recent software and hardware improvements have targeted increasing the dynamic range of these instruments. This has been accomplished by charging the Z-focus lens to decrease the intensity of the primary ion beam. Alternate scans are taken at the normal and attenuated Z-focus lens settings, and peaks are sketched in using software algorithms when the intensity reaches a threshold. However, it is unclear if these improvements have any affect on the ability of LC-TOF-MS to provide quantitative data.

Antipsychotics have been used for treating psychological disorders since the 1950's; however, the second generation antipsychotics (SGAs) were found to have more advantages than first generation antipsychotics (FGAs) in the 1980's. It has been shown that SGAs such as olanzapine, risperidone, and clozapine are effective in treating positive and negative symptoms in schizophrenia.[7] Chronic exposure to FGAs often results in cholinergic imbalances in the striatum and abnormalities in motor function. Given that cognition is now recognized as a key factor that influences long term outcome in schizophrenia, [8-10] it is important to determine if there is a correlation between plasma levels of antipsychotics and cognitive function. Improved clinical monitoring of these drugs will be possible if such a correlation exists. The therapeutic ranges of these drugs in plasma are low (ng/ml levels)[11-13] and would require an analytical method with high sensitivity.

Some antipsychotics have been detected using HPLC-UV .[14-21] However, most HPLC-UV methods have lower limits of quantitation (LOQs) in the mid to upper ng/ml range which is higher than the therapeutic range for many of these compounds. Methods have also been published to detect antipsychotics in biological matrices using HPLC-MS/MS.[22-29] These methods report LOQs in the upper pg to lower ng/ml range. The goals of this study were to evaluate the Water's LCT Premier™ as a quantitation tool and to compare the performance of TOF-MS vs. tandem mass spectrometry (MS/MS) for a method that was previously validated for antipsychotics in rat plasma.[22] Since the data at the higher concentration range was software-generated, the precision and accuracy at the higher concentration range was an area of interest.

EXPERIMENTAL

Chemicals and Reagents

Risperidone (RISP), clozapine (CLOZ), olanzapine (OLAN) and haloperidol (HAL) were donated by Eli Lilly& Co. (Indianapolis, IN, USA). Paliperidone (PAL) was donated by Janssen Research Foundation (Beers, Belgium). Leucine enkephalin and the internal standard midazolam (IS) were obtained from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Isopropyl ether was purchased from Acros Organics (Morris Plains, NJ, USA). Ammonium formate and ammonium phosphate were obtained from Sigma (St. Louis, MO, USA). Formic acid was purchased from J.T. Baker (Phillipsburg, NJ, USA). Deionized water was generated from a Continental deionized water system (Natick, MA, USA).

Liquid chromatography

An Agilent 1100 Series HPLC system (Palo Alto, CA, USA) equipped with a degasser, binary pump, autosampler, and thermostated column compartment was used. A separate Agilent 1100 Series binary pump was used to introduce the leucine enkephalin into the reference probe. A Waters Atlantis^{TM} dC-18 (2.1 × 30 mm, 3µm) (Milford, MA, USA) with a 4.0 × 2.0 mm Phenomenex Security Guard C8 column (Torrance, CA, USA) was used for the chromatographic separation. The compounds were separated by gradient elution using mobile phases of 5 mM ammonium formate in water, pH 6.1 (mobile phase A) and acetonitrile (mobile phase B). The flow rate was 0.30 ml/minute using the following gradient (minutes, % B): (0,15) (1,15) (5,50) (10,72) (10.5, 80) (13.5, 80) (14, 15) (20, 15). A 15 µl injection was loaded onto the column. The first 5 minutes of the LC flow was diverted to waste, while data was acquired up until 12 minutes. The last 8 minutes of each run was also diverted to waste.

Mass Spectrometry

A Waters LCT Premier^{TM} mass spectrometer was operated in electrospray ionization (ESI) positive V-optics mode. The capillary voltage was 3500 V and the cone voltage was 35 V. The source temperature and desolvation temperatures were 130°C and 350°C respectively. Cone gas and desolvation gas flows were 50 and 500 L/hr respectively. The MCP plate voltage was 2700 V. Spectra were acquired from *m/z* 100-600 at a rate of 1 sec/spectrum. The dynamic range enhancement (DRE) function was used and leucine enkephalin was used as the reference spray. A 0.50 µg/ml solution in 50:50 acetonitirle:water was infused into the reference probe at a rate of 10 µl/min. The protonated C_{13} isotope peak was used for the lock mass (*m/z* 557.2804) and the protonated C_{12} peak was used for the attenuated lock mass (*m/z* 556.2117). One scan from the reference probe was taken for every 5 scans from the analyte probe. Ten scans from the reference were averaged for the calculation of the DRE corrected peaks. The *m/z* 557.2804 corrected for the accurate mass, and the *m/z* 556.2117 was used for the DRE function.

Calibration standards and quality control standards

Stock solutions were prepared by dissolving the RISP, OLAN, PAL, HAL, and CLOZ in methanol to yield a mixture with a final concentration of 1 mg/ml (see Figure 6.1 for structures and MW). Standard solutions were prepared by diluting the stock solutions in 5 mM ammonium fomate:acetonitrile (70:30, v/v). The final concentrations were 20, 100, 250 , 500, 1,000, and 2,000 ng/ml. The final concentrations of the quality control (QC) standards were 50, 300, and 1,500 ng/ml. The IS was prepared in the same manor and the final concentration was 100 ng/ml. Blank plasma samples were spiked with the standards to yield final concentrations of 2, 10, 25, 50, 100, and 200 ng/ml. The spiked concentration of the IS was 10

ng/ml. The final concentrations of the QC samples were 5, 30, and 150 ng/ml. Stock solutions were stored at -20°C. Fresh standard solutions were prepared each validation day.

Sample collection

All procedures were reviewed and approved by the Medical College of Georgia Committee on Animal Use for Research and the Veterans Affairs Medical Center Subcommittee on Animal Use and were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by US National Institute of Health. Male albino Wistar rats (Harlan, Indianapolis, IN, USA) 2-3 months old were housed individually in a temperature-controlled room (25ºC), maintained on a 12-h light/dark cycle with free access to food. Rats were thus treated with HAL (2.0 mg/kg/day), RISP (2.5mg/kg/day) and OLZ (10.0mg/kg/day) orally in drinking water for periods greater than two weeks to reach steady-state concentrations. These doses were chosen based on previous studies in which behavioral and time-dependent neurochemical effects were detected.[8, 10] These doses also resulted in plasma levels that approximated the levels often associated with antipsychotic effects in humans.[30] Plasma samples were collected and kept frozen at -70 ºC until analysis.

Sample preparation

The extraction method was adapted from the method published by Zhang, et al.[22] To 250 µl of rat plasma, 25 µl of the standard mix, 25 µl of the IS, and 200 µl of 5 mM sodium phosphate were added. After briefly vortexing, 3 ml of isopropyl ether were added for extraction. The samples were vortexed for 5 minutes, followed by centrifugation for 10 minutes at 2,000 × g. The organic layer was evaporated using a vacuum centrifuge. The residue was reconstituted in 100 µl of methanol:20 mM ammonium formate (pH 3.86 with

formic acid) (70:30, v/v). The samples were sonicated for 1 minute, briefly vortexed, and centrifuged for 5 minutes at 16,000 × g before LC injection.

Method validation

Six-point calibration curves were acquired by plotting peak area ratios of RISP, OLAN, PAL, HAL, and CLOZ to the IS against analyte concentration. The curves were acquired daily for 3 days (n=5 for intra-day and n=15 for inter-day). Precision and accuracy were determined for the 3 QC points (5, 30, and 200 ng/ml) and the LOQ (2 ng/ml). The LOQ values were determined by observing where precision and accuracy values did not exceed 20%. Precision was reported as percent relative standard deviation (%RSD) and accuracy was reported as percent error (%Error).

Recoveries for each analyte were also determined. Absolute recovery was determined by dividing the analyte/IS ratio in the plasma by the response of the standard solution in methanol:20 mM ammonium formate (pH 3.86 with formic acid) (70:30, v/v). Relative recovery was determined by dividing the response of the analyte/IS ratio in the plasma by the response of the analyte/IS ratio in plasma that was spiked after the extraction process. The matrix effects were determined using the method reported by Matuszewski, et al.[31] and were calculated to be the response of the plasma spiked after the extraction divided by the response of the standard solution in methanol:20 mM ammonium formate (pH 3.86 with formic acid) (70:30, v/v).

RESULTS AND DISCUSSION

Dynamic range enhancement

The normal Z-focus lens was optimized to where the intensity of the HAL ion was the greatest. HAL was chosen because it had the lowest response. The Z-focus lens was set at the

highest setting for attenuated mode. A 1 µg/ml mixture of the antipsychotics (in 50:50 acetonitrile:water) was infused into the analyte probe. A PEEK tee was used to introduce the mixture with 85:15 5mM ammonium formate:acetonitrile.

When calibration data was acquired in normal mode, the response was only linear from 2 to 25 ng/ml. At concentrations above 25 ng/ml, the chromatographic peaks were flat. When the data was acquired in DRE mode, the response was linear from 2 to 200 ng/ml. It was also found that increasing the normal Z-focus lens setting resulted in a greater increase in the dynamic range. When the normal Z-focus lens was increased to a higher setting, the response was linear to 1,000 ng/ ml. However, the LOQ was increased to 5 ng/ml. The Z-focus lens is a parameter that can be optimized to produce the linear range that is desired according to the samples that are going to be analyzed. We chose to set the normal Z-focus lens to obtain the lowest LOQ possible and to cover the therapeutic range of the analytes.

Method validation

Specificity

Standard mixtures were injected to determine the retention times of the analytes. Figure 6.2 shows a chromatogram of a 30 ng/ml standard. The retention times were 7.0, 7.7, 8.3, 8.6, 9.2 and 9.6 for PAL, RISP, OLAN, IS, HAL, and CLOZ, respectively. Ion chromatograms were extracted from the total ion chromatograms (TIC) for each analyte. The specificity for each analyte was improved when a smaller mass window was used. When a mass window of 100 mDa was used, there was cross-talk between the CLOZ (*m/z* 327.1376) and the IS (*m/z* 326.08) extracted ion chromatograms. A mass window of 50 mDa was chosen to eliminate the cross-talk and improve specificity. Figure 3 shows a blank plasma

chromatogram and the respective extracted ion chromatograms for each analyte. It can be seen that there are no interferences from endogenous compounds in the plasma.

Accurate mass measurement

Accurate mass measurements were acquired by averaging ten scans across each chromatographic peak for a 25 ng/ml spiked plasma sample. The measured masses were within 1.2 mDa of the theoretical masses and within 3.19 ppm accuracy. The data is summarized in Table 6.1.

Linearity

Standard solutions were made fresh each validation day and a linear calibration curve was generated for each analyte. The linear range was 2 to 200 ng/ml for each analyte. The ratio of the analyte peak area to the internal standard peak area was plotted against concentration. A weighting factor of $1/x^2$ was used to fit each curve.

Accuracy and precision

Precision and accuracy measurements were acquired for the 3 QC points and the LOQ for each compound. The accuracy and precision data is summarized in Table 6.2. Values for the intra-day precision and accuracy ranged from 3.32-9.62% and 4.00-12.61%, respectively. Inter-day precision and accuracy ranged from 4.12-13.55% and 5.53-12.97%, respectively. These results were acceptable according to the current FDA bioanalytical validation guidelines.

Recovery and matrix effects

Absolute recovery, relative recovery, and matrix effects data are summarized in Table 6.3. Relative recoveries ranged from 50.72 to 98.99%. The relative recovery for olanzapine was lower than the other drugs. Matrix effects were somewhat higher for CLOZ, HAL, and

OLAN, however it did not prevent the method from passing validation or covering the therapeutic range of the analytes.

Application of the method

The LC-TOF method was applied to rat plasma from rats that where chronically treated with RISP, HAL, and OLAN (n=5 per treatment). The concentration data is summarized in Table 6.4. The data was compared to data obtained by the LC-MS/MS method reported by Zhang, *et al*.[22] for the same samples. Figure 6.4 shows extracted ion chromatograms for the rats treated with the respective drugs. In comparing the LC-TOF data with the LC-MS/MS data, the results RISP, PAL, and HAL were lower, while the OLAN numbers were higher. This may be due to the differences in the ion suppression observed for RISP, PAL, and HAL and ion enhancement for OLAN between the methods. However, a paired two-sample t-test was used (α=0.05) showed there was no significant difference between the results from the LC-TOF and the LC-MS/MS method.

Comparison of time-of-flight to triple quadrupole mass spectrometry

One advantage of using TOF-MS over MS/MS is the high resolution capability. Typically triple quadrupole instruments have unit mass resolution, whereas TOF instruments have resolution of at least 10,000 FWHM. This high resolution capability allows for resolution of chromatographic peaks from background interferences, or from other compounds with the same unit mass. This method was able to provide high quality exact mass data at 25 ng/ml demonstrating that the DRE function does not adversely affect this capability of the TOF-MS. TOF-MS is also an excellent tool for screening to verify the presence of a compound or compounds of interest in complex matrices, such as plasma. Because there is no scanning

involved using TOF-MS, several spectra can be acquired at a fast rate. However, it does appear that the MS/MS method remains about 10-20 times more sensitive.

The TOF-MS and MS/MS methods have similar accuracy and precision. Again it does not appear that the DRE function has diminished the quantitative capabilities of the TOF-MS system. Another advantage of TOF-MS is that precursor-to-product ion transitions do not have to be determined or optimized as they are when using MS/MS. This may result in shorter method development times. Overall, it appears that TOF-MS can be used successfully for multianalyte bioanalysis from a complex biological matrix such as plasma.

Conclusion

We report a validated method to determine 5 antipsychotic drugs in rat plasma. The linear dynamic range was improved by using the DRE function on the instrument. Using the DRE function, the linear range was 2 to 200 ng/ml. The method is specific for RISP, PAL, HAL, CLOZ, and OLAN. The percent errors and RSDs for accuracy and precision were acceptable and were all less than 14%. This method was successfully applied to plasma samples from rats that were chronically treated with RISP, HAL, and OLAN. These results were compared to results obtained using a method using LC-MS/MS. Both sets of results were found to be not significantly different when using a paired two-sample t-test.

Acknowledgements

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Table 6.1. Accurate mass data for antipsychotics in rat plasma (average of 10 scans for 25 ng/ml spiked plasma sample)

Drug	Theoretical Mass	Measured Mass	Mass Difference (mDa)	Mass Difference (ppm)
Olanzapine	313.1487	313.1497	1.00	3.19
Clozapine	327.1376	327.1372	-0.40	-1.22
Haloperidol	376.1479	376.1483	0.40	1.06
Risperidone	411.2196	411.2184	-1.20	-2.92
Paliperidone	427.2145	427.2137	-0.80	-1.87
Midazolam (IS)	326.0860	326.0860	0.00	0.00

Table 6.2. Intra-day (n=5) and inter-day (n=15) precision (%RSD) and accuracy (%Error) of drugs in rat plasma

		Intra-day			Inter-day		
Drug	**Theoretical Concentration (ng/ml)**	**Measured Concentration (ng/ml)**	**%RSD**	**%Error**	**Measured Concentration (ng/ml)**	**%RSD**	**%Error**
PAL	2	1.87 ± 0.08	4.26	6.49	1.89 ± 0.13	7.02	7.56
	5	5.02 ± 0.29	5.77	4.00	5.27 ± 0.34	6.54	7.27
	30	31.52 ± 1.35	4.27	5.58	32.84 ± 1.70	5.17	9.64
	150	145.77 ± 7.95	5.45	4.58	134.87 ± 9.72	7.20	10.67
RISP	2	1.85 ± 0.06	3.32	7.55	1.96 ± 0.20	10.04	7.92
	5	5.11 ± 0.32	6.24	5.47	5.49 ± 0.40	7.36	10.90
	30	32.29 ± 1.38	4.29	7.68	33.16 ± 1.36	4.12	10.53
	150	135.84 ± 9.75	7.17	9.60	130.82 ± 8.50	6.50	12.84
CLOZ	2	1.97 ± 0.15	7.54	6.62	1.97 ± 0.27	13.55	11.10
	5	5.52 ± 0.28	5.13	10.47	5.65 ± 0.37	6.49	12.97
	30	32.26 ± 1.35	4.17	7.54	32.86 ± 1.59	4.85	9.62
	150	131.08 ± 7.74	5.90	12.61	130.92 ± 6.30	4.81	12.72
HAL	2	1.78 ± 0.14	7.66	10.76	1.83 ± 0.19	10.18	10.16
	5	5.39 ± 0.46	8.60	10.47	5.55 ± 0.30	5.49	11.90
	30	33.01 ± 1.39	4.20	10.03	33.10 ± 1.47	4.44	10.33
	150	134.28 ± 5.40	4.03	10.48	135.64 ± 10.27	7.57	10.82
OLAN	2	1.99 ± 0.19	9.62	7.08	2.02 ± 0.20	9.85	8.19
	5	4.74 ± 0.25	5.34	5.88	5.34 ± 0.62	11.60	10.70
	30	28.47 ± 1.17	4.10	5.10	30.33 ± 2.09	6.89	5.53
	150	156.28 ± 8.03	5.14	5.08	145.80 ± 14.65	10.05	8.44

Table 6.3. Absolute recovery, relative recovery, and matrix effects (mean ± SD) of drugs in rat plasma

Drug	Concentration (ng/ml)	Absolute recovery (%)	Relative recovery (%)	Matrix effect (%)	Type of effect
PAL	5	68.05 ± 2.66	77.58 ± 3.67	87.91	12.09% suppression
	30	84.57 ± 5.52	87.42 ± 5.86	96.81	3.19% suppression
	150	79.36 ± 1.42	88.06 ± 2.84	90.18	9.82% suppression
RISP	5	79.62 ± 3.88	82.02 ± 5.10	97.40	2.60% suppression
	30	88.68± 6.51	85.84 ± 4.36	103.29	3.29% enhancement
	150	90.45 ± 1.42	91.85 ± 1.58	98.49	1.51% suppression
CLOZ	5	64.52 ± 5.16	83.37 ± 7.14	77.67	22.33% suppression
	30	78.59 ± 3.55	90.90 ± 3.03	86.44	13.56% suppression
	150	77.23 ± 1.69	95.14 ± 2.57	81.19	18.81% suppression
HAL	5	61.32 ± 2.88	86.17 ± 6.63	71.52	28.48% suppression
	30	69.38 ± 3.60	87.12 ± 2.25	79.63	20.37% suppression
	150	67.43 ± 3.88	98.99 ± 4.43	75.92	27.08% suppression
OLAN	5	58.12± 2.66	50.72 ± 3.33	114.85	14.85% enhancement
	30	74.98 ± 4.49	59.80 ± 2.28	125.35	25.35% enhancement
	150	67.43 ± 3.88	62.85 ± 2.62	107.25	7.25% enhancement
IS	10	91.10 ± 6.10	93.93 ± 0.52	97.00	3.00% suppression

Table 6.4. Rat plasma concentrations of the drugs after chronic treatment in drinking water (n=5)

Drug	LC-TOF concentration (ng/ml)	LC-MS/MS concentration (ng/ml)
RISP	4.96 ± 4.09	5.91 ± 3.58
PAL	17.87 ± 15.59	27.84 ± 15.45
HAL	10.87 ± 7.03	13.32 ± 7.07
OLAN	81.49 ± 68.01	56.78 ± 63.67

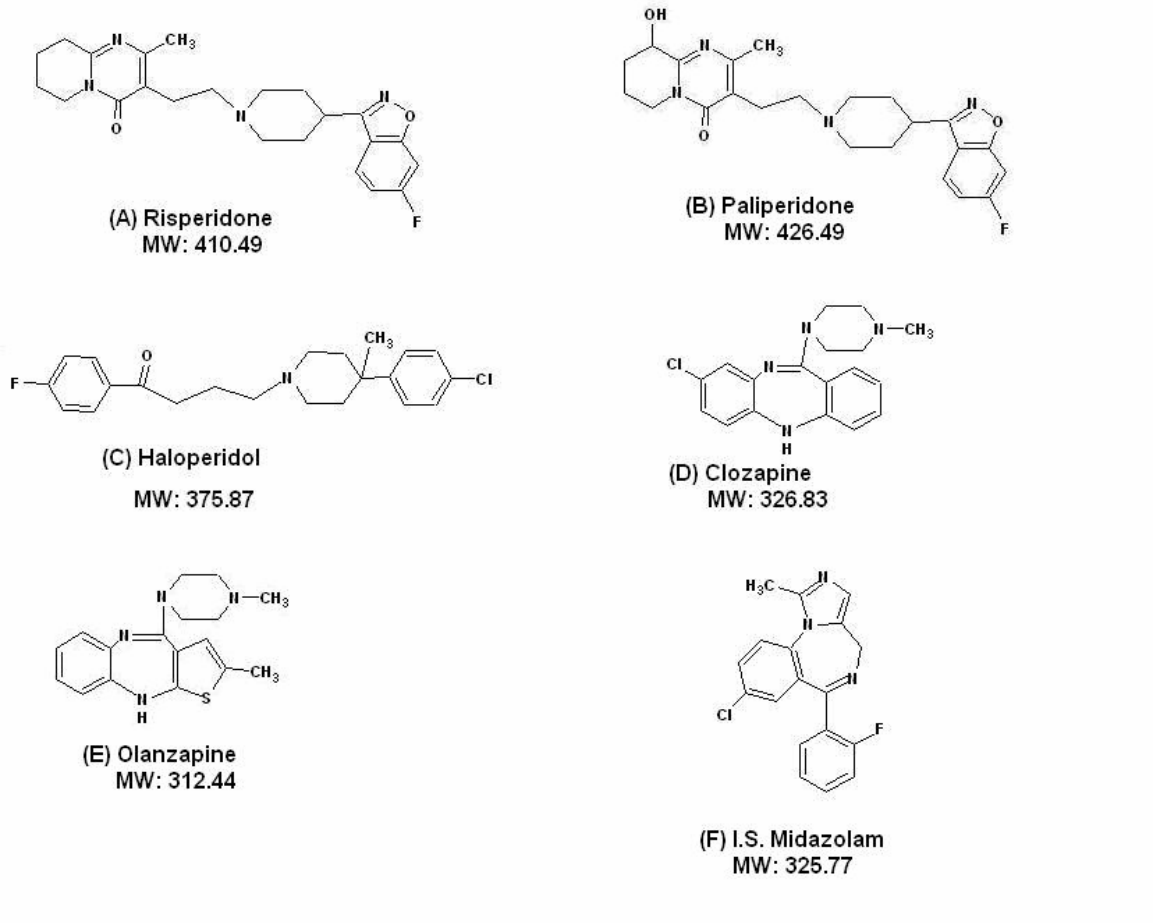

Figure 6.1. Chemical structures of (A) RISP, (B) PAL, (C) HAL, (D) CLOZ, (E) OLAN, and (F) I.S.

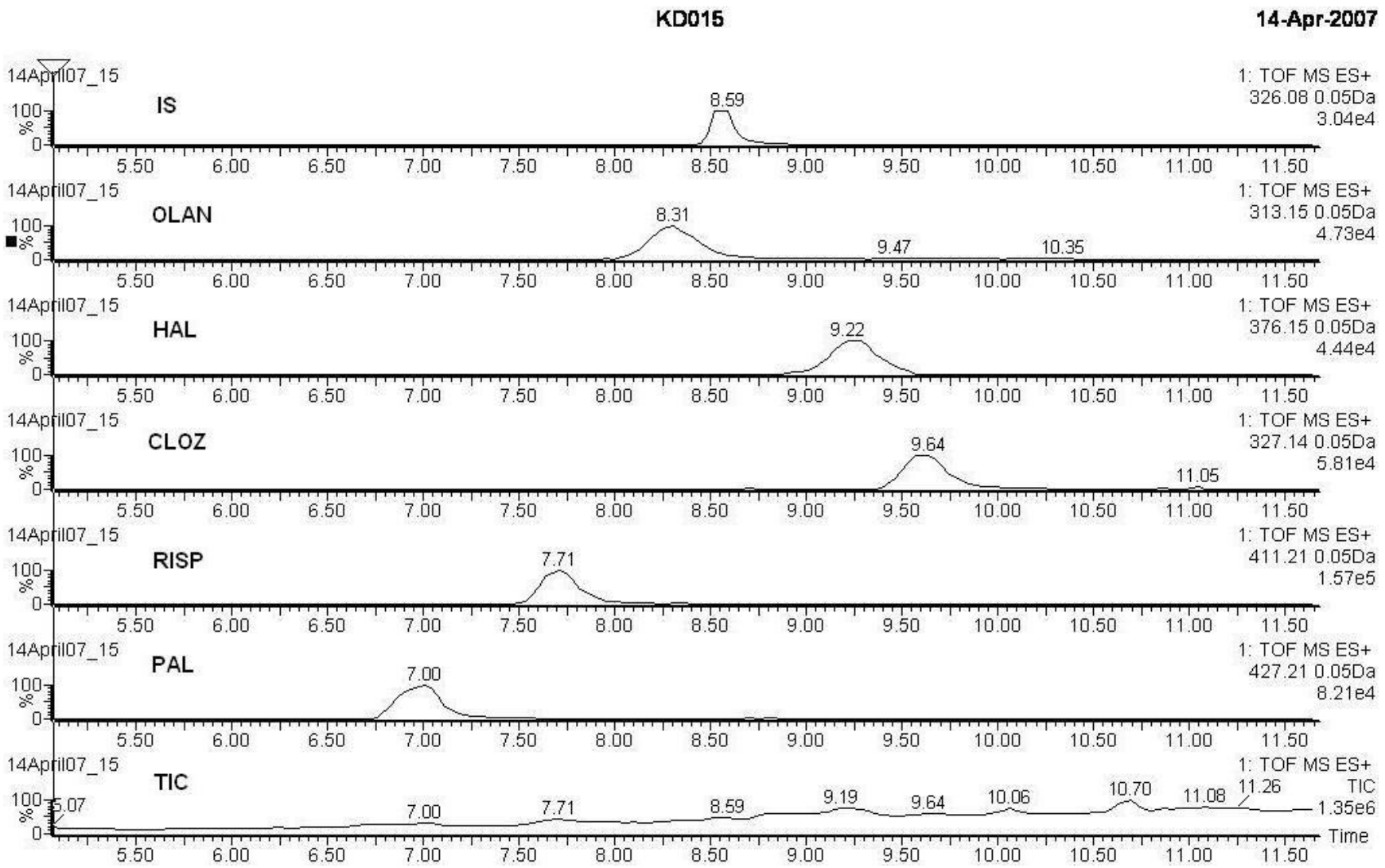

Figure 6.2. TIC and extracted ion chromatograms for 30 ng/ml standard solution.

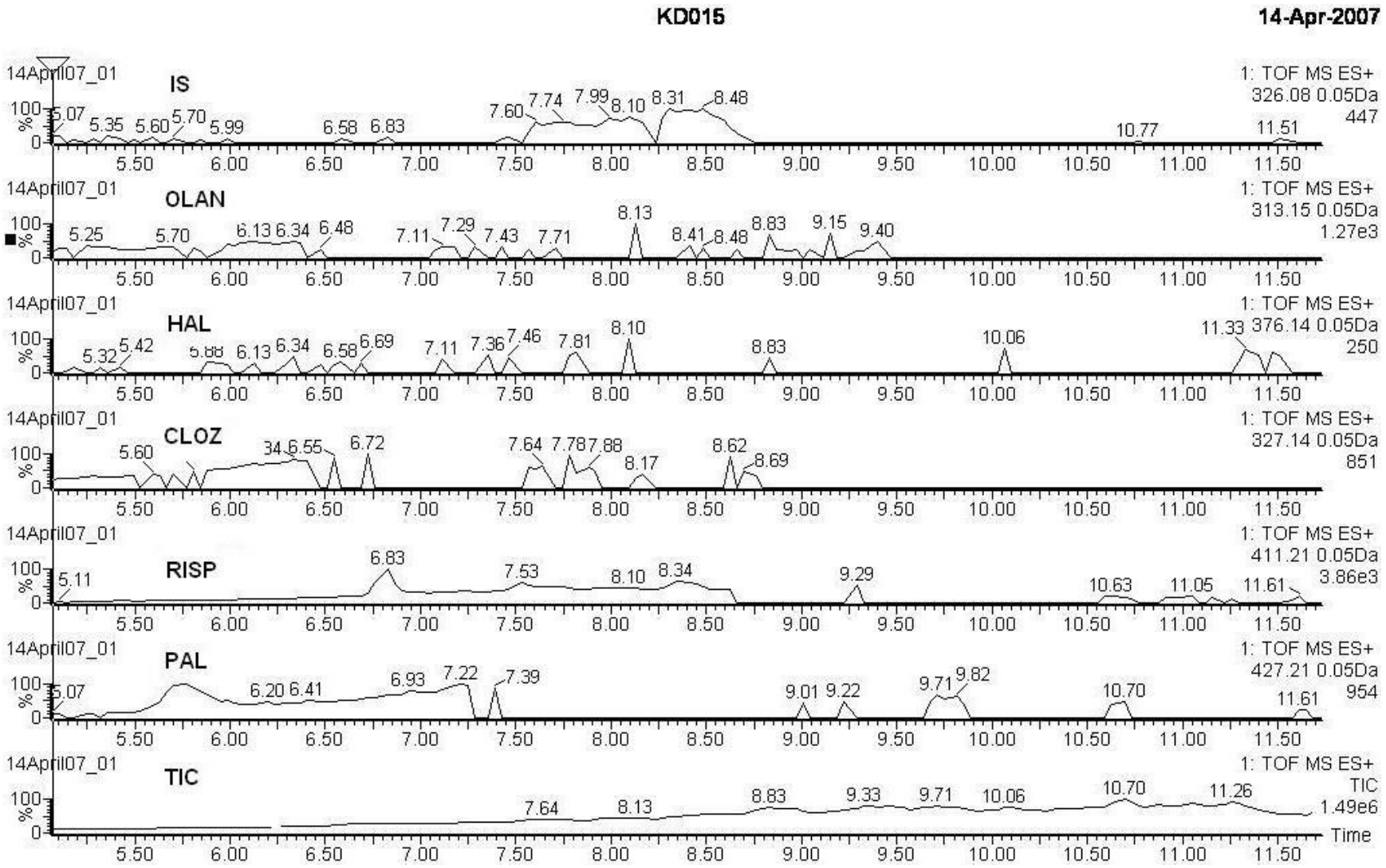

Figure 6.3. TIC and extracted ion chromatograms for blank plasma

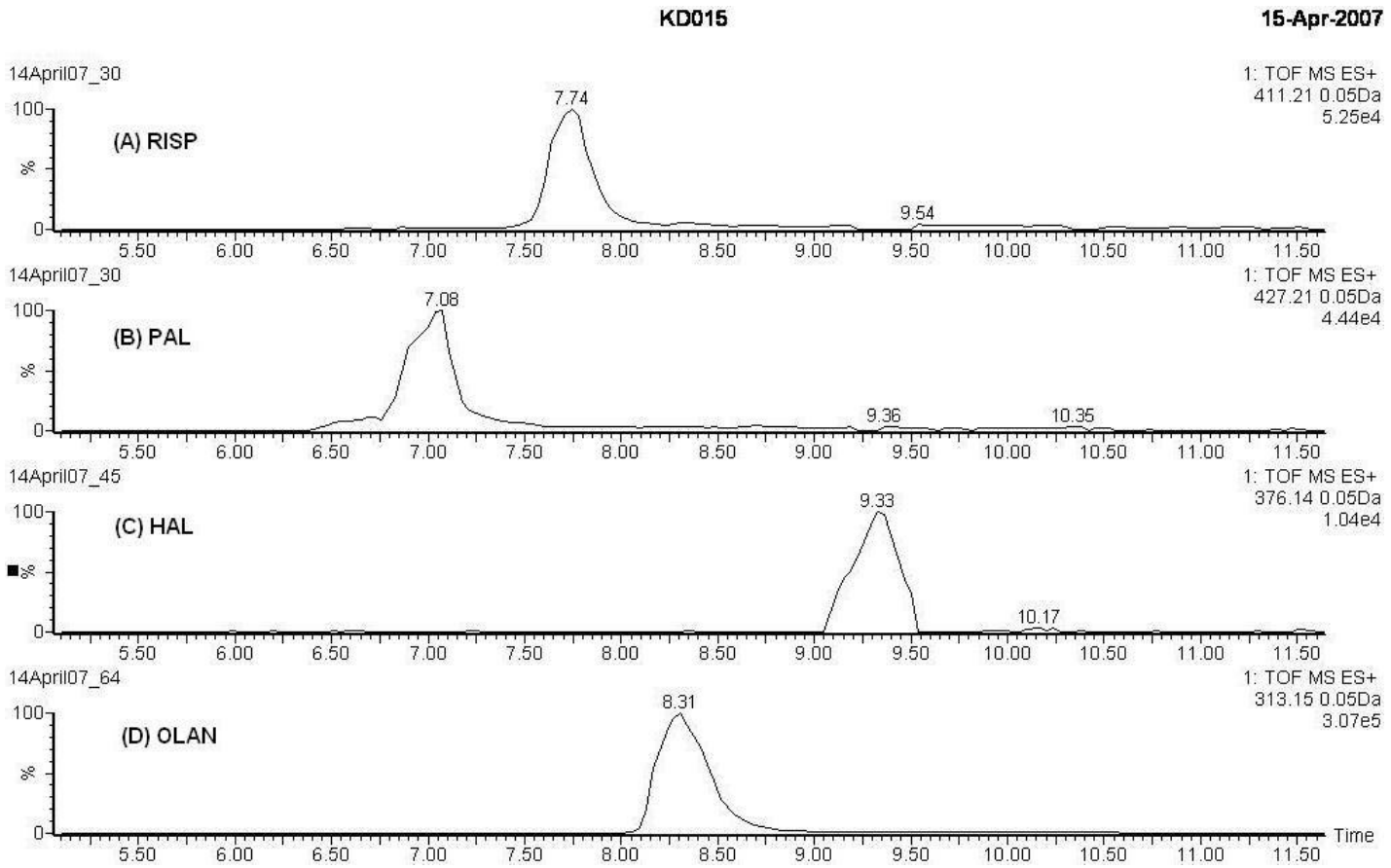

Figure 6.4. Extracted ion chromatograms from rats chronically treated with (A) RISP with the active metabolite (B) PAL, (C) HAL, and (D) OLAN.

Chapter 6

CONCLUSIONS

Chlorpyrifos is still being used agriculturally and it is important to assess the damage in cognitive function due to exposure. The methods presented are highly specific and sensitive for the detection of CPF and its metabolites in the rat. The coupled-column LC system for the brain tissue reduced the sample preparation time and was able to detect all three compounds in one injection. The method for detection in rat blood used a rapid liquid/liquid extraction and was able to detect all three compounds in less than 10 minutes per sample. Both validated methods were acceptable with the current FDA requirements for bioanalytical validation. They were also successfully applied to real samples from rats that were dosed with chlorpyrifos.

A TOF-MS method was also validated for the quantitation of antipsychotic drugs in rat plasma. The dynamic range enhancement (DRE) function was used to improve the dynamic range. It was shown that the DRE function did not affect the quantitative data, or the accurate mass data. The validation data was acceptable with the current FDA requirements for bioanalytical validation. The method was successfully applied to real samples from rats that were exposed to antipsychotic drugs. The results were compared to results using MS/MS from the same rats. The data was found to be not significantly different.

These analytical methods were rapid, specific, and sensitive for their respective analytes. The TOF-MS method can be used for quantitation as well as accurate mass acquisitions. These methods can be used to accurately determine the extent to which there is a correlation between plasma and/or brain levels and cognitive function in the rat.

APPENDIX

APPLICATIONS OF REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY FOR PHARMACEUTICAL INVESTIGATIONS[1]

[1] Shelbourn, T., Williamson, L., Gillespie, T., and Montgomery, R. Submitted to *Journal of Analytical Atomic Spectrometry*

Abstract

Reverse-phase chromatographic methods have been developed for the analyses of various pharmaceutical compounds using sequential photodiode array (PDA) and inductively coupled plasma mass spectrometric (ICP-MS) detection. The objective of this work was to investigate the utility of elemental detection by HPLC/ICP-MS in pharmaceutical investigations. Interfacing the ICP-MS instrument to the HPLC/PDA system was straightforward. The eluent from the PDA was pumped directly into the ICP-MS nebulizer using standard sample introduction components at flow rates ranging from 0.5 to 1.5 mL/min. Two separate sets experiments were carried out. Initial speciation experiments were conducted on the B supplements cyanocobalamin (vitamin B12), biotin and thiamine (vitamin B1) using isocratic separations. ICP-MS chromatograms were generated by simultaneously monitoring cobalt at *m/z* 59, phosphorus at *m/z* 31, and sulfur at *m/z* 34. Linear regression data were obtained for cyanocolbalamin, thiamin hydrochloride and biotin. Elemental detection was used to resolve co-eluting thiamine and cyanocobalamin peaks in a rapid isocratic separation. The signal for biotin in the sulfur chromatogram was determined to be much larger than that of the corresponding UV chromatogram at 250nm. In the second set of experiments, an active pharmaceutical ingredient (API) was hydrolyzed with base in order to monitor the parent compound and two degradation products, degradation product 1 (DP1) and degradation product 2 (DP2). The API molecule contains bromine, chlorine, and sulfur, DP1 contains bromine and sulfur, and DP2 contains chlorine. The ions monitored for analysis of API, DP1 and DP2 were *m/z* 79 for bromine, *m/z* 35 for chlorine, and *m/z* 34 for sulfur. The calculation of relative amounts of API, DP1 and DP2 from the bromine, chlorine and sulfur chromatograms was performed. The data suggests that the same calculation performed from the UV chromatogram

introduces a bias due to the difference in extinction coefficients for each compound. The capacity for monitoring UV absorbance and elemental ions sequentially was demonstrated to provide enhanced analytical specificity without requiring interpretation of complex mass spectra.

Introduction

Applications for reverse-phase HPLC with sequential UV absorbance and elemental detection by ICP-MS for the speciation of pharmaceutical compounds containing a transition metal (cobalt) and/or the nonmetallic heteroatoms sulfur, phosphorus, chlorine, and bromine were investigated. Multiple elements were monitored simultaneously by ICP-MS in order to fully utilize specificity of elemental detection. An objective of this investigation was to demonstrate the use of elemental detection by ICP-MS with minimal modification of typical packed-column HPLC isocratic and gradient methods. Background signal from polyatomic isobaric interferences at *m/z* 31, 34, 35, and 79 were attenuated by using a hexapole collision cell charged with a mixture of 1% ammonia/helium.

Proof of concept investigations of this technique were performed using the B supplements cyanocolbalamin (B12) which contains cobalt and phosphorus, thiamin (B1) which contains sulfur, and biotin, which contains sulfur. These data were collected using isocratic HPLC methods. These compounds were chosen for method development as several HPLC-UV methods have been published to detect water-soluble B vitamins (Kledjus, 2004, Markopoulou, 2002, Heudi, 2006, Chatzmichalakis, 2004, Moreno 2000). Two methods have been reported to detect water-soluble B vitamins, including cyanocolbalamin, thiamin, and biotin using UV detection. (Heudi 2004, Li 2001). ICP-MS detection has previously been used to detect vitamin B12, (Makarov, 1999) by monitoring cobalt at *m/z* 59. For these experiments, UV and elemental detection were used in serial. Cyanocolbalamin chromatographs were acquired while

monitoring UV absorbance with the PDA, along with phosphorus (*m/z* 31) and cobalt (*m/z* 59) simultaneously using the ICP-MS.

Following the work with the B supplements, the application of the method to the API compound was investigated. These data were collected using a gradient HPLC method. The parent molecule was hydrolyzed with dilute base under elevated temperature to produce DP1 and DP2. One mole of the API molecule contains one mole of bromine, 2 moles of chlorine, and 2 moles of sulfur. One mole of compound DP1 contains 1 mole of bromine and 2 moles of sulfur. One mole of DP2 contains 2 moles of chlorine. When hydrolyzed, one mole of API will form one mole of each degradation product, DP1 and DP2. See Table 1 for structures for each compound used for these investigations.

Experimental

HPLC system

A Waters 2695 chromatographic system equipped with a Waters 996 photodiode array (PDA) detector (Milford, MA) was used for these studies. An Agilent Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 3.5 µm) was used for the B-supplement experiments. An Agilent Zorbax Eclipse XDB-C8 column (150 × 4.6 mm, 3.5 µm) was used for the analysis of API, DP1 and DP2. All of the connections were made with 0.007 inch i.d. PEEK tubing. The eluent from the PDA detector cell was plumbed directly into the ICP-MS sample introduction system.

ICP-MS system

A Thermo Elemental X-Series ICP-MS (Waltham, MA) configured with a hexapole collision cell was used as the elemental detection apparatus. The ICP-MS was configured with a Glass Expansion (West Melbourne, Victoria, Australia) Conikal® concentric glass nebulizer and a Thermo glass impact bead spray chamber cooled to -10° C using a Peltier cooling block. The

spray chamber was drained using the integrated ICP-MS system peristaltic pump. A Thermo 'High Performance Interface' (HPI) platinum-tipped sample cone (18mm Pt insert) was paired with a Thermo HPI nickel Microskimmer® skimmer cone. A one-piece quartz torch with a 1.5mm internal diameter injector tube was used throughout. The ICP-MS chromatographic data were acquired and processed using Thermo PlasmaLab® software in the transient time resolved acquisition (TRA) mode. The ICP-MS workstation was configured with a trigger card and cable in order to facilitate automatic data acquisition from the Waters HPLC system.

Reagents, standards and samples

Cyanocobalamin (B12), thiamine hydrochloride (B1), and biotin were obtained from Sigma-Aldrich (St. Louis, MO). API material was obtained from Eli Lilly research facilities (Indianapolis, IN). HPLC grade methanol and acetonitrile were purchased from Mallinckrodt (Phillipsburgh, NJ). Ammonium acetate, acetic acid, and potassium phosphate monobasic were purchased from Sigma-Aldrich. Phosphoric acid was purchased from Mallinckrodt. Sodium hydroxide (1.0 N) was purchased from Red Bird Services (Batesville, IN). Milli-Q water was used throughout (Millipore, Bedford, MA).

Individual stock solutions of cyanocolbalamin, thiamin hydrochloride and biotin were prepared at 1000 µg/mL in water. Ammonium hydroxide was added drop-wise to the biotin stock until dissolution. Fresh calibration standard solutions were prepared daily by serially diluting the stock solutions in water. The standards contained a mixture of the 3 analytes ranging in concentrations from 5-100 µg/mL.

A 1.0 mg/mL API solution was prepared in 0.1 N NaOH. This solution thermally stressed at 80°C for 72 hours in order to form degradation products DP1 and DP2. This solution was injected directly.

The 25 mM ammonium acetate buffer was prepared by dissolving ammonium acetate in water and adjusting the final pH to 4.0 with acetic acid. The 25 mM potassium phosphate buffer was prepared by dissolving potassium phosphate monobasic in water and adjusting the final pH to 2.5 with phosphoric acid.

Instrumental conditions

HPLC conditions

See Table 2.

ICP-MS conditions

A forward power setting of 1.4 KW and nebulizer flow of 0.90±0.05 L/min was used throughout for this work. The ICP-MS was able to tolerate HPLC flow rates up to 1.5 mL/min. The HPLC flow rate was optimized according to peak symmetry. Oxygen gas was titrated into the argon nebulizer gas in order to eliminate carbon deposits on the sample cone and torch injector tube. The collision cell was used for all experiments performed for this work in order to attenuate the respective polyatomic isobaric interferences for the analytical ions of interest (see Table 3).

The collision cell was charged with a mixture of 1% ammonia/99% helium. System optimization was performed by spiking the mobile phase with a suitable source of the element of interest. Spiked and un-spiked mobile phases were alternately introduced into the ICP-MS using separate channels of the quaternary HPLC pump. The analytical column was replaced with a section of 0.007 inch ID PEEK tubing. For optimizing parameters for gradient elution, a 50%

mixture of acetonitrile and 0.25mM phosphate buffer was introduced using separate channels of the HPLC pump. Analyte signal-to-background assessments for optimization were made using the ICP-MS real time display. Compromise parameters were required as multiple elements were acquired simultaneously.

Results and discussion

Analysis of B-Supplements

Linear regression statistics for the four B-supplement analytes are presented in Table 4. The following series of chromatograms illustrate a potential advantage of monitoring elemental signals by ICP-MS. These data were acquired using isocratic method I. The cyanocolbalamin, thiamin, and biotin concentrations of the solution are 100 µg/mL. The biotin molecule exclusively consists of aliphatic carbon-carbon bonds and, therefore, has a small extinction coefficient. Correspondingly, the peak is very small in the UV chromatogram. However, as biotin contains 13.1% sulfur by weight, the biotin peak is very pronounced in the ICP-MS sulfur (*m/z* 34) chromatogram.

The following series of chromatograms illustrate the ability of the ICP-MS to resolve co-eluting compounds based upon their unique element content. These data were acquired using isocratic method II. Cyanocolbalamin and thiamin co-elute in the 250nm UV chromatogram at approximately 3.2 minutes. By sequentially monitoring sulfur (*m/z* 34), phosphorus (*m/z* 31) and cobalt (*m/z* 59), the thiamin peak is isolated in the ICP-MS sulfur chromatogram, and the cyanocolbalamin is isolated in the ICP-MS phosphorus and cobalt chromatograms. This capacity to resolve molecules based upon elemental content has effectively cut the run time from 14 minutes to 6 minutes.

Analysis of API, DP1 and DP2

The series of chromatograms in Figure 3 illustrate potential application of reverse phase HPLC/ICP-MS for monitoring and related substances response quantification of degradation products of a pharmaceutical compound that contain nonmetallic heteroatoms. It is standard practice in the pharmaceutical industry to report the relative amount of impurities and/or degradation products as a percent of total integrated peak area of the chromatogram.

In order to perform this calculation using the ICP-MS chromatograms, the chlorine (*m/z* 35) peak area for DP2 was corrected for the relative instrument response of bromine, sulfur, and chlorine in the API ICP-MS chromatograms (see Table 5). This was necessary as the DP2 molecule contains chlorine, but does not contain sulfur or bromine.

The instrument responses for bromine, sulfur and chlorine were also corrected for the percent weight of each element in each of the three compounds by dividing the peak areas by the respective weight fraction. For example, API is 18.3% bromine by weight. The raw bromine peak area was determined to be 1173000 area counts. This value was divided by the bromine/API weight fraction, 0.183, to arrive at the corrected area of 6409836 area counts.

The percent of each compound determined from the UV chromatograms recorded at 250nm and the ICP-MS chromatograms are presented in Table 6. The results presented for the UV chromatogram are calculated directly from the integrated peak areas. The results presented for the ICP-MS chromatograms represent an average of the chlorine peak area calculated separately from both the relative bromine and sulfur responses.

Hydrolysis of one mole of API will result in one mole each of degradation products DP1 and DP2, in a 1:1 ratio. It is likely that the significant difference in relative peak areas in the UV chromatogram between the two degradation products is due to differences in extinction

coefficients of the three compounds. Note that the UV chromatogram in Figure 3 was recorded at the maximum absorbance for API by the PDA detector. This is presented to illustrate further the difference in extinction coefficients for API, DP1 and DP2. As the ICP-MS elemental responses are independent of the source of the molecular species from which they originate, this mode of detection could potentially provide more accurate percent relative degradation product results than typical UV detection.

In this experiment, the ratio of compound DP1 to DP2 is much closer to the theoretical 1:1 ratio using the ICP-MS data than the relative percent area directly calculated from the UV chromatogram. The calculations from the ICP-MS chromatograms are straightforward provided that the structure of each compound is known.

It is also recognized that the organic content of the mobile phase affects the relative response of an element. Generally, the elemental response will decrease with increased organic content, as in the case of a gradient elution, due to reduced ionization of the monitored element in the plasma. Additionally, the baseline in an elemental chromatogram of a gradient separation could change due to formation of an isobaric polyatomic species consisting of atoms contained in the mobile phase. An example is the carbon-based polyatomic species at *m/z* 31 increasing in a phosphorus chromatogram (See Table 3). This may increase the inaccuracy in the direct calculation of relative peak areas in element chromatograms and render the technique untenable for that purpose.

The Thermo PlasmaLab® ICP-MS software makes provisions for the use of a timeslice internal standard. This entails the introduction of an elemental standard solution to the mobile phase flow through a post-column T fitting. The instrument monitors the timeslice internal standard response continuously versus time and automatically applies the changes in response to

the analyte transient TRA signals. Attempts were made in this work to correct changes in analyte transient TRA signals by incorporating an indium timeslice internal standard post-column via a T-fitting. A syringe pump was used to introduce the internal standard solution. This internal standard did not appropriately correct for changes in instrument response due to increased organic content from the mobile phase gradient and was not utilized for these studies.

Conclusions

Reverse phase HPLC with sequential UV and elemental detection by ICP-MS has proved to be a useful technique for the investigation of pharmaceutical molecules containing the heteroatoms sulfur, phosphorus, bromine, and chlorine, as well as organometallic compounds containing a transition metal such as cobalt. Multiple isotopes can be monitored simultaneously in sequence with UV detection without altering the HPLC method to accommodate the ICP-MS instrumentation. Interfacing the ICP-MS system with a standard HPLC/PDA system was straightforward. Isocratic and gradient mobile phases were introduced directly into the ICP-MS at flow rates ranging from 0.5 to 1.5 mL/min using standard ICP-MS sample introduction components without detriment to the performance of the system. Enhanced specificity by simultaneous multi-element detection can provide resolution of co-eluting compounds provided that they contain a heteroatom that can be monitored using ICP-MS. With sequential PDA/ICP-MS detection, this can be accomplished without the necessity of interpreting complex mass spectra. ICP-MS instrument responses are independent of the molecular species from which they originate which may facilitate investigation of a complex mixture where one or more compounds do not have a chromophore. However, the relative sensitivity of the elemental detection of a compound depends upon many factors including the percent composition of the element and the relative abundance of the elemental isotope that is being monitored. For some

pharmaceutical applications, elemental detection by ICP-MS may not provide sufficient sensitivity to adequately monitor degradation products down to 0.1% of the parent peak, which is typically the regulatory requirement. For monitoring elements using ICP-MS with a gradient separation, it is possible that the instrument response of the element will change with changes in the mobile phase composition due to solvent loading the plasma. For accurate quantification and correct relative response calculations for gradient separations, the incorporation of an appropriate timeslice internal standard may be required.

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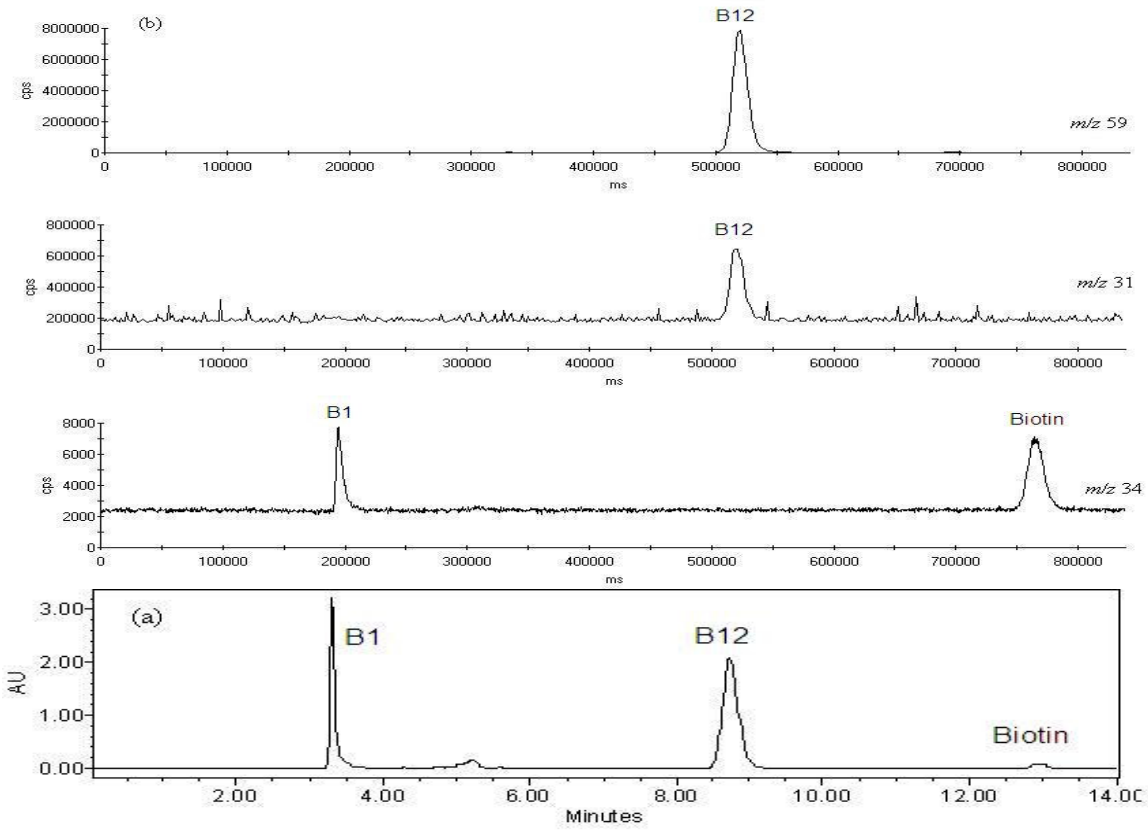


Figure 1. B-Supplement Chromatograms, Isocratic HPLC Method I.

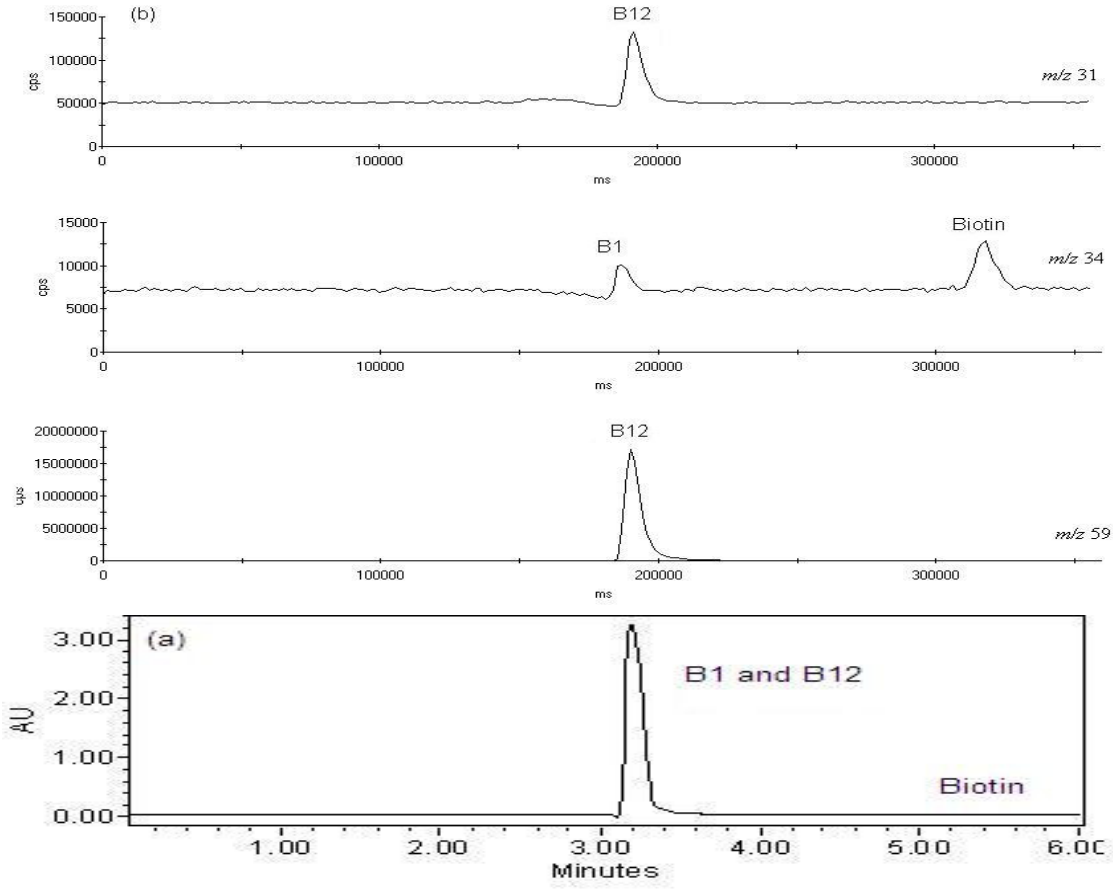


Figure 2. B-Supplement Chromatograms, Isocratic HPLC Method II.

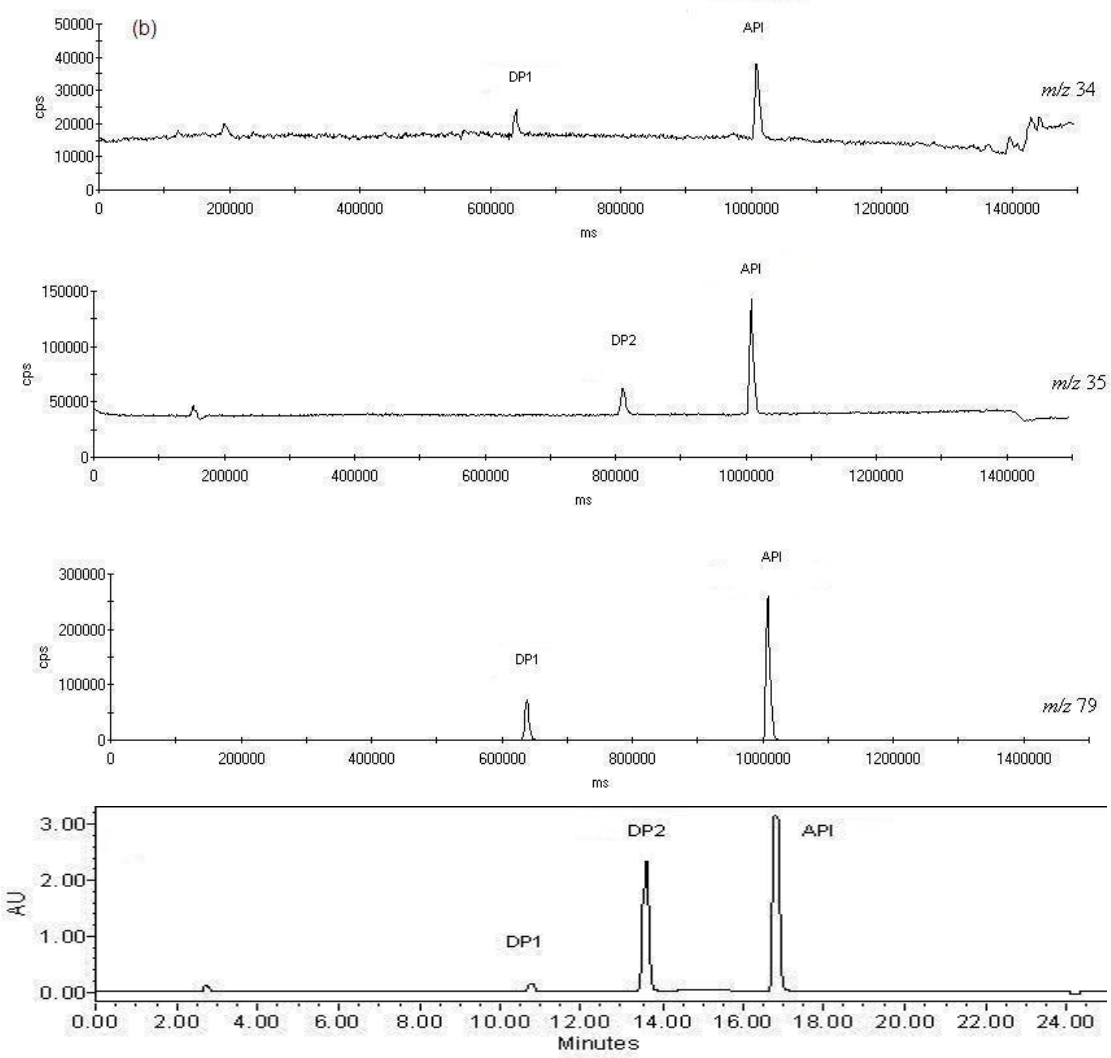


Figure 3. API, DP1 and DP2 Chromatograms, Gradient HPLC Method.

Table 1. Analyte Formulae and Structures

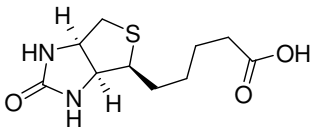
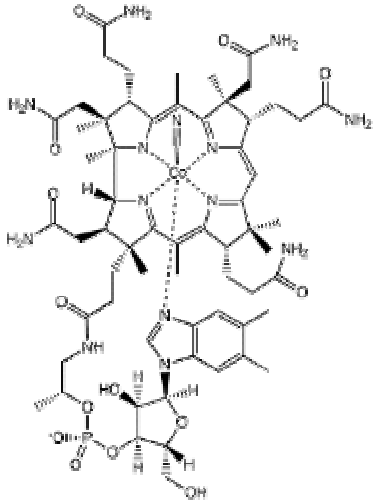
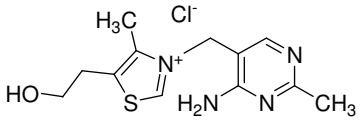
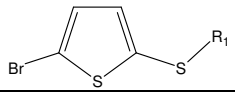
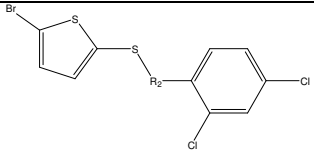
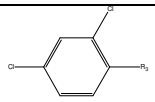
Compound Identification	Molecular Formula	Structure
Biotin	$C_{10}H_{16}N_2O_3S$ 244.31 g/mol 13.1% S	
Cyanocolbalamin (Vitamin B12)	$C_{63}H_{88}CoN_{15}P$ 1355.37 g/mol 4.4% Co 2.3% P	
Thiamin (Vitamin B1)	$C_{12}H_{17}N_4OS$ 265.36 g/mol 12.1% S	
DP1	26.7% S 33.3% Br	
API	14.7%S 16.2% Cl 18.3% Br	
DP2	37.1% Cl	

Table 2. HPLC Conditions

Parameter	C18 (Isocratic Method I)	C18 (Isocratic Method II)	C8 (Gradient Method)
Column	Agilent Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 3.5 µm)	Agilent Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 3.5 µm)	Agilent Zorbax Eclipse XDB-C8 column (150 × 4.6 mm, 3.5 µm)
Injection Volumer, µL	50	50	20
Flow Rate, mL/min	0.5	0.5	0.5
Solvent A composition	25mM Ammonium Acetate Buffer, pH 4.0	25mM Ammonium Acetate Buffer, pH 4.0	25mM Potassium Phosphate Buffer, pH 2.5
Solvent B composition	Methanol	Methanol	Acetonitrile
Program	75% A/25% B	60% A/40% B	Initial: 80%A/20%B 0-20 minutes:20%A/80%B 20.1-25 minutes: 80%A/20%B
Duration of run, min	14	6	25
Column Temperature	Ambient	Ambient	35º C

Table 3. Element Ions and Associated Polyatomic Interferences

Ion	Abundance	Isobaric Polyatomic Interferences
^{34}S	4.21%	$O^{18}O^{+}$
^{35}Cl	75.40%	$^{19}OHO^{+}$, $OH^{18}O^{+}$
^{79}Br	50.60%	ArK^{+}
^{31}P	100%	$CH_3O^{+}{}_{(1)}$, OHN^{+}, $O^{15}N^{+}$, $^{19}OHC^{+}$

Table 4. Linear Regression Statistics for B-Supplements by Elemental Detection

Analyte	Ion Monitored	Concentration Range (µg/mL)	R^2	Sensitivity (slope)	Intercept (µg/mL)
B1	^{34}S	20-100	0.990206	473	-4.960070
B12	^{59}Co	5-100	0.999922	3736371	0.010445
B12	^{31}P	5-100	0.999929	12863	-0.547058
Biotin	^{34}S	5-100	0.998868	1048	-1.293714

Table 5. ICP-MS Peak Area Calculations for API, DP1 and DP2

Compound	Bromine Peak Area (Weight Corrected)	Sulfur Peak Area (Weight Corrected)	Chlorine Peak Area (Weight Corrected)	API Br/Cl Ratio	API S/Cl Ratio	Corrected Chlorine Peak Area (Br/Cl)	Corrected Chlorine Peak Area (S/Cl)
API	6409836	805442	2516049	2.5476	0.3201	N/A	N/A
DP1	1034535	121273	N/A	N/A	N/A	N/A	N/A
DP2	N/A	N/A	324259	N/A	N/A	826075	103802

Table 6. Relative Percentages of API, DP1, and PD2

Compound	Relative %, UV Chromatogram (250nm)	Relative %, ICP-MS Chromatograms
API	69	78
DP1	21	12
DP2	10	10