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LC World Talk

SHIMADZU'S NEWSLETTER FOR THE HPLC GLOBAL COMMUNITY

Applications of Ultra Fast HPLC

Detailing the Prominence UFLC System in the Analysis of Benzodiazepines and Analgesics

2D-HPLC for Bioanalysis "Co-Sense for BA" System

Unique "Shim-pack MAYI Series" of Pretreatment Columns Makes the Difference

Metabolite Identification Software

MetID Solutions Software for Metabolite Identification

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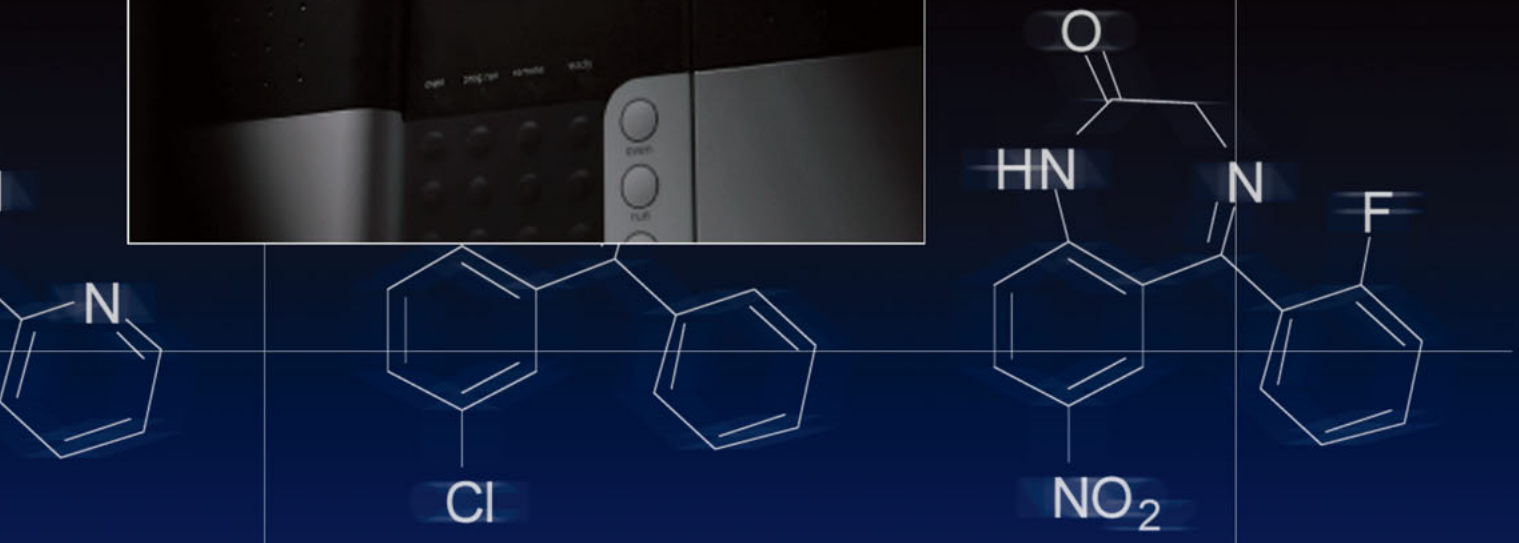


The Prominence UFLC delivers the speed and reproducibility necessary for today's laboratory setting.

Prominence UFLC
ULTRA FAST LIQUID CHROMATOGRAPH

APPLICATIONS OF ULTRA FAST HPLC

Detailing the Prominence UFLC System in the Analysis of Benzodiazepines and Analgesics

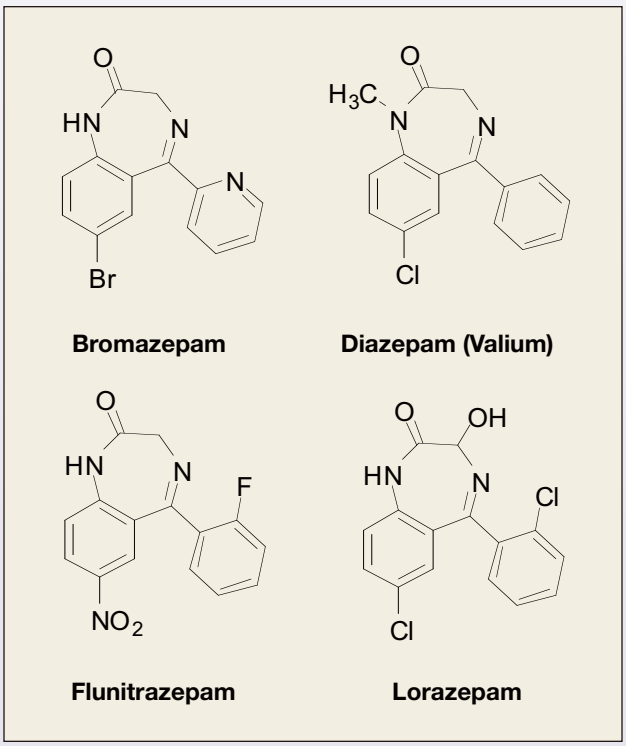
The demand for increased efficiency and speed in pharmaceutical analysis extends through drug discovery and development to clinical screening and trials. The need to meet these throughput requirements without compromising the data required in quality assurance/quality control (QA/QC) applications has highlighted the important technical challenge of providing faster separation in high performance liquid chromatography (HPLC).

In past issues, we have introduced and discussed several factors to consider when examining the issue of Ultra Fast Liquid Chromatography (UFLC). Primary among these factors is the goal of UFLC, which is not high pressure; high pressure is simply a consequence of using a column packed with a small particle (sub-2 µm). The goal is increased throughput, meaning how many samples can be analyzed per day, per hour, or even per minute. In order to truly achieve high throughput, not only does the run time of a single chromatogram need to be optimized, but the total cycle time of the assay must be addressed and optimized as well.

To address this stated goal, the Prominence UFLC (Ultra Fast Liquid Chromatograph) system has been developed. Prominence UFLC, in combination with the XR Series of high-speed columns, enables users to shorten analysis time drastically and easily without extremely high pressure, while maintaining the high separation efficiency of conventional columns and system performance features such as reproducibility, carryover and durability.

Here, we introduce two recent applications that showcase the Prominence UFLC and its ability to deliver the speed and reproducibility necessary in today's laboratory setting.

Benzodiazepines have sedative, hypnotic, and muscle relaxant powers and are used to treat a variety of conditions that include insomnia, anxiety, panic attacks, seizures, and alcohol or opiate-withdrawal symptoms. Benzodiazepines have largely replaced barbiturates due to a lower potential for abuse and a reduced chance of lethal overdose.



Benzodiazepine Analysis

Experimental Conditions

Instruments:	Shimadzu Prominence UFLC
Column:	Shim-pack XR-ODS (3.0mmI.D. x 50 mmL, 2.2µm)
Mobile Phase:	A: H_2O B: Acetonitrile
Gradient:	B: Concentration 25% (initial)⟶ 80% (1.30 min)⟶ 25% (1.40min)
Flow rate:	1.5 mL/min
Column temp:	40°C
Injection vol:	5 µL
Detection:	UV 254 nm
Runtime:	1.75 minutes

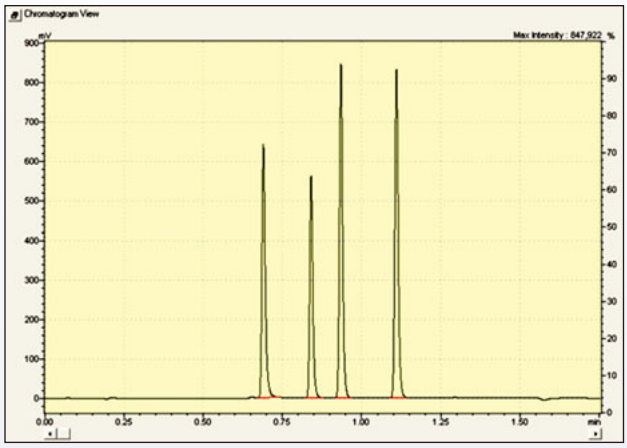


Figure 1: UFLC Chromatogram of four benzodiazepams (100ug/mL). 1: Bromazepam, 2: Flunitrazepam, 3: Lorazepam, 4: Diazepam

Compound	Retention Time	RT % RSD	Peak Area % RSD
Bromazepam	0.689	0.10	0.16
Flunitrazepam	0.839	0.10	0.12
Lorazepam	0.933	0.10	0.08
Diazepam	1.110	0.09	0.18

Table 1: Retention Time and Peak Area Reproducibility (n=10)

Results

Four benzodiazepines were successfully separated and detected using gradient UFLC conditions. Excellent retention time and peak area reproducibility was obtained under UFLC conditions. The total analytical run time, including column re-equilibration and injection cycle time, was less than 2 minutes, while achieving excellent retention time and peak area reproducibility—all at pressure less than 5,000 psi.

Analgesics Analysis

Experimental Conditions

Instruments:	Shimadzu Prominence UFLC
Column:	Shim-pack XR-ODS (3.0mmI.D. x 50mm, 2.2µm)
Mobile Phase:	A: 0.1% Phosphoric acid B: Acetonitrile B: concentration 10% (initial)⟶ 45% (0.60min)⟶ 60% (1.20min)⟶ 10% (1.21min)
Flow rate:	1.25mL/min
Column temp:	40˚C
Injection vol:	10 µL
Detection:	240 nm
Run time:	1.9 minutes

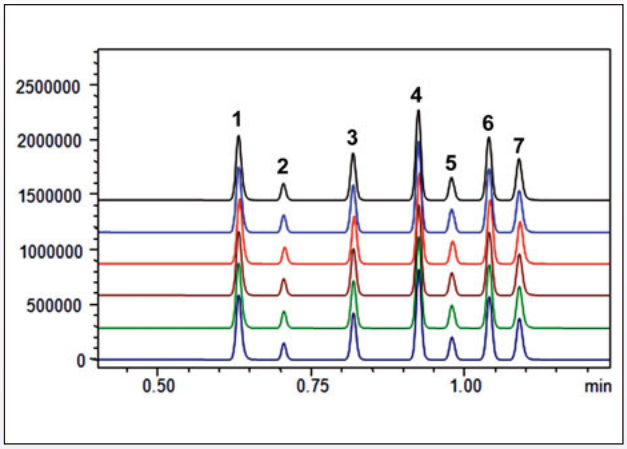


Figure 2: UFLC chromatogram of analgesics (25µg/mL). 1: Acetaminophen, 2: Caffeine, 3 : 2-Acetamidophenol, 4: Acetanilide, 5: Acetylsalicylic Acid, 6: Phenacetin, 7: Salicylic Acid

	R.T. %RSD	Peak Area %RSD
1. Acetominophen	0.098	0.029
2. Caffeine	0.105	0.033
3. 2-Acetamidophenol	0.082	0.034
4. Acetanilide	0.067	0.024
5. Acetylsalicylic acid	0.063	0.096
6. Phenacetin	0.064	0.035
7. Salicylic acid	0.058	0.172

Table 2: Retention time and Peak Area Reproducibility (n=6)

Results

The over-the-counter analgesics were successfully separated with excellent retention time and peak area reproducibility. The total analytical cycle time, including column re-equilibration, was around 2 minutes.

Conclusion

The demands for faster analysis times will continue to grow as researchers attempt to increase sample throughput. While speed is certainly important, the integrity of the data is also vital. Many discussions about UFLC will come around to data reproducibility. Much of this discussion stems from the fact that the specialized systems designed for dealing with the extreme pressures seen with sub-2 µm particle columns may exhibit some decreased performance with respect to system reproducibility.

As seen in these two examples, the retention time RSD performance shows that the pumps on the system are capable of delivering a consistent and reproducible gradient. This allows the user to create a very specific retention time window in which to look for particular peaks, thus aiding in identification. The peak area RSDs show that the autosampler is capable of a reproducible injection and also suggest that carryover is not a problem.

Today's HPLC systems are used for a broad range of applications that can include UFLC, traditional HPLC, QA/QC work, Mass Spectrometry front end, multi-dimensional chromatography, on-line sample clean up, set up as a column switching system for method development and many others. Some of these techniques may require a specialty system to get the ultimate in performance for the method—e.g. a bio-inert system. However, many others can be accomplished with a single, well-engineered, component-based HPLC system.

A modular system based on sound engineering principles, including pumping options that can deliver a wide flow rate range with precise delivery resolution, combined with an autosampler capable of accurate and precise sampling and excellent carryover performance, and including the necessary system accessories for continued expansion of capabilities, will offer superior performance for both UFLC and traditional HPLC.

THE WORLD'S MOST ADVANCED LIQUID CHROMATOGRAPH

Now with Empower™ Control

HPLC systems are currently used in a wide variety of fields, but the application environments have been changing dramatically. These changes are occurring because of factors such as a more intensely competitive environment for product development, resulting from the trend toward corporate globalization, and because of the increasing influence of laws and regulations, represented most prominently by FDA's GxP and 21 CFR Part 11. In particular, with advances in networking technology, systems are shifting from being decentralized individual-use instruments to being part of a shared centralized network of instruments, primarily to better facilitate regulatory management. Customers are now seeking a new type of HPLC that not only allows centralized data management, but also provides centralized control of instruments and devices in order to realize improvements in the efficiency and reliability of analyses.

Responding to the growing needs to use LC-20A Prominence™ Series HPLC in a client/server compatible multi-vendor environment, Shimadzu has been working with various software vendors to incorporate hardware control into their software. Recently, Shimadzu added Empower™ and Empower2™ (hereafter Empower™) to this suite of laboratory software, enabling full control for LC-20A Prominence HPLC.

Instrument	Shimadzu Model	
System Controller	CBM-20A	
	CBM-20Alite (housed inside a pump or autosampler)	
Pump •Support of Multi-Pump Configurations for Multi-Dimensional LC		LC-20AD, LC-20AT, LC-20AB (binary pump)
Autosampler •Support of Various Sample Rack Configurations		SIL-20A, SIL-20AC (with cooled option)
•Support of the Optional Autosampler Active Rinse Control		Rack Changer, Rack Changer C (with cooled option)
Column Oven	CTO-20A, CTO-20AC (with cooled option), CTO-10ASvp	
Detector	UV-Vis	SPD-20A, SPD-20AV
	Fluorescence	RF-10AXL
	PDA	SPD-M20A (3D data acquisition available)
	Others via Analog	PC-55N A/D Board (up to 2 channels)
Rotary 2/6-Position Valve	FCV-12AH (installed in CTO-20A / CTO-20AC ovens), FCV-13AL, FCV-14AH (installed in CTO-20A / CTO-20AC ovens)	
Solenoid Valve	LPGE Solenoid Valve Unit (installed in LC-20AD / LC-20AT)	
	FCV-10AL, FCV-10ALvp, FCV-11AL, FCV-11ALS, FCV-15AL	
Sub-controller	Option Box vp	
Support of Shimadzu Ultra Fast Liquid Chromatograph (Prominence UFLC)		

Table 1: Shimadzu equipment controllable by Empower™ software

A list of Shimadzu instruments that can be controlled by Empower software is provided in Table 1. Shimadzu's LC Driver can control up to 4 systems connected to a single Empower data acquisition server or 2 systems with PDA detectors, installed with personal (stand-alone type), workgroup (network type) or enterprise (network type) versions of Empower. If one of the connected systems is a PDA detector, then 2 more non-PDA systems can be controlled.

Empower software communicates with the Shimadzu System Controller (CBM) via an RS-232 or TCP/IP connection found on the rear panel of CBM-20A and CBM-20Alite. The other HPLC modules are connected to the CBM via optical cables as usual. PDA detectors require an Ethernet cable connection.

Article continued on back cover

2D-HPLC FOR BIOANALYSIS "CO-SENSE FOR BA" SYSTEM

The Co-Sense for BA system features the unique "Shim-pack MAYI Series" of pretreatment columns and enables automated sample pretreatment for better reproducibility of bio-sample analysis.

LC/MS with high sensitivity and selectivity is a powerful tool for pharmacokinetic, clinical and metabolic studies. Biological samples such as plasma, serum and urine are pretreated to minimize the influence by proteins and matrices in the sample before injection into LC/MS. Recently, several restricted access media (RAM) columns have been developed to enable on-line pretreatment of biological samples. Internal surface reversed-phase columns[1-4], mixed functional phase silica[5], semi-permeable surface silica[6], and diol silica[7,8] have been reported as approaches to enable automated sample pretreatment.

A methyl cellulose-immobilized reversed-phase column MC-ODS (Shim-pack MAYI-ODS) was prepared by modification of porous silica with methyl cellulose, followed by modification of the internal surface with ODS[9]. The external surface covered with methyl cellulose prevents adsorption with proteins while small molecules can be retained in the internal surface by hydrophobic interaction with the ODS groups. Shim-pack MAYI-ODS columns enable direct injection of bio-samples, such as plasma, by using a column-switching technique[9,10]. In the reversed-phase RAM column, polar compounds have weak retentive properties, since the retention is due to hydrophobic interactions, and the recovery rate of polar compounds is reduced. MC-SCX (Shim-pack MAYI-SCX)[11,12] and Shim-pack MAYI-SAX have ion-exchange groups inside the pores and can be successfully applied to the analysis of hydrophilic compounds in plasma.

We have discussed the principle and potential applications of the Co-Sense for BA system, which is configured from a column-switching system and a MAYI pretreatment column, in previous *LC World Talk* issues and application notes. Here, we summarize the features and applications of Co-Sense for BA.

Shim-pack MAYI Series

Shim-pack MAYI series columns are on-line pretreatment columns for HPLC which are designed to extract and concentrate analytes in bio-samples. The packing material is composed of totally porous, high-purity spherical silica particles. The outer surfaces of the silica particles are coated with methyl cellulose polymer, and the inner surface of the pores are chemically bonded with stationary phase such as C18. The MAYI series has a wide variety of stationary phase, and the chemistries of the MAYI series are listed in **Table 1**.

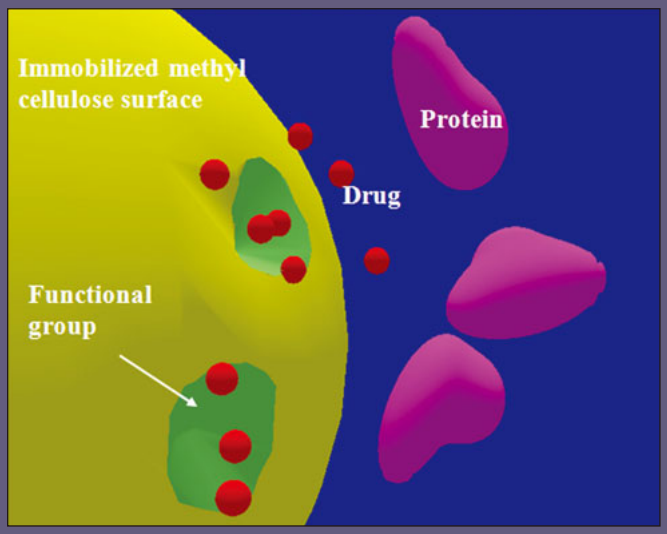
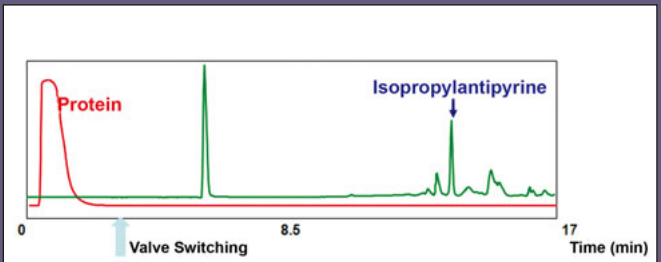


Figure 1: An image of the particle surface in a MAYI column.



Sample:	Plasma spiked with isopropylantipyrine
Loading solution:	0.1% phosphate/Acetonitrile (95/5)
Detection:	Isopropylantipyrine: 275nm (Green trace)
	Plasma matrix: 280nm (Red trace)

Figure 2: Deproteinization of a plasma sample by Shim-pack MAYI ODS (G).

Column	Inner Surface Modification
Shim-pack MAYI-ODS(G)	C18
Shim-pack MAYI-C1(G)	C1
Shim-pack MAYI-C4(G)	C4
Shim-pack MAYI-C8(G)	C8
Shim-pack MAYI-C14(G)	C14
Shim-pack MAYI-SCX(G)	Sulfonate group
Shim-pack MAYI-SAX(G)	Trimethylammonium group

Table 1: The list of Shim-pack MAYI series columns.

Proteins in the bio-samples are discharged from the MAYI column by size exclusion, while small target molecules can penetrate into the pores and are trapped on the stationary phase inside the pores **(Figure 1)**.

By incorporating a MAYI column as a pretreatment column in a column-switching system, proteins in samples are removed automatically, and targeted small compounds can be analyzed.

Deproteinization of samples and the analysis of targeted small compounds can be performed automatically and seamlessly **(Figure 2)**.

System Configuration

A typical configuration of Co-Sense for BA is shown in **Figure 3**. Sample injected from the autosampler flows to the MAYI column by Pump C. Target analytes can be trapped and concentrated on the MAYI column, while proteins are discharged from it. After the deproteinization process is complete, the target compounds retained on the trap column are backflushed to the analytical column by turning the valve position, to be separated on the analytical column and detected with the appropriate detector (UV, MS, MS/MS, etc).

Generally, a column-switching system is composed of two pumps for the binary gradient formation, a pump for loading the sample, a 6-port valve, an autosampler, a column oven and a detector as main modules. In the case of Co-Sense for BA, the solvent switching valve at pump C and an additional pump (pump D) for sample dilution are often used for more effective sample analysis, as shown in **Figure 3**. The solvent switching valve enables washing of the pretreatment line with C2 (or C3, C4) solvent to reduce contamination while target compounds are analyzed. The dilution pump is useful to accelerate the weak interaction between proteins and analytes, and results in an improvement of the recovery rate.

The two-valve configuration as shown in **Figure 4** allows the system to perform as a binary gradient system or

a Co-Sense for BA system with simply a left valve turn. The left valve is used to plumb the autosampler in the flow path for the Co-Sense for BA system or the binary system. It is simply an autosampler stream selection valve.

Method Development for MAYI-ODS

Deproteinization

The time required for discharging proteins from the MAYI column depends on the column size, flow rate, dilution factor and loading solvent. The 6-port valve connected to the MAYI column and the analytical column should be turned to the MAYI column position to prevent clogging in the analytical column and tubing until after the protein discharge process on the MAYI column is completed. The elution curve of proteins from the MAYI column was monitored at UV 260nm (PDA is not preferable). The time required for the protein absorption to decrease to 1m Abs after injection of plasma is shown in **Table 2**. It requires approximately 2 minutes to discharge proteins from MAYI-ODS (G) when the dilution factor is 8, flow rate is 3mL/min, and the injection volume is less than 50µL.

Injection volume	Flow Rate of the Loading Solution			
	1mL/min	2mL/min	3mL/min	4mL/min
5µL	2.8min	1.2min	0.8min	0.7min
10µL	2.9min	1.5min	1.0min	0.8min
20µL	-	1.8min	1.2min	1.0min
50µL	-	2.5min	1.7min	1.3min
100µL	-	3.4min	2.2min	1.8min

Table 2: Time for discharge of plasma proteins from Shim-pack MAYI ODS (G). Time required for that protein absorption at 260nm decreased to 1m Abs after plasma injection. Dilution factor: 8 (C flow/D flow =1/7), Loading solution: 10mM ammonium acetate/ acetonitrile = 95/5, MAYI-ODS (4.6mmI.D. x10mmL).

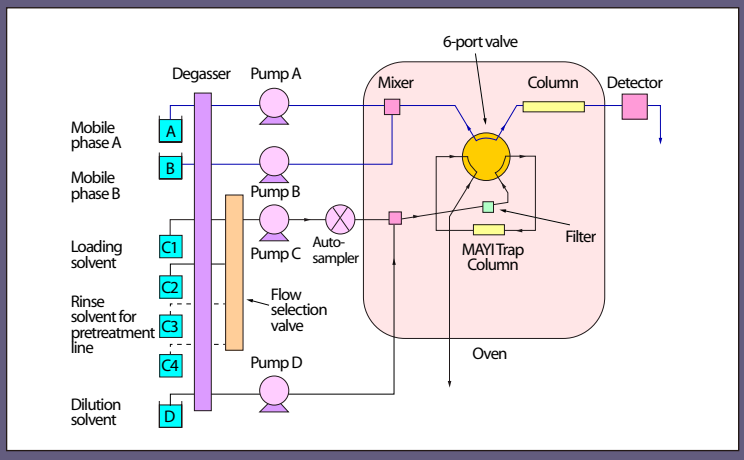


Figure 3: Basic configuration of Co-Sense for BA system.

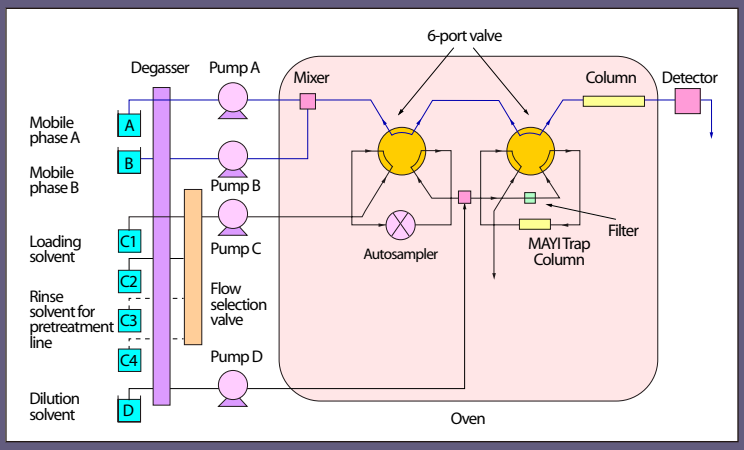


Figure 4: Two-valve configuration of Co-Sense for BA system.

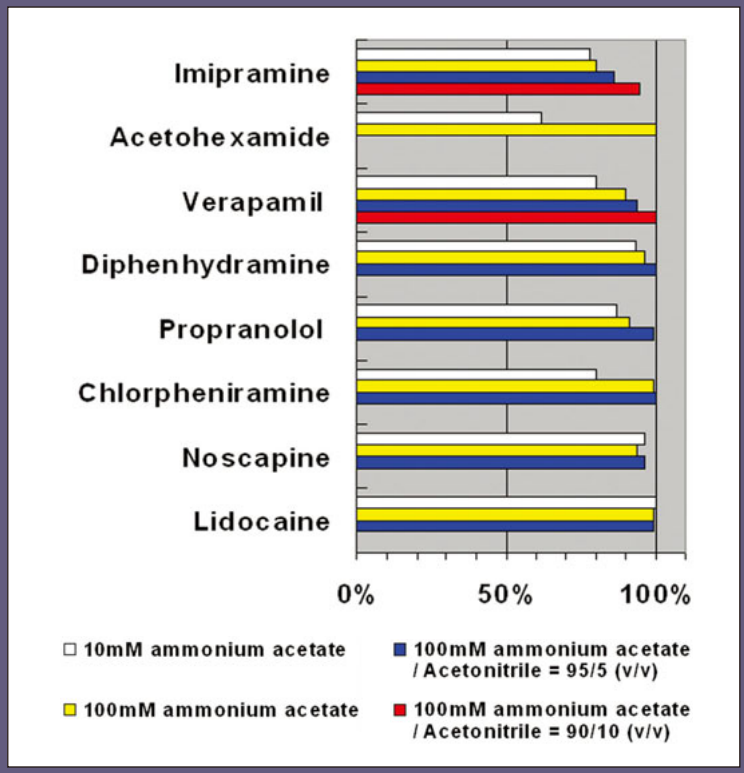


Figure 5: Recovery rate of drugs in plasma on different loading solvent (spiked 0.5µg/mL, injection volume: 50µL).

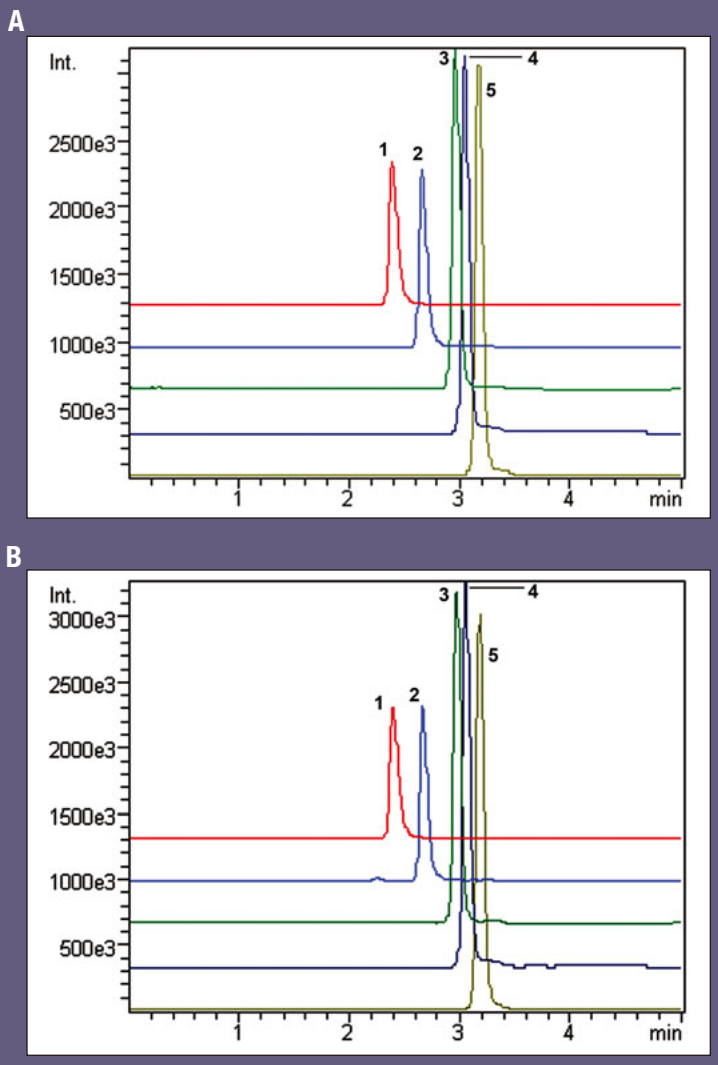


Figure 6 a & b: SIM chromatograms of 5 drugs: (a) standard, (b) plasma spiked. 1: metoprolol; 2: propranolol; 3: lidocaine; 4: dibucaine; 5: bupivacaine. Concentration of each compound: 1 µg/mL, Injection volume: 5µL.

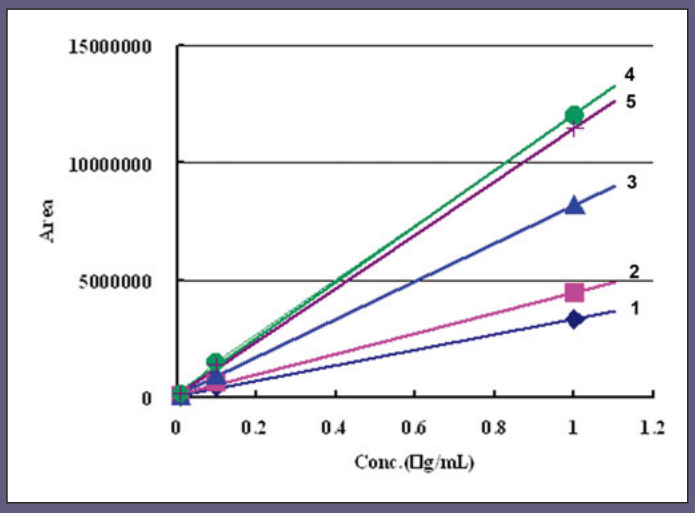


Figure 7: Calibration curves of 5 drugs. Rat plasma spiked with drugs was injected (10 mL). 1: Metoprolol - r=0.99954; 2: Propranolol - r=0.99958; 3: Lidocaine - r=0.99981; 4: Dibucaine - r=0.99978; 5: Bupivacaine - r=0.99986. 10 - 1000ng/mL, 10mL, n=5.

Loading Solvent (Pretreatment Mobile Phase)

The loading solvent (C1 mobile phase in **Figures 3 and 4**) has a role for delivering injected samples to the trap column. Low molecular substances such as drugs exist in conjunction with proteins in bio-samples. The drugs are sometimes removed with proteins during the deproteinization process on MAYI-ODS, resulting in poor recovery of drugs.

It is important to weaken the bond between proteins and drugs to get high recoveries by optimizing the loading solvent. Optimization of ion intensity and organic content in the loading solvent is an effective factor for recovery improvement **(Figure 5)**.

Typically, a 10% or lower concentration of acetonitrile can be added to the loading solvent to get a high recovery rate.

Since the pI of albumin in plasma samples is near 4-5, it is desirable that the pH of the loading solvent is adjusted to 2-3 or 6-7 to prevent clogging by albumin precipitation. In addition, using the same loading solution as mobile phase A for the analysis is a convenient way to save labor.

Furthermore, Co-sense for BA has an extra pump (Pump D) to dilute the injected sample with loading solvent by an on-line function. Injected samples can be automatically diluted with optimal loading solvent to separate the drugs from proteins. On-line dilution will be effective to help the improvement of recovery rate, when the loading solvent doesn't provide a sufficient recovery rate. Generally, the same solvent as loading solvent (C1) is used for dilution.

Application

MAYI-ODS

The MAYI column is an on-line pretreatment column that achieves highly efficient deproteinization and long-term stability through optimization of particle and coating technology, and also enables wide applications on bio-samples. Five basic drugs spiked with plasma were analyzed within a 6-minute cycle by Co-Sense for BA with LC/MS **(Figure 6)**.

These drugs were monitored with an MS detector in an ESI positive mode using selected ion monitoring (SIM) on the [M+H]+ ions of each drug component. The method conditions are shown in **Table 3.**

The plasma sample provided a similar elution profile to the standard sample. This analytical method showed excellent performance on repeatability, recovery rate and linearity **(Table 4 and Figure 7)**.

Figure 8 shows a SIM chromatogram of warfarin and ketoprofen in human plasma. The drugs in bio-samples can be analyzed easily with an optimized method **(Table 5)** for automated pretreatment.

MAYI-SCX and SAX

The Shim-pack MAYI-ODS can be applicable for a wide variety of applications, such as drug analysis, since it has ODS as a stationary phase and can trap many compounds in bio-samples. However, ODS can sometimes make it difficult to retain hydrophilic compounds on the MAYI-ODS since the retention depends on hydrophobic interaction.

MAYI-SCX and SAX have ion exchange groups as stationary phase in the pore to enable trapping of hydrophilic compounds.

MAYI-SCX was applied for the analysis of plasma containing atenorol, which is very hydrophilic (1-Octanol / H_2O partition coefficient: 0.05). Atenorol was retained on the sulfo group in the MAYI-SCX, when 0.1% acetic acid was used for the extraction mobile phase, then could be eluted from the MAYI-SCX and separated from matrices on the analytical column by an acetic ammonium mobile phase **(SIM chromatogram: Figure 9, method: Table 6)**.

Figure 10 shows a SIM chromatogram of tricyclic antidepressants in plasma with the analytical conditions listed in **Table 7**.

These compounds can be trapped not only by the MAYI-ODS, but also by the MAYI-SCX due to the tricyclic antidepressant's own basic behavior.

The MAYI-SAX, which has a trimethylammonium stationary phase, works as a strong anion exchange column. Aspirin and salicylic acid in plasma were analyzed with the MAYI-SAX column **(Figure 11, Table 8)**. Retention (or elution) of carboxylated compounds on the MAYI-SAX depends on the pH of the loading solvent, which flows through the MAYI-SAX. Aspirin was trapped on the MAYI-SAX column at pH=4, and eluted from the column at pH=2.

Pretreatment column:	Shim-pack MAYI-ODS (G) (4.6mmi.d. x 10mm)
Extraction mobile phase:	10mM ammonium acetate/ acetonitrile = 95/5
Flow rate:	3mL/min
Column temperature:	45ºC
Injection volume:	5µL or 10 µL
Extraction time:	1min
Dilution factor:	8 fold
Analysis column:	Phenomenex Mercury MS (4.0mmi.d. x 10mm, 5µm)
Mobile phase:	A: 10mM ammonium acetate, B: acetonitrile
Gradient program:	5%B(0-0.5min) ⟶ 90%B(3-4min) ⟶ 5%B(4.01min) ⟶ STOP(5min)
Flow rate:	0.8mL/min
Split ratio:	1 : 3 (mass spectrometer : waste)
Column temperature:	45ºC
Ionization:	Electrospray
Probe voltage:	+4.5kV
Nebulizing gas flow:	4.5L/min

Table 3: Analytical conditions

Compound	RSD, %(n=5)		Recovery, %
	Standard	Plasma spiked	
Metoprolol	1.2	1	95
Propranolol	1	1.4	97.4
Lidocaine	0.9	2.6	94
Dibucaine	1.6	2.5	107.7
Bupivacaine	0.7	1.5	97.1

Table 4: Repeatability and recovery of each test compound (1µg/mL, 5µL).

Pretreatment column:	Shim-Pack MAYI-ODS (G) (4.6mmI.D.x10mmL.)
Extraction mobile phase:	10mM (ammonium) formate buffer (pH=3.7) / acetonitrile = 90/10
Flow rate:	3.0mL/min
Column temperature:	40°C
Injection volume:	10µL
Extraction time:	2min
Dilution factor:	8
Analysis column:	Shim-pack FC-ODS (75mmL×4.6mmI.D.)
Mobile phase:	A: 10mM (ammonium) formate buffer (pH=3.7),B : acetonitrile
Gradient program:	B50%(0min) ⟶ B50%(2min) ⟶ B90%(5min) ⟶ B90%(8min) ⟶ B50%(8min) ⟶ Stop(10min)
Flow rate:	0.8mL/min
Split:	non split
Column temperature:	40°C
Ionization:	Electrospray
Drying gas:	0.2MPa
Probe voltage:	4.5kV
Nebulizing gas flow:	4.5L/min

Table 5: Analytical conditions

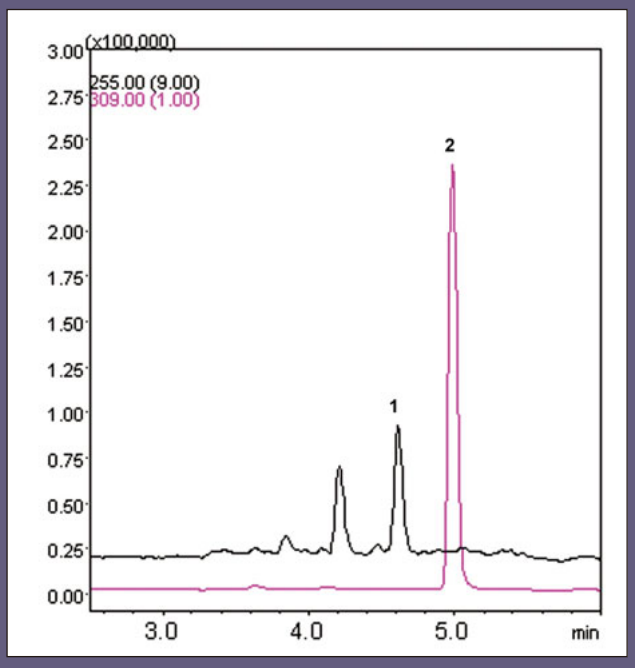


***Figure 8:** SIM chromatograms of warfarin and ketoprofen in spiked plasma. 1: Ketoprofen; 2: Warfarin. Concentration of each compound: 100ng/ mL, injection volume: 10mL.*

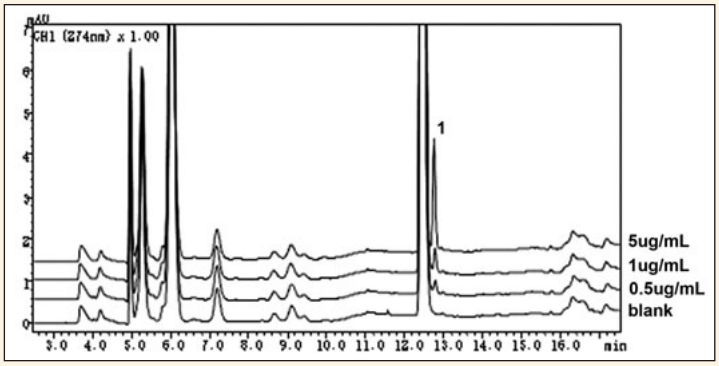


***Figure 9:** Chromatogram of atenorol spiked in plasma; 1: Atenorol.*

Pretreatment column:	Shim-Pack MAYI-SCX (G) (4.6mmI. D.x10mmL.)
Extraction mobile phase:	0.1% Acetic acid
Flow rate:	3.0mL/min
Column temperature:	40°C
Injection volume:	10µL
Extraction time:	2min
Dilution factor:	8
Analysis column:	Shim-pack VP-ODS (150mmL×4.6mmI.D.)
Mobile phase:	A : 100mM acetic (ammonium) buffer (pH=4.7) B : acetonitrile
Gradient program:	B2%(5min) ⟶ B35%(14min) ⟶ B75%(14.01min) ⟶ B75%(18min)
Flow rate:	1.0mL/min
Column temperature:	40°C
Detection:	UV 274nm

Table 6: Analytical conditions

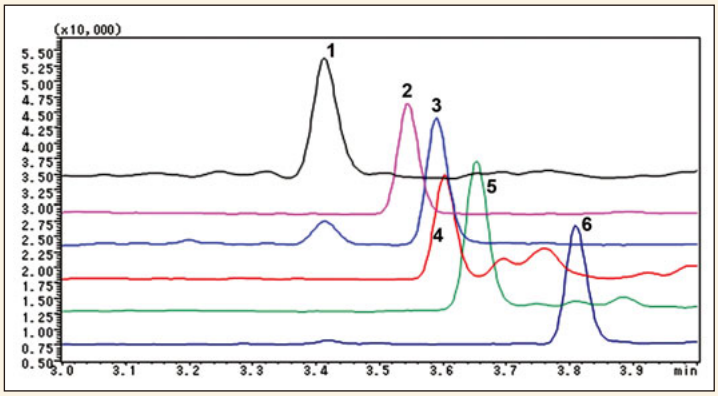


***Figure 10:** SIM Chromatogram of 6 drugs spiked in plasma. 1: Doxepin (m/z: 280), 2: Desipramine (m/z: 267), 3: Imipramine (m/z: 281), 4: Nortriptyline (m/z: 264), 5: Amitriptyline (m/z: 278), 6: Clomipramine (m/z: 315). Concentration of each compound: 10ng/ mL, injection volume: 20µL.*

Conclusion

The Shim-pack MAYI series shows the ability for protein removal through the use of a unique coating technique and optimized packing materials. The Co-Sense for BA system with MAYI pretreatment columns enables automated pretreatment of bio-samples such as plasma and, as a result, can eliminate the manual labor required for the pretreatment process. MAYI columns can be applicable to a wide variety of applications due to the versatility, durability, and different varieties of stationary phase. The system provides better reliability for quantitative analysis because it eliminates manual sample preparation errors or inconsistencies. Co-sense for BA is a very powerful tool for the automated pretreatment of bio-samples.

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12] S. Kawano, M. Takahashi, T. Hine, E. Yamamoto, N. Asakawa, Rapid Commun. Mass Spectrom. 19(2005) 2827-2832.

Pretreatment column:	Shim-Pack MAYI-SCX (G) (4.6mmI. D.x10mmL.)
Extraction mobile phase:	0.1% Acetic acid
Flow rate:	3.0mL/min
Column temperature:	40°C
Injection volume:	20µL
Extraction time:	1min
Analysis column:	Gemini C18 (50mmL×2.0mmI.D.)
Mobile phase:	A: 100mM acetic (ammonium) buffer (pH=5.0) B: acetonitrile
Gradient program:	B20%(1min) ⟶ B90%(4min)
Flow rate:	0.5mL/min
Column temperature:	40°C
Detection:	ESI (LCMS-2010EV)

Table 7: Analytical conditions

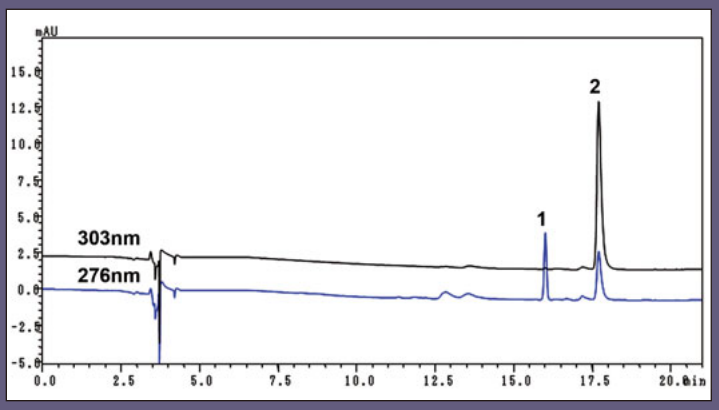


***Figure 11:** Chromatogram of atenorol spiked in plasma; 1: Aspirin, 2: salicylic acid. Concentration of each compound: 10mg/mL, injection volume: 10mL.*

Metabolite Identification Software

MetID Solutions software for metabolite identification: An integrated solution for accelerating metabolite detection and identification using high mass accuracy MS^n data

The key restriction in metabolite identification is the need for experts to interpret large LC/MS data sets quickly and efficiently. In MetID Solutions we have integrated data-dependent acquisitions, correlation analysis using partial least squares regression analysis, isotope labeling, a mass defect filter and in silico modeling to help accelerate metabolite reporting in a single data browser. This approach was investigated as a possible alternative to existing methods of identifying metabolites in non-radio-labeled pharmacodynamic studies.

The study of how a drug is absorbed, distributed, metabolized and eliminated by the body is not only a critical part of the drug discovery process, it is also one of the most costly. Metabolism can dictate the rate of absorption into the body, lead to the production of new and possibly toxic species, or activate the drug.

As a result, many pharmaceutical companies are now conducting metabolite identification studies in the early phases of drug candidate selection.

MS has emerged as an ideal technique for the identification of such structurally diverse metabolites.

In this paper, we highlight the choices and decisions involved in metabolite identification for tolcapone and how they can be merged into a systematic approach.

Why study metabolite identification?

Metabolite identification research is a core part of drug development programs and has several objectives:

- Assure human safety of a development candidate
- Assure that all human metabolites have been adequately assessed in the safety testing of animals
- Protect intellectual property
- Assist back-up programs
- Characterize specific locations on the NCE (new chemical entity) structure that become altered in the metabolic process. Using this knowledge of possible toxicity (or insufficient metabolic stability) is needed to design drugs with improved pharmacokinetic parameters
- Design out metabolism-related non-clinical and clinical liabilities in the back-up compounds

Methods

The potential of this approach has been investigated for the analysis of in vitro metabolites of tolcapone (a well-characterized drug that exhibits extensive metabolism).

A parent drug (20 uM) was incubated with rat S9 liver fractions, and samples were taken at t=0, t=1h and t=4h. All samples were in 50% acetonitrile.

Samples were measured by electrospray ion trap-time of flight mass spectrometry and data mined using a partial least squares algorithm that has been applied to spectral comparison.

The LCMS-IT-TOF (Shimadzu Corporation) acquired MS^1 and MS^2 data in both positive and negative ion modes; each scan segment was 110msec.

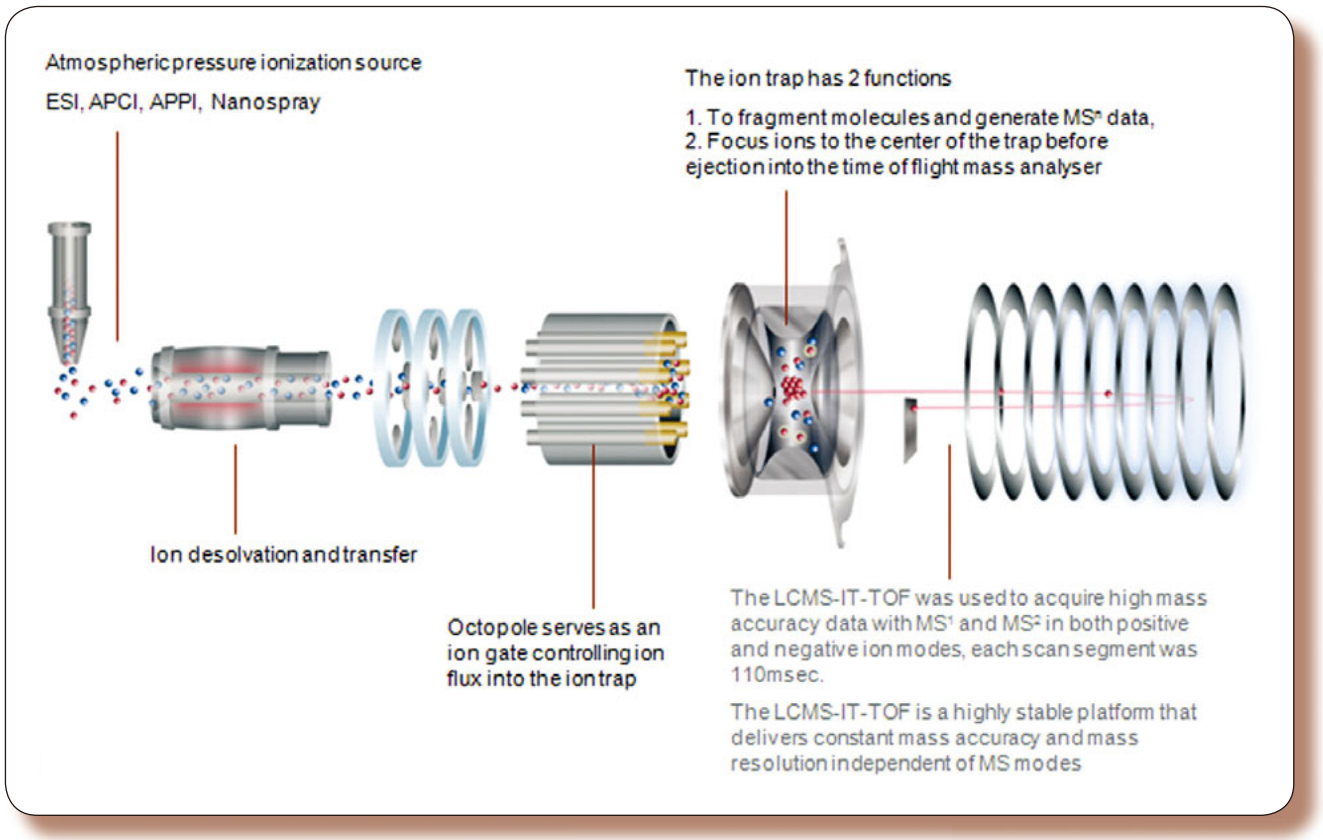
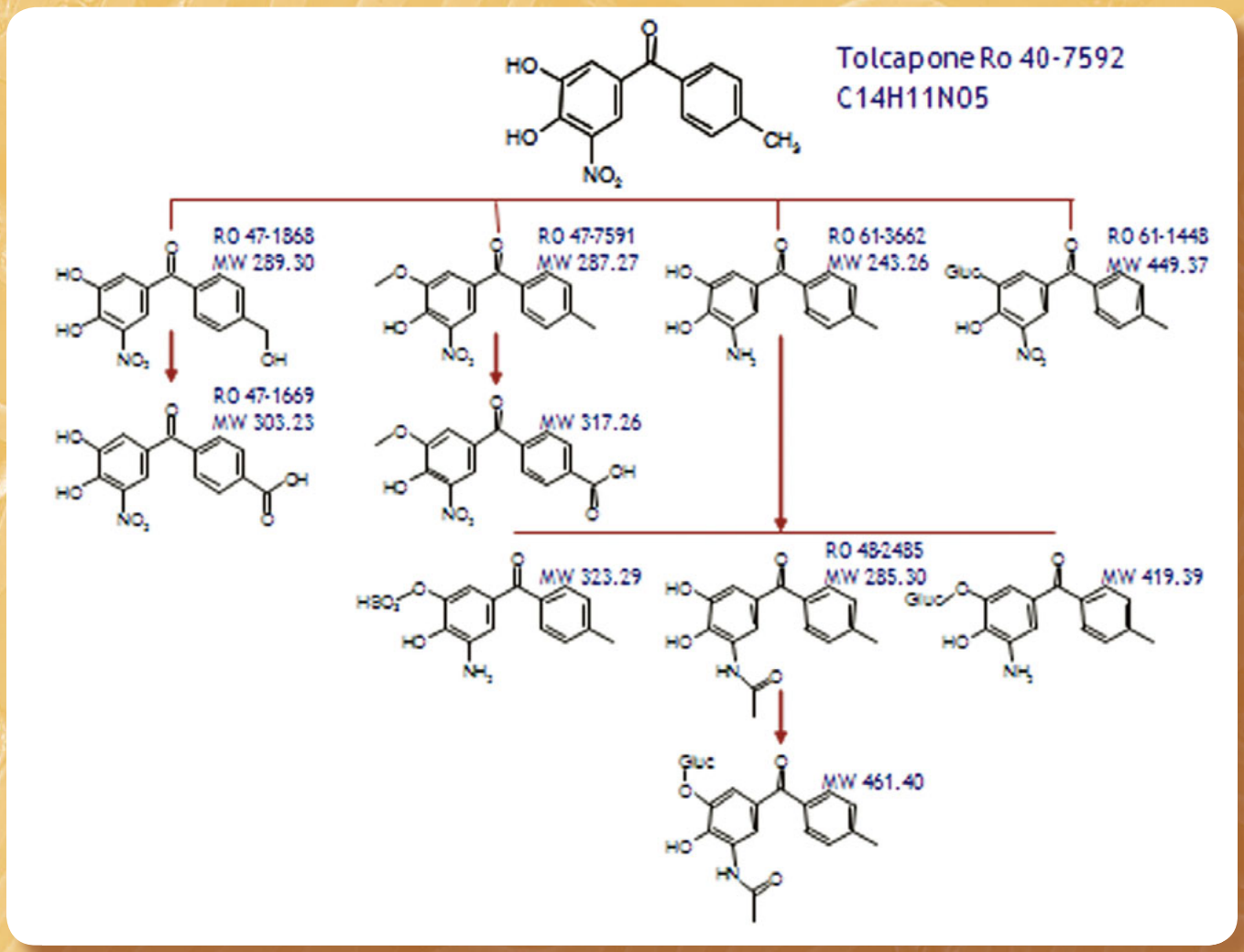


Figure 1: Schematic of the LCMS-IT-TOF mass spectrometer.



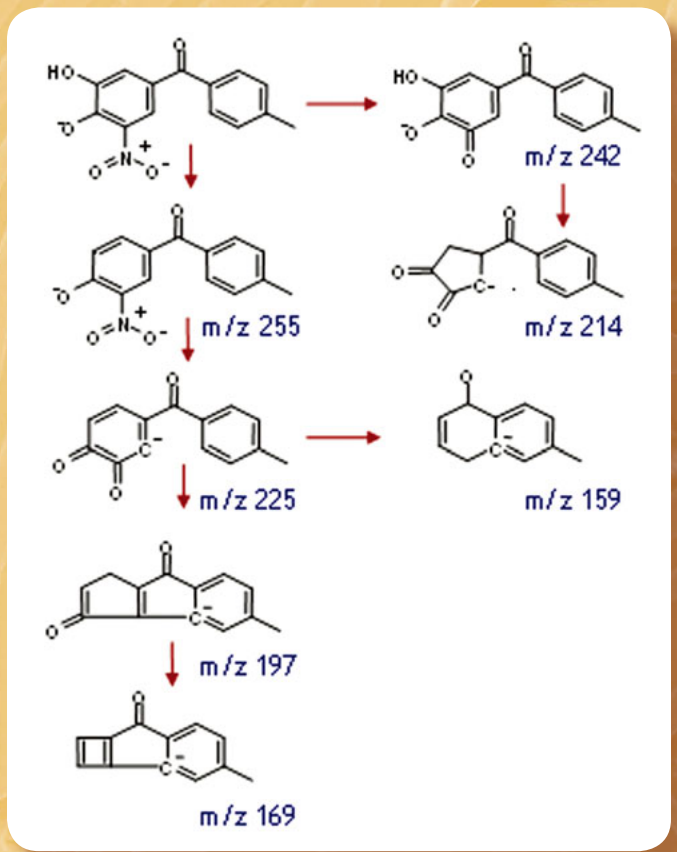


Figure 2: Metabolism of tolcapone in human (R. Gasser et al, Br J Clin Pharmacol, 48(4); (1999) 513-520). In rat S9 liver fractions, the expected metabolites include glucuronides [RO 61-1448] and oxidation products [RO 47-7591].

Figure 3: Proposed fragmentation pathway for tolcapone in negative ion.

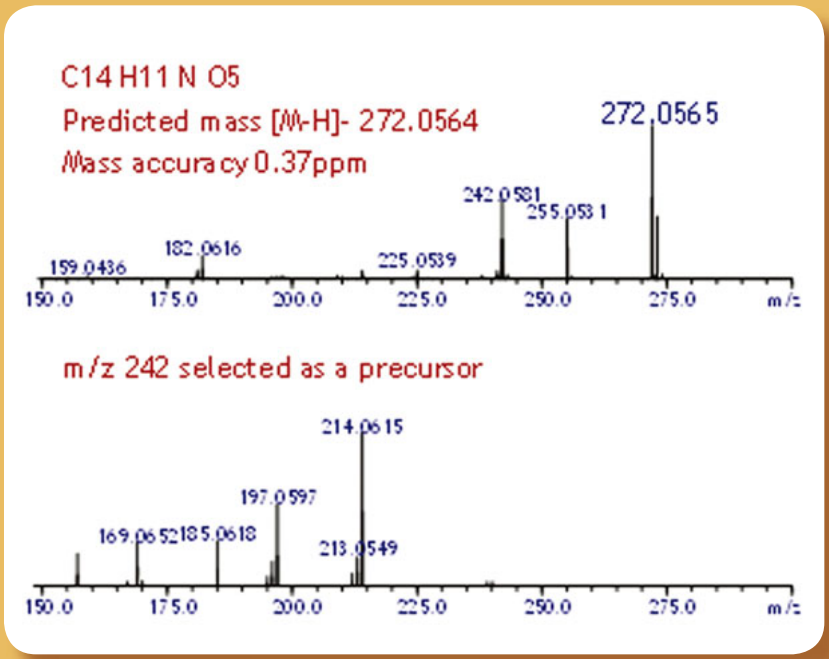
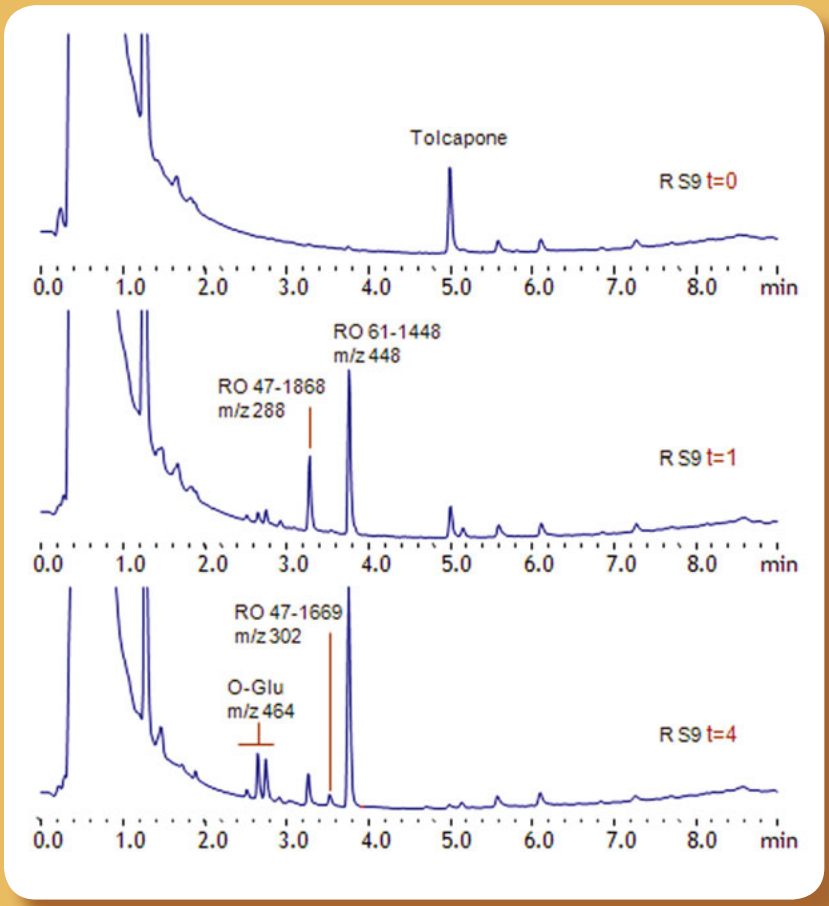


Figure 4: Tolcapone MS and MS/MS mass spectrum. Dependent on the collision energy applied to the ion trap we can influence the nature of the precursor ion selection for MS^2. In this example, m/z 242.0581 was selected as the precursor ion to show the fragmentation pathway to m/z 214.0615.

Figure 5: Following the incubation of tolcapone with rat S9 liver fractions, a series of metabolite signals increased with time (UV detection, 260nm; all traces are normalized to t=0).



Tolcapone

Tolcapone (3,4-dihydroxy-4'-methyl-5-nitrobenzophenone; Ro 40–7592; Figure 3) is a novel, reversible, orally active inhibitor of the enzyme catechol-O-methyltransferase (COMT) that has been developed for use as an adjunct to levodopa therapy.

The pharmacological action of tolcapone arises from its function as a substrate for COMT. In addition to the catechol structure, tolcapone contains two electron withdrawing substituents and easily delivers a proton (pKa=4.5); the resulting anion has a high affinity for COMT (IC50 in rat liver 36 nM) and displaces other catechols (such as catecholamines and levodopa) from the catalytic center of the enzyme.

Metabolite Detection

In the case of tolcapone incubated with rat S9 liver fractions, the metabolites include a series of glucuronides and oxidation products.

Target masses –
parent drug molecule in negative ion
Tolcapone
m/z 272.0564
$C_{14}H_{11}NO_5$

Target masses –
expected metabolites in negative ion
RO 47-1868
Oxidation
m/z 288.0514
$C_{14}H_{11}NO_6$

RO 47-1669
Primary alcohol to carboxylic acid
m/z 302.0315
$C_{14}H_9NO_7$

RO 61-1448
3-O-,D-glucuronide
m/z 448.0885
$C_{20}H_{19}NO_{11}$

O-Glucuronide (3 isomers)
m/z 464.0834
$C_{20}H_{19}NO_{12}$

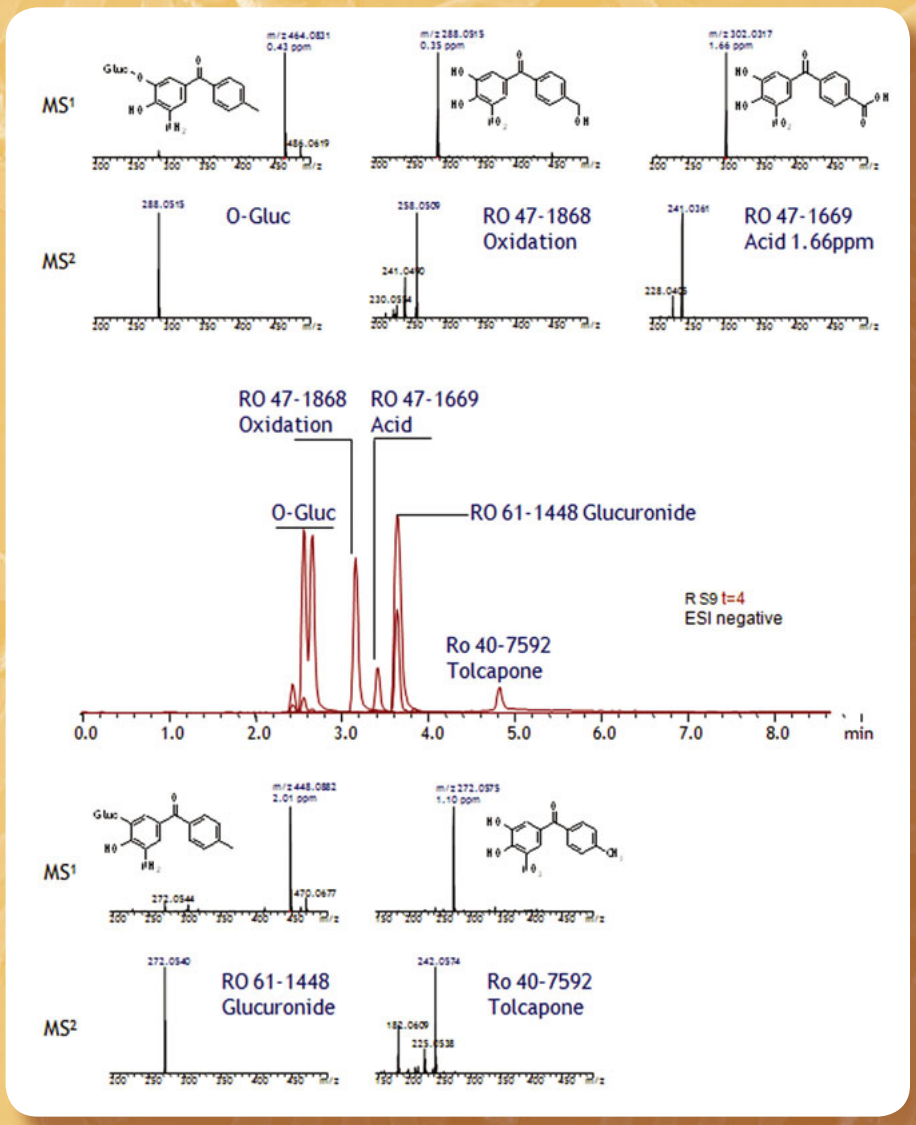


Figure 6: Tolcapone metabolites present in rat liver S9 fraction detected by high mass accuracy MS and MS/MS data-dependent acquisitions.

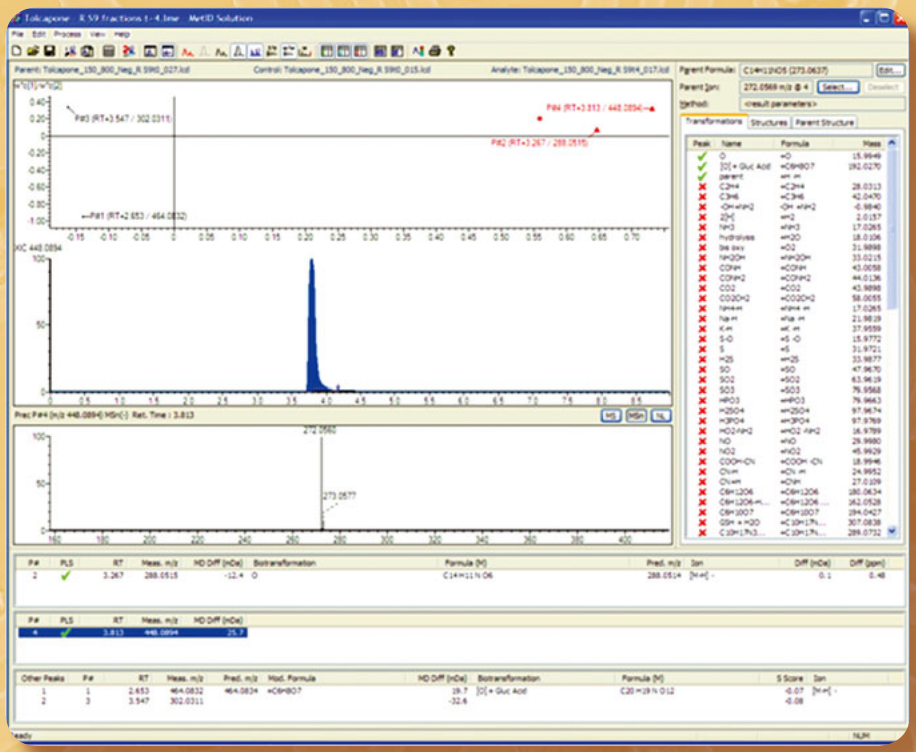


Figure 7: MetID software showing metabolites of tolcapone detected by high mass accuracy MS^n acquisitions.

MS and MS/MS data enable the MetID Solutions software to correlate common parent fragment ions and neutral loss data with possible metabolites. This is a key advantage in searching for metabolites that have not been accounted for in predictive drug metabolism software or global biotransformation lists.

MetID Solutions Software

To bring together a series of tools for metabolite detection and identification, we have designed a single, unified environment to help visualize and confirm metabolites.

Key tools in metabolite detection software:

- Supports control file subtraction (this helps to remove common endogenous components and highlight metabolite components).
- Supports global and local biotransformation lists (which can be integrated with drug prediction software such as Meteor from LHASA). The mass target lists are highly specific and highly flexible as the mass accuracy window can be user-defined and applied to customized lists.
- Mass defect filters (MDF) (both positive and negative mass defect). The MDF approach attempts to discriminate metabolite ions from matrix ions based on the similarity of the mass defect values of a drug and its metabolites. For example, glutathione conjugation leads to a mass defect of +68 mDA compared to the parent drug.
- Isotope cluster analysis. Searches for specific isotope distributions within a spectrum highlighting compounds that contain characteristic isotope distributions such as chlorine or bromine.
- Correlation analysis between parent drug MS^n ion signals and possible metabolites. Partial least squares regression analysis (PLS) is used to correlate fragment ion and neutral loss data from the parent drug molecule with likely metabolites. If common ion signals are detected (either as neutral loss or a fragment ion), there is a strong likelihood that the component is a metabolite.
- Integrated formula prediction software. To increase the confidence in metabolite identification, the software automatically uses formula prediction routines on MS^n data streams. This is a unique approach in component identification and results in a highly effective candidate list filtered using high accuracy MS^n data.

Advantages of Partial Least Squares Regression Analysis (PLS) in Metabolite Identification

There are five main advantages:

- A control file is not required to exclude endogenous species or artifacts.
- As PLS uses fragment ion and neutral loss data, the results are independent of conventional peak-picking routines.
- It is far quicker compared to peak-processing routines.
- It is far more effective at identifying unexpected metabolites compared to conventional peak processing.
- Maximizes information-rich data sets acquired using high mass accuracy MS^n data.

Continued from page 6.

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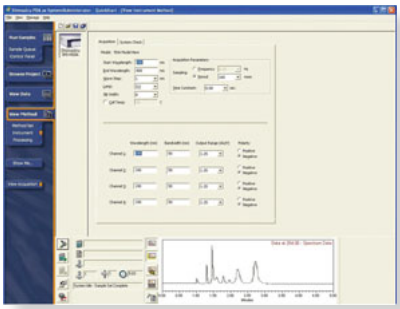

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