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LC World Talk

SHIMADZU'S NEWSLETTER FOR THE HPLC GLOBAL COMMUNITY

A Mecca of New Technology Development and Strategic Alliances: 2D-HPLC and the NIH

Unprecedented progress made in researching genomics and proteomics demands concurrent progress for better tools and technology.

A Mecca of New Technology Development and Strategic Alliances: 2D-HPLC and the NIH

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Dr. Kowalak and Mr. Masuda at NIMH. These disciplines have since given birth to a myriad of "new" specialized disciplines referred to as the "omics"

IT IS QUITE AMAZING TO WITNESS THE INTENSE PACE of progress in research advances in the arena of drug discovery. The relentless quest and shifts in synthesis and screening strategies to optimize the process of introducing drug candidates into the discovery pipeline continue to be an intriguing saga. Millions of compounds have been synthesized by various strategies adopted by major pharmaceutical industry over time and while each strategy has had limited success, these successes are technologically milestones ahead of where the industry was decades ago, and far behind where the industry can go with new tools and technology. The major reasons for shifts in drug discovery strategies are obviously and more recently due to the great impact and contributions from genomics and proteomics.

Improvement in drug discovery has always unequivocally benefited from a synergy of contributions from multiple disciplines. These disciplines have since given birth to a myriad of "new" specialized disciplines referred to as the "omics" sciences that are trickling into the very strategies for drug discovery, while concomitantly unveiling the characteristics of protein structure, function, mechanisms of protein-protein interaction, biomarkers, and protein profiles of normal and disease states. Determining what genes and their respective proteins do gives way to the fundamental fields of functional genomics and proteomics.

Discovery of one omic seems to propagate another and so on, but do not shoot the messenger—"transcriptomics."

This means that the unprecedented progress made in researching genomics and proteomics demands concurrent progress for better tools and technology. The first wave of drug discovery demands on analytical companies a decade ago propagated development of automated high-throughput solutions. The second tsunami cresting at this point will again require the same, but in the area of automated multidimensional LCMS in order to put all of the "omics" together again. And once again, Shimadzu continues discovering the opportunities to collaborate with key players in genomics and proteomics technology development. The Shimadzu saga of collaboration with strategic alliances continues, namely at the NIH.

A Mecca for funding and multidisciplinary research on proteomics investigation is currently ongoing at the NIH and some of its twenty-eight independent research divisions, including the various satellite campuses, which staff some of the finest researchers in the world. In addition to active research on campus, the NIH congressionally appropriates funding for outsourcing research projects that constantly stimulate cutting-edge research and new technology development worldwide. As an analytical instrument vendor, Shimadzu has always had its finger on the pulse of cutting-edge research, especially in the areas of drug discovery and development, and has fostered collaborations for new technology development opportunities, as in the past with major Pharma such as Bristol-Meyers Squibb (Fall Issue of LC World Talk, 2002). As exciting, Shimadzu Corporation is currently involved with a public cooperative research program between research institutions and businesses or other laboratories, under the US Department of Health and Human Services, to collaborate with key research scientists at NIH.

Shimadzu Corporation Collaboration with NIMH

Located on the main campus of NIH in Bethesda are the divisions of NIMH (National Institute of Mental Health) and NCI (National Cancer Institute), and housed in NIMH is the Laboratory of Neurotoxicology (LNT) headed by Dr. Sanford P. Markey. The LNT is a center for proteome research at NIMH focusing on cutting-edge R&D exploring innovative techniques and technologies to refine peptide and protein research to identify proteins and biomarkers associated with normal and disease states. Studies involve looking at and identifying the subtle but significant changes in protein expression and profiles early on in neurological disease states. Answering these types of questions will certainly lead to a better understanding of the mechanisms of disease states.

Shimadzu Marketing Center (SMC) was introduced to Dr. Markey and Lab Manager Dr. Jeffrey A. Kowalak at LNT last year by our Shimadzu colleagues in the local Mid-Atlantic Regional Office about the time genomics was at full throttle. Dr. Nishimura (SMC) realized this would be a perfect opportunity to work with Dr. Markey and submitted a proposal for a Cooperative Research and Development Agreement (CRADA). Shortly thereafter, Mr. Masuda joined LNT from R&D, Shimadzu Corporation, Kyoto, Japan, to directly assist with collaborative efforts in technology development for proteomics, namely micro 2D-HPLC, at LNT.

Shimadzu has always had its finger on the pulse of cutting-edge research, especially in the areas of drug discovery and development, and has fostered collaborations for new technology development opportunities

1D and 2D-HPLC/MS configurations using the LC-VP Series HPLC modules with extremely new low flow configuration for the pumps to increase sensitivity for ESI/MS.

Multi-Dimensional HPLC (2D-HPLC MS/MS): A Tool for Proteomics

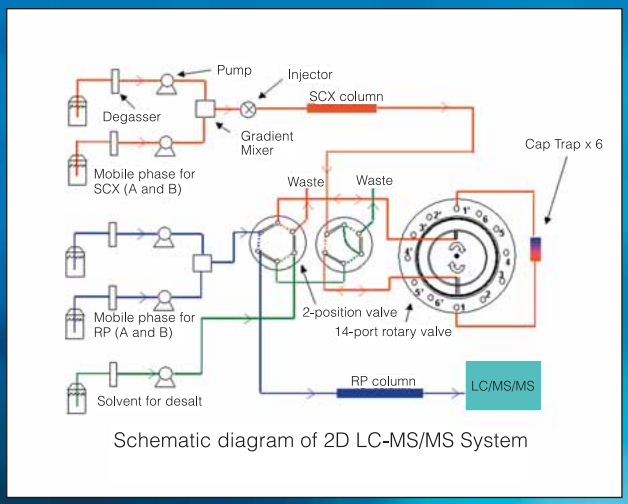
The goal was to improve and automate peptide and protein analysis through the use of HPLC and ESI tandem mass spectrometry to provide increased sensitivity, reproducible chromatographic resolution, and sequence information of complex mixtures of polypeptides. Typical methodology for protein and peptide separations has depended, and still widely depends, on Gel Electrophoresis, better known as SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis). The separation of peptides and proteins is based upon charge and size. After separation of the first dimension gel, the components can then be further separated on a second gel, providing a "peptide map." SDS-PAGE remains a very powerful tool for the discipline, and researchers still use gel electrophoresis. Many have used SDS-PAGE in the first dimension followed by HPLC-MS. In Dr. Markey's lab, they wanted to explore the potential of an automated on-line micro 2D-HPLC MS/MS system and demonstrate that it would provide a powerful tool for proteome analysis and an alternative to typical 2D-SDS-PAGE. The micro 2D-LCMS/MS system would deliver increased sensitivity, reproducible chromatographic resolution necessary for analysis of complex mixtures of polypeptides, and sequence information.

To demonstrate the analyses, Dr. Markey decided to use a typical model system of cultured cells: one batch of cells were control cells and the other set of cells was exposed to a particular xenobiotic, which would alter the profile of proteins normally synthesized and, therefore, create a postulated difference in analysis profiles. At the end of cell culture, the cells would be lysed, providing a complex protein mixture to be analyzed by one of two ways:

1. A first-dimension separation of a complex protein mixture using SDS-PAGE with in-gel digestion of the protein to peptides for subsequent analysis using reversed-phase separation coupled with ESI-LCMS, and
2. Direct digestion of the protein mixture followed by completely automated 2D-LCMS where the first dimension was a strong cation exchanger followed by desalting on a trap column (almost a two step focusing of the peptides), and in the second dimension by reversed-phase separation coupled with ESI-LCMS (see the schematic diagram for the proteome analysis and the schematic diagram of the micro 2D-LCMS system along with typical chromatograms on page 5).

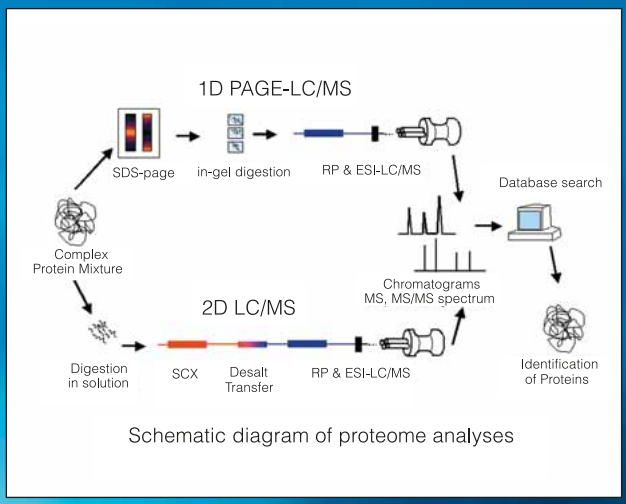
Basically, the micro 2D-HPLC system incorporates 6 capillary trapping pre-columns mounted on a 14-port valve to capture sample fractions after sequential elution from a micro-bore strong cation exchange column (SCX). The SCX fractions trapped on the Cap Trap columns (Michrom BioResources) are effectively desalted and sequentially transferred by reverse-phase gradient elution onto a pre-equilibrated capillary reversed-phase column.

To briefly summarize the results, many more proteins were identified using the micro 2D-LCMS system than found in the 1D-SDS-PAGE LCMS analysis system; however, proteins in the 1D system were characterized by less peptides per



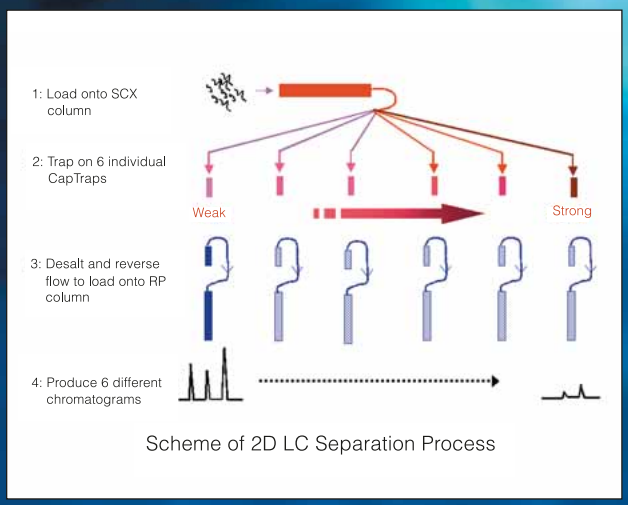
Schematic diagram of 2D LC-MS/MS System

Figure 1



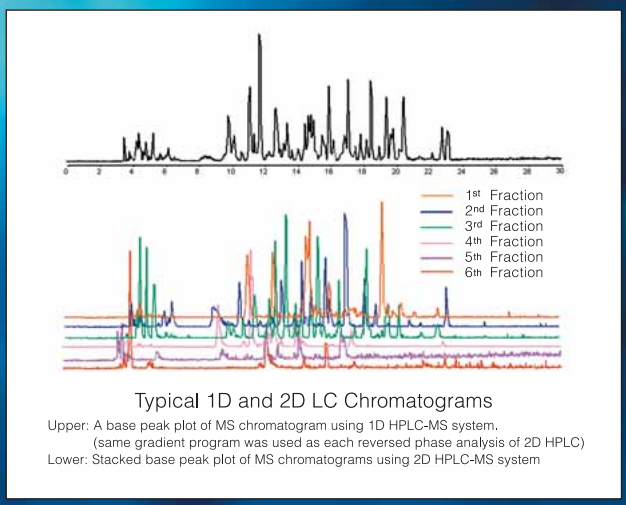
Schematic diagram of proteome analyses

Figure 2



Scheme of 2D LC Separation Process

Figure 3



Typical 1D and 2D LC Chromatograms

Upper: A base peak plot of MS chromatogram using 1D HPLC-MS system.
(same gradient program was used as each reversed phase analysis of 2D HPLC)
Lower: Stacked base peak plot of MS chromatograms using 2D HPLC-MS system

Figure 4

protein, probably due to the complexity of the analyte mixture. More importantly, key proteins known to exist in the experimental condition were only detectable on the 2D-LCMS system (data not shown). Lowering pump flow rates with special modifications allowed for highly reproducible shallow gradients and increased sensitivity in the ESI tandem MS.

Shimadzu Corporation continues to work with Dr. Markey and Dr. Kowalak to improve sensitivity and refine the micro 2D-HPLC MS to incorporate nano flow technology for proteomics at NIMH, and has received an extension on the CRADA arrangement. The world of "omics" has dramatically impacted and redefined the world of drug discovery and this appears to be just the beginning. Shimadzu welcomes the challenge to collaborate in this new era of "omics" because it is certainly here to stay.

Data, laboratory photos, HPLC schematics were provided courtesy of LNT/NIMH (NIH).

Acknowledgements

Part of this study was provided by a CRADA (Cooperative Research and Development Agreement) between the National Institutes of Health and Shimadzu Corporation. SMC and Shimadzu Corporation extend kind thanks and appreciation to the NIH and Drs. Markey and Kowalak (NIMH) for this opportunity to collaborate.

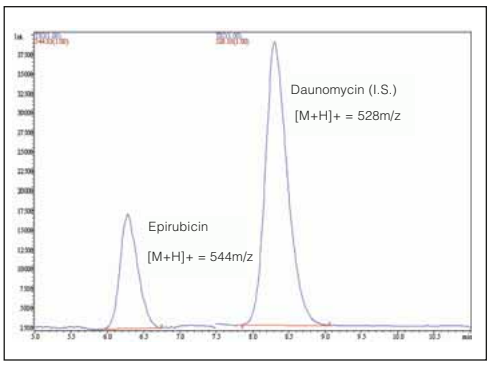
by D.J.Murry[1]; R.J.Classon[2]
[1]Purdue University, Dept of Pharmacy Practice, West Lafayette, IN;
[2]Shimadzu Scientific Instruments, Columbia, MD

Validating LC/MS Methods for Clinical Drug Monitoring

There is a growing need to perform analysis of pharmaceutical compounds from biological fluids using benchtop LC/MS instruments. The latest generation of single quadrupole instruments enables routine, reliable quantitative determination of target molecules with high sensitivity and accuracy, along with excellent reproducibility and linearity over a wide range of concentrations. A growing number of LC/MS methods are being developed to support in-vitro analysis and to meet GLP requirements for use in clinical applications.

The applications shown here are examples currently being used in clinical trials and for in-vivo determination of low levels of pharmaceuticals in human plasma. The LC/MS techniques are being used for infants, children and adult patients. In order to obtain stable, robust calibrations, Solid Phase Extraction (SPE) is used for sample preparation prior to analysis by LC/MS.

The applications described here used Atmospheric Pressure Chemical Ionization (APCI) to minimize interferences and to reduce ion suppression effects. APCI offers simple operation, produces only singly charged ions, and does not produce alkalai metal adducts.



Graph 1—Mass Chromatogram of Epirubicin from Plasma at 10 ng/mL concentration

Application 1: Epirubicin

Epirubicin is a chemotherapy agent used in treating a variety of malignancies, including soft-tissue sarcomas. Anthracyclines like Epirubicin and Doxorubicin have been used in cancer treatment for over 20 years. The exact mechanism of action is not totally understood, but appears to be primarily related to intercalation of the planar ring with DNA and subsequent inhibition of DNA and RNA synthesis.

The anthracyclines are known to have significant cardiac toxicity at high doses. Patients taking Epirubicin and Doxorubicin are at increased risk of toxicity. Since cancer treatments often involve multiple drug combinations, the cumulative dose may be lower in patients who have received other cardiotoxic agents. Arrhythmias, irreversible cardiomyopathy and the risk of congestive heart failure, along with the problems of dose accumulation, make a strong rationale for monitoring drug exposure in individual patients. Studies have often shown only half the Epirubicin needed to inhibit sarcoma growth is administered. Traditional assays for Epirubicin use HPLC with fluorescence detection.

Method

Plasma samples were spiked with known amounts of Epirubicin ranging from 1ng/mL to 1000 ng/mL. Daunomycin was used as an internal standard. Spiked plasma samples (1mL) were first acidified by the addition of 20 mL of phosphoric acid and then purified using SPE. The extraction cartridges (OASIS HLB) were wetted with methanol and then H_2O (1 mL each) prior to loading the plasma sample. Plasma was loaded directly onto the SPE cartridges, followed by washing with 2 mL of H_2O to elute salts and polar impurities. The target compounds were eluted with methanol acidified with formic acid (2% v/v). The eluate was dried under nitrogen gas, and reconstituted with mobile phase (75 ml). After concentration of the target compounds (13x enrichment), analysis was performed on a Synergi Polar RP (Phenomenex) C18 column. Epirubicin eluted at 6 minutes and daunomycin eluted at 8 minutes. Total run time was 11 minutes. A bypass valve was used to divert solvent flow prior to and after the elution of the target compounds. Analysis was carried out by APCI LC/MS.

The calibration set included 13 spiked plasma extracts covering the range of 1 to 1000 ng/mL for Epirubicin. Three control samples with three replicates each were analyzed as part of each calibration for confirmation.

Table 1: Analytical conditions for LC-MS analysis of Epirubicin

LC/MS Instrument:	Shimadzu LCMS-2010
Column:	Synergi Polar RP (Phenomenex) C18 250 x 2 mm, 4-micron
Mobile Phase:	A. Aqueous phase: H_2O + 0.05% TFA B. Organic phase: 50:50 Acetonitrile:methanol + 0.03% formic acid Isocratic elution 60% B
Flow Rate:	0.2 mL/min
Injection Volume:	20 µL
Column Temperature:	40°C
Probe Voltage:	4.5 kV (APCI-Positive mode)
Probe Temperature:	350°C
CDL Temperature:	200°C
Nebulizer Gas Flow:	2.5 L/min (nitrogen)

Discussion

Fifteen different plasma lots were used in validating this method. There were no interfering peaks present at the elution times of the target compounds; thus, a simple 11-minute isocratic method was found to be acceptable. This LC/MS method has been validated with a 200-sample validation set. Over 300 patient samples have been analyzed using this method. Serial samples (n = 10) were obtained over a 24-hour period following administration. Drug recovery of spiked samples was typically in the 95-105% range at typical 2 ng/mL dose levels. The limit of quantitation was 0.5 ng/mL of plasma. Linearity was 0.998 throughout the therapeutic range of 1 ng/mL to 1000 ng/mL. Inter-day and intra-day C.V. values were less than 10% at all therapeutic levels. Frozen samples were stable for up to two months.

The method is presently being used to batch process samples from clinical administration of Epirubicin in treating soft tissue sarcomas. Samples are obtained over a 24-hour period following cycle one of therapy, analyzed to determine Epirubicin concentrations, and dosage modifications made to achieve a target systemic exposure for subsequent treatment cycles.

Application 2: Methylphenidate

Attention-Deficit/Hyperactivity Disorder (ADHD) affects between 3 and 6% of all school-age children. It is marked by impulsivity, hyperactivity, and inattention. The typical onset time is between 3 and 7 years of age.

Although there are a number of therapeutic treatments for ADHD, the most common is the use of Methylphenidate. This drug has been used for over 40 years and accounts for 90% of the stimulants used for treatment of ADHD. The compound is believed to work by inhibiting dopamine re-uptake by binding to the dopamine transporter. It also inhibits norepinephrine and serotonin re-uptake.

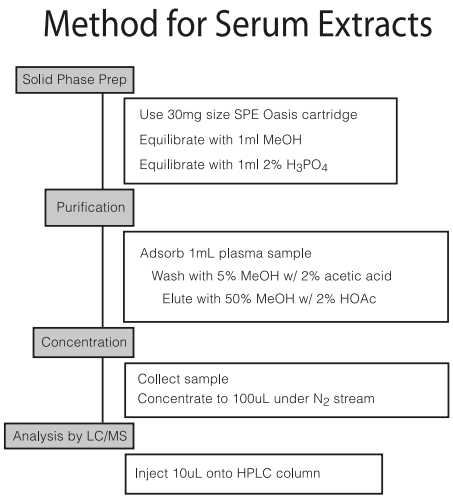
Approximately 60-70% of affected children respond to treatment.

Unfortunately, there are occasionally adverse effects with methylphenidate, including insomnia, nervousness, jitteriness, headache, palpitation and tachycardia. In order to minimize these effects, dosage must be carefully controlled. Typical dosage is 0.1 to 1 mg/kg. Metabolism of the drug in children is rapid, with average half-life in the 2-hour range. Food intake and other drugs affect absorption so individual uptake must be monitored.

The traditional methods for this analysis use GC/MS. Derivatization is required to detect the primary metabolite. LC/MS allows direct detection of the drug and metabolite with excellent linearity and sensitivity.

Method

Plasma samples were spiked with known amounts of methylphenidate and ritalinic acid. Deuterated (d-5) ritalinic acid was used as an internal standard. Spiked serum samples were then purified using SPE. The extraction cartridges were wetted and acid equilibrated prior to use. Plasma was loaded directly onto 30 mg size Oasis brand SPE cartridges, followed by washing with 5% methanol to elute salts and polar impurities. The target compounds were eluted with 50% methanol. After concentration of the target compounds (10x enrichment), analysis was performed on a Phenomenex Luna C18 column (150 x 4.6 mm, 3 microns). Gradient conditions were 20-75% over 5 minutes, 1.5 minutes at 75%, and 5 minutes equilibration. Total run time was 12 minutes. A bypass valve was used to divert solvent flow prior to and after the elution of the target compounds. Detection was carried out using LC/MS with APCI ionization.



Graph 2—Solid Phase Extraction of plasma samples

Experimental

Whole blood was taken from healthy blood donors and collected in heparinized tubes. Protein was removed by acidification and centrifugation for 5 minutes at 3000 RPM. Analyses were conducted to verify the stability of the drug in plasma. The SPE cartridges were equilibrated with methanol and aqueous phosphoric acid prior to use. Plasma was loaded on the SPE cartridges directly without dilution. The cartridge was washed with MeOH:H_2O (5:95%) acidified with 2% acetic acid. The analytes were eluted with 50% MeOH/50% H_2O with 2% acetic acid.

LC/MS

A Shimadzu LCMS-2010 quadrupole mass spectrometer operated with an APCI interface. Probe temperature was 400°C and desolvation (CDL temperature) was 200°C. All target ions were analyzed in positive mode. Acquisition time was 0.2 second for each ion. Ion grouping was used to minimize time offset between measurements. All other mass spectrometer settings were the default autotune values.

The [M+H]+ ion for methylphenidate was detected at 234 m/z while ritalinic acid was detected at 220 m/z, and the d-5 Ritalinic acid at 225 m/z.

The calibration set consisted of 10 standard concentrations made up and extracted from plasma. Concentrations ranged from 50 pg/mL to 100 ng/mL for methylphenidate and from 2 to 1000 ng/mL for ritalinic acid. Blank plasma runs were run before starting calibration and after the last samples. Calibration curves were generated for the ratio of the internal standard to the target ion. Linearity (r2) for the methylphenidate was 0.997 over the range from 50 pg/mL to 100 ng/mL. Ritalinic acid linearity (r2) was 0.998 over the range of 2-1000 ng/mL.

Table 2: Analytical conditions for LC-MS analysis of Methylphenidate

LC/MS Instrument:	Shimadzu LCMS-2010
Column:	Column: Luna C18 150 x 4.6 mm, 3µm (Phenomenex)
Mobile Phase:	A. Aqueous phase: H_2O + 0.1% acetic acid B. Organic phase: MeOH + 0.1% acetic acid Gradient elution 20% B--75% B in 6.5 minutes
Flow Rate:	0.8 ml/min flow rate
Injection Volume:	20 °L
Column Temperature:	40°C
Probe Voltage:	+4.5 kV (APCI-Positive mode)
Probe Temperature:	400°C
Nebulizer Gas Flow:	2.5 L/min (Nitrogen)

Discussion

A fast LC/MS procedure has been developed to support clinical trials and in-vitro metabolism experiments. 200 samples were run in developing this method. Drug recovery of spiked samples ranged from 95-100% at typical 2 ng/mL dose levels. The limit of quantitation was 0.05 ng/mL of plasma (corresponds to 5 pg injected on-column). Linearity was typically 0.997 throughout the range of 0.05 ng/mL to 100 ng/mL. Intra-day and inter-day coefficient of variation was less than 10% for both low and high controls.

Conclusions

These results demonstrate that the Shimadzu LCMS-2010 is a valuable instrument for clinical use. Both of the methods described provide sensitivity from the low- to mid-picogram range from human plasma with excellent quantitative reproducibility.

Quantitative results were stable and reliable, and rivaled the best practices in use today. Linearity was 3 orders of magnitude or better for spiked plasma extracts. Sample preparation for target compounds from plasma can be carried out using inexpensive SPE cartridges. Internal standards were used to provide the accuracy and precision required for robust and reliable quantitation.

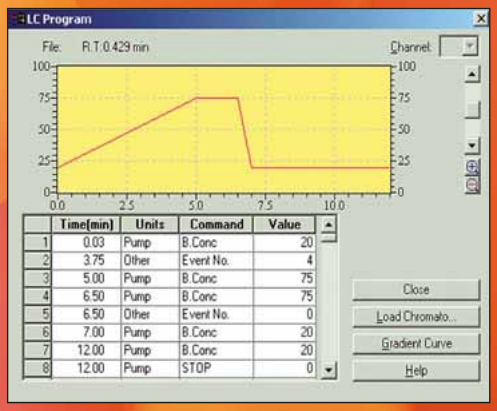
The sensitivity of LC/MS makes this an ideal detector for low levels of pharmaceutical products in biological fluids. The use of APCI interface allows simple gas phase ionization of the target analytes without needing pH adjustment for mobile phase, which allows more flexibility in terms of chromatographic separations. APCI also reduces the formation of adducts and artifacts.

Other recent clinical applications of LC/MS:

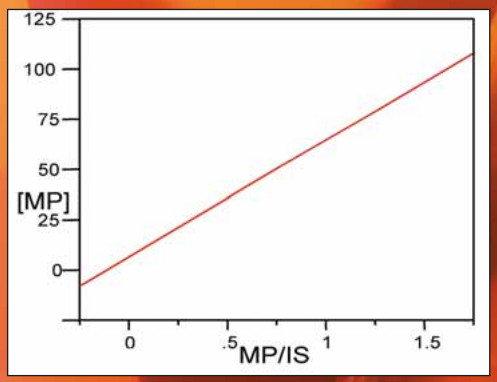
- Azithromycin
- Budesonide
- Cisplatin
- Fluticasone
- Paclitaxel
- Parthenolide
- Beclamethasone
- Camptothecin
- Ethinyl Estradiol
- Ifosfamide
- Docetaxel

References

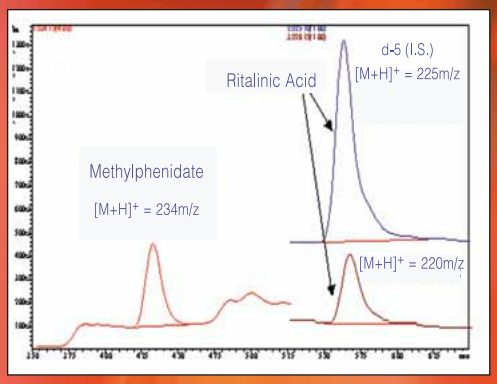
Doerge DR, Fogle CM, Paule MG, McCullagh M, Bajic S. "Analysis of methylphenidate and its metabolite ritalinic acid in monkey plasma by liquid chromatography/electrospray ionization mass spectrometry." *Rapid Communications in Mass Spectrometry* 14, 619-623 (2000).



Graph 3—Gradient conditions for HPLC



Graph 4—Methylphenidate calibration versus internal standard



Graph 5—Mass Chromatogram of Methylphenidate and metabolite from Plasma at 2 ng/mL concentration

Dr. Anthony Lemmo, Director of Research, right, and his Research Associate, Dr. Cameron Olbert, in front of the 8-channel isocratic High-Throughput HPLC System at the TPI facility

TransForm Pharmaceuticals Maximizes Innovation Through Collaboration:

Multiplexed High-Throughput HPLC

S. M. Wishnies[1], C. Olbert[2], M. Nishimura[1], A. V. Lemmo[2], Y. Kohno[3] and T. Adams[4]

[1]Shimadzu Marketing Center; [2]TransForm Pharmaceuticals, Inc., Lexington, MA; [3]Shimadzu US Manufacturing; [4]Shimadzu Mid-Atlantic Region

Robust on-line automated high-throughput parallel HPLC was a demand and the challenge put forth by TransForm Pharmaceuticals, Inc. to analytical instrument companies. Shimadzu Corporation stepped up to the plate to answer. This is what this business is all about: "TransForming" a demand and a simple phone call into a customer/vendor alliance.

TransForm Pharmaceuticals, Inc. (TPI), founded in late 1999 with initial technology from Millennium Pharmaceuticals (NASDAQ:MLNM), is a privately held company now located in Lexington, Massachusetts. TPI is reinventing the pharmaceutical industry's approach to form and formulation, with a novel set of high-throughput automated platform technologies, powered by state-of-the-art informatics and a scientific and managerial team with extensive experience in pharmaceuticals. TPI uses these capabilities to optimize drug forms and/or formulations, and increase the clinical and commercial value of pharmaceutical products across the entire pharmaceutical value chain.

In R&D, TPI works with partners to help them make better candidate selection decisions, and reduce attrition and development time and cost. For later stage and marketed products, TransForm helps partners enhance product life cycle management by discovering new forms and/or formulations that optimize bioavailability and solubility, broaden intellectual property protection, and create the potential for new dosage forms and/or combination products. TransForm is also using these capabilities to develop its own proprietary product portfolio.

To achieve these goals, TPI has been aggressive in pursuing and developing sound relationships and technology with instrumentation partners like Shimadzu for issues such as high-throughput HPLC analysis of a large number of compounds. With the eminent demand for high-throughput, rapid parallel analysis and screening of potential drug candidates in discovery and development, TPI focused their resources on the pharmaceutical issues and approached Shimadzu to address the HPLC technology enhancements and automation challenges. The first course of action was to determine feasibility for the task at hand and then implement a quick solution.

TPI and Shimadzu met to discuss the applications and technology TPI needed for high-throughput HPLC. Shimadzu Marketing Center (SMC), a willing collaborator with progressive companies like TPI on providing customized solutions, accepted the opportunity to work

with them on their throughput demands. TPI's Research Director, Anthony Lemmo, PhD, and his Senior Research Associate, Cameron Olbert, put forth the issues, details, and timeline involved for SMC to explore feasibility studies. TPI had worked out and optimized an isocratic HPLC method; however, they needed to multiplex the assay to increase productivity and do this without having to purchase multiple independent HPLC systems. So, the demand was to multiplex additional HPLC pumps and UV detectors beyond the commercially available HPLC configuration. These are opportunities Shimadzu thrives on and the flexibility of the LC-VP series HPLC component allows for customization.

In due time, all parties involved realized that parallel HPLC analysis would expedite screening of TPI's compounds; additionally, automated multiplexed analysis would increase the utility and productivity not only for their drug discovery efforts, but also at other bottlenecks in drug and pharmaceutical process development. Optimally, this system would interface successfully with multiple Shimadzu HPLC pumps and UV detectors. The parallel approach would also provide an economical solution since only one multiplexed system would be required instead of individual HPLC systems.

See Figure 1.

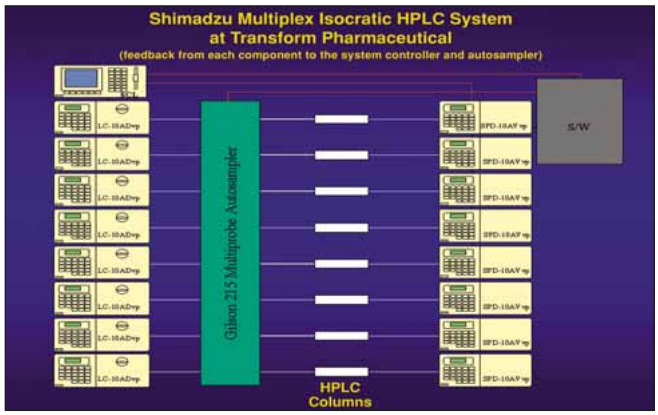


Figure 1—Schematic of the Shimadzu 8 LC-10ADvp pump/8 LC Isocratic HPLC Configuration with Gilson 8-probe autosampler

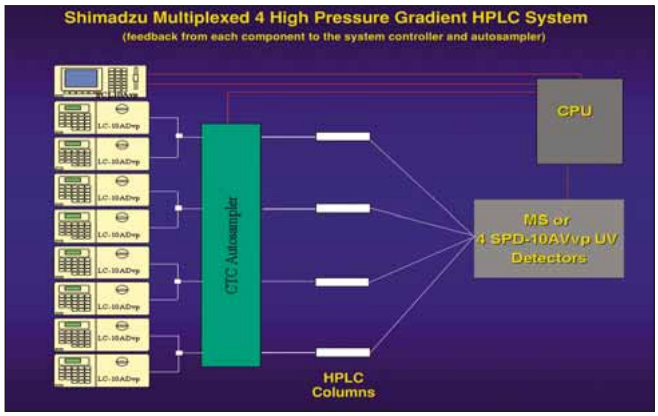


Figure 2—Schematic of the Shimadzu 8-pump delivery of a 4 high-pressure gradient HPLC system

In a relatively short time, Shimadzu produced a multiplexed HPLC system. A new parallel multiplexed HPLC system was developed to allow for simultaneous analysis on 8 HPLC columns. The system can also be configured with 8 analytical pumps for simultaneous delivery of 4 channels of high-pressure gradients to a "muxed" mass spectrometer. At this time, TPI is using the system in the 8 pump/8 SPD detector isocratic configuration.

Another advantage of the system is sensitivity. Detector signal is not compromised by being optically manipulated or electronically split 8 ways (tremendous loss of sample energy). TPI has validated the system with particular attention to linearity, reproducibility, and channel-to-channel performance on all 8 SDP-10AVvp detectors.

The attractiveness of the system is the flexibility to construct a number of multiplexed configurations using only one system controller, which can then be interfaced with a variety of equipment depending on the requirements of the application. Arrays of Shimadzu multiplexed chromatographic configurations are possible which allow for rapid and high-throughput analysis in drug discovery and development. The system can also be configured to simultaneously run 4 high-pressure gradients at highly reproducible micro flow rates up to traditional analytical flow rates.

See Figure 2.

All of the multiplexed systems are smart in that if there are any errors on a pump or detector, there is built-in error feedback to the system controller to stop the runs, etc. so that precious samples are not wasted. The other inherent beauty of the system is that if multiplexing is no longer required, the components can be broken down and reconfigured into standalone operating HPLC components or systems. You cannot do that with fixed 4 and 8-channel systems and achieve the same performance.

Currently, one of TransForm's high-throughput automated platforms, the multiplexed Shimadzu HPLC, is being employed to analyze a large number of drug formulations generated in these platforms to evaluate them for solubility, stability and performance. With a combination of fast chromatography and the multiplexed system, TransForm is currently collecting chromatographic data on 20,000 samples per week. The system has proven to be robust and dependable, and has dramatically increased throughput and productivity.

Acknowledgements

Shimadzu Marketing Center deeply appreciates the opportunity to work for TPI and Drs. Olbert and Lemmo at TransForm. We also thank Ross Sematore, Field Sales from Shimadzu's Boston Office, who was key to forging this relationship with TPI. Finally, without the technical engineering expertise of Dr. Yutaka Kohno (Shimadzu US Manufacturing and R&D, Canby, OR), this project would have been extremely difficult.

Cool Solutions for Hot Stuff

Spicy food has gained great popularity in the United States, as evidenced by a proliferation of ethnic restaurants and unprecedented sales of condiments like salsas and hot sauces containing chili peppers. The burning sensation of peppers belonging to the genus capsicum is due to capsaicinoids, whose structures are based on that of capsaicin, trans-8-methyl-N-vanillyl-6-none-namide. The capsaicinoids are found primarily in the pepper's inner lining and placenta, the internal "ribs" that run down the middle and along the sides. The naturally occurring capsaicinoids are c capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin.[1,2]

by Craig S. Young, M.S.

Traditionally, the heat level of peppers is expressed using the Scoville Organoleptic Scale, devised by pharmacist Wilbur Scoville in 1912. With it, heat levels are assigned according to the amount of sweetened water dilution necessary to neutralize the heat sensation. The more dilution needed, the higher the apparent heat level.[2,3] Peppers' heat levels vary widely from zero units for bell peppers to a mouth-searing 300,000 Scoville Heat Units (SHU) for habaneros. Pure capsaicin and dihydrocapsaicin comprise generally 80–90% of the total capsaicinoid content in peppers and each comes in at 16,000,000 SHU.[3] As one would imagine, the original Organoleptic Scale is subjective, time-consuming and costly.

HPLC with UV detection at 280nm has been widely accepted as a superior alternative since it provides an accurate, repeatable and economical test. Using HPLC, peppers' heat levels are still expressed in SHU. AOAC method 995.03 permits such a determination of capsaicinoids by HPLC.[4] However, as is typical of absorbance detectors, UV misses many non-capsaicinoid pepper components that also contribute to flavor.

Since its inception, Evaporative Light Scattering (ELS) detection has emerged as a valuable complement to spectroscopic detectors for HPLC.[5-7] The ELSD makes a light scattering measurement of all semi- and non-volatile particles that have been dried of mobile phase through evaporation. Because its response is independent of the light absorbing properties of molecules, it can reveal sample components that UV detectors miss and provide a more accurate profile of relative component abundance than can be had with a spectroscopic detector.[7] Together, multi-wavelength UV and ELSD make a powerful, complementary pair for analyzing the flavor constituents of chili peppers.

Method

A fresh pepper cross-section weighing 4g (flesh and ribs without seeds) was diced into 1/8" pieces and placed into a 20mL scintillation vial. 10mL 90/10 ethanol/water was added and the mixture was sonicated with shaking for 1 hour. The extract was syringe filtered (nylon; 0.2µm).

Capsaicinoids were identified by authentic injection of the standards. Experiments using ELSD-LT drift tube temperatures from 28-40°C showed no variation in detector response to the capsaicinoids, indicating that a precise evaporation temperature is not critical to detection accuracy and reproducibility. The hydrophobic components of peppers, including the capsaicinoids, are separated by their hydrophobicities on a reversed-phase C18 column.

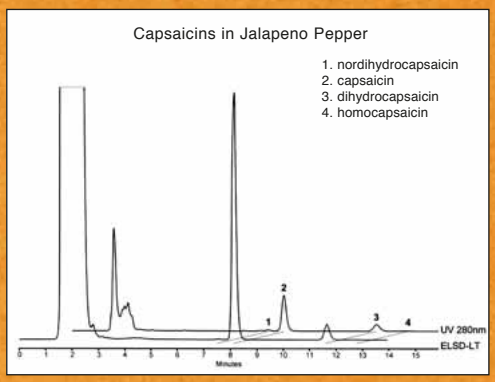


Figure 1—Determination of capsaicinoids in serrano peppers by isocratic method.

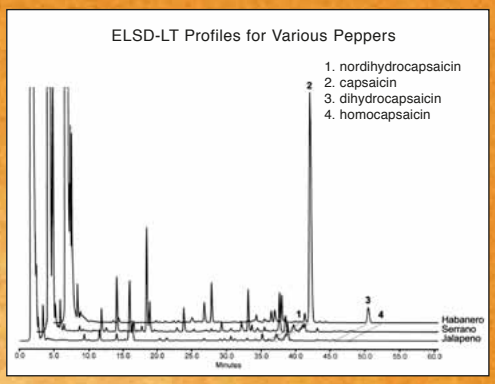


Figure 2—Comparison of gradient chromatograms obtained for extracts of habanero, serrano and jalapeno peppers.

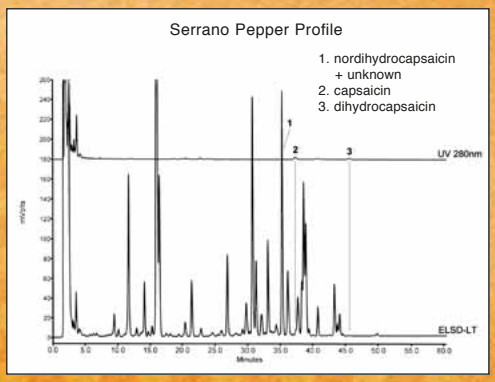


Figure 3—Comparison of UV 280nm and ELSD-LT response to the flavor constituents of the serrano pepper.

Conditions

Column:	Premier C18, 5µm, 150x4.6mm
Flow rate:	1mL/min.
Column Temp:	30°C
Mobile Phase A:	1% acetic acid, B: acetonitrile
Gradient (Time, %B):	(0,25)(60,50)(61,100)(62,100)(63,25)(70,25)
Injection Volume:	30µL
Detector:	Shimadzu ELSD-LT (Gain 10; Press. 350kPa; Temp. 40°C)

Results and Conclusion

Both UV 280nm and ELSD-LT may be used for the isocratic determination of the capsaicinoids alone. This is illustrated in Figure 1 for a simple analysis of serrano pepper.

As shown in Figures 2 and 3, the ELSD-LT reveals a complex mixture of hydrophobic constituents, presumably glycosides, which absorb weakly at UV 239nm with significant baseline drift, and not at all at 280nm. The majority of these constituents were found to be common to Habenaro, Serrano and Jalapeno peppers. Two unresolved non-capsaicinoid constituents at ~15.5min. were non-chromophoric between 190–500nm but their signals were prevalent by ELSD. In Figure 3, the ELSD-LT reveals a non-chromophoric co-eluting constituent with nordihydrocapsaicin.

This data supports the use of 280nm UV for determining only the capsaicins as it is highly selective for those constituents. The ELSD-LT offers numerous advantages, including better baseline response for the gradient method and detection of all flavor constituents, both chromophoric and non-chromophoric, in one run.

Equipment Used for all Experiments

Pump(s):	LC-10ADvp
Injector:	SIL-10ADvp
Column Oven:	CTO-10A/Cvp
Detector(s):	SPD-M10Avp PDA 190 – 900nm plumbed in series with ELSD-LT
Data System:	CLASS-VP 7.2 @10Hz
Solvent Degasser:	DGU-14A
Columns:	Shimadzu Premier C18, 5µm, 150 x 4.6mm

References

1. Hoffman, P. G. et. al., J. Agric. Food Chem., 31, 1326-1329 (1983).
2. Dong, M. W., Today's Chemist At Work, 9, No. 5, 17-20 (2000).
3. Restek Corporation, Food and Flavor Application Note #59199.
4. AOAC Official Methods of Analysis (2000), Method 995.03.
5. S.C. Churms, J. Chromatography. A, 720, 75-90, (1996).
6. B. Herbreteau, M. Lafosse, L. Morin-Allory, and M. Dreux, J. Chromatography. 505, 100, (1990).
7. Young, C. S., Cereal Foods World., 47(1), 14-16 (2002).

The burning sensation of peppers belonging to the genus capsicum is due to capsaicinoids

Compliance Corner is a new section to LC World Talk that will be dedicated to updates and activities related to regulatory issues. The editor also welcomes questions to be addressed in the next issue. Please e-mail questions to LCWorldTalk@shimadzu.com. Please visit our Web site at www.shimadzu.com.

Shimadzu Co-Sponsors Compliance Seminars for 2 Consecutive Years in Tokyo, Japan:

The Regulatory Landscape may Change Again with the New Draft Guidance for Industry on 21 CFR Part 11

Both Dr. Nishimura (right) and Mr. Mutin (left) from SMC were keynote speakers at the IVT seminars. Both focused on compliance issues and on CLASS-Agent 21 CFR Part 11 compliant software, an integral companion to the Lab Solutions software.

Time has trickled away for industry to comment on the new Draft Guidance to Part 11, Electronic Records and Signatures issued by the FDA in February 2003. However, timing of the release date for the new draft guidance could not have been more serendipitous for Shimadzu as Shimadzu Marketing Center (SMC) and the Liquid Chromatography Business Unit (LCBU), Shimadzu Corporation, co-sponsored the Compliance Seminars held by the Institute of Validation Technologies (IVT) for the second consecutive year in Tokyo, Japan. By serendipitous, it was very fortunate that seminar speakers (co-sponsors, consultants and major pharma industry representatives) and John Kirchner (IVT host) were able to meet and discuss the impact the new draft guidance would have on the pharma industry prior to the seminars so that materials could be added to their presentations or revised accordingly to benefit the attendees. The seminar was held just one week after the release of the new draft guidance.

The change in the "compliance atmosphere" appears to have precipitated mainly in the form of a risk-based approach assessment with an increased "mix" of GAP analysis throughout the period of the new draft guidance front. The point of the meteorological analogy made here between the weather in reference to the new draft guidance is implicit: at the end of the day, it is a guidance and could change just like the weather, so do not forget when and how to apply the predicate rule of 21 CFR Part 11! Compliance to 21 CFR Part 11 has not disappeared.

Seminar attendance, driven by the new draft guidance, was exceptional this year and so were the guest speakers. Shimadzu featured presentations from three key speakers, including Shimadzu's resident experts on FDA compliance issues, Drs. Hayakawa (LCBU) and Nishimura (SMC), followed by a member of the SMC staff, Alex Mutin, who focused on compliance issues and on CLASS-Agent 21 CFR Part 11 compliant software, an integral companion to the Lab Solutions software.

Most of the speakers adhered to their original scripts, but some restructured their seminar materials to address and deduce what impact the new draft guidance would have on the regulatory landscape and pharmaceutical industry as a whole.

IVT compliance seminars co-sponsored by Shimadzu were well attended.

There seems to be a false sense of security with some that the pressure is off now, at least for a while, with regard to compliance; however, continuing to practice the predicate rule would be a wise thing to do. Perhaps there will be practical relief provided by the new draft guidance, but it may be beneficial to carefully review and understand the draft guidance while keeping full perspective on the predicate rule requirements because they will be enforced for records that are subject to Part 11. Just a quick review of general issues involving the "new draft guidance" (basic interpretation from the new draft guidance to Part 11 issued in February 2003):

- Part 11 still applies when using records in electronic format; however, there are certain additional conditions to this that were listed in the new draft guidance
- Write an SOP for your decision to maintain either paper or electronic records
- Do not forget about the Audit Trail and computer-generated time-stamped audit trails
- Record Retention: again determine through risk assessment the value of records to be retained - see the predicate rule and follow it
- Legacy Systems: the effective date of 21 CFR Part 11 was August 20, 1997. Prior to that date, the FDA will usually not enforce the Part 11 requirements
- Hybrid systems: paper and electronic records can co-exist providing that the predicate rule requirements are met; also, content and meaning of both must be preserved (combinations of paper and e-records, signatures, including handwritten signatures committed to e-records)

The new draft guidance is a promising effort to make better sense of Part 11 and to make it somewhat more economically feasible. Shimadzu continuously strives toward providing simple, effective, and economically reasonable solutions to compliance with data acquisition and archiving software packages regardless of the final outcome of the guidance (Lab Solutions and CLASS-Agent software). Shimadzu will also be there to assist customers with their compliance needs even after the rule is appended. We are in the compliance business for the long term.

Guest speakers Drs. Christopher Reid (Integrity Solutions Limited, Middlesbrough, England), Erik Muegge (Alcon Labs, USA) and Ludwig Huber (Agilent) shared their opinions and comments on the new draft guidance.

Drs. Koyazaki (left) and Nishimura greeting attendees at the IVT seminars.

Shimadzu Scientist Awarded Nobel Prize for Chemistry

Koichi Tanaka Rewarded for Revolutionary Mass Spectrometry Technique

Koichi Tanaka

Mr. Koichi Tanaka of Shimadzu Corp, Kyoto, Japan, was jointly awarded one half of the prize with John B. Fenn of Virginia Commonwealth University for their development of soft desorption ionization methods in mass spectrometric analyses of biological macromolecules. Tanaka's approach has become fundamental in the standard methods (MALDI, SELDI and DIOS) for structural analyses of proteins, DNA and carbohydrates, which make it possible to characterize the components of biological systems. Many of the laser desorption technologies on the market today have their foundations in Mr. Tanaka's work.

This year's Chemistry Prize focuses on powerful analytical methods for studying biological macromolecules, such as proteins. Mr. Tanaka is the architect of a soft ionization technique that the Royal Swedish Academy of Sciences describes as "hovering through blasting." In 1987, at an Oriental mass spectrometry symposium(1), Tanaka proved that protein molecules could be ionized using the soft laser desorption technique. A year later, the scientific community saw the full publication of his seminal paper(2).

Combining MALDI with a time-of-flight mass spectrometric detection has signified a monumental step in molecular weight determination of biological macromolecules. The Royal Swedish Academy of Sciences has recognized Tanaka's technology for what it is—a cornerstone of proteomics.

1) Proceedings of the Second Japan-China Joint Symposium on Mass Spectrometry, Matsuda H., Liang, X-T., eds.; Bando Press, Osaka, pp. 185-188 (1987).

2) Rapid Communications in Mass Spectrometry 2, 151-153 (1988), Koichi Tanaka, Hiroaki Waki, Yutaka Ido, Satoshi Akita, Yoshikazu Yoshida and Tamio Yoshida

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3. Kanda-Nishikicho 1-chome, Chiyoda-ku, Tokyo 101, Japan
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Cable Add.: SHIMADZU TOKYO
Overseas Telex No.: 0232-3291 (Answer Code: SHMDT J)
http://www.shimadzu.com

SHIMADZU SCIENTIFIC INSTRUMENTS, INC.
7102 Riverwood Drive, Columbia, MD 21046, USA
Phone: 410-381-1227 or 800-477-1227 • FAX: 410-381-1222
http://www.ssi.shimadzu.com

SHIMADZU EUROPE LTD., OPERATION CENTER
Albert-Hahn-Strasse 6-10, D-47269 Duisburg, F.R. Germany
Phone: 49(203)7687-0 • FAX: 49(203)7666-25
http://www.shimadzu.com.de

SHIMADZU (ASIA PACIFIC) PTE LTD.
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