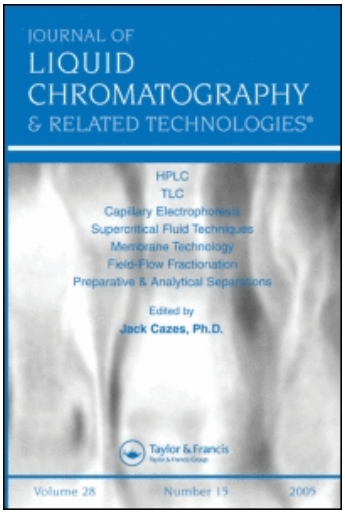

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

DEVELOPMENT AND VALIDATION OF A LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY METHOD, USING SILICA COLUMN AND AQUEOUS-ORGANIC MOBILE PHASE, FOR THE ANALYSIS OF CLONIDINE AS LOW AS 10 pg/mL IN HUMAN SERUM

Mary Pelzer[a]; Thomas Addison[a]; Wenbao Li[a]; Xiangyu Jiang[a]; Naidong Weng[a]
[a] Covance Laboratories, Inc., Madison, WI, U.S.A.

Online publication date: 05 August 2002

J. LIQ. CHROM. & REL. TECHNOL., 25(7), 1019–1032 (2002)

DEVELOPMENT AND VALIDATION OF A LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY METHOD, USING SILICA COLUMN AND AQUEOUS-ORGANIC MOBILE PHASE, FOR THE ANALYSIS OF CLONIDINE AS LOW AS 10 pg/mL IN HUMAN SERUM

Mary Pelzer, Thomas Addison, Wenbao Li, Xiangyu Jiang, and Naidong Weng*

Covance Laboratories, Inc., 3301 Kinsman Boulevard, Madison, WI 53704, USA

ABSTRACT

A bioanalytical method using liquid chromatography–tandem mass spectrometry (LC/MS/MS) was developed and validated for the determination of clonidine in human serum. Clonidine and internal standard clonidine-d_4 were extracted from alkalized human serum using liquid/liquid extraction. The extract was evaporated to dryness, reconstituted, and injected onto a Betasil silica column (50 mm × 3 mm) using an aqueous-organic mobile phase consisting of acetonitrile–water–formic acid (80 : 20 : 1, v/v/v). The flow rate was 0.7 mL/min. The

*Corresponding author. E-mail: naidong.weng@covance.com

chromatographic run time was 2.5 min per injection, with retention time of 1.7 min for clonidine. The detection was by monitoring clonidine at m/z 230 → 213 and IS at m/z 236 → 219, respectively. The standard curve range was 10–1000 pg/mL. The low limit of quantitation (LLOQ) is 10 pg/mL. The inter-day precision and accuracy of the quality control (QC) samples were <6.4% relative standard deviation (RSD) and <7.9% relative error (RE). The recovery of extraction was 95% for clonidine and 90% for the internal standard.

INTRODUCTION

Clonidine is a widely used antihypertensive drug. The chemical structures of clonidine and internal standard clonidine-d_4 are shown in Figure 1. Clonidine stimulates alpha-adrenoreceptors in the brain stem. This action results in reduced sympathetic outflow from the central nervous system and in decreases in peripheral resistance, renal vascular resistance, heart rate, and blood pressure (1). The side effects of orally dosed clonidine such as dry mouth, drowsiness, dizziness, constipation, and sedation are believed to be related to the fluctuation of the clonidine concentration in the blood stream. Therefore, controlled release dosage forms, such as transdermal therapeutic systems capable of minimizing concentration fluctuation and maintaining a steady state over a prolonged period of time, were developed. Newer controlled release dosage forms using a smaller quantity of clonidine but achieving equal or better therapeutical efficacy are under investigations (2,3). A sensitive and reliable bioanalytical method is required to analyze samples generated from clinical trials. For our study, a method that can reliably measure as low as 10 pg/mL of clonidine in human serum was needed.

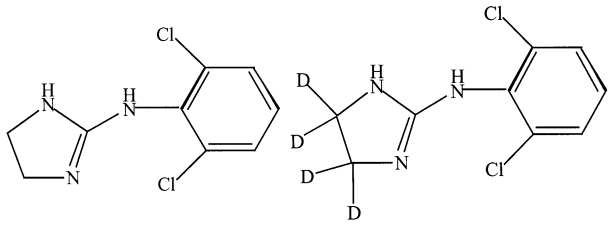

Figure 1. Chemical structures of clonidine and internal standard (clonidine-d_4).

Numerous analytical methods, including radio-immunoassay (4), HPLC (3), GC (5–8), GC/MS (9–13), and LC/MS/MS (14) have appeared in the literature to analyze clonidine in biological fluids. None of the published methods could reliably measure clonidine concentration lower than 50 pg/mL. Methods using non-mass spectrometric detection are tedious and may not have the desired selectivity. The published LC/MS/MS method in a symposium provided the additional selectivity offered by the tandem mass spectrometer, but the method details were not provided. That LC/MS/MS method has the reported sensitivity of 50 pg/mL.

In this paper, we present a highly sensitive LC-MS-MS method for the assay of clonidine in human serum. Particular effort was made to improve the method sensitivity by our novel approach of using a silica column and aqueous-organic mobile phase.

EXPERIMENTAL

Chemicals and Reagents

Clonidine (purity 100%) was from USP (Rockville, MD, USA), and internal standard clonidine-d_4 (isotopic purity 100%) was supplied by Cambridge Isotope Laboratories (Andover, MA, USA). Upon receipt, these reference standards were stored at room temperature and protected from light. Ethyl ether and ammonium hydroxide of ACS reagent grade were from Fisher Scientific (St. Louis, MO, USA). Acetonitrile, methanol, formic acid (FA), and water of HPLC grade were also from Fisher Scientific. Control human serum was from Biochemed (Winchester, VA, USA) and was stored in a freezer at –20°C.

Calibration Standards, Quality Control (QC) Samples, and Sample Extraction

Standards and QC samples were made from two separate stock solutions (1 mg/mL) of clonidine in methanol. Working calibration-spiking solutions at concentrations of 100, 200, 500, 1000, 2000, 5000, 8000, and 10,000 pg/mL were prepared in methanol. The stock and intermediate solutions were stored at 2–8°C, foil-covered. Solutions were stable for at least the duration of the validation, approximately 2 weeks. QC samples at levels of 10.0, 30.0, 150, 750, and 2000 pg/mL were prepared by adding an appropriate amount of clonidine QC intermediate solutions in methanol to blank human serum. The amount of methanol in QC samples was always less than 0.7% (v/v) and serum properties

were not changed. Aliquots of all QC samples were taken and stored frozen at –20°C.

Calibration standards were prepared by taking aliquots of 100 μL of each calibration intermediate solution into 16 × 125 mm glass tubes with screw caps containing 1.0 mL of blank serum. Aliquots (1.0 mL) of QC and blank serum samples were transferred into the glass tubes and 50 μL of internal standard solution (10 ng/mL in methanol) was added to all tubes except blank serum samples. Methanol (150 μL) was added to blank samples and 100 μL of methanol was added to the QC samples. Ammonium hydroxide solution (1 N, 50 μL) was added to each tube and samples were vortex-mixed for 30 seconds. Ethyl ether (5 mL) was added to each tube and samples were vortex-mixed at high setting for 3 minutes. The tubes were then centrifuged at 2500 rpm at room temperature for 5 minutes using a Beckmann Centrifuge Model J6-MC (Fullerton, CA, USA). The lower aqueous layer was frozen in a dry ice/acetone bath and the upper organic layer was decanted into a 13 × 100 mm glass tube. The organic solvent was evaporated to dryness at 40°C under 10-psi nitrogen in a Zymark TurboVap LV Evaporator (Hopkinton, MA, USA). The samples were reconstituted with 200 μL of 0.1% formic acid in acetonitrile by vortex mixing for 2 minutes. The samples were transferred to limited-volume polypropylene vials. The advantage of using a reconstitution solution with elution strength weaker than the mobile phase has been discussed (15).

LC/MS/MS

The LC/MS/MS system consisted of a Shimadzu HPLC system (Kyoto, Japan) and a PE Sciex API 3000 tandem mass spectrometer (Concord, Ontario, Canada) with (+) ESI. The analytical column, Betasil silica of 5 μm, 50 × 3.0 mm I.D., was from Keystone Scientific (Bellefonte, PA, USA). The mobile phase was acetonitrile–water–formic acid (80 : 20 : 1, v/v/v). The injection volume was 25 μL; run time was 2.5 min; flow rate was 0.7 mL/min. Autosampler carry-over was determined by injecting the highest calibration standard then an extracted blank sample. No carry-over was observed. Without any column-regeneration, one column could be used for at least 1000 injections of the extracted samples.

Sensitivity of the multiple reaction mode (MRM) was optimized by testing with an infusion of 0.1 μg/mL clonidine or clonidine-d_4 in a mixture of acetonitrile, water, and FA (50 : 50 : 1, v/v/v). The Ionspray needle was maintained at 5 KV. The turbo gas temperature was 400°C and the auxiliary gas flow was 8.0 L/min. Nebulizing gas, curtain gas, and collision gas flows were at instrument settings of 10, 10, and 10, respectively. The declustering potential (DP) and focusing potential (FP) were at 46 V and 200 V, respectively. The mass spectrometer was operated under MRM mode with a collision energy (CE) of

24 eV. The transitions (precursor to product) monitored were m/z 230 → 213 for clonidine, and 236 → 219 for IS. The dwell time was 300 msec. Both quadrupoles were maintained at unit resolution.

Macintosh MassChrom 1.1, software was used for data processing and MacQuan version 1.6 was used for data analysis. A weighted $1/\text{concentration}^2$ linear regression was used to generate calibration curves from standards and calculate the concentrations of quality control samples.

Validation of the LC/MS/MS Method

The method was validated by three consecutive analytical batches on three separate days. Each calibration batch contained a single set of calibration standards and six replicates of QC samples at each of the three concentration levels. One validation batch also included LLOQ QC samples (10 pg/mL), and over the curve QC samples (2000 pg/mL), which were diluted 5 fold with control blank serum prior to analysis. Each batch also contained other test samples, such as processing and storage stability samples. QC samples and other test samples were interspersed among calibration standards. An extracted blank sample was always placed after the ULOQ (upper limit of quantitation) standard to determine carry-over of the LC/MS/MS system. One curve contained 96 samples to simulate the length of clinical sample analysis.

Analyte stability was tested by subjecting QC samples to 3 freeze-thaw cycles, and maintained at room temperature for 24 hours prior to extraction. Stability of clonidine extracts stored at autosampler conditions (10°C) for 24 hours was also determined.

Recovery was determined by comparing the peak areas of the analyte extracted from serum with those of post-extraction matrix recovery samples fortified with clonidine.

RESULTS AND DISCUSSION

Development of the LC/MS/MS Method

The selection of silica column and aqueous-organic mobile phase for quantitative analysis of clonidine was based on our previous experiences with other polar compounds (16,17). Bare silica columns operated with aqueous-organic mobile phases are a viable means of analyzing polar compounds in biological fluid. Traditionally, reversed-phase LC/MS/MS methods were used for analysis of analytes in biological fluids, although reversed-phase LC/MS/MS for analysis of polar analytes can be a challenge. Highly aqueous mobile phase is

used to retain polar analytes on the reversed-phase columns, but should be avoided in order to achieve spraying conditions at the LC/MS interface necessary for adequate sensitivity. Acidic mobile phases are often used to ensure that the basic analytes are in their protonated forms. Protonation is the most important means of ionization in positive ion electrospray mass spectrometry. However, protonated analytes will have even poorer retention on reversed-phase column and may be subject to matrix suppression.

Here, we present use of LC/MS/MS on silica columns with aqueous-organic mobile phase to analyze polar analytes such as clonidine in biological fluids. The mobile phase consisted of acetonitrile–water–formic acid (80 : 20 : 1). The acetonitrile content (80%) in the mobile phase is significantly higher than what is typical (<20%) when using a reversed-phase C_{18} column. The sensitivity is improved because of the increased organic content in the mobile phase. The significant amount of water (20%) in the mobile phase signifies our novel use of a silica column. Unlike classic normal-phase LC, where the trace amount (in the ppm range) of water in the mobile phase had to be strictly controlled, our mobile phase can be very easily prepared and the LC/MS/MS condition is completely compatible with the contemporary biological sample extraction techniques. The acidic mobile phase nature ensures the protonation and sensitivity of the basic analyte such as clonidine in the mobile phase.

Figures 2–4 show the chromatograms of 10.0 pg/mL (LLOQ) in serum, 1000 pg/mL (ULOQ) in serum, and serum blank, respectively. The sensitivity and specificity of the method is demonstrated. All six lots of human serum were shown to be free of interference for clonidine and the IS. No interference from clonidine to internal standard and vice verse was observed. The mass transition 230 → 213 was monitored for clonidine and the clonidine-d_4 mass transition monitored would normally be 234 → 217. However, the IS did show an interference from isotopic effect using that transition. Therefore, the transition 236 → 219 was chosen as the IS channel. No isotopic effects were observed in the 236 → 219 channel. The silica column demonstrated superior stability under the chromatographic conditions. At least 500 samples can be analyzed on a single silica column.

Validation Results

Extraction recoveries were determined by comparing the peak areas of extracted QC samples with peak areas of matrix recover samples prepared by adding compounds to post-extraction serum blanks at the corresponding concentrations. The extraction recoveries for clonidine and internal standard clonidine-d_4 were determined at 30 pg/mL, 150 pg/mL, and 750 pg/mL concentrations. The mean recoveries of clonidine were 98% (n = 6, RSD = 8.5%) at 30 pg/mL, 93% (n = 6, RSD = 3.0%) at 150 pg/mL, and 95%

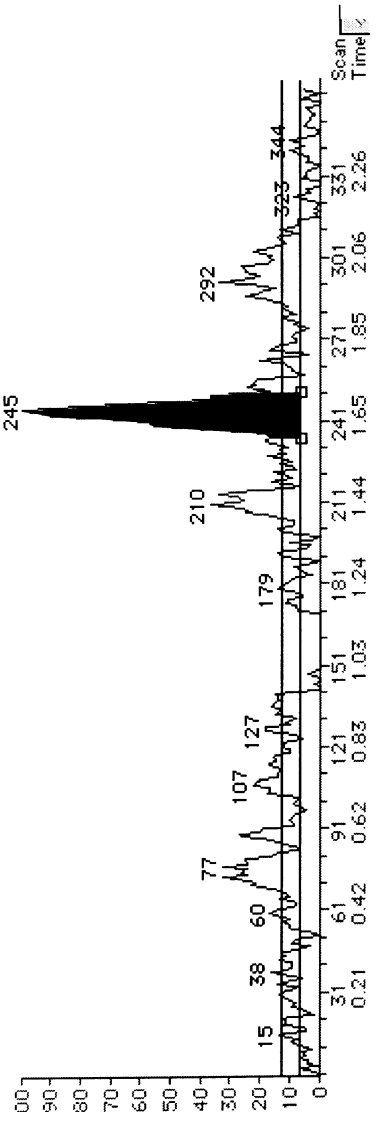

Figure 2. Chromatogram of a blank serum sample spiked with clonidine at LLOQ (10 pg/mL).

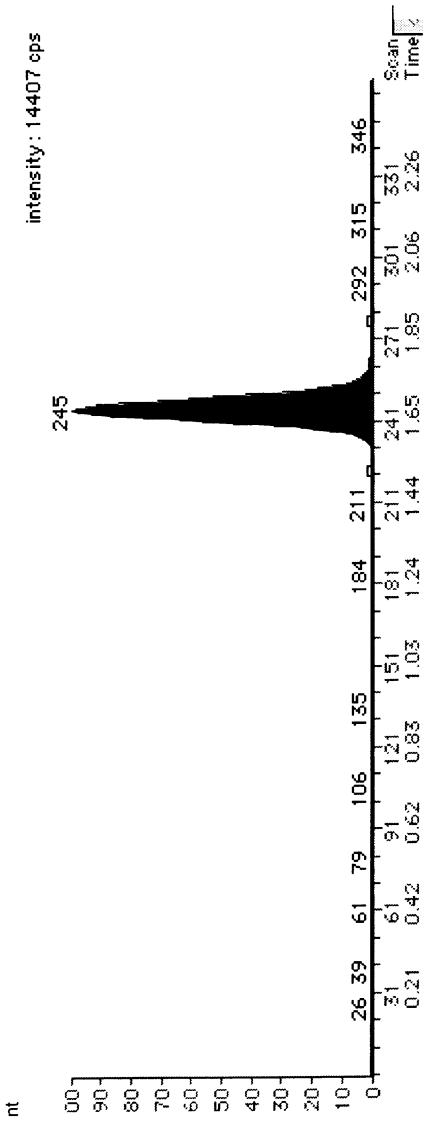

Figure 3. Chromatogram of a blank serum sample spiked with clonidine at ULOQ (1000 pg/mL).

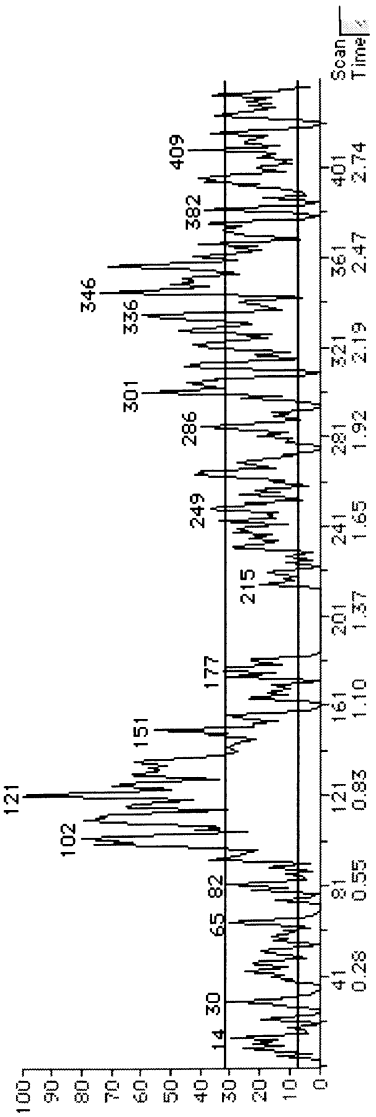

Figure 4. Chromatogram of a blank serum sample.

Table 1. Precision and Accuracy of Calibration Standards

(pg/mL)	10.0	20.0	50.0	100	200	500	800	1000	Slope	r^2
Batch 1	9.91	20.2	50.5	101	201	491	808	980	3.18E − 03	0.9999
Batch 2	10.1	19.4	49.5	99.3	211	488	787	1020	3.29E − 03	0.9995
Batch 3	10.1	19.4	49.9	103	197	505	832	960	3.21E − 03	0.9995
Mean	10.0	19.7	50.0	101	203	495	809	987	3.23E − 03	0.9996
RSD (%)	1.1	2.3	1.0	1.8	3.6	1.8	2.8	3.1	1.7	
RE (%)	+0.4	−1.7	−0.1	+1.1	+1.5	−1.1	+1.1	−1.3		

Table 2. Precision and Accuracy of Quality Control Samples

	Intraday (n = 6)					Interday (n = 18)		
(pg/mL)	10.0	30.0	150	750	2000*	30.0	150	750
Mean	9.77	30.5	162	774	2165	31.1	162	780
RSD (%)	14.9	8.0	2.2	1.9	4.5	6.4	3.1	2.9
RE (%)	–2.3	+1.7	+7.7	+3.1	+8.3	+3.7	+7.9	+3.9

*Samples were diluted five fold with blank serum prior to analysis.

(n = 6, RSD = 1.7%) at 750 pg/mL. The overall recovery for clonidine was 95%. The overall recovery for the IS, which was 500 pg/mL in serum, was 90% (n = 18, RSD = 1.7%). Acceptable and consistent recoveries were obtained for both clonidine and internal standard.

The accuracy and precision data were generated using 3 validation batches. One of the batches were assayed using a different lot of analytical column to assess ruggedness of the method. Calibration curve parameters and data are listed in Table 1. The correlation coefficients of the three validation curves were all >0.999. The standards were linear in the range of 10–1000 pg/mL, using weighted ($1/\text{concentration}^2$) least-square linear regression. The precision and accuracy data for QC samples are summarized in Table 2. The data show that this method is consistent and reliable with low RSDs and REs values. For the LLOQ QC samples, the RSD (n = 6) of the measured concentration was 14.9%. The relative error of the mean of the measured concentrations was −2.3%.

The stability tests were designed to cover the anticipated conditions that the clinical samples may experience. Stability during sample handling (repeated freeze-thaw and bench-top) and for processed sample extracts was tested and established. The results are summarized in Table 3. Three freeze/thaw cycles and ambient temperature storage of the QC samples for up to 24 hours prior to

Table 3. Stability of the Samples

	Concentration (pg/mL)		
	30.0	150	750
Control (n = 18)			
Mean	31.1	162	780
RSD (%)	6.4	3.1	2.9
3 Freeze/thaw cycles (n = 3)			
Mean	30.5	167	797
RSD (%)	1.9	2.6	1.7
As % of Control	98.1	103	102
24 hours bench-top (n = 3)			
Mean	31.4	167	798
RSD (%)	8.2	0.3	0.7
As % of Control	101	103	102
24 hours extract (n = 3)			
Mean	34.3	167	821
RSD (%)	1.1	1.4	2.0
As % of Control	110	103	105

extraction, appeared to have little effect on the quantitation. QC samples stored in a freezer at –20°C remained stable through the course of the validation. Extracted calibration standards and QC samples were allowed to stand at 10° for 24 hours prior to injection. No effect on quantitation of the calibration standards or QC samples was observed.

CONCLUSION

An easy, sensitive, and reliable LC/MS/MS method for the measurement of clonidine in human serum has been successfully developed and validated. A silica column and an aqueous-organic mobile phase were used to improve the sensitivity. The LLOQ is 10 pg/mL and the analytical run time is only 2.5 minutes per sample.

REFERENCES

1. *Physicians' Desk Reference*®, 54th Ed.; Medical Economics Company, Inc.: Montvale, NJ, 2000; 794–795.
2. Fujimura, A.; Sasaki, M.; Harada, K.; Kumagai, Y.; Ohashi, K.; Ebihara, A. Influences of Bathing and Hot Weather on the Pharmacokinetics of a New Transdermal Clonidine, m-5041t. J. Clin. Pharmacol. **1996**, *36* (10), 892–896.
3. Rosansky, S.; Johnson, K.L.; McConnell, J. Use of Transdermal Clonidine in Chronic Hemodialysis Patients. Clin. Nephrol. **1993**, *39* (1), 32–36.
4. Arndts, D.; Stahle, H.; Forster, H.J. Development of a Ria for Clonidine and Its Comparison with the Reference Methods. J. Pharmacol. Methods **1981**, *6* (4), 295–307.
5. Nazarali, A.; Baker, G.B.; Boisvert, D.P. Analysis of Clonidine in Biological Tissues and Body Fluids by Gas Chromatography with Electron-Capture Detection. J. Chromatogr. **1986**, *380* (2), 393–400.
6. Velasquez, M.T.; Rho, J.; Maronde, R.F.; Barr, J. Plasma Clonidine Levels in Hypertension. Clin. Pharmacol. Ther. **1983**, *34* (3), 341–346.
7. Edlund, P.O. Determination of Clonidine in Human Plasma by Glass Capillary Gas Chromatography with Electron-Capture Detection. J. Chromatogr. **1980**, *187* (1), 161–169.
8. Chu, L.C.; Bayne, W.F.; Tao, F.T.; Schmitt, L.G.; Shaw, J.E. Determination of Submicrogram Quantities of Clonidine in Biological Fluids. J. Pharm. Sci. **1979**, *68* (1), 72–74.

9. Yamahata, T.; Dote, S.; Ozawa, Y.; Nishikawa, H.; Maeda, S. Determination of Clonidine in Human Plasma by Gas Chromatography-Electron-Impact Mass Spectrometry. J. Chromatogr. B. **1994**, *653* (1), 92–97.
10. Girault, J.; Fourtillan, J.B. Quantitative Measurement of Clonidine in Human Plasma by Combined Gas Chromatography/Electron Capture Negative Ion Chemical Ionization Mass Spectrometry. Biomed. Environ. Mass Spectrom. **1988**, *17* (6), 443–448.
11. Murray, S.; Davies, D.S. Bis(trifluoromethyl)aryl Derivatives for Drug Analysis by Gas Chromatography Electron Capture Negative Ion Chemical Ionization Mass Spectrometry. Application to the Measurement of Low Levels of Clonidine in Plasma. Biomed. Mass Spectrom. **1984**, *11* (8), 435–440.
12. Murray, S.; Waddell, K.A.; Davies, D.S. The Measurement of Clonidine in Human Plasma and Urine by Combined Gas Chromatography Mass Spectrometry with Ammonia Chemical Ionization. Biomed. Mass Spectrom. **1981**, *8* (10), 500–502.
13. Arrendale, R.F.; Stewart, J.T.; Tackett, R.L. Determination of Clonidine in Human Plasma by cold On-Column Injection Capillary Gas Chromatography-Selected-Ion Monitoring. Mass Spectrom. **1988**, *432*, 165–175.
14. McMullin, M.M.; Diamont, F.X.; Monahan, R. Analysis for Clonidine by LC-MS-MS, and a Comparison of Patient Values Using LC-MS-MS Versus GC-NPD. Clin. Chem. **1998**, *44* (6), A86.
15. Naidong, W.; Chen, Y-L.; Shou, W.; Jiang, X. Importance of Injection Solution Composition for LC-MS-MS Methods. J. Pharm. Biomed. Anal. **2001**, *26* (5/6), 753–767.
16. Naidong, W.; Shou, W.; Chen, Y-L.; Jiang, X. Novel Liquid Chromatographic-Tandem Mass Spectrometric Methods Using Silica Columns and Aqueous-Organic Mobile Phases for Quantitative Analysis of Polar Ionic Analytes in Biological Fluids. J. Chromatogr. B **2001**, *754*, 387–399.
17. Shou, Z.; Jiang, X.; Beato, B.B.; Naidong, W. A Highly Automated 96-Well Solid Phase Extraction and Liquid Chromatography/Tandem Mass Spectrometry Method for the Determination of Fentanyl in Human Plasma. Rapid Commun. Mass Spectrom. **2001**, *15*, 466–476.

Received December 17, 2001
Accepted January 14, 2001
Manuscript 5703