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Automated 96-Well SPE and LC−MS−MS for Determination of Protease Inhibitors in Plasma and Cartilage Tissues

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Bioanalytical methods based on automated solid-phase extraction (SPE) and high-performance liquid chromatography with electrospray tandem mass spectrometry (LC−MS−MS) have been developed and utilized for the determination of MMP inhibitors in plasma and cartilage tissues. The SPE methods were automated using a 96-well extraction plate and a 96-channel programmable liquid-handling workstation. The LC−MS−MS methods were developed using a rapid gradient LC separation, followed by sample introduction through an ionspray interface in the positive ion mode and tandem mass spectrometric detection with selected reaction monitoring. In the optimized SPE methods, crude plasma or ground cartilage supernatant samples were loaded onto an SPE plate to remove proteins and other interfering components in the matrixes to render relatively clean extracts for LC−MS−MS analysis. Compared to the simple plasma protein precipitation method, the automated SPE method afforded significant time-saving in sample preparation and improved sensitivity in MS detection. The methods were validated and successfully applied to the analysis of protease inhibitors in plasma and cartilage tissues.

Hydroxamic acid-based matrix metalloprotease (MMP) inhibitors are actively being explored as potential drug candidates for the treatment of cancers, arthritic disorders, and other connective tissue-related diseases. In the screening and selection of these compounds for the targeted disease indications, the pharmacokinetic properties of each compound can play a key role in making a decision on whether to advance a compound for further studies. In support of pharmacokinetic and animal efficacy studies, rapid, sensitive, and selective analytical methods are required for the routine determination of drug levels in biological fluids and tissues. Since a drug must have sufficient amounts available in the blood stream and be delivered to the site of action to produce its therapeutic effects, it is important to develop reliable bioanalytical methods to measure drug levels in plasma (or blood) and the target organs. When the hydroxamic acid-based compounds are targeted for arthritic disorders, the drug concentrations in articular cartilage tissues need to be determined since a certain drug level is required to block MMP activities in cartilage. High-performance liquid chromatography−tandem mass spectrometry (LC−MS−MS) has been widely used for the analysis of drug compounds in biological matrixes because of its excellent specificity and high sensitivity. However, the electrospray ionization process in a typical LC−MS−MS method is very sensitive to the interferences from the potential coelution of some endogenous components in the biological fluids and animal tissues, affecting ionization efficiency, reproducibility, and accuracy of the analysis.[1−3] To minimize these interferences and improve overall ruggedness, an effective sample cleanup procedure can be utilized to make the best use of the LC−MS−MS methodology.

Traditionally, we have used a simple plasma protein precipitation procedure for plasma sample preparation. This method is universal for all plasma samples. However, this approach only removes plasma proteins, leaving other plasma matrix components in the sample. When an electrospray or ion spray interface is employed for sample introduction and a rapid assay time is desired for higher throughput, coeluting matrix components can cause ion suppression and reduce the ion intensity of the analyte. This effect can also decrease reproducibility and accuracy of the assay. Sample preparation has been a key step for accurate and reliable LC−MS−MS assays and has been the subject of several recent reviews.[4−6] Solid-phase extraction (SPE) has been a widely utilized technique for biological sample preparation not only because of its ease for automation but also because of its ability to selectively remove interfering matrix components. Recent development and commercialization of 96-channel robotic liquid-handling workstations[7] as well as a wide selection of 96-well SPE sorbents[8] allow the rapid development and automation of SPE methods to eliminate traditional time-consuming and labor-intensive biological sample preparation steps for plasma[9,10] and urine samples.[11] The

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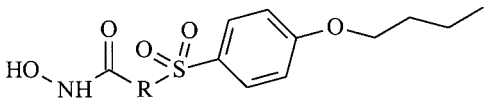
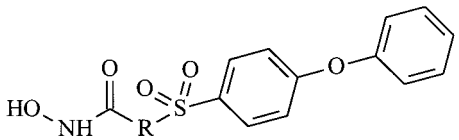


Compound I



Compound II

Figure 1. Structures of **I** and **II**.

advantages of sample preparation by SPE for LC−MS and LC−MS−MS analyses include the removal of nonvolatile components and a relatively clean extract with reduced interfering matrix components, allowing rapid LC−MS−MS runs and improved sensitivity of MS detection. SPE automation in a 96-well format significantly reduces sample preparation time, increases sample throughput, improves reproducibility, and frees the analyst from repetitive manual preparation. This paper reports the development and application of automated 96-well SPE and LC−MS−MS methods for the determination of two hydroxamic acid-based MMP inhibitors in plasma and cartilage tissues.

EXPERIMENTAL SECTION

Materials. Hydroxamic acid-based protease inhibitors, compounds **I** and **II** (see Figure 1), as well as isotopically labeled analogues of the analytes (as internal standards), IS-**I** and IS-**II**, were synthesized by Procter & Gamble Pharmaceuticals (Mason, OH). HPLC-grade acetonitrile, methanol, and formic acid were purchased from J. T. Baker (Phillipsburg, NJ). Blank rat plasma was obtained from Rockland (Gilbertsville, PA) while articular cartilage was obtained from Procter & Gamble Pharmaceuticals.

Sample Preparation. Stock solutions of both the compounds and internal standards were prepared in 20% acetonitrile in water to give concentrations of 1.0 mg/mL and 100 μg/mL, respectively. Plasma standards were made by diluting the respective stock solutions in blank rat plasma to yield nine standard solutions ranging from 0.1 to 200 ng/mL. Quality control (QC) samples were prepared as described for the calibration standards to give four concentration levels: 0.6 (low-level QC), 6 (mid-level QC), 150 (high-level QC), and 1000 ng/mL (over-the-curve QC). Cartilage standards were prepared by adding appropriate amounts of the stock solution to 1.5 mL of ground cartilage supernatant (obtained from grinding 5 mg of wet cartilage tissue with 1.5 mL of methanol) to yield 11 concentration levels in a range of 0.1−300 ng/mL. The QC cartilage samples were also made in the same manner as the cartilage standards to give four concentration levels: 0.6, 30, 220, and 1000 ng/mL. All above plasma and cartilage standards and QC samples were subject to automated 96-well SPE prior to being injected into the LC−MS−MS system.

Automated 96-Well SPE Extraction Apparatus and Procedures. A 96-channel programmable liquid-handling workstation (Quadra 96, model 320, Tomtec, Hamden, CT) and Waters Oasis HLB extraction plate (Milford, MA) were utilized for the automation of SPE procedures for plasma and cartilage samples. The 96-well sample source plate was first prepared by transferring 100-μL aliquots of plasma samples including calibration standards and quality control samples to an empty plate. Then 50-μL aliquots of internal standard dissolved in 20% acetonitrile in water (100 ng/mL) were added to the 96-well sample source plate by the Quadra 96. A vacuum manifold system was used during the entire SPE process with a low vacuum maintained at 10-in. Hg. The SPE plate was placed on top of the vacuum manifold while a waste tray (collecting eluate from conditioning, loading, and washing steps) or a collection plate (collecting the eluate from elution step) was put inside the manifold. The Quadra 96 was programmed to perform the SPE procedures as described below. The SPE plate was first conditioned with 200 μL of methanol and water sequentially. Then the Quadra 96 aspirated 130-μL aliquots of the sample from the source plate to load onto the SPE plate. The SPE plate was then washed with 400 μL of 5% methanol in water to a waste tray and eluted with 400 μL of 95% acetonitrile and 0.1% formic acid in water to a 96-well autosampler collection plate filled with empty LC vials. The collection plate was then placed in a heating block at 50 °C and evaporated to dryness under nitrogen. The dry extract residues were reconstituted with 100 μL of 20% acetonitrile in water by the Quadra 96. Each aspiration step by the Quadra 96 was proceeded with 50 μL of air gap to aid in complete and accurate dispensing.

For cartilage samples, 500 μL of methanol was added to each crude cartilage sample (an average wet weight of 5 mg) placed in a 5-mL glass tissue grinder (DUALL 22, Kontes Glass Co., Vineland, NJ) with a pestle connected to and driven by an overhead motor drive unit. After 1 min of grinding, the supernatant was taken and the above grinding step was repeated three times. The pooled cartilage supernatant was diluted in Milli-Q water and transferred to a sample source plate for subsequent SPE. The SPE was performed in the same manner as that described for the plasma samples except that 10% methanol in water was used as a wash solution.

Liquid Chromatography. HPLC was performed with Gilson 305, 306 pumps (Norwalk, CT) and a Leap CTC HTS PAL autosampler (Carrboro, NC). Samples were injected from the 96-well autosampler plates with an injection volume of 20 μL. The LC separation was achieved on Phenomenex (Torrance, CA) Luna C8(2) column (2.0 × 50 mm, 3 μm, 100 Å) with a 3-min linear gradient elution from mobile phase A (acetonitrile/water/formic acid; 5:95:0.1; v/v/v) to mobile phase B (acetonitrile/water/formic acid; 80:20:0.1; v/v/v). A constant mobile-phase flow rate was maintained at 0.4 mL/min. The effluent from the column was directly transferred to the TurboIonSpray interface for MS detection.

TurboIonSpray Tandem Mass Spectrometry. Tandem MS was carried out on a Perkin-Elmer Sciex API 3000 triple-quadruple tandem mass spectrometer (Toronto, ON, Canada) using a TurboIonSpray interface in the positive-ion mode. The TurboIonSpray source consisting of a nebulizing ion spray probe and a heated turbo probe was operated with the following key parameters: The turbo probe temperature was set at 450 °C with a drying nitrogen gas flow of 8 L/min; the ion spray voltage was maintained at +2200 V while the orifice plate potential (OR) was

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Table 1. Intra- and Interassay Precision, Accuracy, and Recovery Data for Plasma Samples of Compound **I** after Automated 96-Well SPE ($n = 6$)

concn (ng/mL)	precision (% RSD)		interassay accuracy (%)	recovery (%)
	intraassay	interassay		
0.6	8.7	6.2	95.4	89.5
6.0	4.3	2.3	103.0	92.1
150	1.6	3.1	97.8	87.3
1000	2.8	3.0	102.0	91.7

Table 2. Intra- and Interassay Precision, Accuracy, and Recovery Data for Cartilage Samples of Compound **II** after Automated 96-Well SPE ($n = 6$)

concn (ng/mL)	precision (% RSD)		interassay accuracy (%)	recovery (%)
	intraassay	interassay		
0.6	3.3	4.3	103.0	91.6
30	1.2	2.1	97.3	90.4
220	3.0	2.4	108.0	93.1
1000	4.1	3.8	107.1	92.2

set at +31 V and the focusing ring potential at +180 V; the nebulizer and curtain nitrogen gas flow rates were 1.2 and 0.95 L/min, respectively (corresponding to the instrument settings of 9 and 8). The tandem MS analysis was performed by selected reaction monitoring (SRM) with the following key parameters: The first quadrupole (Q1) was set to monitor the precursor ions $(M + H)^+$ at m/z 359 and 365 for **I** and IS-**I**, m/z 379 and 365 for **II** and IS-**II**, respectively. The third quadrupole (Q3) was adjusted to monitor product ions at m/z 298 and 304 for **I** and IS-**I** and m/z 233 and 219 for **II** and IS-**II**. Unit mass resolution was utilized for both Q1 and Q3 mass-resolving quadrupoles (full peak width at half-height at ~0.7 Da). A collision energy of 32 V and a collision nitrogen gas thickness of 1.8×10^{15} molecules/cm^2 (instrument setting, 4) were selected for collisionally activated dissociation in the second quadrupole (Q2). The dwell time was 300 ms for each SRM channel with a 5-ms pause between scans. All ion source and tandem MS instrument parameters were optimized for high sensitivity by infusing a standard solution of 2 μg/mL at a flow rate of 10 μL/min using an infusion pump (Harvard Apparatus, South Natick, MA).

RESULTS AND DISCUSSION

Automated 96-Well SPE. The selection of the Oasis HLB extraction plate as the SPE plate of the choice for our application was made based on the best recovery, least variability, and optimal selectivity using the same SPE conditions across different types of SPE sorbents, including C8 and C18 stationary phases. The SPE conditions were optimized manually using portions of a 96-well plate.

For plasma samples, the wash solvents of various methanol percentages with and without formic acid added were evaluated. Results indicated that higher methanol percentage or addition of formic acid lowered the analyte recovery. A wash solvent consisting of 5% methanol in water without formic acid was found optimal in terms of selectivity and recovery. However, an elution solvent of 95% acetonitrile containing 0.1% formic acid was found to give the best recovery. This is most likely attributed to the reduced

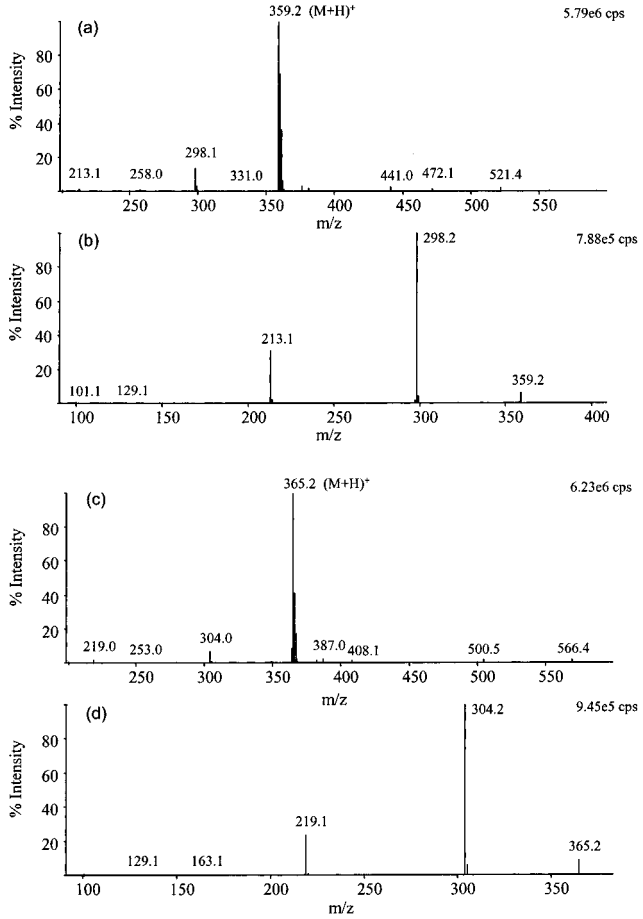

Figure 2. Positive ion spray full-scan mass spectra of **I** (a) and IS-**I** (c), as well as product ion scan mass spectra of precursor ions at m/z 359 for **I** (b) and m/z 365 for IS-**I** (d).

retention of the protonated molecules when formic acid was added in the elution solvent. Comparison of results from various wash and elution solvent volumes also indicated that 400 μL of solvent for both wash and elution yielded the highest recovery. For cartilage samples, the optimal SPE conditions were found to be the same as those used for the plasma samples except that 10% methanol in water was used as the wash solution because it gave better selectivity while yielding the same recovery as 5% methanol in water. The SPE procedures helped remove nonvolatile salts and matrix interferences, allowing faster LC runs compared to the protein precipitation approach. Additionally, since the SPE procedures were automated using a 96-channel robotic liquid-handing workstation (Quadra 96), a significant time-saving was achieved compared to the simple plasma protein precipitation procedure, where an additional vortex−centrifugation step was needed to separate plasma proteins from the analyte even with the use of the Quadra 96 for automation.

The extraction recovery was assessed by comparing the peak area ratios of the analyte to the internal standard between the samples spiked with the analyte both before and after extraction of the same levels, with the internal standard spiked postextraction in both cases. The recovery values at various concentration levels under the optimal SPE conditions are shown in Tables 1 and 2. The extraction recovery was averaged at 90% for the plasma samples of **I** and 92% for the cartilage samples of **II**.

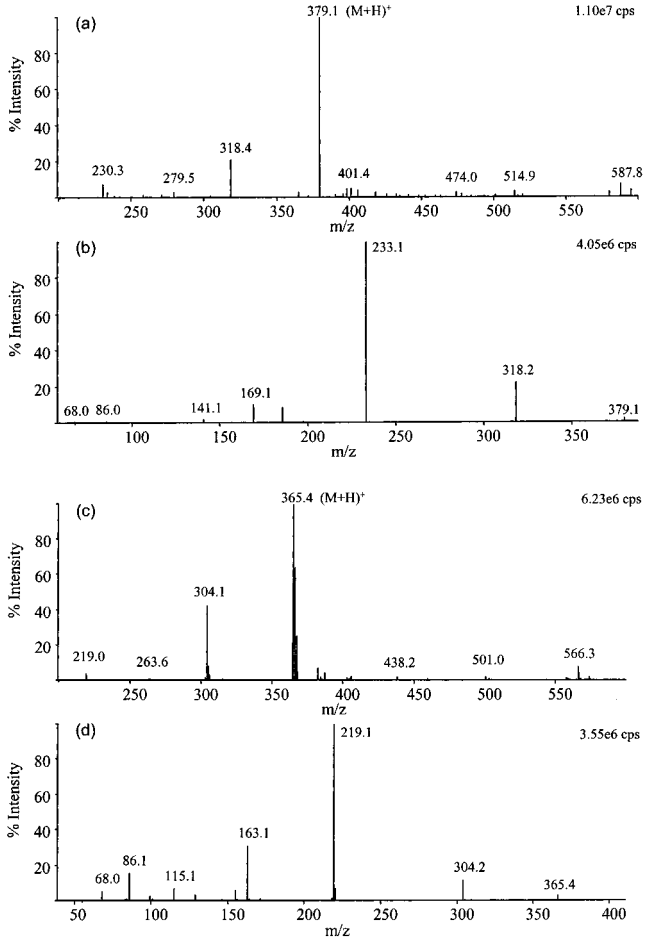

Figure 3. Positive ion spray full-scan mass spectra of **II** (a) and IS-**II** (c), as well as product ion scan mass spectra of precursor ions at *m*/*z* 379 for **II** (b) and *m*/*z* 365 for IS-**II** (d).

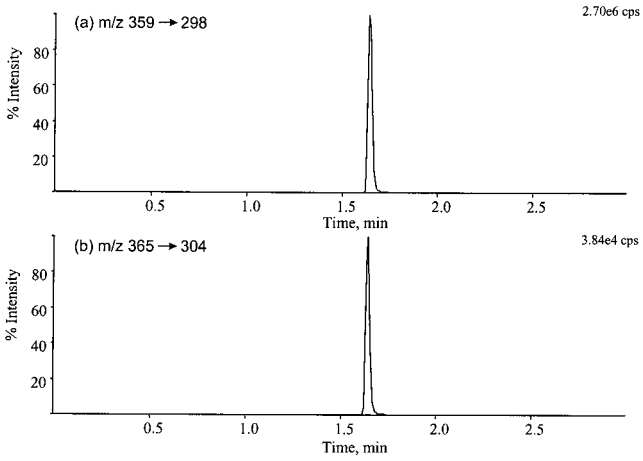

Figure 4. SRM chromatograms of plasma samples of **I** (a) and IS-**I** (b) at a concentration of 5 ng/mL.

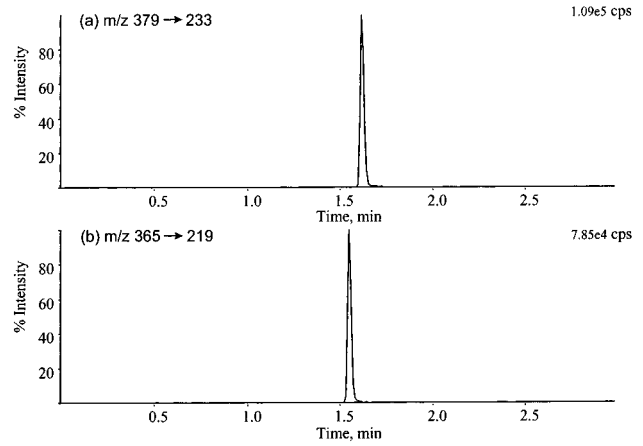

Figure 5. SRM chromatograms of cartilage samples of **II** (a) and IS-**II** (b) at a concentration of 5 ng/mL.

Liquid Chromatography and Mass Spectrometry. A rapid gradient elution was developed and employed to increase the sample throughput of the methods. The fast LC−MS−MS runs were afforded because of the relatively clean sample extracts from the SPE in which interferences from the biological matrixes were drastically reduced, resulting in less demands on separation and thus shorter chromatographic run time. The full-scan ion spray mass spectra of **I** and **II** and their corresponding product ion scan spectra from the protonated molecular ions, along with the mass spectra of their respective internal standards, are shown in Figures 2 and 3. These mass spectra were obtained from LC−MS−MS runs of **I** and **II** containing their respective internal standards. As shown in Figure 2a,c, there were base peaks at *m*/*z* 359 and 365, corresponding to the protonated molecular ions $(M + H)^+$ of **I** and its stable-isotope labeled internal standard IS-**I**. In Figure 2b,d, the dominant product ions were *m*/*z* 298 and 304 from their corresponding precursor ions at *m*/*z* 359 and 365, respectively. Therefore, the SRM transitions were selected at *m*/*z* 359 → 298 and 365 → 304 for best sensitivity. Similarly, the SRM transition pairs were set to *m*/*z* 379 → 233 and 365 → 219 for **II** and IS-**II** based on the mass spectra in Figure 3, where *m*/*z* 379 and 365 were the protonated molecular ions of **II** and its internal standard whereas *m*/*z* 233 and 219 were the predominant product ions of their respective precursor ions.

The representative SRM chromatograms for **I** (*m*/*z* 359→298) and its internal standard (*m*/*z* 365→304) in plasma are shown in Figure 4a and b, respectively. Both the analyte and internal standard were eluted at 1.67 min. For **II** in cartilage, the analyte was eluted at 1.62 min while the internal standard was at 1.57 min, as indicated in the SRM chromatograms (*m*/*z* 379 → 233 for **II** and *m*/*z* 365 → 219 for its internal standard) in Figure 5a and b, respectively.

Precision and Accuracy. Intraassay precision and accuracy were determined by analyzing blank plasma and cartilage supernatant matrixes spiked with the respective compounds and internal standards at different concentrations with six replicate injections. The intraassay precision ranged 1.6−8.7% RSD for **I** in plasma samples and 1.2−4.1% RSD for **II** in cartilage samples. The interassay precision was less than 6.2% RSD for **I** in plasma and 4.3% RSD for **II** in cartilage, respectively. The interassay accuracy was also assessed at different concentration levels, ranging from 95.4 to 103% for **I** and 97.3−108% for **II**. All these assay results are detailed in Tables 1 and 2.

Specificity and Linearity of Calibration. For the plasma samples of **I** and the cartilage samples of **II**, the specificity was evaluated by examination of the SRM ion chromatograms at the transitions for both the analyte and internal standard. As shown in Figures 6 and 7, no interference was observed in all SRM

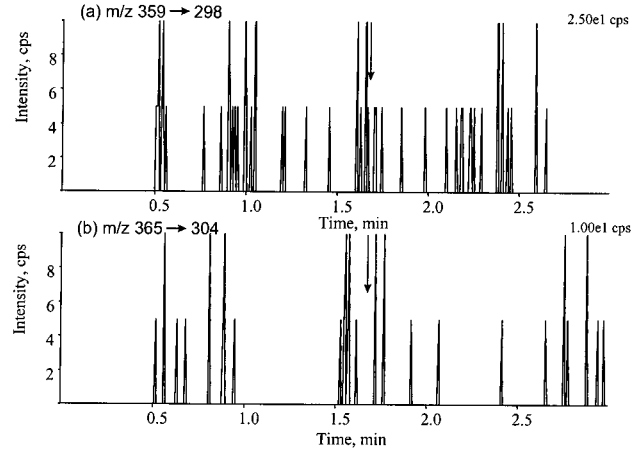

Figure 6. SRM chromatograms of a double blank plasma sample at transitions *m*/*z* 359 → 298 (a) and 365 → 304 (b). Arrows indicate the expected retention times of the analyte and internal standard.

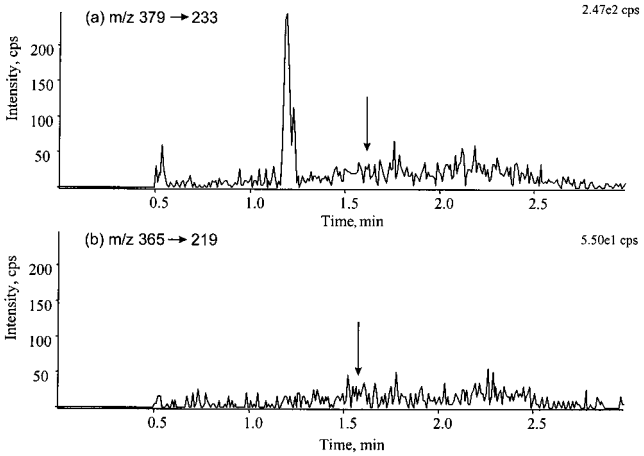

Figure 7. SRM chromatograms of a double blank cartilage sample at transitions *m*/*z* 379 → 233 (a) and 365 → 219 (b). Arrows indicate the expected retention times of the analyte and internal standard.

channels from double blank samples (neither the analyte nor the internal standard was present) of plasma and cartilage extracts at their respective retention times.

The calibration curve was constructed by a weighted linear least-squares regression of standard concentrations against the peak area ratios of the analyte to the internal standard. For the plasma samples of **I**, the peak area ratios for the standards typically gave a linear calibration curve in the range of 0.2−200 ng/mL with the correlation coefficient of greater than 0.998 using a $1/x$-weighted linear regression model. For the cartilage samples of **II**, the calibration curve was linear in the range of 0.5−300 ng/mL with the correlation coefficient of greater than 0.999 using a $1/x^2$-weighted linear regression model.

Analyte Stability and Limits of Detection and Quantitation. The stability of **I** and **II** was evaluated in dissolution solvent (20% acetonitrile in water) and in their respective matrixes (plasma or cartilage). It was found that **I** was stable for at least 3 months in plasma and **II** was stable for 2 months in cartilage at −20 °C. The compounds were also stable at ambient temperature in their respective matrixes and dissolution solvents for the entire duration of sample preparation.

The lower limits of detection (LODs), at a signal-to-noise ratio of 3, were 0.2 ng/mL for **I** in plasma and 4 ng/g (or 0.1 ng/mL) for **II** in cartilage. The lower limits of quantitation (LOQs), at a signal-to-noise ratio of 10, were 0.6 ng/mL for I in plasma and 12 ng/g in cartilage.

CONCLUSIONS

The combination of automated 96-well SPE and LC−MS−MS provided increased sample throughput and improved sensitivity for the analysis of MMP inhibitors in plasma and cartilage tissues. The high throughput and effective sample cleanup were realized using a 96-well SPE plate and a 96-channel robotic liquid-handling workstation. The automation of SPE in 96-well format reduced the time needed for sample preparation and freed up the analyst for other tasks. The resulting relatively clean sample extracts afforded the development of rapid and sensitive LC−MS−MS assays. The high sensitivity and specificity of the assay methods were accomplished using liquid chromatography with ion spray tandem mass spectrometry in the positive ion SRM mode. The limits of detection for the two hydroxamic acid-based compounds were 0.2 ng/mL in plasma and 4 ng/g in cartilage tissues. The developed bioanalytical methods were sensitive, selective, and reproducible, allowing rapid determination of protease inhibitor compounds in plasma and cartilage tissues in an automated 96-well high-throughput format.

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