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Review

Separation and identification methods for metalloproteinase inhibitors

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Abstract

Metalloproteinase inhibitors are being explored for the treatment of a wide variety of human diseases including cancers, arthritis, cardiovascular disorders, human immunodeficiency virus infection, and central nervous system illnesses. This review provides an overview of various analytical sample preparation, separation, detection, and identification techniques employed for the quantitative and qualitative determination of these inhibitor compounds. Special emphasis is placed on biological sample preparation by automated solid-phase extraction, liquid–liquid extraction, and protein precipitation by centrifugation or filtration. Other sample preparation methodologies are also evaluated. Applications of high-performance liquid chromatography, gas chromatography, and capillary electrophoresis to the quantitative determination of metalloproteinase inhibitors are described. Examples of qualitative analysis of metalloproteinase inhibitors by hyphenated liquid chromatography with mass spectrometry and nuclear magnetic resonance are also presented. The advantages and limitations of these separation and identification methodologies as well as other less frequently employed techniques are assessed and discussed. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Metalloproteinase inhibitors

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1. Introduction

Metalloproteinase is one of the four most actively studied classes of proteinases — aspartic, cysteine, metallo, and serine — that are responsible for the hydrolysis of peptide bonds of various proteins regulating a variety of physiological and pathological processes [1,2]. The inhibition of these proteinase enzymes has been shown to effectively treat various human diseases. Metalloproteinases are a class of metal (typically zinc) containing enzymes catalyzing amide bond hydrolysis. Among them, matrix metalloproteinases (MMP), tumor necrosis factor-α convertase (TACE), angiotensin-converting enzyme (ACE), and neutral endopeptidase (NEP) are all zinc-containing metalloproteinases. While the inhibitors of ACE and NEP are mainly targeted at cardiovascular disorders such as hypertension, the inhibition of TACE is explored for the treatment of inflammatory diseases such as rheumatoid arthritis and multiple sclerosis. In contrast, MMP inhibitors are being developed to treat cancers, inflammatory disorders, and degenerative diseases [2]. MMPs are a family of structurally related zinc-containing enzymes that degrade and remodel structural proteins in the extracellular matrix, such as collagens, proteoglycans, fibronectin, elastin, and laminin. There are currently at least 20 known MMPs that mediate the remodeling and breakdown of connective tissues. These enzymes are inhibited naturally by endogenous proteinase inhibitors, mainly a family of tissue inhibitors of metalloproteinases (TIMPs). The MMPs are finely regulated by TIMPs in normal tissue remodeling. However, in the disease state, inappropriate expression and activation of MMPs cause an imbalance, leading to tissue degradation. Many disease processes start with degradation of connective tissues in the extracellular matrix. Therefore, MMPs are implicated in a wide variety of diseases including rheumatoid arthritis and osteoarthritis [3], cancers [4], multiple sclerosis [5], congestive heart failure [6], periodontal diseases [7], gastric ulceration [8], and human immunodeficiency virus (HIV) infection [9].

Cancer is one of the most actively pursued therapeutic areas where MMPs are involved in many disease stages. In cancer, most deaths come from the results of tumor metastasis. For the metastasis to occur, the degradation of extracellular matrix and basement membranes is the prerequisite for the tumor cells to get to the blood vessel and systemic circulation. Although MMPs are expressed in both the normal and tumor cells, the activities of many MMPs are found to be elevated in the tumor-surrounding tissues. Therefore, blocking the activities of the MMPs responsible for the damage of extracellular matrix is the focal point for cancer therapeutics, and some clinical data have shown the efficacy of such a therapy. In cancer, the altered delicate balance between matrix synthesis and degradation leads to increased matrix turnover, facilitating tumor cell invasion, metastasis, and angiogenesis. In addition, it has been shown that the inhibition of MMPs plays a role in tumor cell survival as evidenced by the induction of apoptosis in melanoma cells [10,11]. Another actively investigated area is the role of MMPs in HIV and AIDS. It has been shown that the HIV regulatory protein, Tat, upregulates the production of MMP-9 which is associated with local tissue damage in acquired immunodeficiency syndrome (AIDS) progression [12]. The MMP inhibitors have also been found to block HIV replication [9]. In addition, the neurodegenerative effects of HIV infection have been reported to be associated with the activity of MMP-2 in the brain [13]. Therefore, MMP inhibitors are being explored for the potential treatment of HIV infection and AIDS.

In this review, the main focus will be placed on the separation and identification of metalloproteinase inhibitors in the drug discovery and development process. The key requirement for a potent inhibitor of metalloproteinases is a functional group capable

of chelating the active site zinc ion strongly. Fig. 1 shows the representative structures of four major classes of MMP inhibitor compounds — hydroxamic acids, carboxylic acids, thiols, and phosphinic acids — representing four different zinc binding groups. Among these structural classes of matrix metalloproteinase inhibitors, hydroxamic acid- and carboxylic acid-based compounds are the most extensively explored. The quantitative and qualitative determination of these compounds is critical in all stages of drug discovery. Pharmaceutical characterizations such as assessment of bulk drug purity, solubility, and stability, as well as identification of degradation products and impurities are routinely performed. Bioanalytical analyses in support of ADME (absorption, distribution, metabolism, and excretion), pharmacokinetic, and toxicokinetic studies are crucial in the drug selection and optimization processes. Chromatographic separation techniques have played a key role in these pharmaceutical and bioanalytical assays. This article will provide an overview of sample preparation, separation, detection, and identification methods employed for the analysis of metalloproteinase inhibitors in various types of samples.

2. Sample preparation

Sample preparation is an important part of quantitative and qualitative analysis of drug compounds in various complex matrices. Traditionally, high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection is the most popular separation technique for routine analysis of drug compounds in biological samples. In recent years, liquid chromatography with tandem mass spectrometry (LC–MS/MS) has been gaining popularity and has become the method of choice for trace analysis of drug molecules in biological samples because of its excellent specificity, speed, and sensitivity. However, the ion suppression due to matrix interferences are also most pronounced in the methodology, leading to the problems associated with precision, accuracy, and reproducibility [14,15]. Many sample preparation techniques have been developed for processing complex samples. Among them, protein precipitation (PPT), solid-phase extraction (SPE) and liquid–liquid extraction (LLE) are the three most widely employed techniques in preparing biological samples for chromatographic analysis. A comparison of these methodologies is schematically presented in Fig. 2. SPE has been a widely utilized technique for biological sample preparation. Recent developments of 96-channel robotic liquid handling workstations [16] as well as a wide selection of 96-well SPE sorbents [17] afford the rapid development and automation of SPE methods for body fluids [18,19]. In contrast, PPT has been the main methodology employed for blood, plasma, or serum sample preparations because of its simplicity and universality. PPT has been semi-automated using a robotic liquid handler and 96-well plates [20]. Recent development of fully automated PPT by filtration using 96-well filter plates and a robotic liquid handler has made the PPT method speedy and even more attractive [21,22]. LLE is the most widely used sample cleanup method in organic synthesis and is a common sample preparation technique for chromatography. Recent developments in automation in the 96-well plate format have made LLE an attractive alternative to SPE and PPT for preparation of complex samples [23,24]. Especially when ESI–MS detection is employed, LLE is a preferred sample preparation technique to SPE and PPT for it renders the least amount of ion suppression in ESI–MS detection [25], leading to improved reproducibility and accuracy of the assay results. However, LLE may not be suitable for ultratrace analysis of very polar compounds due to potentially low analyte recovery from LLE.

2.1. Protein precipitation and filtration

Protein precipitation has been the most widely employed sample preparation technique for blood, plasma and serum samples. Currently, two main methods in PPT have been routinely employed: centrifugation and filtration. The protein precipitation by centrifugation method (shown in Fig. 2a) has been widely adopted and reported in the literature for plasma sample preparation of matrix metalloproteinase inhibitors [21,26,27]. In a comparative study of different sample preparation methods [21], a semi-automated protein precipitation by centrifugation method was developed and employed for the preparation of two carboxylic acid-based metalloproteinase inhibitors in plasma. In this method, 300 μl

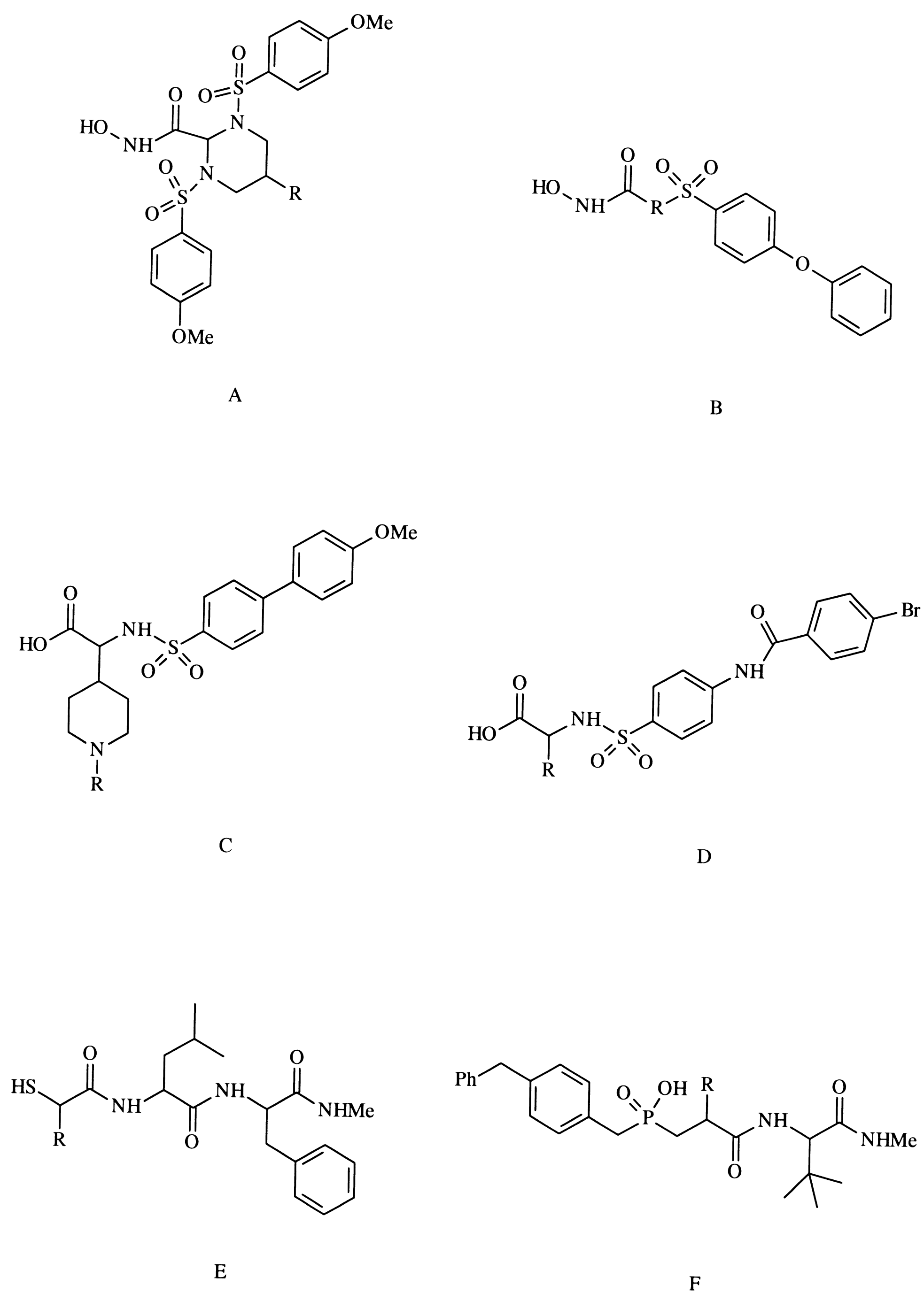

Fig. 1. Representative structures of hydroxamic acid- (A and B), carboxylic acid- (C and D), thiol- (E), and phosphinic acid- (F) based metalloproteinase inhibitors.

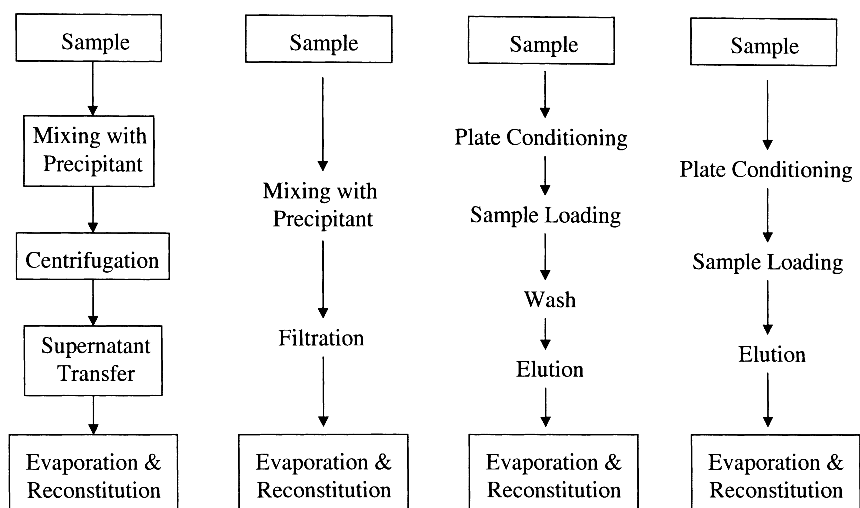

Fig. 2. Schematic representation of 96-well sample preparation methodologies: Protein precipitation by centrifugation (a), protein precipitation by filtration (b), automated solid-phase extraction (c), and automated liquid–liquid extraction (d). Note that the process denoted by a box is a disjointed off-line step.

aliquots of acetonitrile were added to 100 μl aliquots of plasma samples in a 96-well plate using a 96-channel liquid handler. The resulting mixtures were vortexed and centrifuged. The supernatant was then evaporated to dryness and reconstituted in acetonitrile–water (30:70, v/v) for final LC–MS/MS analysis. The analyte recovery was better than 82% for the two compounds studied. A protein precipitation by centrifugation method was also employed for the determination of a matrix metalloproteinase inhibitor in human plasma [26]. The MMP inhibitor was investigated in the clinical trials for the treatment of refractory metastatic cancer. Plasma samples were prepared by adding 1000 μl of acetonitrile to 250 μl of plasma with the resulting mixture vortexed and centrifuged. The supernatant was then dried and reconstituted in acetonitrile–oxalic acid (10 m*M*, pH 2.2) (45:55, v/v) for HPLC–MS analysis. The analyte recovery ranged from 52.1 to 58.7% in a concentration range of 500–8000 ng/ml. A manual protein precipitation procedure was also described for the quantitation of a carboxylic acid-based metalloproteinase inhibitor by HPLC–UV [27]. A 1.0 ml aliquot of acidified acetonitrile containing internal standard was added to a 0.5 ml aliquot of plasma, the resulting plasma samples were vortexed and then centrifuged. The resultant supernatant was transferred to HPLC vials for analysis.

Protein precipitation by filtration is rather new for the preparation of plasma samples [21,22]. The use of the 96-well filtration plates eliminated the two disjointed steps involved in the centrifugation method, i.e., vortex and centrifugation, as shown in Fig. 2b. The procedure is fully automated using the 96-well filter plate and a 96-channel robotic liquid handler. Compared to the centrifugation method, this method is faster, simpler, and easier to perform. The protein precipitation by filtration method has been used in the preparation of plasma samples for pharmacokinetic and in vivo animal efficacy model studies of matrix metalloproteinase inhibitors [21]. In this work, different organic solvents — methanol, ethanol and acetonitrile — were compared for effectiveness of protein precipitation and recovery of the analytes. Methanol was found best suited for the filtration method because of its ease of filtration without clogging the filter and minimal protein breakthrough. Two carboxylic acid-based matrix metalloproteinase inhibitors with high (>99%) and low (<80%) plasma protein binding were evaluated

using the filtration method. Analyte recovery of more than 90% was obtained for both compounds. The 96-channel liquid handler sequentially aspirated plasma and methanol, and then dispensed the mixture into the 96-well filter plate (0.45 μm pore size). The filtrate was then evaporated to dryness and reconstituted for chromatographic analysis. In this comparative study of plasma sample preparation by centrifugation and filtration, the analyte recovery was found higher in the automated filtration method than in the manual or semi-automated centrifugation method, with comparable precision and accuracy among all three methods for the two MMP inhibitors studied. In conclusion, the automated 96-well filtration method is preferred over the manual or semi-automated centrifugation method for its ease, simplicity, and speed.

2.2. Solid-phase extraction

Solid-phase extraction is a commonly employed sample preparation method for drug compounds in complex matrices. SPE is used to selectively remove interfering matrix components, leading to reduced chromatographic method development time and improved assay selectivity, accuracy, and sensitivity. Today, SPE is fully automated using a 96-channel robotic liquid handling workstation and a 96-well SPE plate, as shown in Fig. 2c. The development and application of automated 96-well SPE and LC–MS/MS methods were reported for the determination of two hydroxamic acid-based MMP inhibitors in plasma and cartilage tissues [18]. In this work, a 96-channel liquid handling workstation and Waters Oasis HLB extraction plate were utilized for the automation of SPE procedures for plasma and tissue 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 liquid handler. The SPE plate was sequentially conditioned with 200 ml of methanol and water, loaded with 130 ml of the sample from the source plate, washed with 5–10% methanol in water, and eluted with 95% acetonitrile in water containing 0.1% formic acid. A vacuum manifold system was used at low vacuum during the entire SPE process. The analyte recovery was in the range of 90–94% in plasma and 90–97% in cartilage tissue. The selective removal of interfering matrix components resulted in an improved lower limit of detection in its LC–MS/MS assays.

An SPE method has also been developed and employed for the sample preparation of a metalloproteinase inhibitor, BB94, in human plasma for LC–MS analysis [28]. In another study, a thiadiazole metalloproteinase inhibitor (PNU-107859) and its metabolites in rat biliary fluid were pretreated using SPE, where Varian Bondelut SCX cartridges were used to remove impurities in the matrix for HPLC–UV analysis [29].

2.3. Liquid–liquid extraction

Liquid–liquid extraction has been a preferred sample preparation method for chromatographic analysis because of its clean extracts. Recent development and application of a semi-automated 96-well LLE methodology [23,24] have demonstrated that LLE is an attractive alternative to SPE in complex biological sample preparation. Recent developments in automated 96-well LLE methodology [30,31] have avoided the use of three disjointed steps in the semi-automated LLE method: phase mixing by vortexing, phase separation by centrifugation, and phase transfer by aqueous layer freezing, as shown in Fig. 2d. Because of that, this automated methodology has resulted in significantly reduced sample preparation time and labor in LLE.

Plasma samples of two carboxylic acid-based matrix metalloproteinase inhibitors with high and low levels of plasma protein binding were prepared by the automated LLE methodology using a 96-well LLE plate and a 96-channel robotic liquid handling workstation [31]. The 96-well LLE plate was constructed of a 96-well filter plate filled with inert diatomaceous earth particles, allowing continuous and efficient extraction of analytes between the aqueous biological sample and the organic extraction solvent. In the optimized LLE method, a formate buffer solution was first loaded into a 96-well LLE plate. Then crude plasma samples and a water-im-

miscible organic solvent, methyl ethyl ketone, were sequentially added to the LLE plate so that LLE took place in the interface between the two liquid phases on the surface of individual particles in each well. The organic eluate containing extracted analytes was evaporated and reconstituted for LC–MS/MS analysis. This fully automated LLE methodology eliminated several disjointed steps involved in a manual or semi-automated LLE method, and was faster and simpler. It provided clean sample extracts for improved ESI–MS/MS detection. The organic solvent, methyl ethyl ketone, was used as both the organic extraction solvent and protein denaturant for enhanced analyte recovery. For the two metalloproteinase inhibitor compounds studied, the analyte recovery was found to be better than 70%. This automated LLE methodology is universal and can be employed for sample preparation of other biological fluids.

LLE has also been used in the preparation of a metalloproteinase inhibitor, marimastat, in human plasma [32]. In this work, the marimastat and its internal standard in plasma were extracted into ethyl acetate at pH 6.6 followed by back extraction into 0.1 *M* ammonia solution, which was neutralized by addition of 0.15 *M* hydrochloric acid prior to LC–MS analysis. In another study of metalloproteinase inhibitor, AG3340, in mouse plasma [33], LLE was performed using methyl *tert*.-butyl ether as the organic extraction solvent. The extracts containing the compound and its stable isotopically labeled internal standard were evaporated and reconstituted in acetonitrile–water–formic acid (15:85:1, v/v/v) for LC–MS/MS analysis.

2.4. Others

Other sample preparation techniques can also be employed for chromatographic analysis of metalloproteinase inhibitors in complex matrices. These include ''dilute and shoot'', on-line SPE, solid-phase microextraction (SPME), supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), and microwave-assisted extraction (MAE). The ''dilute and shoot'' method is frequently used in urine samples and other complex samples without endogenous large biomolecules such as proteins. On-line SPE is generally used for complex samples including plasma and urine samples [34,35]. This method involves minimal off-line sample treatment and allows an unattended operation. The limitations of on-line SPE include the extended sample run time relative to off-line SPE and potential sample stability issues resulted from the long storage time in the autosampler. SPME selectively extracts the analytes of interest from complex matrices, offering improved resolution and sensitivity [36]. The extraction in SPME is slow and low recovery is generally expected due to its equilibrium extraction nature as opposed to exhaustive extraction in SPE. SFE is another useful sample preparation technique. It has been used in many areas including pharmaceuticals to reduce solvent use and improve extraction selectivity and speed [37]. Under supercritical conditions, the solubility and diffusivity of the analyte in extraction fluid are increased, leading to improved extraction. However, polar analytes are difficult to extract by SFE. ASE and MAE are extraction techniques based on the elevated temperature and pressure of extraction solvent to improve extraction speed and efficiency [38,39]. They can be especially useful for sample preparation of drugs in animal tissues for chromatographic analysis, provided that the analytes are stable under those extraction conditions.

3. Separation and quantitation

A variety of separation techniques can be employed for the quantitative determination of metalloproteinase inhibitors in various matrices. These techniques include high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE), capillary eletrochromatography (CEC), supercritical fluid chromatography (SFC), and thin-layer chromatography (TLC). Among them, HPLC is the most widely utilized method due to its simplicity and general applicability to the vast majority of drug molecules, including metalloproteinase inhibitors. Other separation techniques are good alternatives to HPLC in certain situations where HPLC is not a suitable or efficient technique for the samples of interest.

3.1. High-performance liquid chromatography

Undoubtedly, HPLC is the most widely used separation technique for pharmaceutical applications. Its ability to separate thermally labile and nonvolatile analytes has made HPLC the method of choice for the analysis of most drug compounds. HPLC is readily interfaced and routinely utilized with various detection systems such as ultraviolet, mass spectrometry, fluorescence (FL), and electrochemical (EC) detection. UV detection is still the most widely used while FL or EC detection is employed for improved specificity and sensitivity. Evaporative light scattering detection (ELSD) is often utilized for purity determination. For determination of trace drug levels in biological samples, MS detection is the most commonly used and preferred for its sensitivity, specificity and speed.

Many metalloproteinase inhibitor compounds are relatively polar, which has made reversed-phase HPLC a suitable method for their determination. Bulk drug purity has been routinely assessed by HPLC [40–45]. A reversed-phase HPLC method was employed to determine the purity of phosphinic peptide metalloproteinase inhibitors in crude products [43]. In this work, the compounds and impurities were separated on an HPLC column RCM C_{18} by linear gradient elution with a water–acetonitrile–TFA mobile phase system, and detected by UV/Vis detection. Reversed-phase HPLC methods were also employed to isolate and purify carboxylic acid-based MMP inhibitors [44]. The purity of the inhibitor compounds was determined using a Waters Symmetry C_{18} column (4.6×200 mm) with a water–acetonitrile–formic acid mobile phase, and detected by a photodiode array (PDA) detector. The purity of some hydroxamic acid-based MMP inhibitors was assessed by several HPLC methods using a VYDAC C_{18} column (4.6×250 mm) with a water–acetonitrile–phosphoric acid mobile phase system [45].

Both UV and ELSD detectors have been increasingly employed in tandem to assess bulk drug purity. HPLC–UV–ELSD has been utilized to determine the purity of several metalloproteinase inhibitor compounds in the author's laboratory. In those experiments, a generic linear gradient separation method was employed, where samples were separated on a Waters symmetry C_{18} column (3.9×150 mm) by linear gradient elution using a water–acetonitrile–formic acid mobile phase. The eluent from the HPLC column was monitored immediately by a photodiode array detector, followed by detection with an ELSD detector. Fig. 3 shows the representative UV and ELSD chromatograms of the bulk drug sample of a metalloproteinase inhibitor from a single HPLC–UV–ELSD run.

In general, the same HPLC method developed for purity assessment is also suitable for compound stability determination. A minor modification of the

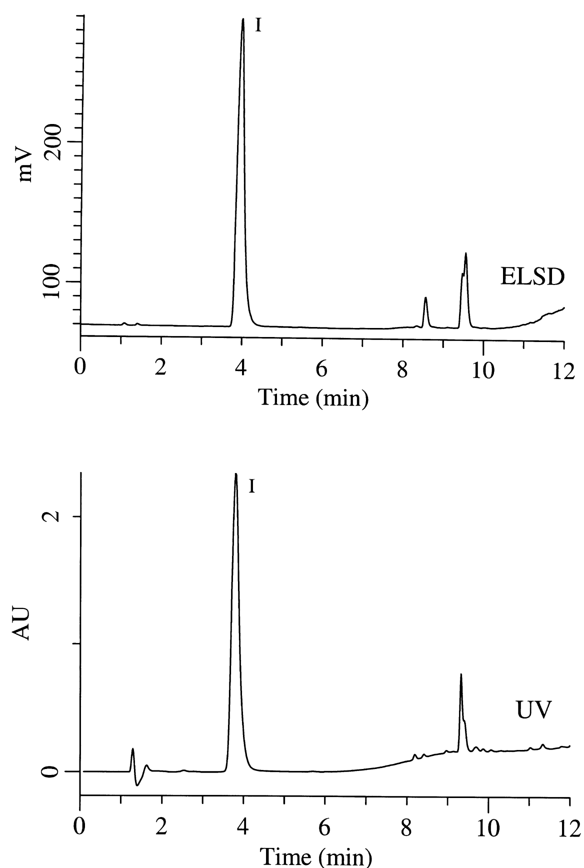

Fig. 3. Representative ELSD (upper trace) and UV (lower trace) chromatograms of an MMP inhibitor compound (I) from a single LC–UV–ELSD run. Conditions: Waters symmetry C_{18} column (3.9×150 mm, 5 μm particle size); 12 min linear gradient elution from mobile phase A (acetonitrile:water:formic acid, 5:95:0.1, v/v/v) to mobile phase B (acetonitrile:water:formic acid, 95:5:0.1, v/v/v) at a flow-rate of 1.0 ml/min; UV at 210 nm; ELSD detector set at a nitrogen gas flow-rate of 1.8 l/min and a drift tube temperature of 40°C in the impactor-on mode.

method may be required sometimes to separate the parent compound and its degradation products. The samples can be incubated in the autosampler and injected onto the HPLC at a preset time interval. However, when analyte stability is evaluated in plasma or some in vitro assay media where sample solutions contain proteins and other large molecules, sample pretreatment such as protein precipitation is needed prior to HPLC analysis to avoid column clogging. A column-switching method using a restricted access media (RAM) column was employed for the determination of MMP inhibitors in plasma by direct injection of plasma samples for an unattended around-the-clock operation [35]. These column-switching methods involved the use of a RAM precolumn, a column-switching valve, and an analytical column, allowing the complete automation of sample preparation and HPLC. The plasma samples were directly injected onto a precolumn packed with semipermeable surface (SPS)/octadecyl silane (ODS) stationary phase and then backflushed onto an ODS analytical column using a 6-port column-switching device. The drug stability of three hydroxamic acid-based metalloproteinase inhibitors in rat plasma was determined using both the automated and traditional HPLC methods. The results obtained from the automated column-switching methods were in good agreement with those from the traditional off-line manual protein precipitation method. In addition to the elimination of labor-intensive and time-consuming sample preparation procedures, the column-switching methods allowed on-line analyte enrichment and accurate determination of drug stability in plasma with detection limits in the range of 10–20 ng/ml. In this column-switching method, the analyte peaks were well separated from the degradant and plasma matrix component peaks. The calibration curves of the three compounds were linear from 0.1 to 100 μg/ml. The precision was less than 4% RSD (intra-day) and 8% RSD (inter-day), while the accuracy was found to be greater than 95% for all three compounds.

HPLC has also been employed for the determination of plasma protein binding of many drug compounds in conjunction with the use of ultrafiltration [46,47]. In the study of plasma protein binding of carboxylic acid-based metalloproteinase inhibitors in the rat [47], HPLC with UV and FL detection was utilized to determine the bound and unbound drug concentrations in plasma. Since the compounds of interest contained fluorophores, FL detection was used to improve the selectivity and lower limits of quantitation of the separation methods. The UV and FL detectors were connected in series to record both UV and FL chromatograms. The HPLC–UV–FL system allowed separation and detection of both fluorescent and non-fluorescent compounds in a single run. In those experiments, samples were prepared in both rat plasma and phosphate-buffered saline. The ultrafiltrates of the plasma and phosphate samples were obtained by centrifugation of the samples in an ultrafiltration device and transferred to HPLC vials for analysis. HPLC assays were carried out on an HPLC system with a PDA detector and a fluorescence detector. The samples were separated on a Waters XTerra column (4.6×30 mm) by linear gradient elution and monitored by both UV (240–285 nm) and FL (excitation: 250–300 nm; emission: 350–400 nm) detectors. The lower limits of detection were 5 ng/ml by UV and 0.5 ng/ml by FL. The representative UV and FL chromatograms of an MMP inhibitor compound in the plasma filtrate are shown in Fig. 4.

HPLC has been applied to the areas of in vitro drug adsorption and metabolism. Many metalloproteinase inhibitor compounds were evaluated for their oral absorption using a tissue permeation chamber system and the drug levels in both the donor and receiver chambers were measured by HPLC. Metabolic stability of metalloproteinase inhibitors was also assessed using liver slices, hepatocytes, liver microsomes, and hepatic S9 fractions using HPLC methods [48]. In the author's laboratory, HPLC with UV and radioactivity (RAD) detection was employed to determine the levels of parent compounds and their metabolites in liver slice metabolism studies using two ^{14}C radiolabeled metalloproteinase inhibitor compounds. Each labeled compound was incubated with the rat, rabbit, dog, monkey, or human liver slices at various concentrations in tissue culture media and sampled at different times. The retrieved samples were then separated on a Waters Symmetry C_{18} column (3.9×150 mm) by linear gradient elution with a water–acetonitrile–formic acid mobile phase system. The effluent was detected immediately by a PDA detector

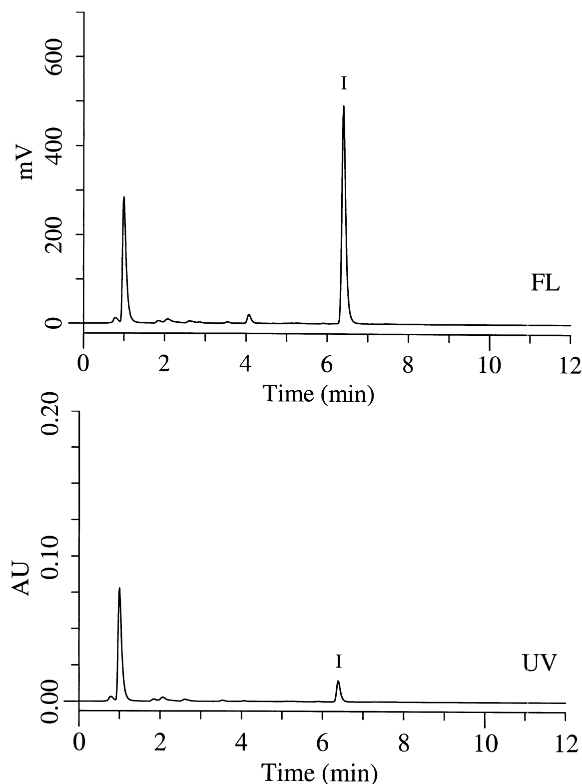

Fig. 4. Representative FL (upper trace) and UV (lower trace) chromatograms of the plasma filtrate sample of a metalloproteinase inhibitor compound (I) from a single LC–UV–FL run. Conditions: Waters XTerra column (4.6×30 mm, 2.5 μm particle size); linear gradient elution from mobile phase A (methanol–water–formic acid; 5:95:0.1, v/v/v) to mobile phase B (methanol–water–formic acid; 95:5:0.1, v/v/v) at a flow-rate of 1.2 ml/min; UV, 280 nm; FL, 280 nm excitation, 360 nm emission.

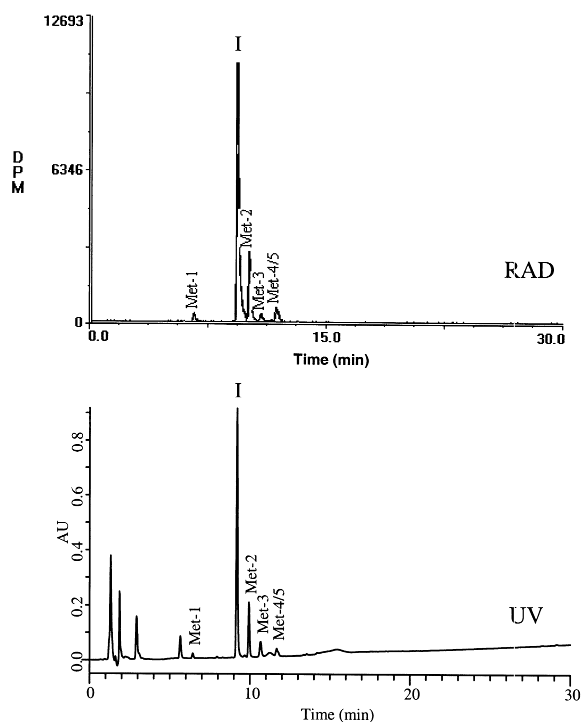

Fig. 5. Representative RAD (upper trace) and UV (lower trace) chromatograms of the liver slice incubation sample of an MMP inhibitor (I) and its metabolites from a single LC–UV–RAD run. Conditions: Waters Symmetry C_{18} column (3.9×150 mm, 5 μm particle size); 30 min linear gradient elution from mobile phase A (acetonitrile–water–formic acid, 5:95:0.1, v/v/v) to mobile phase B (acetonitrile–water–formic acid, 95:5:0.1, v/v/v); flow-rate, 1.0 ml/min; UV, 247 nm; scintillation cocktail flow, 3 ml/min (3:1, scintillator to eluent ratio); RAD flow cell, 0.5 ml.

and then by a RAD detector which was connected in series with the UV detector. The effluent from the UV detector was first mixed with a scintillation cocktail, followed by the radioactivity measurement in a RAD flow cell. The standard calibration curve was linear from 0.1 to 100 μg/ml with a lower limit of quantitation of 100 ng/ml for both compounds. The representative UV and RAD chromatograms of an in vitro metabolism sample of compound I and its metabolites from a single LC–UV–RAD run are shown in Fig. 5. The developed HPLC–UV–RAD methods were successfully employed to determine the metabolic stability and hepatic clearance rates of the two metalloproteinase inhibitors.

In the drug delivery area, HPLC was used to measure drug concentrations in a tissue penetration chamber system to evaluate the permeability of metalloproteinase inhibitors in cartilage tissues [49]. Four MMP inhibitors were investigated in the study. Reversed-phase HPLC methods were developed and utilized to measure the drug concentrations in both the donor- and receptor-side samples. The separation was performed on an HPLC system with a PDA detector. The samples were separated on a Waters Symmetry C_{18} column (150×3.9 mm, 5 μm particle size) with linear gradient elution using an acetonitrile–water–formic acid mobile phase system and UV detection at 245 nm. The HPLC methods developed were very selective for all four compounds and no chromatographic interferences between the parent drug peak and other matrix component peaks

were found. The calibration curves of the four compounds were linear from 0.1 to 100 μg/ml. The precision based on six repetitive injections was less than 3% RSD (intra-day) and 5% RSD (inter-day) for all four compounds. The accuracy was found to be greater than 97% with the detection limit of 10 ng/ml. In the author's laboratory, HPLC–UV–EC methods were also employed to measure drug levels in dosing solutions and in vitro assay media. The EC detector was used to improve detection selectivity and sensitivity for certain metalloproteinase inhibitors containing electroactive functionalities. The samples were typically separated on a Waters Symmetry C_{18} column (150×3.9 mm) by gradient elution with an acetonitrile–phosphate buffer mobile phase system and detected sequentially by a PDA detector and a multi-channel electrochemical detector. In this EC detection, all samples were monitored on eight channels from eight electrodes set at different potentials ranging from +200 mV to +1500 mV. The compound of interest can be identified based on its retention time and oxidation (reduction) profile. Fig. 6 shows representative EC (four selected channels are displayed) and UV chromatograms of a mixture of three metalloproteinase inhibitors in a dosage formulation.

HPLC is also the most widely employed technique in the analysis of metalloproteinase inhibitors in body fluids such as plasma in pharmacokinetic, toxicokinetic, and in vivo animal or human efficacy studies. Among many HPLC detection techniques, UV and MS are the two most widely employed because of their sensitivity and applicability. In MS detection, ESI and APCI are the two ionization sources routinely employed in API for relatively polar or ionizable analytes. ESI can be used for nonvolatile, thermally labile, or large molecules while APCI is for small molecules with a certain degree of volatility and thermal stability. Since ESI is very sensitive to ion suppression from matrix interferences, APCI can be a good alternative to ESI for certain complex biological samples. Although APCI is less sensitive to ion suppression, it may cause thermal decomposition of the analyte or have lower sensitivity in some situations.

Bioanalytical methods based on automated 96-well SPE and LC–MS/MS were developed and utilized for the determination of two hydroxamic acid-based

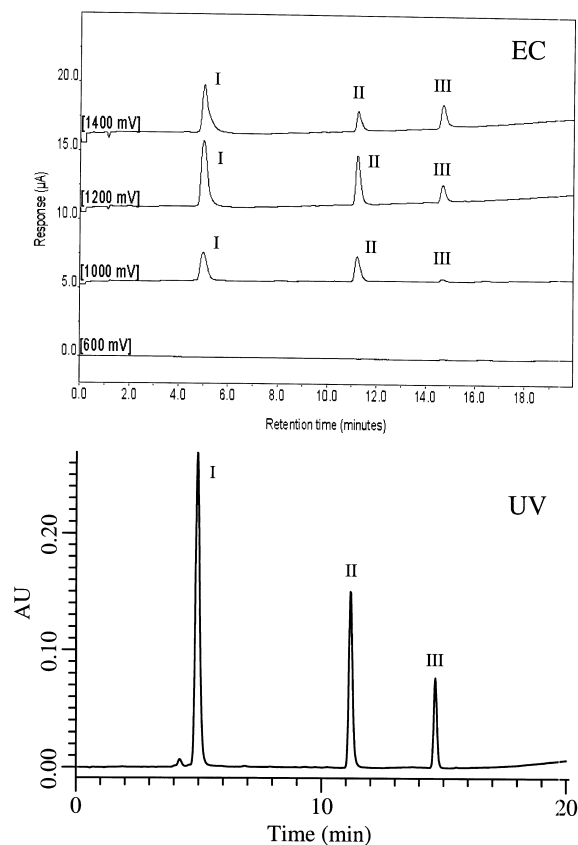

Fig. 6. Representative EC (upper traces) and UV (lower trace) chromatograms of a mixture of three metalloproteinase inhibitors in a dosage formulation from a single LC–UV–EC run. Conditions: Waters Symmetry C_{18} column (150×3.9 mm, 5 μm particle size); 20 min gradient elution from mobile phase A (acetonitrile–phosphate buffer (20 m*M*, pH 3), 10:90, v/v) to mobile phase B (acetonitrile–phosphate buffer (20 m*M*, pH 3), 70:30, v/v); flow-rate, 1.0 ml/min; UV, 240 nm; EC, 600–1400 mV.

metalloproteinase inhibitors in plasma and cartilage tissues [18]. The HPLC separation was performed on Phenomenex Luna $C_8(2)$ column (2.0×50 mm, 3 μm particle size) using short linear gradient elution with an acetonitrile–water–formic acid mobile phase system. The effluent from the column was directly transferred to the TurboIonSpray interface for MS/MS detection. Both the plasma and tissue samples of the two compounds were prepared by automated 96-well SPE methods. The limits of detection for the two compounds were 0.2 ng/ml in plasma and 4 ng/g in cartilage tissues. A linear calibration curve

was obtained in the range of 0.2–200 ng/ml for the plasma samples and 0.5–300 ng/ml for the cartilage samples. Both the intra- and inter-assay precisions were determined with less than 9% RSD while accuracy was within 97–108% for both compounds. 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.

In another study of MMP inhibitors [31], an LC–MS/MS method was developed and employed for the separation and quantitation of two carboxylic aid-based compounds in rat plasma. The plasma samples were prepared by a fully automated liquid–liquid extraction methodology and separated on a Phenomenex C_8(2) column (2.0×50 mm, 3 μm particle size) using short gradient elution with an acetonitrile–10 m*M* bicarbonate buffer (pH 7.4) mobile phase system. The effluent from the column was directly transferred to the TurboIonSpray interface for MS/MS detection. The intra- and inter-assay precisions of less than 11% RSD were observed for both compounds while assay accuracy was in the range of 96–111%. Linear calibration curves were obtained for both compounds in the range of 0.1–1000 ng/ml with the limit of quantitation of 0.6 ng/ml. The developed bioanalytical method, based on the automated LLE and negative ion spray LC–MS/MS, was sensitive, selective, and reproducible, allowing rapid and accurate determination of the two metalloprotease inhibitor compounds in plasma. A similar LC–MS/MS method was also utilized for the determination of two other carboxylic acid-based MMP inhibitors in plasma in a comparative study of different protein precipitation methods [21]. The plasma samples were prepared using both the PPT by centrifugation and PPT by filtration methods. The resulting samples were separated on Phenomenex Luna C_8(2) column (2.0×50 mm, 3 μm particle size) and detected by negative ESI–MS/MS in the SRM mode. Comparable precision and accuracy results were obtained from both methods. However, the PPT by filtration method is preferred because of its simplicity and speed. The representative SRM chromatograms of compound I and its internal standard from the plasma filtrate are shown in Fig. 7.

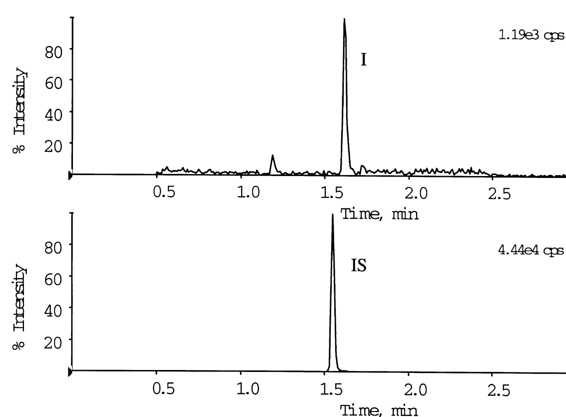

Fig. 7. Representative SRM LC–MS/MS chromatograms of the plasma filtrate sample of a metalloproteinase inhibitor compound (I) and its internal standard (IS). Conditions: Phenomenex C_8(2) column (2.0×50 mm, 3 μm particle size) with 2.5 min gradient elution from mobile phase A (acetonitrile–bicarbonate buffer (10 m*M*, pH 7.4), 20:80, v/v) to mobile phase B (acetonitrile–bicarbonate buffer (10 m*M*, pH 7.4), 80:20, v/v) at a flow-rate of 0.4 ml/min; Turboprobe temperature, 450°C; ionspray voltage, −4,200 V; orifice plate potential, −51 V; focusing ring potential, −140 V; collision energy, 32 V.

LC–MS has been employed to determine an oral metalloproteinase inhibitor, marimastat, in human plasma for pharmacokinetic studies [32]. In this work, separation and quantitation of marimastat in plasma were carried out on an HPLC C_{18} column using a methanol–water mobile phase system with APCI–MS detection in the SRM mode. The method was validated with inter-batch precision of less than 12% RSD and inter-batch accuracy of 89%–105%. The standard calibration curve was linear with the lower limit of detection of 3 ng/ml.

In an antitumor efficacy study of an oral metalloproteinase inhibitor (AG3340) in nude mice, an LC–MS/MS method was developed and employed to determine drug concentrations in plasma [33]. Samples prepared by LLE were separated on a Zorbax SB C_{18} column (2.1×50 mm) with 4.5 min gradient elution using an acetonitrile–water–acetic acid mobile phase system at a flow-rate of 0.2 ml/min. The positive ion ESI–MS detection in the SRM mode was achieved on a triple quadrupole tandem mass spectrometer. The calibration curve was linear from 0.1 to 500 ng/ml with the correlation coefficient of greater than 0.998. The lower limit of quantitation was obtained at 0.5 ng/ml.

HPLC–MS method has also been used to de-

termine a metalloproteinase inhibitor COL-3, a chemically modified tetracycline, in human plasma [26]. In the study, LC–MS was performed on an LC system with an MSD detector. Plasma samples of COL-3 were separated on a Waters Symmetry C_{18} column (2.1×150 mm) with a Waters Symmetry C_{18} guard column (2.1×10 mm). An APCI interface was used in the positive ion mode with SIM detection at two ions for the analyte, one being the quantifying ion at m/z 372.1 and the other qualifying ion at m/z 326.1. The mass spectra of both the analyte (COL-3) and internal standard (chrysin) are shown in Fig. 8. The selected ion chromatograms of the analyte and internal standard were obtained, as shown in Fig. 9, for quantification. For five drug levels ranging from 50 to 8000 ng/ml, the precision was less than 14.9% RSD in the intra-run assays and less than 13.4% in

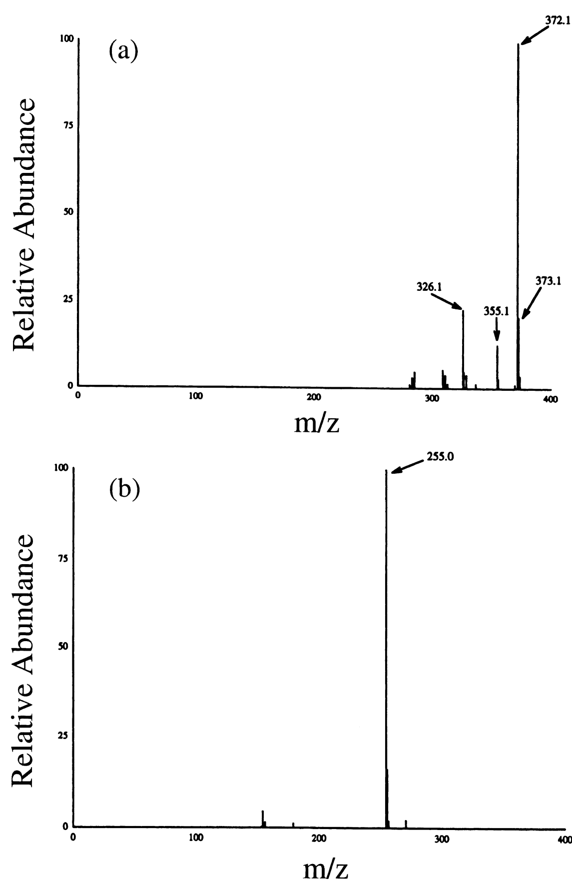

Fig. 8. Mass spectra of COL-3 (a) and chrysin (b). Reprinted with permission from Ref. [26].

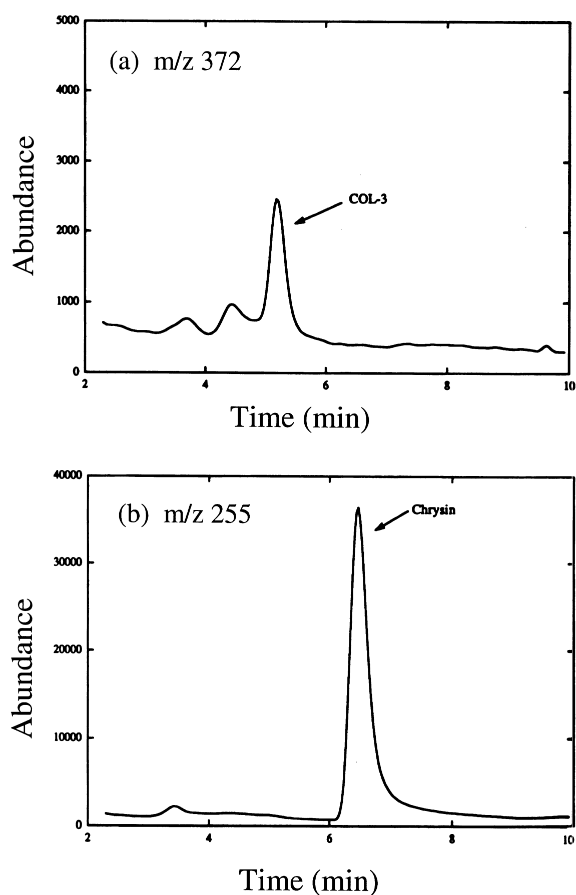

Fig. 9. Selected ion chromatograms of COL-3 (a) and chrysin (b) in human plasma. Conditions: Waters Symmetry C_{18} column (2.1×150 mm, 3.5 μm particle size) with gradient elution from mobile phase A (oxalic acid (10 m*M*, pH2.2)–acetonitrile, 55:45, v/v) to mobile phase B (oxalic acid (10 m*M*, pH2.2)–acetonitrile, 10:90, v/v) at a flow-rate of 0.3 ml/min; drying gas and vaporizer temperatures at 170°C and 300°C; corona current and potential at 4 μA and 3300 V; fragmentor at 90 V. Reprinted with permission from Ref. [26].

the inter-run assays, while the accuracy ranged from 81.7 to 106.9% in the intra-run assays and 81.8–103.1% in the inter-run assays. The recovery was better than 52% for COL-3 at 500–8000 ng/ml and 71.8% for the internal standard at 25 ng/ml. In this method, oxalic acid, a metal blocking agent, was used as an additive in the mobile phase to improve peak shape and sensitivity. In addition, the ratio of the qualifying and quantifying ions was employed to confirm the COL-3 peak in patient samples. It was

demonstrated that the developed method was sensitive and reliable for analyzing the plasma samples in a concentration range of 30–10 000 ng/ml.

An HPLC–UV method was reported to determine a series of structurally related metalloproteinase inhibitors in rat plasma [50]. These compounds in rat blood samples were extracted with acetonitrile, dried, and reconstituted in an appropriate mobile phase for HPLC analysis. The samples were separated on a Phenomenex Prodigy C_{18} column with isocratic elution at 0.5 ml/min using a mobile phase consisting of acetonitrile and a running buffer (0.25% triethylamine in 25 m*M* ammonium phosphate buffer) at different ratios for different compounds. The analytes were detected at UV 241–248 nm using a multi-wavelength detector. The methods were successfully employed to quantitate drug levels in plasma from pharmacokinetic and in vivo anti-tumor efficacy studies.

An HPLC–UV method was described for the determination of a metalloproteinase inhibitor and its three metabolites in human clinical studies [27]. The samples were separated on a Beckman Ultrasphere C_8 column (250×4.6 mm) with gradient elution using an acetonitril–acetate buffer mobile phase system. The analyte and metabolites were detected by a UV/Vis detector. The chromatographic peaks of the analyte, internal standard, and three metabolites were well resolved as shown in Fig. 10. In the concentration range of 0.1–50 μg/ml for the parent compound and 0.1–3 μg/ml for the three metabolites, the intra-day precision was less than 4.8% RSD while the intra-day accuracy ranged from 95 to 105%. The inter-day precision was less than 7.4% RSD and inter-day accuracy was from 96 to 105%. The method had a detection limit of 0.05 μg/ml for the parent compound and its three metabolites.

Other studies involving HPLC analysis were also found in the literature. Isolation and identification of a metalloproteinase inhibitor, PNU-107859, and its metabolites, were achieved by HPLC–UV on a Zorbax Rx-C_8 column (4.6×250 mm) with isocratic elution using a mobile phase consisting of acetonitrile–water–trifluoroacetic acid (25:75:0.1, v/v/v) [29]. The eluent was detected at a UV wavelength of 311 or 220 nm on a diode array detector at a flow-rate of 1.0 ml/min. Applications of LC–MS were also reported in the studies of other metallo-

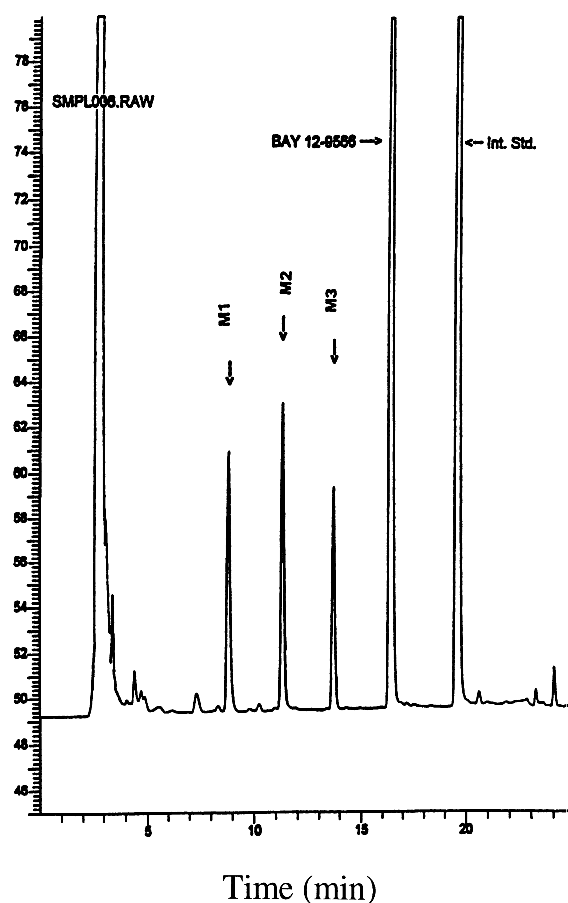

Fig. 10. HPLC chromatogram of plasma standard containing BAY12-9566 and metabolites M1, M2, and M3. Conditions: Beckman Ultrasphere C8 column (250×4.6 mm, 5 μm particle size) with gradient elution from 40% A–60% B to 10% A–90% B (A, 100 m*M* acetate buffer, pH 3.5; B, 10% acetate buffer, 90% acetonitrile) at a flow-rate of 0.8 ml/min; UV at 290 nm. Reprinted with permission from Ref. [27].

proteinase inhibitors, such as BB94 [28] and RO32-3555 [51] in human plasma, AG3340 [52] in rat blood and bile samples, and a series of carboxylic acid-based compounds in rat plasma [53].

3.2. Capillary electrophoresis and capillary electrochromatography

Capillary electrophoresis is a separation technique of high efficiency with low sample and solvent consumption. It has been widely used for separation of both large and small drug molecules [54,55].

Although limited by its lower sensitivity and reproducibility compared to HPLC, CE has been chosen as an alternative means of analysis of metalloproteinase inhibitors in purity and stability evaluations as well as in vitro tissue permeation studies, where in vitro oral absorption or in vitro drug delivery properties can be determined. In the author's laboratory, capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) were employed as alternatives to HPLC for separations of some special samples such as enantiomeric mixtures and very polar or ionic compounds. In the analysis of a hydroxamic acid-based metalloproteinase inhibitor in an in vitro tissue permeability study, a CZE method was developed using a Waters AccuSep fused-silica capillary (75 μm×55 cm) and a phosphate running buffer. The matrix components were well separated from the main compound as indicated in Fig. 11. In another study of purity determination of a mixture of two isomeric metalloproteinase inhibitors, an MECC method was developed using a Phenomenex CZESep-200/glycerol-coated capillary column (75 μm×60 cm) and an electrolyte system of sodium dodecyl sulfate (SDS) and phosphate. The two isomers were well resolved and the impurities were separated from the main analytes in the MECC method, as illustrated in Fig. 12. CE is also an excellent technique for chiral

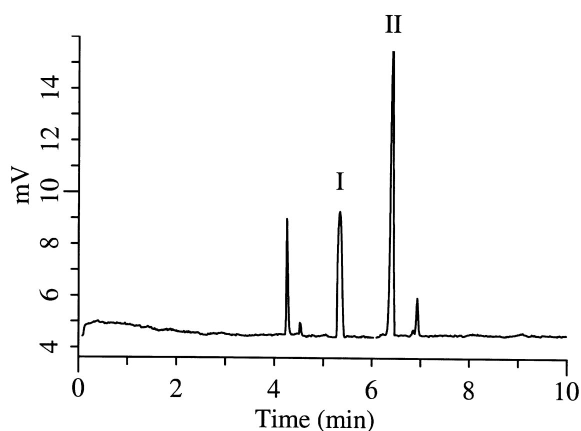

Fig. 12. Separation of two isomeric MMP inhibitors (I and II) by MECC. Conditions: Phenomenex CZESep-200/glycerol-coated capillary column (75 μm×60 cm, 53 cm effective length); electrolyte system, 50 m*M* SDS in 10 m*M* phosphate buffer (pH 7.2); electrokinetic injection, 5 s; negative sample voltage, 15 kV; negative run voltage, 20 kV; UV, 254 nm.

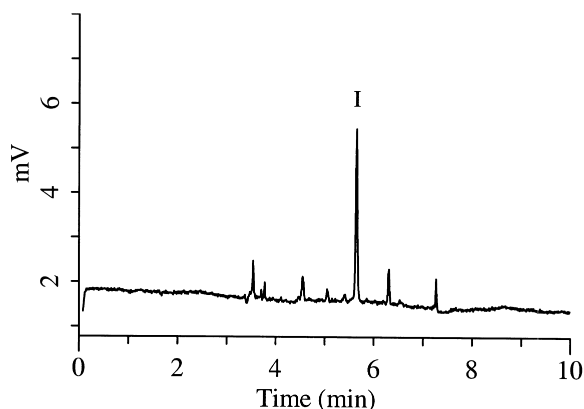

Fig. 11. A CZE electropherogram of the in vitro tissue permeability sample of a metalloproteinase inhibitor compound (I). Conditions: Waters AccuSep fused-silica capillary (75 μm×55 cm, 48 cm effective length); running buffer, 12 m*M* phosphate (pH 7.2); hydrostatic injection, 5 s; positive run voltage, 30 kV; UV, 254 nm.

separation. A CE method was developed to determine the enantiomeric purity of a hydroxamic acid-based metalloproteinase inhibitor compound. In the method, the separation of the enantiomers was achieved on a Waters AccuSep fused-silica capillary (75 μm×55 cm) using a chiral selector, hydroxypropyl-β-cyclodextrin, in a running buffer system. The two enantiomers were well resolved under those conditions, as shown in Fig. 13.

Capillary electrochromatography is generally viewed as a hybrid technique combining both high selectivity from HPLC and high peak capacity from CE. CEC is a good alternative to HPLC and CE for analysis of complex samples. However, CEC is still a new and evolving technique, requiring a specially modified CE system or a dedicated CEC instrument capable of performing both isocratic and gradient elution. Special handling and conditioning of the packed capillary are needed to prevent the gas bubble formation in the capillary, which has been a common problem leading to many failed CEC runs. The utility of CEC has been recently reviewed [54]. In the author's laboratory, a CEC method was developed to separate a mixture of three metalloproteinase inhibitors. In this method, the mixture sample was separated on a Unimicro Electropak ODS capillary column (100 μm×40 cm) and de-

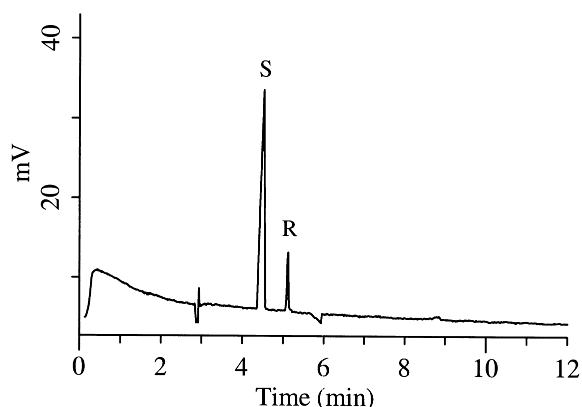

Fig. 13. Chiral separation of R- and S-enantiomers of a metalloproteinase inhibitor by CE. Conditions: Waters AccuSep fused-silica capillary (75 μm×55 cm, 48 cm effective length); running buffer, 10 m*M* hydroxypropyl-β-cyclodextrin (a chiral selector) in 20 m*M* phosphate at pH 8.0; hydrostatic injection, 10 s; positive run voltage, 20 kV; UV, 254 nm.

tected by UV. The three compounds were well resolved in the CEC method, as shown in Fig. 14.

The advent and continued development of 96-channel CE system [56] should further broaden the utility of CE for determination of drug molecules in a high-throughput format to efficiently support combinatorial chemistry and many other drug discovery areas such as enzyme inhibitory assays and in vitro absorption and metabolism screens. This multiplexed

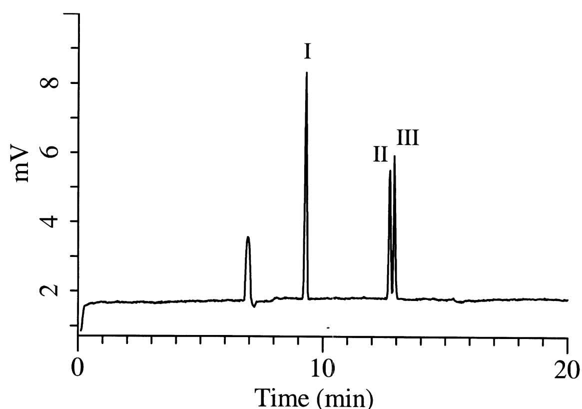

Fig. 14. A CEC electrochromatogram of a mixture of three metalloproteinase inhibitors. Conditions: Unimicro Electropak capillary column (100 μm×40 cm, 3 μm non-porous ODS particles); isocratic elution, acetonitrile–5 m*M* phosphate buffer at pH 7.4 (70:30, v/v); electrokinetic injection, 10 s; sample voltage, 15 kV; run voltage, 25 kV; UV, 254 nm.

capillary array CE technique allows the analysis of 96 samples simultaneously, which should greatly increase the sample throughput and accelerate the drug discovery process.

3.3. Gas chromatography

Since most metalloproteinase inhibitors can be analyzed by HPLC, gas chromatography was not widely employed for the determination of these compounds. As a result, few GC methods were developed and reported in the literature. Most metalloproteinase inhibitors can not be directly analyzed by GC because they contain polar functional groups with active hydrogens such as –COOH, –OH, –NH, and –SH, causing poor thermal stability, low volatility, and strong interactions with column packing materials. Analytical derivatization, such as silylation, alkylation, and acylation, is generally required to improve analyte volatility and thermal stability. Because of these required additional sample pretreatment steps involving analyte derivatization and extraction in GC, HPLC is the preferred technique for separation and quantitation of metalloproteinase inhibitor compounds. However, when the analyte of interest is readily vaporized at reasonable temperatures without thermal decomposition, GC is an excellent technique for its high separation efficiency and speed.

A GC–MS method was developed and employed for the determination of metabolic stability of hydroxamic acid-based metalloproteinase inhibitors in an in vitro metabolism study [57]. In this work, the metabolic stability of the compounds were assessed through the analysis of hydroxylamine (HA) released during the incubation of the compounds with liver microsomes. In the metabolism assay, the hydroxamic acid compounds were metabolized to their corresponding carboxylic acids, releasing HA as a common metabolic product. Therefore, HA monitoring was used to determine the metabolic hydrolysis of the hydroxamic acid-based compounds in rat and human liver microsomes. In the GC–MS method, HA was derivatized to acetone oxime by addition of acetone to the liver microsomal supernatant, followed by direct injection of the supernatant into the GC–MS, with detection of the oxime by selected-ion-monitoring (SIM). The stable isotopically

labeled [^{15}N]HA was used as an internal standard to improve precision and accuracy. The two inhibitors were first incubated with the rat and human microsomal fractions. The incubation mixtures were then centrifuged and the supernatant was isolated and taken for subsequent derivatization with acetone. The separation of calibration standards, quality controls, and study samples was carried out on a GC–MS system using a Restek Stabilwax capillary column (30 m×0.25 mm, 0.5 μm film thickness). The full scan spectra of derivatized HA and [^{15}N]HA were acquired over a mass range from m/z 10–200. The SIM analysis was conducted using molecular ions for the HA and [^{15}N]HA derivatives, respectively. Both the analyte and internal standard, coeluted but monitored at two separate SIM channels, were well separated from any matrix interferences. The peak area ratios for the standards gave a linear calibration curve in the range of 0.1–50 μg/ml. The accuracy was found to be in the range of 95.4–102.1% with precision in the range of 2.1–4.0% RSD for the 0.1, 1 and 10 μg/ml QC samples. Replicate injections of the same sample gave RSD values of less than 4% (intra-day) and 8% (inter-day) at all QC levels. The absolute recoveries for the analyte at three QC concentration levels were greater than 99.5%. The limit of quantitation was 33 ng/ml. The GC–MS method was selective, simple, reproducible, and sensitive for the determination of the hydrolysis of hydroxamic acid compounds, where hydrolysis was the major metabolic pathway. Since many of these hydroxamic acid-based metalloproteinase inhibitor compounds were found to be rapidly hydrolyzed to the corresponding carboxylic acids, the methodology was used to rank order hydroxamic acid analogs based on their susceptibility to hydrolysis in the drug screening and selection process.

3.4. Others

Supercritical fluid chromatography and thin-layer chromatography are the other two useful separation techniques that can be employed for the determination of drugs in complex matrices. Compared to HPLC, SFC is inherently faster due to its higher flow-rates afforded by low solvent viscosity. SFC is often a good alternative to HPLC for solving difficult problems such as chiral separations. SFC technique and its applications have been recently reviewed [37]. TLC is a quick, convenient, and inexpensive technique widely employed by organic chemists for monitoring reaction products, by-products, intermediates, and impurities. The main advantage of TLC is its ability to assay many samples in parallel on a single TLC or high-performance TLC (HPTLC) plate, leading to a large increase in sample throughput compared to the column techniques such as HPLC or GC. Its limitations include lower separation efficiency and capacity compared to the column methodologies. However, TLC or HPTLC should be useful when high sample throughput is required, such as in the purity assessment of combinatorial libraries. TLC methods were developed and employed for the purity determination of phosphinic peptide metalloproteinase inhibitors using Merck silica gel 60F254 plates with chloroform–methanol–acetic acid or toluene–ethyl acetate–acetic acid as developing solvent systems [43]. The plates were visualized with a UV lamp using the AMC reagent or ninhydrin spray. Similar TLC methods were also developed to monitor the synthesis of carboxylic acid-based MMP inhibitors using Merck 60F254 silica gel plates, which were visualized with UV light, iodine vapors, or 5% phosphomolybdic acid in 95% ethanol [44]. Other TLC methods were also reported in the purity assessment of metalloproteinase inhibitors [42,45,53].

4. Qualitative analysis

Qualitative analysis of drug samples is typically carried out using hyphenated techniques combining the separation power of chromatography with the identification capability of spectroscopy. HPLC–MS and HPLC–NMR are the two most widely employed hyphenated technologies in the pharmaceutical industry for simultaneous separation and identification of analytes in complex matrices. Combined use of LC–MS and LC–NMR has been considered as the most efficient approach to solving complex structural problems. Other coupled techniques can also be employed as complementary or alternative techniques to HPLC-based hyphenated methodologies. The choice of a hyphenated technique is mainly dictated by the separation technique that is best

suited for the samples studied. Currently, however, there are few reports published in the literature describing qualitative analysis of metalloproteinase inhibitors, presumably due to proprietary reasons.

4.1. High-performance liquid chromatography with mass spectrometry

HPLC–MS has traditionally been the method of choice for separation and identification of unknown mixtures in simple or complex matrices. In the pharmaceutical industry, LC–MS has been routinely employed for the structure elucidation of drug metabolites, degradation products, and impurities. The instrumentation and applications of LC–MS have been recently reviewed [58]. The main advantages of using LC–MS are its high speed and sensitivity compared to other hyphenated identification techniques such as LC–NMR and LC–IR. The main limitation of LC–MS is that limited structural information is obtained, which often necessitates the use of additional structural identification techniques such as NMR for unambiguous characterization of complex samples.

LC–MS has been employed to separate and characterize a hydroxamic acid-based metalloproteinase inhibitor and its six degradation products in a dosage formulation [59]. LC–MS experiments were carried out on an HPLC system interfaced with a mass spectrometer and a variable-wavelength UV detector. The separation was achieved on a Waters Symmetry C-18 column (3.9×150 mm, 5 μm particle size) using gradient elution with an acetonitrile–water–formic acid mobile phase system. Both the UV chromatogram and MS spectra were obtained from a single run. Mass spectra were obtained using an electrospray ionization interface in the positive ion mode.

The parent compound and its degradation products were well separated by the HPLC method. The resulting UV and total ion MS chromatograms revealed peaks corresponding to the parent compound and its six degradation products. Each single ion chromatogram corresponding to each of seven distinct MH^+ values was obtained. Definitive molecular mass assignments were made for each component, based on molecular adduct ions (MH^+, MNH_4^+, MNa^+) observed in low-cone-voltage (30 V) ESI mass spectra. High-cone-voltage (80 V) ESI mass spectra were also obtained for each component during the same HPLC run. Since the structure of the parent compound was known, the structures of the six degradants were rationalized based on the molecular mass information from low-cone-voltage ESI MS spectra and the fragmentation information from high-cone-voltage ESI MS spectra. One possible structure was assigned to each of the Degradants 1–3. However, more than one possible structure was proposed for Degradants 4–6 because the MS fragmentation data were not definitive. The MS spectra of Degradants 5 and 6 indicated that both compounds had the same molecular mass and yielded identical fragmentation patterns, suggesting that they were structural isomers. Therefore, the same three possible structures were proposed for Degradants 5 and 6. It was apparent that additional characterization information would be required to properly assign structures. In this work, LC–NMR was employed to provide confirmatory structural information on the three degradants that were already identified by LC–MS and further detailed structural information on the other three degradants of which more than one possible structure was proposed.

LC–MS has also been employed in the determination of purity and identity of a series of hydroxamic acid-based metalloproteinase inhibitor compounds synthesized using a combinatorial approach [60]. The compounds were identified using an LC–MS approach, where electrospray ionization in the positive ion mode was utilized and the molecular masses of the compounds were determined by the molecular adduct ions such as MH^+, MNH_4^+, and MNa^+ generated.

In most situations, LC–MS/MS or LC–$(MS)^n$ is preferred over LC–MS for the structure elucidation of unknown mixtures, because multistage MS can provide additional information on fragmented ions, facilitating structural assignments.

4.2. High-performance liquid chromatography with nuclear magnetic resonance

In drug discovery, there are many complex structure elucidation problems that require the use of more than one analytical technique for their solutions. Traditionally, LC–MS has been the method of

choice for the characterization of complex mixtures. However, the structural assignments by LC–MS alone often remain tentative, especially when structural, conformational, and optical isomers need to be identified. In many cases, separate NMR experiments are required to obtain detailed structural information on each component. In doing so, each component has to be separated and isolated from an HPLC run for NMR analysis. With the advent of LC–NMR, this additional fraction collection step is eliminated and the structural characterization work is thus accelerated. Because of recent major advances, LC–NMR has been increasingly utilized to characterize in vitro and in vivo metabolites [61,62], bulk drug impurities [63], and mixture components from combinatorial library synthesis [64,65]. In addition, the combined use of LC–NMR and LC–MS has received much attention lately to facilitate the characterization of in vitro and in vivo metabolites [66,67], combinatorial chemistry mixtures [68], and drug degradation products [59]. The utility of the LC–NMR technology and its applications in drug research and development have been recently reviewed [69,70].

Both LC–NMR and LC–MS have been employed to characterize six degradation products of a hydroxamic acid-based MMP inhibitor in a dosage formulation [59]. In this study, a reversed-phase HPLC method was developed for the separation of the parent compound and its six degradation products. LC–MS was used to obtain the molecular mass and fragmentation information using an electrospray ionization interface in the positive ion mode while LC–NMR was conducted to acquire detailed structural information using a selective solvent suppression pulse sequence in the stop-flow mode.

LC–NMR experiments were carried out on an LC system which was interfaced to a UV detector and an NMR spectrometer in series. The degradation sample was separated on a Waters Symmetry C_{18} column (3.9×150 mm, 5 μm particle size) with linear gradient elution using an NMR grade acetonitrile–deuterated water–formic acid mobile phase system. The eluent from the column was first monitored by a UV detector and then directed to a Bruker 3 mm inverse dual $^{1}H/^{13}C$ LC–NMR probehead for NMR acquisition. Both acetonitrile and water solvent signals were suppressed with a WET pulse sequence. Carbon decoupling was also employed. The LC–NMR experiments were conducted in the stop-flow mode, where LC flow was stopped while the NMR was acquiring data on an LC peak.

LC–MS was first performed to obtain molecular mass and fragmentation information on each component. On the basis of LC–MS data, one possible structure for each one of Degradants 1–3 was assigned. However, more than one possible structure was proposed for each one of Degradants 4–6. It was apparent that additional characterization information from HPLC–NMR would be required to properly assign the structures of Degradants 4–6. LC–NMR experiments were conducted under the identical chromatographic conditions as those in the LC–MS experiments. A UV signal was obtained during the LC–NMR run to correlate the LC peaks with the NMR spectra. The ^{1}H NMR spectra of the parent compound and the first three degradants were obtained and consistent with the structures proposed from the LC–MS experiments. The structures of these compounds were therefore confirmed. For the fourth degradant, two structures were proposed from the LC–MS data: a closed-ring alcohol and an open-ring aldehyde. The molecular mass and fragmentation pattern from LC–MS suggested an alcohol form. However, the NMR spectrum was consistent with the aldehyde instead of the alcohol. It was suggested that the aldehyde, which is the stable form in solution, had undergone a gas phase conversion to the alcohol during electrospray ionization and subsequent collision with surrounding gas molecules in the LC–MS experiment. This exemplified the importance of LC–NMR in elucidating unknown structures in solution and watchouts in LC–MS data interpretation. From LC–MS data, there were three structures proposed for each of the fifth and sixth degradants: a fused tricyclic structure, a cyclic urea, and an open-ring aldehyde. However, the NMR spectra of the degradants were different. The NMR spectrum of Degradant-5 clearly eliminated the open-ring aldehyde structure and the fused tricyclic structure. All resonance peaks in the NMR spectrum of Degradant-5 were easily interpreted by the cyclic urea structure, resulting in the assignment of the urea structure to Degradant-5. Characteristic resonances in the NMR spectrum of Degradant-6 eliminated the fused tricyclic structure and pointed to the open-ring aldehyde structure. Consistent with all other NMR

features, the open-ring aldehyde was assigned to Degradant-6 by LC–NMR. In this work, the LC–NMR data confirmed three degradation products assigned from LC–MS and identified the structures of the other three degradants. This example demonstrated the usefulness of these two complementary techniques for rapid identification of degradation products in dosage formulations. It also showed that the combined use of LC–NMR and LC–MS would provide fast and unambiguous structural characterization of unknown compounds in complex matrices.

4.3. Others

In addition to LC–NMR and LC–MS, other hyphenated separation and identification techniques such as LC–IR, CE–MS, CE–NMR, SFC–MS, SFC–NMR, TLC–MS can also be used for qualitative analysis of drug molecules. They are typically employed in special situations where samples cannot be successfully resolved and characterized by LC–MS and/or LC–NMR. Sometimes these techniques are also employed as complementary techniques to confirm the structural assignments made by LC–MS and/or LC–NMR. These hyphenated techniques should generally be applicable to the simultaneous separation and identification of metalloproteinase inhibitors, although such applications have not been reported in the literature. Separate NMR and MS experiments have been routinely employed to identify and confirm the final products in organic synthesis of metalloproteinase inhibitors [43] and to characterize their metabolites in metabolism studies [29]. Interested readers are referred to the recent literature in hyphenation of chromatography with NMR [69,70], MS [58,71,72], and IR [73] for additional details of instrumentation and applications of these hyphenated techniques.

5. Concluding remarks

Metalloproteinase inhibitors are being explored for the treatment of cancers, HIV, and a variety of other diseases. A wide range of analytical separation, detection, and identification methods have been developed and employed for quantitative and qualitative determination of these inhibitor compounds. Different sample preparation techniques have been also utilized prior to chromatographic analysis to improve precision, accuracy, and reproducibility of the separation methods. Among them, PPT, SPE, and LLE are the three most widely employed sample preparation methodologies used for quantitative chromatographic analysis of metalloprotease inhibitors in complex matrices such as biological fluids and tissues. PPT by centrifugation (semi-automated) or by filtration (fully automated) is the most commonly used method for preparation of plasma and serum samples, while automated SPE or LLE is the best alternative to PPT when matrix interferences prevent sensitive, accurate, and reliable analysis. HPLC with UV and MS detection is the most widely utilized separation method for the determination of metalloproteinase inhibitors in various matrices. Mass spectrometry and nuclear magnetic resonance spectroscopy are the two most often used identification techniques and their hyphenation to HPLC has proven to be most useful for identification of drugs, metabolites, and degradation products in complex matrices. In the future, automated high-throughput sample preparation in conjunction with parallel column HPLC with MS detection will continue to play a key role in quantitative pharmaceutical and bioanalytical analysis of drug molecules including metalloproteinase inhibitors. In addition, the array- and chip-based separation technologies will continue to develop and mature to become mainstream analytical techniques to further improve sample throughput, increase analysis speed, and reduce assay cost.

6. Nomenclature

APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
ASE	accelerated solvent extraction
AIDS	acquired immunodeficiency syndrome
CAD	collision activated dissociation
CE	capillary electrophoresis
CEC	capillary electrochromatography
CZE	capillary zone electrophoresis
EC	electrochemical
ELSD	evaporative light scattering detection
ESI	electrospray ionization

FL	fluorescence
GC	Gas chromatography
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin-layer chromatography
IR	infrared
LLE	liquid–liquid extraction
LOD	limit of detection
LOQ	limit of quantitation
MAE	microwave-assisted extraction
MECC	micellar electrokinetic capillary chromatography
MMP	matrix metalloproteinase
MS	mass spectrometry
NMR	nuclear magnetic resonance
ODS	octadecyl silane
PDA	photodiode array
PPT	protein precipitation
QC	quality control
RAD	radioactivity detection
RAM	restricted access media
RSD	relative standard deviation
SFC	supercritical fluid chromatography
SFE	supercritical fluid extraction
SIM	selected ion monitoring
SPE	solid-phase extraction
SPME	solid-phase microextraction
SPS	semipermeable surface
SRM	selected reaction monitoring
TIMP	tissue inhibitor of metalloproteases
TLC	thin-layer chromatography
UV	ultraviolet

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References

[1] D. Leung, G. Abbenante, D.P. Fairlie, J. Med. Chem. 43 (2000) 305.
[2] M. Whittaker, C.D. Floyd, P. Brown, A.J.H. Gearing, Chem. Rev. 99 (1999) 2735.
[3] T.E. Cawston, Pharmacol. Ther. 70 (1996) 163.
[4] A.F. Chambers, L.M. Matrisian, J. Natl. Cancer Inst. 89 (1997) 1260.
[5] V.W. Yong, Exp. Opin. Invest. Drugs 8 (1999) 255.
[6] F.G. Spinale, M.L. Coker, S.R. Krombach, R. Mukherjee, H. Hallak, W.V. Houck, M.J. Clair, S.B. Kribbs, L.L. Johnson, J.T. Peterson, M.R. Zile, Circ. Res. 85 (1999) 364.
[7] C.M. Overall, O.W. Wieben, J.C. Thonard, J. Periodontal Res. 22 (1987) 81.
[8] U.K. Saarialho-Kere, M. Vaalamo, P. Puolakkainen, K. Airola, W.C. Parks, M.L. Karjalainen-Lindsberg, Am. J. Pathol. 148 (1996) 519.
[9] C.S. Dezzutti, W.E. Swords, P.C. Guenthner, D.R. Sasso, L.M. Wahl, A.H. Drummond, G.W. Newman, C.H. King, F.D. Quinn, R.B. Lal, J. Infect. Dis. 180 (1999) 1142.
[10] M. Ahonen, A.H. Baker, V.-M. Kahari, Cancer Res. 58 (1998) 2310.
[11] N. Johansson, M. Ahonen, V.-M. Kahari, Cell Mol. Life Sci. 57 (2000) 5.
[12] R.M. Lafrenie, L.M. Wahl, J.S. Epstein, I.K. Hewlett, K.M. Yamada, S. Dhawan, J. Immunol. 157 (1996) 974.
[13] D.C.L. Marshall, T. Wyss-Coray, C.R. Abraham, Neurosci. Lett. 254 (1998) 97.
[14] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.
[15] D.L. Buhrman, P.I. Price, P.J. Rudewicz, J. Am. Soc. Mass Spectrom. 7 (1996) 1099.
[16] G.A. Smith, T.L. Lloyd, LC-GC 16 (1998) S22.
[17] D.A. Wells, LC-GC 17 (1999) 600.
[18] S.X. Peng, S.L. King, D.M. Bornes, D.J. Foltz, T.R. Baker, M.G. Natchus, Anal. Chem. 72 (2000) 1913.
[19] H. Zhang, J. Henion, Anal. Chem. 71 (1999) 3955.
[20] A.P. Watt, D. Morrison, K.L. Locker, D.C. Evans, Anal. Chem. 72 (2000) 979.
[21] S.L. King, D.J. Foltz, T.R. Baker, S.X. Peng, in: Proceedings of 48th ASMS Conference on Mass Spectrometry and Allied Topics, Long Beach, California, June 11–16, 2000.
[22] R.A. Biddlecombe, S. Pleasance, J. Chromatogr. B 734 (1999) 257.
[23] M. Jemal, D. Teitz, Z. Ouyang, S. Khan, J. Chromatogr. B 732 (1999) 501.
[24] S. Steinborner, J. Henion, Anal. Chem. 71 (1999) 2340.
[25] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175.
[26] M.A. Rudek, C.L. March, K.S. Bauer, J.M. Pluda, W.D. Figg, J. Pharm. Biomed. Anal. 22 (2000) 1003.
[27] V.K. Agarwal, D.L. Rose, G.J. Krol, J. Liq. Chrom. Rel. Technol. 22 (1999) 1893.
[28] G.J. Beattie, J.F. Smyth, Clin. Cancer Res. 4 (1998) 1899.
[29] M.-S. Kuo, D.A. Yurek, S.A. Mizsak, M.D. Prairie, S.J. Mattern, T.F. DeKoning, J. Pharm. Sci. 88 (1999) 705.
[30] S.X. Peng, C. Henson, M.J. Strojnowski, A. Golebiowski, S.R. Klopfenstein, Anal. Chem. 72 (2000) 261.
[31] S.X. Peng, T.M. Branch, S.L. King, Anal. Chem. 73 (2001) 708.
[32] A.W. Millar, P.D. Brown, J. Moore, W.A. Galloway, A.G.

Cornish, T.J. Lenehan, K.P. Lynch, Br. J. Clin. Pharmacol. 45 (1998) 21.
[33] D.R. Shalinsky, J. Brekken, H. Zou, S. Kolis, A. Wood, S. Webber, K. Appelt, Invest. New Drugs 16 (1999) 303.
[34] S.R. Needham, M.J. Cole, H.G. Fouda, J. Chromatogr. B 718 (1998) 87.
[35] S.X. Peng, M.J. Strojnowski, D.M. Bornes, J. Pharm. Biomed. Anal. 19 (1999) 343.
[36] H. Lord, J. Pawliszyn, J. Chromatogr. A 902 (2000) 17.
[37] R.M. Smith, J. Chromatogr. A 856 (1999) 83.
[38] B.E. Richter, LC-GC 17 (1999) S22.
[39] G. Leblanc, LC-GC 17 (1999) S30.
[40] F.M. Martin, R.P. Beckett, C.L. Bellamy, P.F. Courtney, S.J. Davies, A.H. Drummond, R. Dodd, L.M. Pratt, S.R. Patel, M.L. Ricketts, R.S. Todd, A.R. Tuffnell, J.W.S. Ward, M. Whittaker, Biorg. Med. Chem. Lett. 9 (1999) 2887.
[41] D.H. Steinman, M.L. Curtin, R.B. Garland, S.K. Davidsen, H.R. Heyman, J.H. Holms, D.H. Albert, T.J. Magoc, I.B. Nagy, P.A. Marcotte, J. Li, D.W. Morgan, C. Hutchins, J.B. Summers, Biorg. Med. Chem. Lett. 8 (1998) 2087.
[42] R. Hirayama, M. Yamamoto, T. Tsukida, K. Matsuo, Y. Obata, F. Sakamoto, S. Ikeda, Biorg. Med. Chem. 5 (1997) 765.
[43] J. Buchardt, M. Ferreras, C. Krog-Jensen, J.-M. Delaisee, N.T. Foged, M. Meldal, Chem. Eur. J. 5 (1999) 2877.
[44] M.G. Natchus, R.G. Bookland, M.J. Laufersweiler, S. Pikul, N.G. Almstead, B. De, M.J. Janusz, L.C. Hsieh, F. Gu, M.E. Pokross, V.S. Patel, S.M. Garver, S.X. Peng, T.M. Branch, S.L. King, T.R. Baker, D.J. Foltz, G.E. Mieling, J. Med. Chem. 44 (2001) 1060.
[45] D.E. Levy, F. Lapierre, W. Liang, W. Ye, C.W. Lange, X. Li, D. Grobelny, M. Casabonne, D. Tyrrell, K. Holme, A. Nadzan, R.E. Galardy, J. Med. Chem. 41 (1998) 199.
[46] S.X. Peng, C. Henson, L.J. Wilson, J. Chromatogr. B 732 (1999) 31.
[47] T.M. Branch, S.X. Peng, in: 51st Pittsburgh Conference, New Orleans, Louisiana, 2000.
[48] S.X. Peng, D.M. Bornes, C. Henson, personal communication.
[49] S.X. Peng, E. VonBargen, D.M. Bornes, S. Pikul, Pharm. Res. 15 (1998) 1414.
[50] O. Santos, C.D. McDermott, R.G. Daniels, K. Appelt, Clin. Exp. Metastasis 15 (1997) 499.
[51] N.D. Wood, M. Aitken, S. Harris, S. Kitchener, G.R. McClelland, S. Sharp, Br. J. Clin. Pharmacol. 42 (1996) 676P.
[52] T. Tuntland, K. Zhang, M.K. Ramos, B.V. Shetty, Pharm. Res. 14 (1997) S335.
[53] P.M. O'Brien, D.F. Ortwine, A.G. Pavlovsky, J.A. Picard, D.R. Sliskovic, B.D. Roth, R.D. Dyer, L.L. Johnson, C.F. Man, H. Hallak, J. Med. Chem. 43 (2000) 156.
[54] K.D. Altria, J. Chromatogr. A 856 (1999) 443.
[55] H. Watzig, M. Degenhardt, A. Kunkel, Electrophoresis 19 (1998) 2695.
[56] X. Gong, E.S. Yeung, Anal. Chem. 71 (1999) 4989.
[57] S.X. Peng, M.J. Strojnowski, J.K. Hu, B.J. Smith, T.H. Eichhold, K.R. Wehmeyer, S. Pikul, N.G. Almstead, J. Chromatogr. B 724 (1999) 181.
[58] W.M.A. Niessen, J. Chromatogr. A 856 (1999) 179.
[59] S.X. Peng, B. Borah, R.L. Dobson, Y.D. Liu, S. Pikul, J. Pharm. Biomed. Anal. 20 (1999) 75.
[60] M. Caldarelli, J. Habermann, S.V. Ley, Biorg. Med. Chem. Lett. 9 (1999) 2049.
[61] J.P. Shockor, I.S. Silver, R.M. Wurm, P.N. Sanderson, R.D. Farrant, B.C. Sweatman, J.C. Lindon, Xenobiotica 26 (1996) 41.
[62] U.G. Sidelman, U.B. Braumann, M. Hofmann, M. Spraul, J.C. Lindon, J.K. Nicholson, S.H. Hansen, Anal. Chem. 69 (1997) 607.
[63] J.K. Roberts, R.J. Smith, J. Chromatogr. 677 (1994) 385.
[64] J. Chin, J.B. Fell, M. Jarosinski, M.J. Shapiro, J.R. Wareing, J. Org. Chem. 63 (1998) 386.
[65] J.C. Lindon, R.D. Farrant, P.N. Sanderson, P.M. Doyle, S.L. Gough, M. Spraul, M. Hofmann, J.K. Nicholson, Magn. Reson. Chem. 33 (1995) 857.
[66] A.E. Mutlib, J.T. Strupczewski, S.M. Chesson, Drug Metab. Dispos. 23 (1995) 951.
[67] J.P. Shockor, S.E. Unger, I.D. Wilson, P.J.D. Foxall, J.K. Nicholson, J.C. Lindon, Anal. Chem. 68 (1996) 4431.
[68] R.M. Holt, M.J. Newman, F.S. Pullen, D.S. Richards, A.G. Swanson, J. Mass Spectrom. 32 (1997) 64.
[69] K. Albert, J. Chromatogr. A. 856 (1999) 199.
[70] S.X. Peng, Biomed. Chromatogr. 14 (2000) 430.
[71] S.J. Kok, N.H. Velthorst, C. Gooijer, U.A.Th. Brinkman, Electrophoresis 19 (1998) 2753.
[72] I.D. Wilson, J. Chromatogr. A 856 (1999) 429.
[73] G.W. Somsen, C. Gooijer, U.A.Th. Brinkman, J. Chromatogr. A. 856 (1999) 213.