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Direct determination of stability of protease inhibitors in plasma by HPLC with automated column-switching

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Abstract

Automated column-switching HPLC methods were developed and utilized for the direct analyses of three hydroxamic acid based metalloprotease inhibitors in rat plasma. These column-switching methods involved the use of a restricted-access media (RAM) precolumn and a column-switching valve, allowing the complete automation of sample preparation and HPLC. The plasma samples were directly injected onto a precolumn packed with SPS/ODS stationary phase and then backflushed onto an ODS analytical column using a 6-port column-switching device. The drug stability 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 traditional methods that involve sequential protein precipitation, liquid extraction, solvent evaporation, and sample reconstitution. 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^{-1}. This work represents, for the first time, a drug stability study in plasma by automated column-switching HPLC technique with the use of a RAM column. Our column-switching methods can be readily adapted to any existing HPLC system with minimal hardware modification.

Keywords: Column-switching; RAM column; MMP inhibitors; Drug stability

1. Introduction

Determination of drug stability in plasma plays an important role in the drug discovery and development process. It is not uncommon to find that many potent drug candidates undergo rapid degradation in plasma, resulting in not only short half-lives but also some toxic effects in animals and humans. In order to select the drug candidates that are stable in plasma, it is frequently required to evaluate a large number of drug compounds for their plasma stability. In most cases, HPLC has been chosen as the preferred technique for drug stability determination because of its applicability to a variety of different molecules.

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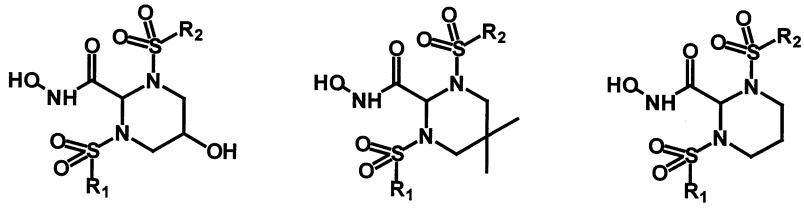
For HPLC analysis of plasma samples, however, sample pretreatment prior to HPLC separation is required to avoid plasma protein precipitation on a regular analytical column. Traditionally, plasma sample preparation is labor-intensive and time-consuming. It involves sequential plasma protein precipitation, centrifugation, extraction, solvent evaporation, and sample reconstitution. For study of drug stability in plasma, one additional step, i.e. incubation of drug plasma samples in a thermostated incubator, is needed. To speed up drug discovery, it is critical to develop a fully automated process for the evaluation of drug stability in plasma. The advent of restricted access media (RAM) based columns [1–3] has made it possible to automate plasma sample preparation and HPLC analysis. Based on the RAM concept, we have developed automated column-switching HPLC methods for the direct analysis of plasma samples utilizing a column-switching device, a RAM based column, and a conventional HPLC column. A detailed review on the use of RAM media in HPLC is given in the literature [4,5]. Briefly, a RAM column is comprised of a hydrophilic outer surface for the elution of large biomolecules with minimal retention and a hydrophobic inner surface for the containment of small analytes. Several different types of such columns have been developed and are commercially available. In the past, the automated column-switching technique was employed to do on-line solid phase extraction (SPE) for plasma and urine samples [6–9], where an SPE column was utilized to clean up the samples. However, the SPE elution procedure is often compound-dependent and may need to be developed separately. By contrast, the elution procedure for a RAM column is independent of compound. A universal elution procedure, e.g. elution with a phosphate buffer, can be applied to different classes of compounds in plasma. Therefore, an automated column-switching method using a RAM column is easy to develop and use compared to using an SPE column. In this work, we chose a semipermeable surface (SPS) column as our RAM column for the development and application of automated column-switching HPLC methods. Like other RAM media, the SPS phase consists of a hydrophilic outer surface, in which a polyoxyethylene polymer is bonded to the silica surface, and a hydrophobic inner surface, in which the conventional phases are bonded underneath the polymer. The main advantage of using an SPS column is that the inner surface of the SPS phase can be varied independently. By combining the use of a RAM column and a column switching device, we are able to develop fully automated one-pump column-switching HPLC methods for the direct analysis of drug stability in rat plasma. These methods are so simple that they only involve an external attachment of a commercially available column-switching valve to an existing HPLC system. No additional hardware modification is needed. This compares favorably to other two-pump column-switching methods where one additional pump needs to be added to an existing HPLC system [10]. Our goal of using the automated column-switching technique is two-fold. One is to eliminate traditional plasma sample preparation procedures associated with drug stability studies and the other is to obtain more data points at shorter intervals for any desired period of time so that drug degradation kinetics can be followed and determined more accurately. Although column-switching technique has been employed previously with RAM columns coupled to regular analytical columns for analyte preconcentration [10–16], this work represents the first time such an arrangement has been used for the study of drug stability in plasma. Normally, the use of RAM columns for the direct analysis of drugs in plasma is limited to the compounds that exhibit good plasma stability.

The drug compounds used in this work are hydroxamic acid based matrix metalloprotease (MMP) inhibitors. These compounds are explored as potential drug candidates for the treatment of cancers, arthritic disorders, and other connective tissue related diseases. This class of compounds is known to be readily hydrolyzed in plasma. Therefore, the stability determination of these drugs in plasma is important in drug screening and selection. In doing so, we developed automated column-switching methods to eliminate traditional time-consuming and labor-intensive sample preparation procedures. This paper reports the



I II III

Fig. 1. Structures of hydroxamic acid based MMP inhibitor compounds I, II, and III.

development and application of automated column-switching HPLC methods for the direct determination of plasma stability of three metalloprotease inhibitors, PGE-1132881 (compound I), PGE-4410186 (compound II), and PGE-9818266 (compound III).

2. Experimental

2.1. Materials

Metalloprotease inhibitors (as shown in Fig. 1), PGE-1132881 (compound I), PGE-4410186 (compound II), and PGE-9818266 (compound III) were obtained from Procter & Gamble Pharmaceuticals (Mason, OH). HPLC-grade acetonitrile, formic acid, monobasic sodium phosphate, and dibasic sodium phosphate were purchased from J.T. Baker (Phillipsburg, NJ). Blank rat plasma was obtained from Rockland (Gilbertsville, PA).

2.2. Solutions

The stock standard solutions of compounds I, II, and III were prepared by dissolving the respective compound in blank plasma to yield a concentration of 100 μg ml^{-1}. The working standard solutions were made by a serial dilution of the stock solution with blank plasma to give concentrations of 100, 50, 25, 10, 1, and 0.1 μg ml^{-1}, respectively. The stability samples were made by dissolving the compound in the blank plasma to give a concentration of 25 μg ml^{-1}. A phosphate buffer was made by dissolving 1.38 g of monobasic sodium phosphate and 14.3 g of dibasic sodium phosphate in 1 l Milli-Q water to yield 50 mM phosphate buffer at pH 7.4. When the automated column-switching HPLC methods were employed, the plasma samples were transferred to HPLC vials in the HPLC autosampler thermostated at 37°C for direct analysis. When a traditional plasma sample preparation procedure was used, aliquots of 1 ml plasma samples were incubated at 37°C and retrieved at different times. Aliquots of 200 μl plasma samples were then treated with two volumes of acetonitrile to precipitate plasma proteins. The resulting samples were centrifuged at $2000 \times g$ for 15 min. Aliquots of 450 μl supernatant solutions were taken and evaporated under nitrogen. The residues were reconstituted with Milli-Q water to yield the same concentrations as the original plasma samples for HPLC analysis. The calibration curves were generated by analyzing the standard samples immediately after they were made to minimize compound degradation.

2.3. Equipment and chromatographic conditions

The HPLC system consisted of a Waters (Milford, MA) 600S controller, a 616 pump, an in-line degasser, a 717 plus thermostatic autosampler, and a 996 photodiode array detector. The column switching device was assembled using a Rheodyne (Cotati, CA) 7750TPMV 6-port column switching valve. The complete system, including the column-switching device, was controlled by the Waters Millennium data system. A Regis (Morton Grove, IL) SPS precolumn (ODS inner phase, 50×2.1 mm i.d., 5 μm) and a Waters (Milford, MA) analytical column (Symmetry C18, 50×2.1

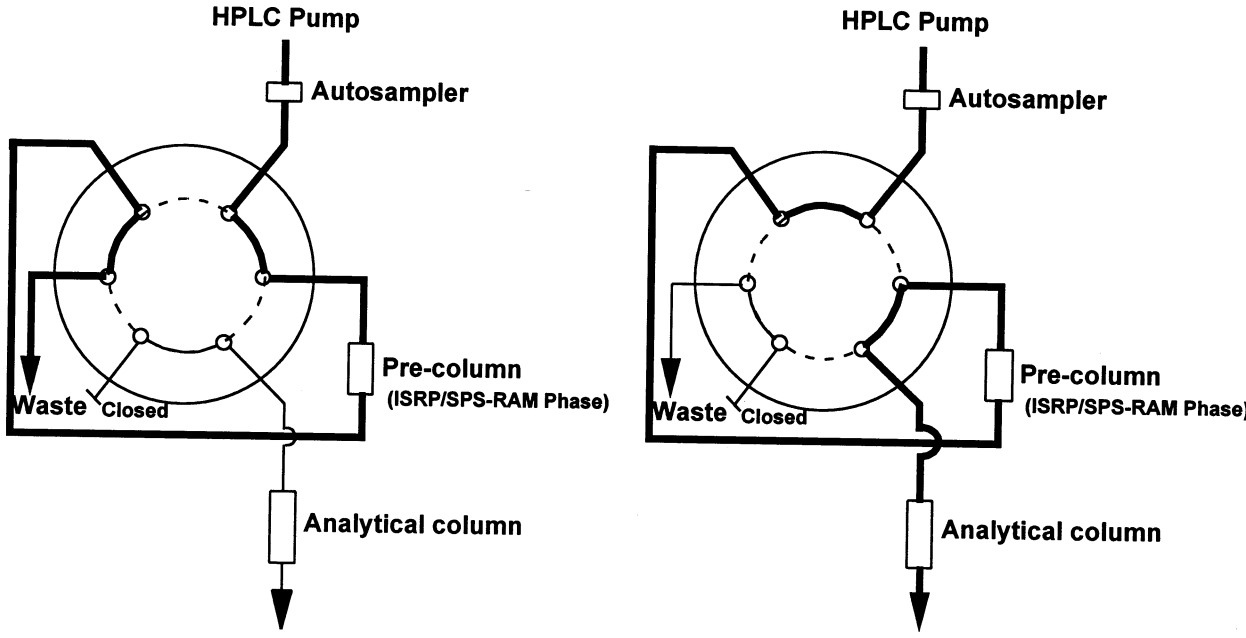


Fig. 2. Diagrams for the one-pump column-switching system. Position A (on the left) is for direct plasma injection and plasma protein removal from precolumn washing. Position B (on the right) is for precolumn backflushing and analyte separation in the analytical column.

mm i.d., 3.5 μm) were utilized. The mobile phase consisted of 5% acetonitrile, 95% water, 0.1% formic acid (mobile phase A) and 80% acetonitrile, 20% water, 0.1% formic acid (mobile phase B). The mobile phase for the precolumn elution was 50 mM phosphate buffer at pH 7.4 in Milli-Q water (mobile phase C). The flow rate was 0.6 ml min^{-1} with UV detection at 245 nm.

Chromatographic conditions and column-switching procedures were as follows. In step I (0–3 min), the column-switching valve was in position A (as shown in Fig. 2). An aliquot of 25 μl of the plasma sample was directly injected onto the SPS precolumn and eluted by mobile phase C consisting of a 50 mM phosphate buffer at pH 7.4 in water. In step I, the plasma proteins were washed away from the precolumn while analytes were retained in the precolumn. In step II (3–5 min), the column-switching valve was in position B (as shown in Fig. 2). The precolumn was backflushed by a linear gradient elution from 100% mobile phase A to the regular initial mobile phase conditions: 30% mobile phase B for compound I; 40% mobile phase B for compound II; and 35% mobile phase B for compound III. In step II, the retained analytes in the precolumn were eluted onto the analytical column for separation. In step III (5–20 min), the column-switching valve was in position B. Both the precolumn and analytical column were eluted by a linear gradient from the initial mobile phase conditions (final mobile phase compositions in step II) to 100% mobile phase B for all three compounds. In step III, all analytes were separated on the regular analytical column. For the HPLC analyses using a traditional plasma protein preparation procedure, aliquots of 25 μl of the reconstituted plasma samples were injected onto a regular analytical column for separation. A 20-min linear gradient elution from the initial mobile phase compositions (30, 40, and 35% mobile phase B for compounds I, II, and III, respectively) to the final mobile phase compositions (100% mobile phase B for all three compounds).

3. Results and discussion

The automated one-pump column-switching HPLC system (as shown in Fig. 2) was developed and employed to study the drug stability of three protease inhibitors in rat plasma. The use of this automated system eliminated a traditional plasma sample preparation procedure involving sequential protein precipitation, centrifugation, liquid extraction, solvent evaporation, and sample reconstitution, allowing the complete automation of sample preparation and HPLC. This one-pump column-switching system can be conveniently adapted to any existing HPLC system. The only modification to an existing HPLC system is to add a 6-port column-switching valve in place of an analytical separation column and then attach a RAM precolumn (an SPS precolumn in this work) as shown in Fig. 2. This one-pump system is especially convenient for routine determination of plasma samples compared to a two-pump column-switching system [10] where one additional pump needs to be purchased and added. The use of the SPS precolumn allows a generic elution method to be developed for the elimination of plasma proteins from plasma samples of different compounds. The main feature of our column-switching system is simple, yet rugged and easy to use.

The specificity of our column-switching methods for all three compounds are demonstrated in Figs. 3–5. Our analyte peaks were clearly 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^{-1} with correlation coefficients of greater than 0.999. The method precision and accuracy were also investigated. The precision expressed as the relative standard deviation (C.V.%) based on eight repetitive injections at a drug concentration of 25 μg ml^{-1} was less than 4% (intra-day) and 8% (inter-day) for all three compounds. The accu-

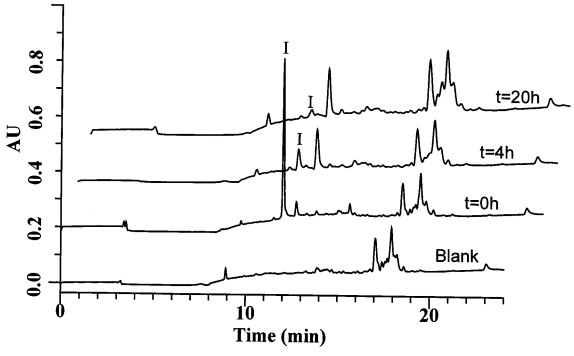


Fig. 3. Chromatograms of the plasma samples of compound I at different times of incubation at 37°C in the thermostated autosampler.

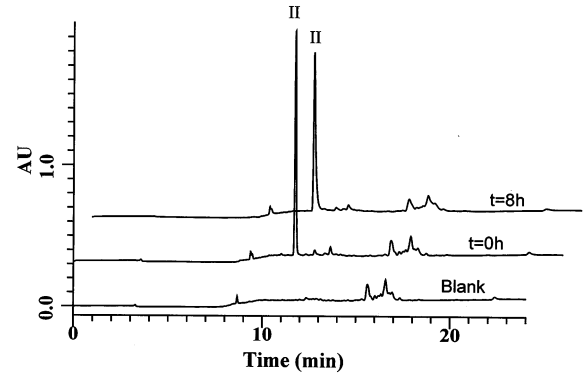


Fig. 4. Chromatograms of the plasma samples of compound II at different times of incubation at 37°C in the thermostated autosampler.

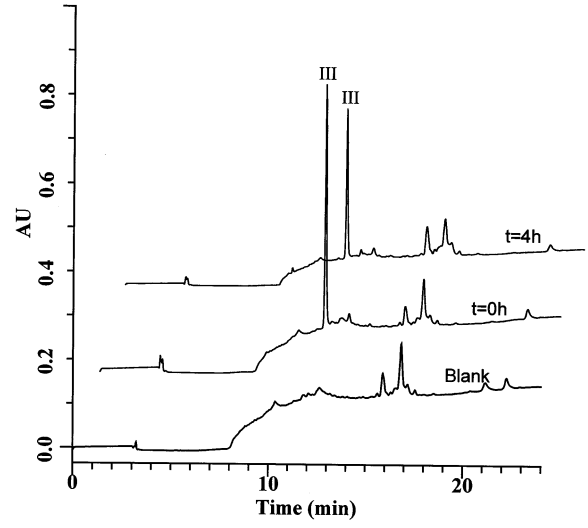


Fig. 5. Chromatograms of the plasma samples of compound III at different times of incubation at 37°C in the thermostated autosampler.

racy was found to be greater than 95% for all three compounds in four drug levels ranging from 0.5 to 100 μg ml^{-1}. The detection limits at a signal-to-noise ratio of 2 were 20 ng ml^{-1} for compounds I and III and 10 ng ml^{-1} for compound II.

In this work, the plasma samples of each drug compound were placed in the HPLC autosampler thermostated at 37°C and directly injected onto the SPS precolumn at different times for the determination of drug stability in rat plasma. The quantitation of each sample was made using a calibration curve of the respective compound. Figs. 3–5 show some representative chromatograms of blank plasma and the incubated plasma samples of the three compounds obtained from the automated column-switching HPLC system. As shown in the figures, the separations of the parent compounds, degradants, and the plasma matrix components were achieved on the analytical C18 column after the on-line removal of the plasma proteins from the SPS precolumn. Compound I was unstable with more than 90% loss of the parent compound after 20 h of incubation at 37°C. In contrast to compound I, compound II was stable with less than 10% loss of the parent drug after 20 h of incubation at 37°C. Compound III was found to be more stable than compound I but less stable than compound II with more than 60% loss of the parent compound after 20 h of incubation.

To evaluate the performance of the automated column-switching system, the drug stability of compounds I (unstable drug) and II (stable drug) in rat plasma was also determined using a traditional plasma sample preparation procedure. The results from both approaches are plotted in Figs. 6 and 7. Not surprisingly, the results from the automated column-switching HPLC were in good agreement with those from the traditional HPLC methods. Clearly, the automated column-switching methods were rugged, accurate, and reliable. Because of the reliability of the methods, we determined the stability of the third compound simply by an automated column-switching method. The results of the drug concentration versus time are plotted in Fig. 8. As seen from Figs. 6–8, the stability of the three drugs was in the following order: Compound II > Compound III > Compound I. Therefore, one can rapidly rank order drug compounds according to their

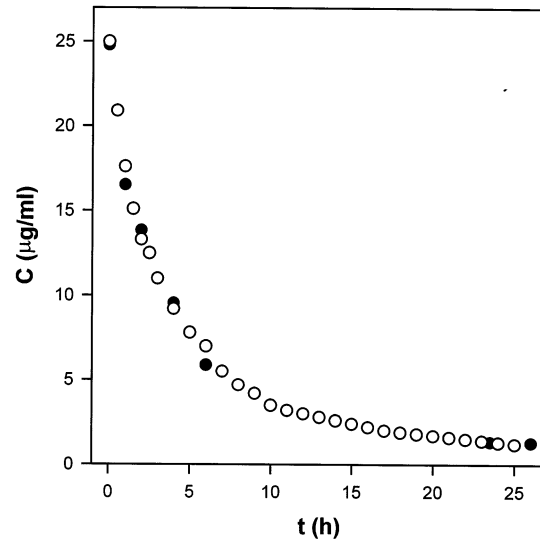


Fig. 6. Drug concentration as a function of incubation time for compound I with (○) and without (●) the use of automated column-switching.

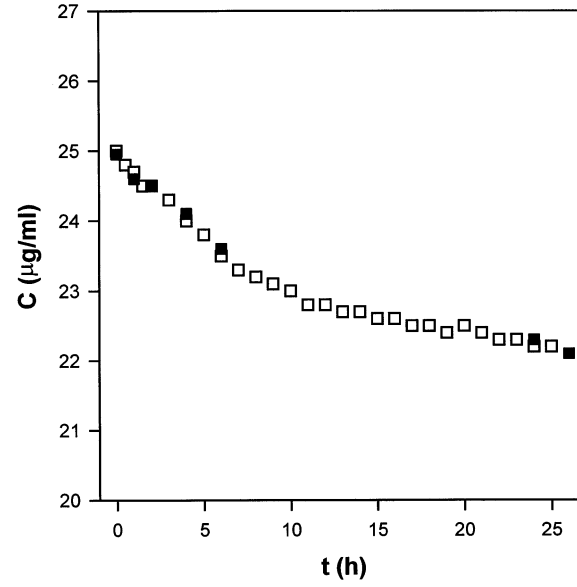


Fig. 7. Drug concentration as a function of incubation time for compound II with (□) and without (■) the use of automated column-switching.

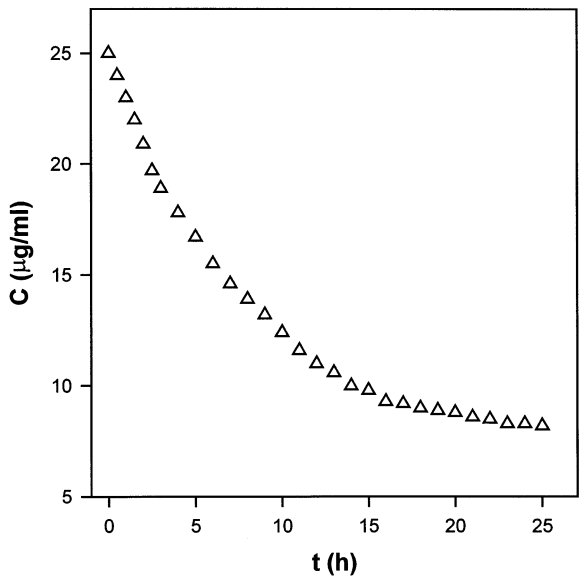


Fig. 8. Drug concentration as a function of incubation time for compound III with the use of automated column-switching.

stability in plasma using an automated column-switching system.

In addition to the elimination of labor-intensive and time-consuming sample preparation procedures, the automated column-switching methods allow unattended overnight HPLC operation for the determination of drug stability in plasma. This automated system is especially useful for drug stability study because drug samples can be incubated in a thermostated HPLC autosampler instead of an external incubator. In this way, drug concentrations can be obtained at various times not only during the day but also during the night, which is often impractical by traditional sample preparation. Hence, more data points can be added to the curve of drug concentration versus time for more accurate determination of drug degradation kinetics and half-life in plasma. In this case, the degradation kinetics for all three compounds were found to follow the first-order reaction kinetics based on the linear relationship between logarithm of drug concentration and incubation time, indicating that drug half-lives are independent of their initial concentrations.

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