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Journal of Chromatography B, 724 (1999) 181–187

JOURNAL OF CHROMATOGRAPHY B

Short communication

Gas chromatographic–mass spectrometric analysis of hydroxylamine for monitoring the metabolic hydrolysis of metalloprotease inhibitors in rat and human liver microsomes

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Received 28 September 1998; received in revised form 23 November 1998; accepted 30 November 1998

Abstract

A gas chromatographic–mass spectrometric (GC–MS) method was developed for the analysis of hydroxylamine (HA) in supernatants obtained from liver microsomes. HA monitoring was used to determine the metabolic hydrolysis of two hydroxamic acid-based matrix metalloprotease inhibitors in rat and human liver microsomes. The hydrolysis of the hydroxamic acids to their corresponding carboxylic acids releases HA as a common metabolic product. 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. The method is simple, reproducible, and sensitive for the determination of the hydrolysis of hydroxamic acid compounds, where hydrolysis is the major metabolic pathway. The methodology can be used for rank ordering and selecting hydroxamic acid analogs based on their susceptibility to hydrolysis.

Keywords: Matrix metalloprotease inhibitors; Hydroxylamine; Metalloprotease

1. Introduction

Matrix metalloprotease (MMP) inhibitors are currently being explored as potential drug candidates for the treatment of cancer, arthritic disorders, and other connective tissue related diseases [1]. As one of the important classes of MMP inhibitors, hydroxamic acid-based compounds are extensively studied for their inhibitory activities against various MMPs. Many of these compounds are found to be metabolized rapidly in vivo, adversely affecting their utility in treating the diseases. Further in vitro and in vivo experiments suggest that the hydrolysis of hydroxamic acids to the corresponding carboxylic acids may be the major route of metabolism of many hydroxamates [2]. Therefore, determination of the susceptibility of these compounds to hydrolysis is critical for the synthesis and selection of metabolically stable drug candidates. Quantitation of the hydrolytic metabolites of a hydroxamic acid, in in vitro or in vivo metabolism samples, is usually accomplished using the specially synthesized corresponding carboxylic acid as a calibration standard in a chromatographic method. For drug screening and selection from a large number of compounds, however, it is

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0378-4347/99/$ – see front matter
PII: S0378-4347(98)00557-X

impractical to prepare the carboxylic acid for each corresponding hydroxamic acid analog evaluated in metabolism assays. Since a common metabolic product, hydroxylamine (HA), is released when a hydroxamic acid is metabolized to the corresponding carboxylic acid (see Fig. 1), it is simple and convenient to measure HA to determine the extent of hydrolysis.

Gas chromatography (GC) with electron capture (EC) detection methods were developed to measure HA in marine and fresh waters [3,4], where HA was converted to nitrous oxide by ferric oxidation and quantitated subsequently by EC detection. GC with nitrogen and flame ionization detection (FID) method was also reported to determine HA in colonic fluid [5], where HA was converted to acetone oxime by reaction with a ketone, which was further separated by a packed glass column and measured by nitrogen and FID detection. Here, we present a simple and sensitive stable-isotope-dilution GC–MS method developed based on the previous method [5] for the determination of HA in rat and human liver microsomal samples. In this method, HA was derivatized with acetone to yield the corresponding acetone oxime (as shown in Fig. 1) and subsequently separated by a capillary column for better separation and quantitated by an MS detector for increased sensitivity. The stable isotope labeled HA was used to improve precision and accuracy. The GC–MS method was employed to evaluate the metabolic hydrolysis of two hydroxamate-based MMP inhibitors (see Fig. 2) in rat and human liver microsomes. Liver microsomes were chosen as our in vitro system because the enzymes that hydrolyze hydroxamic acids are localized primarily in the liver microsomal fraction.

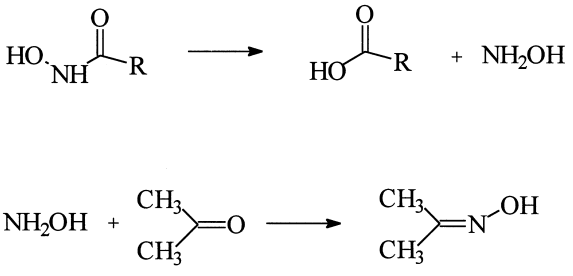
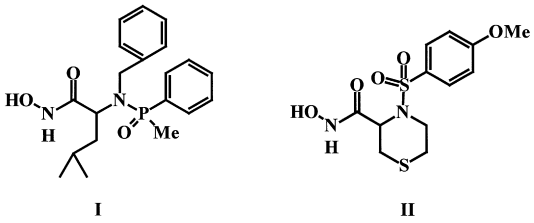


Fig. 1. Reaction schemes for the metabolic hydrolysis of a hydroxamic acid and the conversion of hydroxylamine to acetone oxime.



I II

Fig. 2. Molecular structures of compounds I and II.

2. Experimental

2.1. Materials

Metalloprotease inhibitors (see Fig. 2), compound I (*N*-hydroxy-2-[(methylphenyl-phosphonyl) (benzyl)-amino]-4-methylpentanamide) and compound II (*N*-hydroxy-4-[(4-methoxyphenyl) sulfonyl]-thiomorpholine-3-carboxamide), were obtained from Procter & Gamble Pharmaceuticals (Mason, OH, USA). HA hydrochloride (99.999% purity) and acetone oxime (98% purity) were purchased from Aldrich (Milwaukee, WI, USA). An isotopically labeled [^{15}N]hydroxylamine hydrochloride ([^{15}N]HA, 98+% labeled) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Acetone (HPLC grade) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Human liver microsomes were obtained from In Vitro Technologies (Baltimore, MD, USA). Rat liver microsomes were prepared in house as follows. Sprague-Dawley rats were sacrificed by carbon dioxide asphyxiation. The livers were removed, blotted and weighed. The entire liver was then transferred to ice-cold pH 7.4 homogenization buffer prior to preparation of hepatic microsomes. Hepatic microsomes were prepared by differential ultracentrifugation using standard techniques described in the literature [6]. The final pellet was suspended in a storage buffer containing glycerol (20%, v/v) and stored at ≤ -70°C. The protein concentration of the

final hepatic microsomal suspension was measured spectrophotometrically using a Pierce BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA).

2.2. Liver microsome incubations

Rat and human hepatic microsomal fractions were prepared at a total enzyme concentration of 2 mg/ml in an incubation buffer consisting of 50 m*M* K_2HPO_4 (pH 7.4), 3.0 m*M* $MgCl_2$ and 0.1 m*M* EDTA. MMP inhibitors I and II were incubated at two concentrations (10 and 100 μ*M*) with the rat and human microsomal fractions at 37°C for 0.5 and 1.0 h. The incubation mixtures were then centrifuged at 1,800 *g* for 15 min and the supernatant was isolated and taken for subsequent derivatization with acetone.

2.3. Preparation of standards and quality control samples

The stock solution of HA was prepared by dissolving HA hydrochloride in the blank liver microsomal incubation media to give a concentration of 500 μg/ml. For calibration standards, serial dilutions of the HA stock solution with the blank incubation media were made to give six standard concentrations ranging from 0.1 to 100 μg/ml. Quality control (QC) samples were prepared by spiking blank liver microsomal incubation media with an appropriate volume of the HA stock solution to yield three concentrations at 0.1, 10, and 100 μg/ml. The stock solution of internal standard [^{15}N]HA was prepared in a manner similar to that described for HA to yield a concentration of 500 μg/ml.

2.4. Sample preparation

All the standard and QC samples were treated in the same manner as liver microsomal incubation samples during sample preparation. Aliquots (20 μl) of the [^{15}N]HA stock solution (500 μg/ml) were added to aliquots (960 μl) of the standards, QC samples and liver microsomal incubation samples. Aliquots (20 μl) of acetone were then added to the above solutions to give an internal standard concentration of 10 μg/ml, and form the acetone oxime derivatives of HA and [^{15}N]HA at concentration ratios (unlabeled to labeled) of 10 to 0.01 for the standards and QC samples. The derivatization was conducted at room temperature and the reaction was completed instantaneously. The resulting solutions were transferred to individual autosampler vials for GC–MS analysis.

2.5. Gas chromatography–mass spectrometry

The GC–MS system consisted of a Hewlett-Packard (HP, Palo Alto, CA, USA) Model 5890 gas chromatograph, an HP Model 5970 mass selective detector and an HP Model 7673 autosampler. A Restek (Bellefonte, PA, USA) Stabilwax capillary column (30 m×0.25 mm I.D., 0.5 μm film) was used for the separation. Helium was used as the carrier gas at a column head pressure of 10 p.s.i. (69 kPa). The thermal program consisted of an initial hold at 50°C for 1 min, followed by a linear thermal program to 200°C at a rate of 15°C/min. The injection port was held at 80°C and was operated in the splitless mode (split time=1.0 min) with a 4-mm ID straight Pyrex liner (Restek) lightly packed with deactivated glass wool (Restek). An injection volume of 2 μl was used for all standards and samples. The transfer line was set at 220°C.

The electron impact (EI) mass spectral measurements were performed at an ionization potential of 70 eV. The full scan spectra of derivatized HA and [^{15}N]HA were acquired over a mass range from m/z 10–m/z 200. The selected-ion-monitoring (SIM) analysis was conducted using m/z 73.1 and m/z 74.1 for the HA and [^{15}N]HA derivatives, respectively, with a dwell time of 100 ms for each ion.

2.6. Quantitation of HA

Linear calibration curves were constructed by plotting the peak area ratios (peak area derivatized HA/peak area derivatized [^{15}N]HA) for standards versus HA concentration in the standard. The peak area ratios obtained for QC and liver microsomal incubation samples were used to determine HA levels by interpolation from the linear regression calibration line.

3. Results and discussion

3.1. Full scan mass spectra and selected-ion-monitoring

The full scan mass spectra obtained for HA and [^{15}N]HA derivatives, i.e., acetone oxime and [^{15}N]acetone oxime, are shown in Fig. 3. The spectra were characterized by a prominent molecular ion at *m*/*z* 73 and *m*/*z* 74, respectively, as well as a number of smaller ions. The molecular ion of each derivative was used for SIM in the GC–MS quantitative analysis.

3.2. Selectivity and linearity of calibration

The standards, QC and liver microsomal samples were directly injected into the GC–MS system as

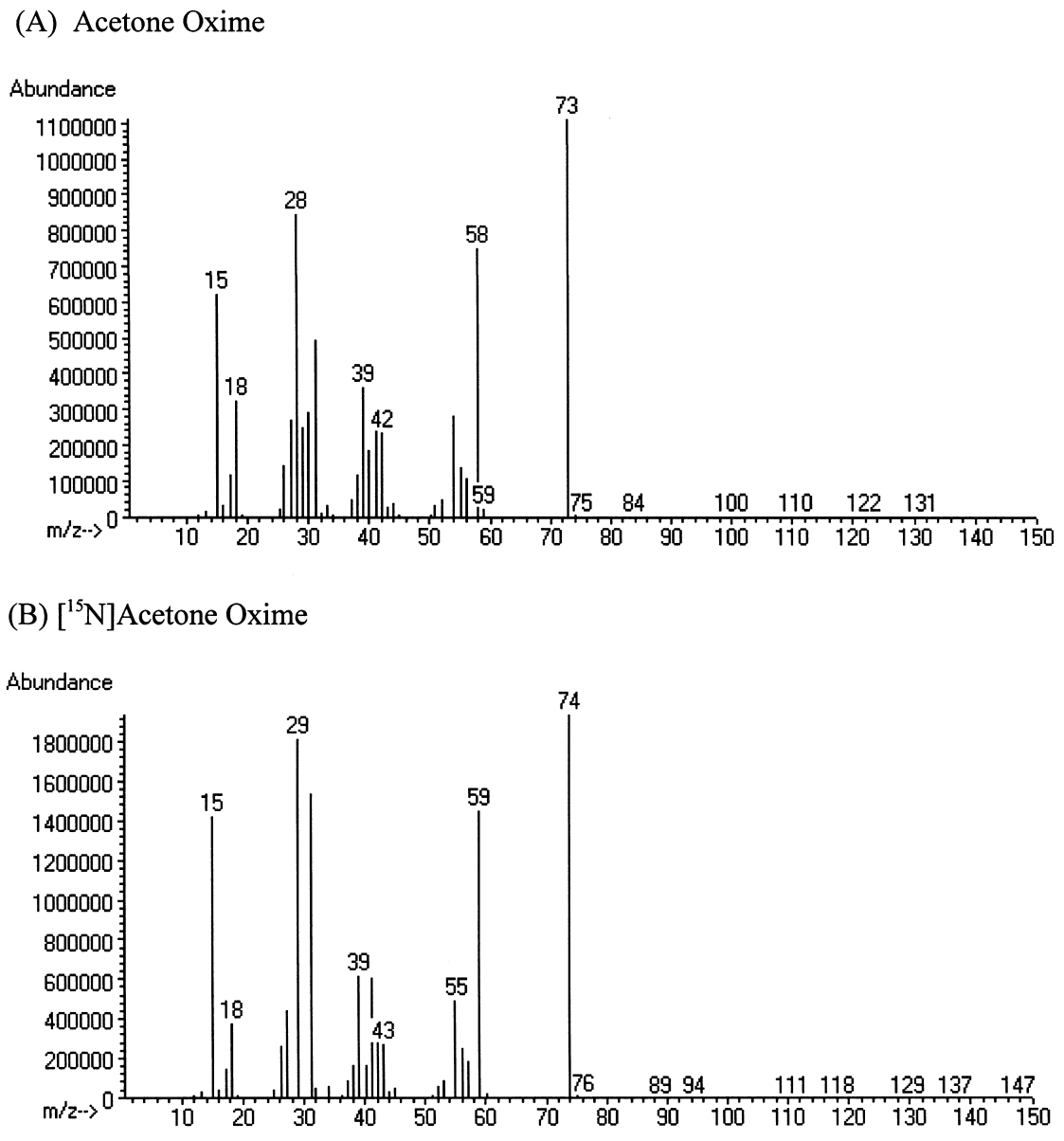


Fig. 3. Electron impact (70 eV) mass spectra for (A) acetone oxime and (B) [^{15}N]acetone oxime.

aqueous samples without prior isolation of the derivatized HA and [^{15}N]HA. Both the analyte and internal standard eluted at 8.13 min and the SIM channel for each ion was free from any matrix interferences (Fig. 4). Since the stable isotope labeled internal standard and the analyte differ by only 1 m/z unit, it was necessary to restrict the range of the standard curve due to the contributions of ^{15}N and ^{13}C ions to the signal of derivatized HA at m/z 74. The peak area ratios for the standards gave a

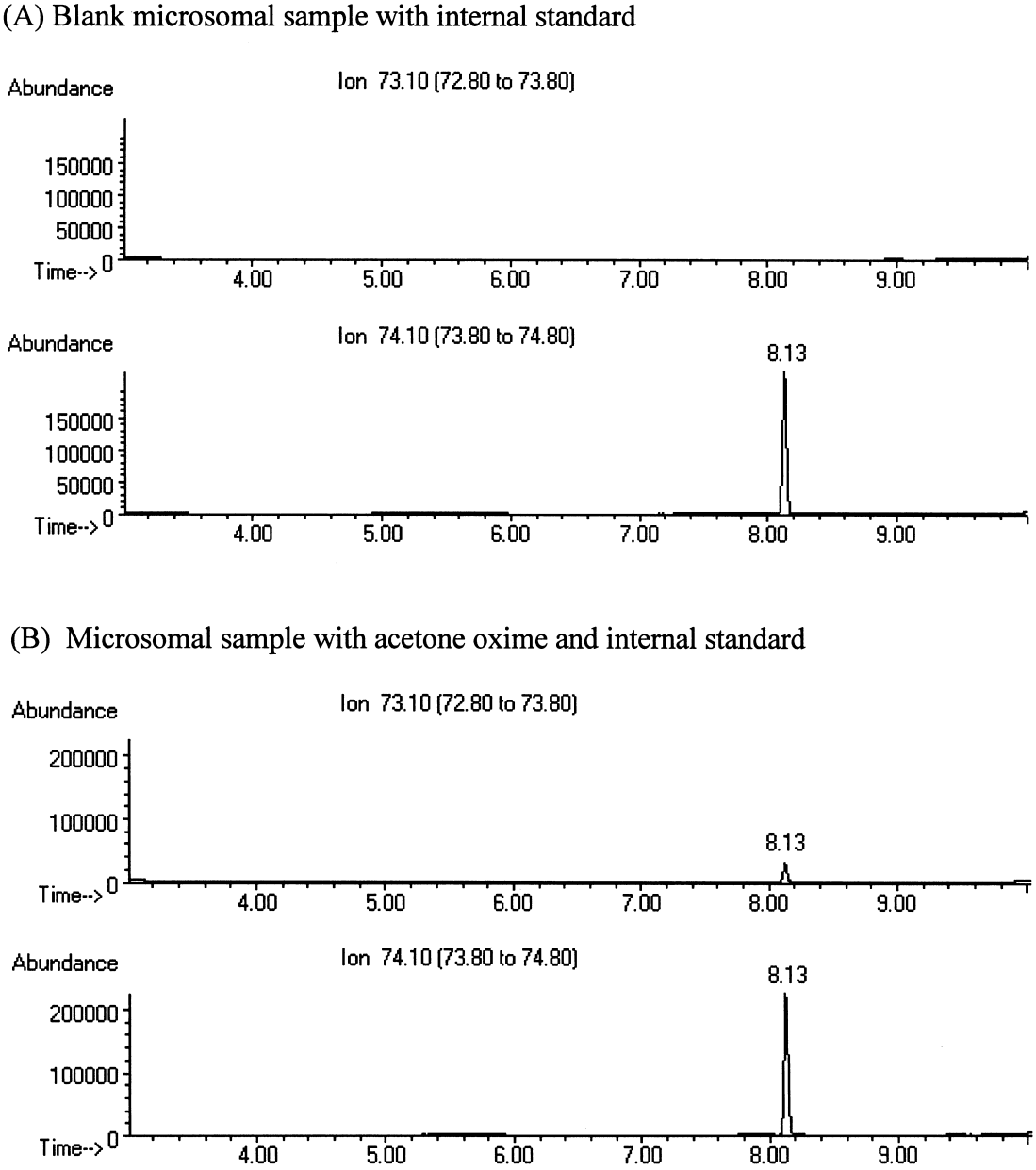


Fig. 4. Selected-ion-monitoring profiles following derivatization with acetone for (A) a blank liver microsomal sample spiked with 10 μg/ml [^{15}N]HA hydrochloride and (B) a blank liver microsomal sample spiked with 1 μg/ml HA hydrochloride and 10 μg/ml [^{15}N]HA hydrochloride.

Table 1
Hydroxylamine levels obtained from rat and human liver microsomal incubations with compounds I and II

Drug concentration (μM)	Incubation time (h)	Compound I ($\mu g/ml$)	Conversion	Compound II ($\mu g/ml$)	Conversion
Rat					
10	0.5	0.30±0.02	43%	0.41±0.02	59%
	1.0	0.32±0.02	46%	0.40±0.02	58%
100	0.5	1.22±0.05	17.6%	2.30±0.10	33.1%
	1.0	1.27±0.05	18.3%	2.32±0.10	33.4%
Human					
10	0.5	0.30±0.02	43%	0.49±0.02	71%
	1.0	0.31±0.02	45%	0.48±0.02	69%
100	0.5	1.40±0.05	20.1%	4.10±0.16	60.0%
	1.0	1.43±0.05	20.6%	4.24±0.16	61.0%

linear calibration curve in the range of 0.1–50 μg/ml with the correlation coefficient of greater than 0.999.

3.3. Accuracy and precision

Intra-assay accuracy and precision were determined by analyzing blank liver microsomal matrix spiked with HA at 0.1, 10, and 100 μg/ml and internal standard [^{15}N]HA at 10 μg/ml ($n=5$). The accuracy was found to be 95.4%, 98.6%, and 102.1%, with RSD values of 4.0%, 2.1% and 3.4% for the 0.1, 1 and 10 μg/ml spiked samples. Replicate injections ($n=8$) of the same sample gave RSD values of less than 4% (intra-day) and 8% (inter-day for three consecutive days) at all spiked levels.

3.4. Absolute recovery, LOD, LOQ, and analyte stability

Absolute recoveries were determined to assess the completeness of the derivatization of HA. They were evaluated at three concentrations (0.1, 10, and 100 μg/ml) using the peak area ratios between the blank incubation samples spiked with HA hydrochloride which was subsequently derivatized with acetone and the blank incubation samples spiked with acetone oxime. The absolute recoveries for the analyte at three concentration levels (0.1, 10, and 100 μg/ml) were greater than 99.5%. The limit of detection (LOD), at a signal-to-noise ratio of 3, was 10 ng/mL. The limit of quantitation (LOQ), at a signal-to-noise ratio of 10, was 33 ng/ml. In addition, the analyte was found to remain stable in the incubation media for at least 3 days at ambient temperature.

3.5. Liver microsome incubations with hydroxamic acid analogs

One of the potential major routes of metabolism of hydroxamic acid analogs is via hydrolysis to yield the corresponding carboxylic acid analog and HA (Fig. 1). Hydroxamic acid compounds I and II (see Fig. 2) were incubated with rat and human liver microsomes over a 1 h period and the levels of HA were measured as an indicator of hydrolytic metabolism. Both compounds were found to be hydrolyzed extensively as indicated by the levels of HA found in the incubation samples, as shown in Table 1. The extent of hydrolysis was affected by the compound structure, the concentration of the compound and the source of the liver microsomes. Compound II was hydrolyzed to a greater extent, at both the 10 and 100 μM concentrations, than compound I by both rat and human liver microsomes. Compound I was found to be hydrolyzed to essentially the same extent by rat and human liver microsomes, while compound II was found to undergo more extensive hydrolysis in human liver microsomes than in rat liver microsomes. For both compounds, the extent of hydrolysis was less for the 100 μM level than for the 10 μM level in both rat and human liver microsomes, suggesting that the hydrolytic enzymes present in the liver microsomes may be saturated by these two compounds at the 100 μM concentration.

4. Conclusions

Methodology was developed for monitoring the extent of hydrolysis of hydroxamic acid analogs in in vitro liver microsomal incubations via the measurement of a common metabolite, HA, using stable-isotope-dilution GC–MS analysis. The simplicity and universal applicability of the method for all hydroxamic acids will facilitate the rapid evaluation of hydrolytic stability for this class of compounds. Some precautions may be taken when applying this method to determining the hydrolysis of hydroxamic acids in animal tissues, biological fluids, or complex matrices, where a higher-molecular-mass ketone may be required for derivatization so that the potential interference in the low mass region can be avoided. In general, the application of the method to the identification of hydroxamic acids with enhanced stability to metabolic hydrolysis should lead to the development of improved orally bioavailable analogs since hydrolysis is one of the major metabolic routes for this class of compounds.

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