Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
33 (2003) 1081–1092
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Determination of hydroxyproline in plasma and tissue using electrospray mass spectrometry

Erick Kindt [a,*], Kristina Gueneva-Boucheva [a], Mark D. Rekhter [b], Jamie Humphries [a], Hussein Hallak [a]

[a] *Department of Pharmacokinetics, Dynamics, and Metabolism, Pfizer Global Research and Development, 2800 Plymouth Road, Ann Arbor, MI 48105, USA*

[b] *Department of Cardiovascular Pharmacology, Pfizer Global Research and Development, 2800 Plymouth Road, Ann Arbor, MI 48105, USA*

Received 25 October 2001; received in revised form 2 May 2003; accepted 14 June 2003

Abstract

A simple and highly specific method that was developed for the determination of hydroxyproline in biological samples is described. This method could potentially be used for monitoring pathological conditions related to collagen degradation, as well as for screening remedial pharmaceuticals for efficacy. Tissue or plasma samples were prepared by hydrolysis and their hydroxyproline content was determined using spiked calibration curves and LC/MS/MS. Specificity of the method was evaluated using an API Time-Of-Flight (TOF) LC/MS to expose potential interferences. The method proposed here appears to be selective, convenient, precise ($< 10\%$ R.S.D.), accurate ($< 10\%$ RE), and sensitive over a linear range of 0.010–10 μg/ml.
© 2003 Elsevier B.V. All rights reserved.

Keywords: Electrospray; Hydroxyproline; Collagen; Tissue; Plasma; Mass spectrometry

1. Introduction

In newly synthesized collagen, many proline residues become hydroxylated to form the pyrrolidinic imino acid hydroxyproline (Fig. 1). These added hydroxyl groups help stabilize the collagen fiber. Because hydroxyproline is located almost exclusively in collagen, where it makes up approximately 10% of this protein [1], its content in various tissues, plasma, and urine is thought to be a reliable indicator of collagen catabolism. In fact, while scientists are contemplating several different parameters like collagen propeptides and pyridinium cross-links [2], there is considerable interest in hydroxyproline levels as an indicator of various pathological conditions related to collagen degradation [3–10].

Although hydroxyproline has been determined with numerous procedures and modifications over the last 50 years [1,3,4,10], efforts towards its convenient bioanalysis have been hampered for two primary reasons—sensitivity and selectivity.

* Corresponding author. Tel.: +1-734-622-2861; fax: +1-734-622-5115.

0731-7085/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0731-7085(03)00359-5

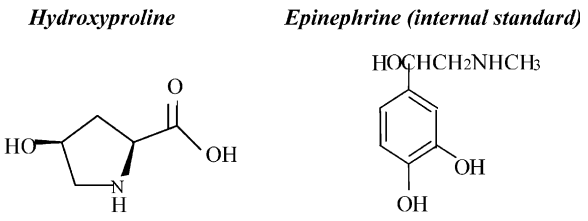


Fig. 1. Structures of hydroxyproline (analyte) and epinephrine (internal standard).

Like other amino acids, hydroxyproline is non-volatile, absorbs only weakly in the ultraviolet-visible region, and possesses no native fluorescence. Therefore, most conventional analytical methodologies have involved some sort of derivatization step to convert hydroxyproline to a detectable form with adequate sensitivity. Because of the variety and complexity of matrices from which hydroxyproline is routinely determined, large numbers of other endogenous interfering components such as amino acids, sugars, and fatty acid salts further complicate its accurate quantification.

This paper describes a novel LC/MS/MS analytical method for hydroxyproline, which is under consideration as a potential tool to study drug efficacy in collagen degradation models [6,7]. Hydroxyproline was chosen as the biomarker of interest because of its relevance in determining changes in tissue collagen content. The method was developed to support the study of a series of matrix metalloproteinase (MMP) inhibitors in various efficacy models related to collagen break down [8,9,11,12]. The development of this biomarker analytical method is discussed.

2. Materials and methods

The quantification of the analytes was performed by LC/MS/MS. Specificity of the method was confirmed using Liquid Chromatography Time-Of-Flight (LC/TOF) mass spectrometry. Tissue or plasma samples were collected and stored at $-20\,^{\circ}C$ until analysis.

2.1. Chemicals and reagents

High performance liquid chromatography (HPLC) grade water and acetonitrile were from Mallinkrodt (Paris, KY). Formic acid (95–97%) was from Aldrich (Milwaukee, WI). Hydrochloric acid (36.5–38%) and acetic acid (glacial) were from EM Science (Gibbstown, NJ). The internal standard epinephrine, *cis*-4-hydroxy-L-proline (natural isomer), and L-leucine were from Sigma (St. Louis, MO). All reagents and analytes were not subject to further purification and were used as obtained.

2.2. Instrumentation and optimization

A Micromass (Manchester, UK) Quattro II™ tandem quadrupole mass spectrometer with a standard Z-spray™ ion source, set to electrospray positive ionization mode, with MASSLYNX™ version 3.4 operating software, was used for all quantitative determinations. Source conditions were typically as follows: capillary 3.5 kV, source temperature 110 °C, and desolvation temperature 280 °C. Multipliers were set to 650 V, and the dwell time for hydroxyproline was 100 ms. For the analytes of interest, precursor-to-product ion transitions were established through direct infusion of each compound into the mass spectrometer. The following ion transitions were used for quantification: hydroxyproline (132 → 68 m/z) and epinephrine (184 → 166 m/z). Sensitivity was then optimized for each compound by varying cone voltage and collision energy in the multiple reaction monitoring (MRM) mode and maximizing signal intensity. For our instrument, at a collision cell pressure of 2×10^{-3} mbar argon, cone and collision voltages were as follows: hydroxyproline (20 V, 20 eV), epinephrine (15 V, 10 eV).

All HPLC TOF mass spectrometry experiments were performed on a Perkin-Elmer Biosystems (Framingham, MA) Mariner™ API-TOF instrument configured with a TurboIonSpray™ source. Instrument settings were as follows: positive ion electrospray, nozzle potential 75 V, spray tip potential 3800 V, nozzle temperature 100 °C, and turbo gas temperature 350 °C. Analyzer conditions were as follows: acquisition rate was 1 spectrum

per 0.12 s, calibration range was 100–1000 m/z, and resolving power was optimized to 5200 (m/Δm, peak width at half height, FWHH). Mass calibration and resolving power were optimized by bracketing a mass range of interest between 100 and 300 m/z with an acquisition rate of 5 s per spectrum, and processing the trace of a hydroxyproline reference standard. A calibration file was created relative to the reference standard theoretical mass, and this calibration file was applied to all data files.

The liquid chromatography system was composed of a Perkin-Elmer (Norwalk, CT) Series-200 autosampler and pump (flow rate 0.3 ml/min). For the quantitative method, the analytical column was a Metachem (Torrance, CA) MetaSil AQ C18 (4.6×150 mm, 3 μ) with a MetaGuard Metasil AQ C18 2.0 mm direct connect (5 μ) guard column. Mobile phase consisted of 0.1% formic acid:acetonitrile (90:10, v/v). Final chromatographic retention times for hydroxyproline and epinephrine (internal standard) were between 4 and 5 min. The selectivity experiments utilizing the TOF mass analyzer used the same LC system as the quantitative method, with the following exceptions: (1) the analytical column was a YMC (Wilmington, NC) Cyano (50×2.0 mm, 3 μm); (2) the mobile phase consisted of 0.1% formic acid:acetonitrile (50:50, v/v); (3) the flow rate was 0.2 ml/min and the injection volume was 10 μl.

2.3. Standard curve and internal standard preparation

A hydroxyproline stock solution, containing 500 μg/ml hydroxyproline in water, was serially diluted in 0.1% acetic acid to prepare a 12-point standard curve ranging from 0.01 to 10 μg/ml. A 5 μg/ml epinephrine (internal standard) working solution was prepared in 0.1% acetic acid.

2.4. Sample and QC preparation

Approximately 30–80 mg of tissue or 50–100 μl plasma sample was put into a 5 ml screw cap Pyrex tube. This was followed by the addition of 5 ml of 6 N HCl. The caps were securely tightened, and the samples were hydrolyzed overnight at 116 °C. Following hydrolysis, tubes were allowed to cool, and 1 ml of the hydrosylate was filtered using a Whatman disposable filter (Puradisc 25 TF) and a 1 ml syringe. Samples were lyophilized, resuspended with 1 ml of distilled water, and frozen at −20 °C until analysis. The reconstituted samples were further diluted at least 20-fold with water during the assay method to keep their concentrations within the standard calibration range. Calculated hydroxyproline concentrations were later multiplied by a dilution factor and normalized by tissue weight or plasma volume for analysis.

For this particular assay development study, quality control samples were prepared by initially diluting a control rabbit cartilage tissue hydrolysate 1000-fold with water. Hydroxyproline stock solution was then diluted 100, 500, and 5000-fold, respectively, with the diluted control tissue hydrolysate, to prepare high (QC3), medium (QC2), and low (QC1) quality controls.

2.5. Assay method

A typical assay protocol for the biomarker consisted of a single 12-point standard curve, blank (0.1% acetic acid) and blank with internal standard samples in singlet, as well as study samples or QC's. Samples and standards were interspersed throughout the assay sequence. Study samples (hydrolysates) were diluted at least 20-fold with water. All of the working standard solutions, diluted study samples or quality control samples, and blank samples were then transferred into separate wells of a 96-well polypropylene autosampler plate in 100 μl aliquots. Subsequently, 10 μl of internal standard solution (or 10 μl of 0.1% acetic acid for the blank sample) was added to all plate wells. The plate was covered, vortexed, and 2 μl was injected into the LC/MS/MS.

3. Results and discussion

Amino acid levels are commonly quantified using colorimetry, HPLC, gas chromatography (GC), and capillary electrophoresis (CE) methods. The numerous methods developed for measuring hydroxyproline by CE and HPLC in biological

samples generally involve some sort of precolumn, post column, or on-column derivatization using UV chromophore or fluorophore reagents. Derivatizing the imino acid into a volatile product is also required for GC analysis. Typical reagents include ninhydrin, phenyl isocyanate (PITC), 4-(dimethylamino)azobenzene-4-sulfonyl (DABSYL) chloride, dimethylamino-naphthylene-1-sulphonyl (DANSYL) chloride, and *o*-phthalaldehyde (OPA) [5]. These techniques have improved assay sensitivity and allowed shorter analysis times, but do have drawbacks related to the chromatographic conditions and derivatizing agent used [13]. For example, ninhydrin has stability problems, DANSYL and DABSYL chloride reportedly present difficulties in quantification, while OPA does not react with imino acids in the absence of oxidizing agents, thus requiring special oxidative steps to convert secondary amino acids into primary ones prior to derivatization. These derivatizing agents also can be light sensitive, require large sample volumes and tedious cleanup steps for excess reagent removal, and demand optimal reaction conditions to obtain reliable and reproducible results [13]. Moreover, some GC and HPLC methods require a two-step derivatization procedure.

Following derivatization, colorimetric methods are the most widely used, but lack adequate sensitivity and as a consequence require relatively large sample volumes [14]. There are also specificity issues with these methods due to interference from the sample matrix [1]. Similarly, most HPLC procedures have complicated chromatographic conditions to remove derivatization byproducts from the LC analytical column and to resolve derivatized primary amino acids, which coexist with the imino acid and are also detected [4]. This could become a serious issue when the method is transferred to a different HPLC system with different operating characteristics.

Although methods which couple LC or CE to mass spectrometry without derivatization have been reported [5], problems exist that are primarily related to limitations in mobile phase or running buffer (e.g. appropriate separation, cycle time, and sensitivity). Bioanalysis using Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS) as a sensitive, selective, and reliable tool for routine quantification has improved this situation [15]. Because of the high intrinsic sensitivity and selectivity of the technique, analysis times have been shortened considerably, and quantification limits have been lowered by one to two orders of magnitude relative to other established methodology. With the capability of distinguishing differences in the characteristic precursor and product mass transitions of each analyte, tandem quadrapole mass spectrometry imparts a degree of specificity that classical chromatography cannot routinely achieve.

The assay described here has proven to be effective for specifically quantifying hydroxyproline with the required sensitivity for utility in drug discovery. It also eliminates some of the troublesome issues of other methodologies. Fig. 2 represents typical selective-reaction-monitoring (SRM) chromatograms for a diluted rabbit cartilage tissue hydrolysate sample calculated to contain 369 ng/ml of hydroxyproline (a), as well as a blank control sample (b). Chromatograms from plasma hydrolysates appeared identical to those from the tissue assay.

Hydroxyproline had a detection limit of 2.5 ng/ml with a signal-to-noise ratio greater than 3. Calibration curve regression was weighted as $1/X^2$ and performed using a linear fit (Fig. 3). The curve equation for hydroxyproline was: $0.00146628X + 0.00295002$. The calibrating standards had a relative standard deviation (R.S.D.) of less than 20%, while correlation coefficients with values greater than 0.99 were common. The intra- and inter-assay accuracy and precision data for the quality control samples are presented in Table 1. Relative error percentages were determined by comparing the unspiked control rabbit cartilage hydrolysate concentrations to those of the quality control samples that were spiked with known concentrations.

Cycle time on the LC/MS/MS is approximately 8 min per sample. The high degree of analyte selectivity provided by SRM negated the necessity for elaborate sample cleanup, or complex HPLC conditions, to resolve the peaks of interest from interfering components. Following the relatively simple sample preparation procedure, 96 samples

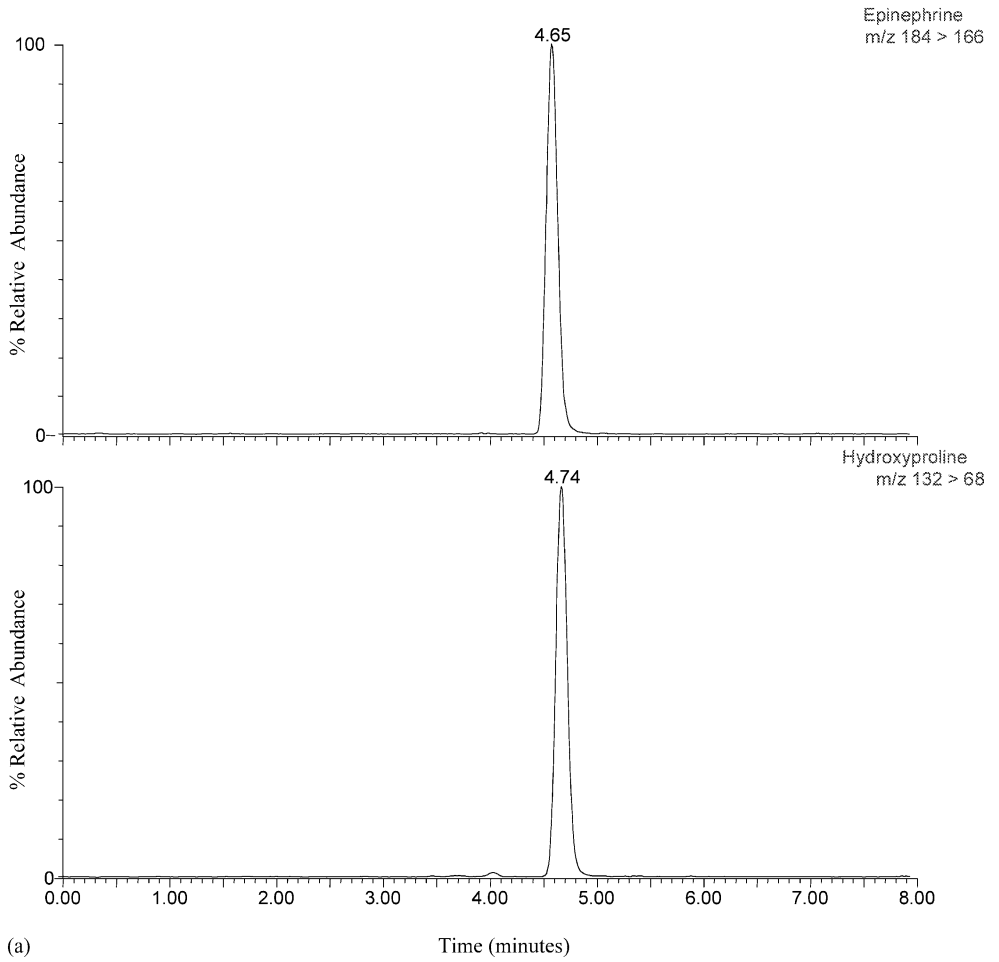
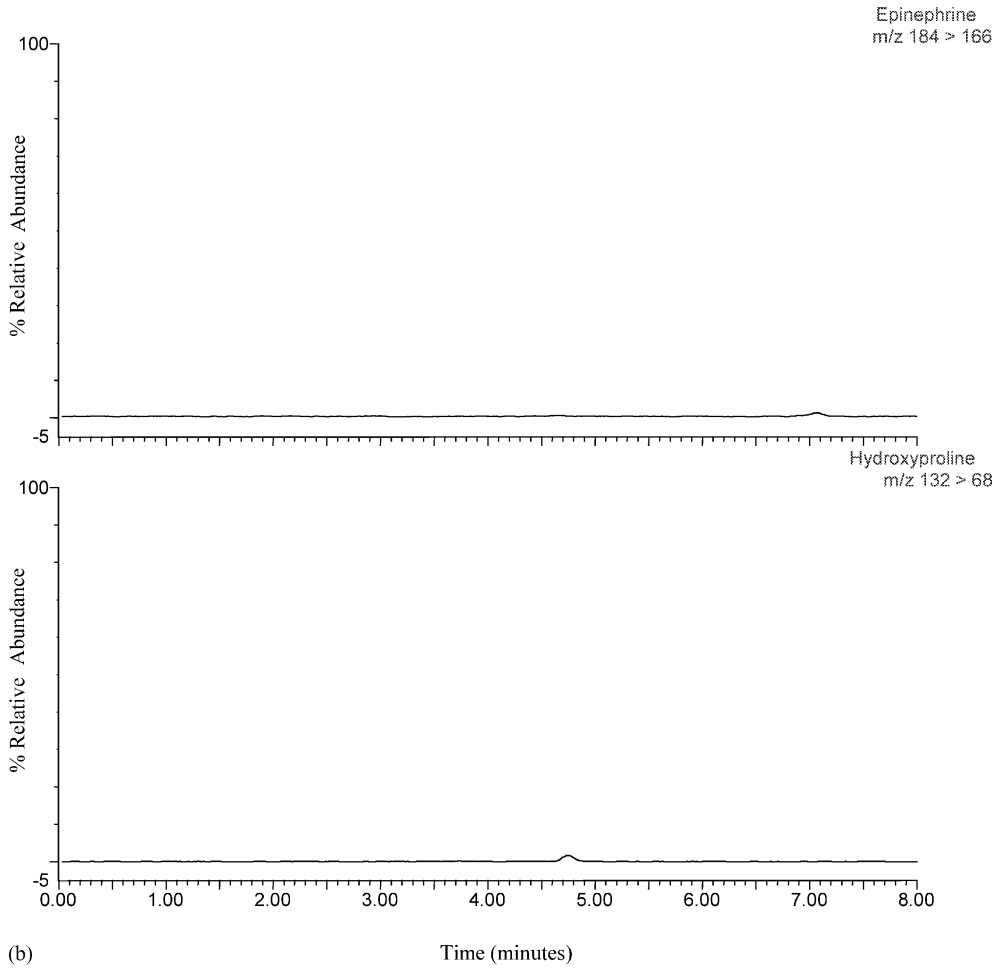


Fig. 2. Representative chromatograms of hydroxyproline and epinephrine in a hydrolyzed rabbit tissue sample following a 2 μl injection (a). The hydroxyproline concentration was calculated to be 369 ng/ml. Blank matrix control sample chromatograms are also illustrated (b).

(including standards, quality controls, and blanks) can be assayed in about 13 h using a single mass spectrometer. Assayed tissue and plasma samples that produced biomarker concentrations within the range of the standard curve are represented in Table 2.

During the initial stages of developing this method, LC/MS/MS detection of hydroxyproline was optimized using a product ion at m/z 86 and an ordinary C18 reversed phase HPLC column. This product ion appeared to give a slightly higher signal-to-noise ratio than others, while the HPLC column offered excellent peak shape and a cycle time of under 1 min per sample (Fig. 4). However, several amino acids and other potential matrix components have a mass spectrometer profile which closely resembles that of hydroxyproline. For example, leucine is isobaric with hydroxyproline (i.e. same nominal mass) and shares a similar fragmentation pattern. Thus, while LC/MS/MS generally provides a high degree of specificity, the absence of interference from other endogenous compounds needed to be verified in this instance.

As a first step to assess the selectivity of the method, a YMC Cyano column was employed to move the hydroxyproline peak further from the



(b)

Fig. 2 (*Continued*)

solvent front (i.e. increase its capacity factor, $k' = (t_r - t_0)/t_0$). This would facilitate greater chromatographic resolution from other potentially coeluting substances. It was observed in the chromatograms of hydrolysate samples that another peak became partially resolved from hydroxyproline. Because this partial resolution did not occur in standard samples, it strongly suggested that another compound was coeluting with hydroxyproline and being detected by the mass spectrometer. The Mariner™ API-TOF mass analyzer was then used to provide high-resolution mass information. Using an external calibration with reference standards, it appeared that the TOF instrument was able to distinguish the m/z values of protonated hydroxyproline and protonated leucine. When a neat sample that contained both of these analytes was injected into the system and an m/z window of 131.965–132.165 was extracted, it produced a chromatogram with a split peak (Fig. 5a). This chromatogram was similar to those that were produced on the quadrupole mass spectrometer following the injection of a hydrolysate sample. Each shoulder of the split peak was scanned separately and produced the spectra for protonated hydroxyproline and protonated leucine illustrated in Fig. 6. When the spectra were applied to extract the exact mass chromatograms, the split peak almost completely resolved into separate leucine and hydroxyproline component peaks

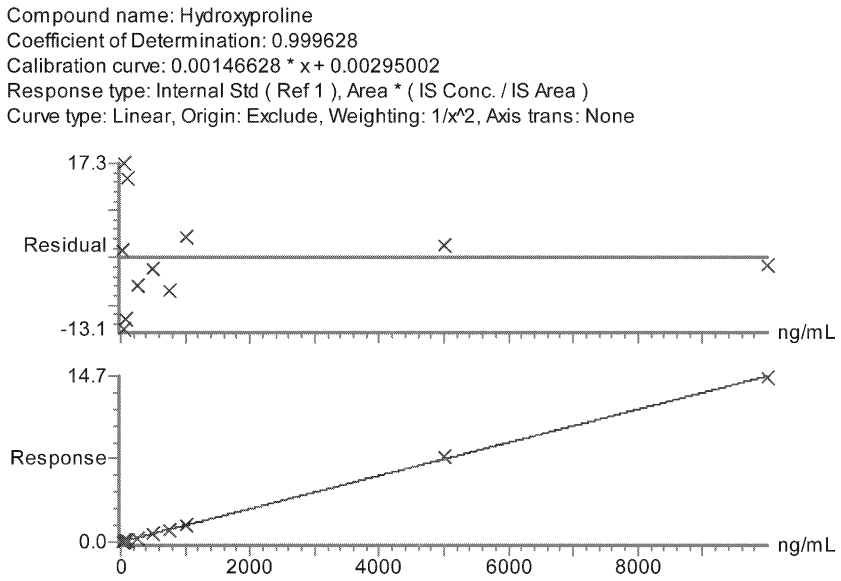


Fig. 3. A typical assay calibration curve with a concentration range of 0.01–10 μg/ml. The regression was weighted as $1/X^2$ and performed using a linear fit. The calibrating standards had a RE of less than 20%, while the correlation coefficient was greater than 0.99.

(Fig. 5b and c). Subsequent exact mass chromatograms that were extracted following the injection of hydrolysate samples revealed a similar result. This confirmed that leucine was most likely cross-interfering with hydroxyproline on the LC/MS/MS system.

The TOF mass spectrometer could not be used for routine sample analysis due to an inadequate limit of detection for hydroxyproline (approximately 500 ng/ml). Therefore, greater chromatographic resolution on the LC/MS/MS system was required to avoid the leucine interference. Svan-

Table 1
Quality control precision and accuracy results

Quality control		Unspiked	QC1	QC2	QC3
Batch run 1	Mean (ng/ml)	399	482	1275	5196
	%R.S.D.[a]	5.92	2.14	1.52	5.49
	%RE[b]	[c]	−3.29	−8.82	−3.76
Batch run 2	Mean (ng/ml)	3.75	520	1314	4906
	%R.S.D.	6.89	2.10	7.93	1.41
	%RE	[c]	9.45	−4.42	−8.73
Batch run 3	Mean (ng/ml)	362	488	1357	5301
	%R.S.D.	1.69	4.89	3.03	2.72
	%RE	[c]	5.47	−0.389	−1.15
Interassay	Mean (ng/ml)	379	495	1323	5164
	%R.S.D.	4.88	4.68	4.91	4.55
	%RE	[c]	3.34	−4.04	−3.99

Note: $N = 5$ each; three separate assays compared.
[a] Percent Relative Standard Deviation.
[b] Percent Relative Error.
[c] Mean concentration used as theoretical.

Table 2
Typical concentration of hydroxyproline in hydrolyzed tissue and plasma samples determined by the LC/MS/MS method (μg/ml)

Sample number	Rat plasma	Rat uterus	Rat aorta	Rat knee cartilage	Mouse aorta
1	0.387	48.1	1.79	24.0	1.10
2	0.285	147	1.09	30.5	4.80
3	0.436	78.7	1.56	32.8	
4	0.345	121	1.11	36.5	
5	0.348	192	1.35	19.7	

Note: all samples were diluted 20-fold prior to analysis.

berg [16] was able to get adequate chromatographic retention and peak resolution on a reversed phase HPLC column when the ion-pairing agent triethlylamine was added to the mobile phase. However, because this type of ion-pairing agent is nonvolatile, it would cause ion suppression (that is, a decrease in ionization efficiency resulting in a loss of analyte sensitivity [17]) and quickly contaminate the mass spectrometer source. The Metasil™ AQ C18 analytical column provided an alternative to overcome this challenge. By possessing a hydrophobic stationary phase that

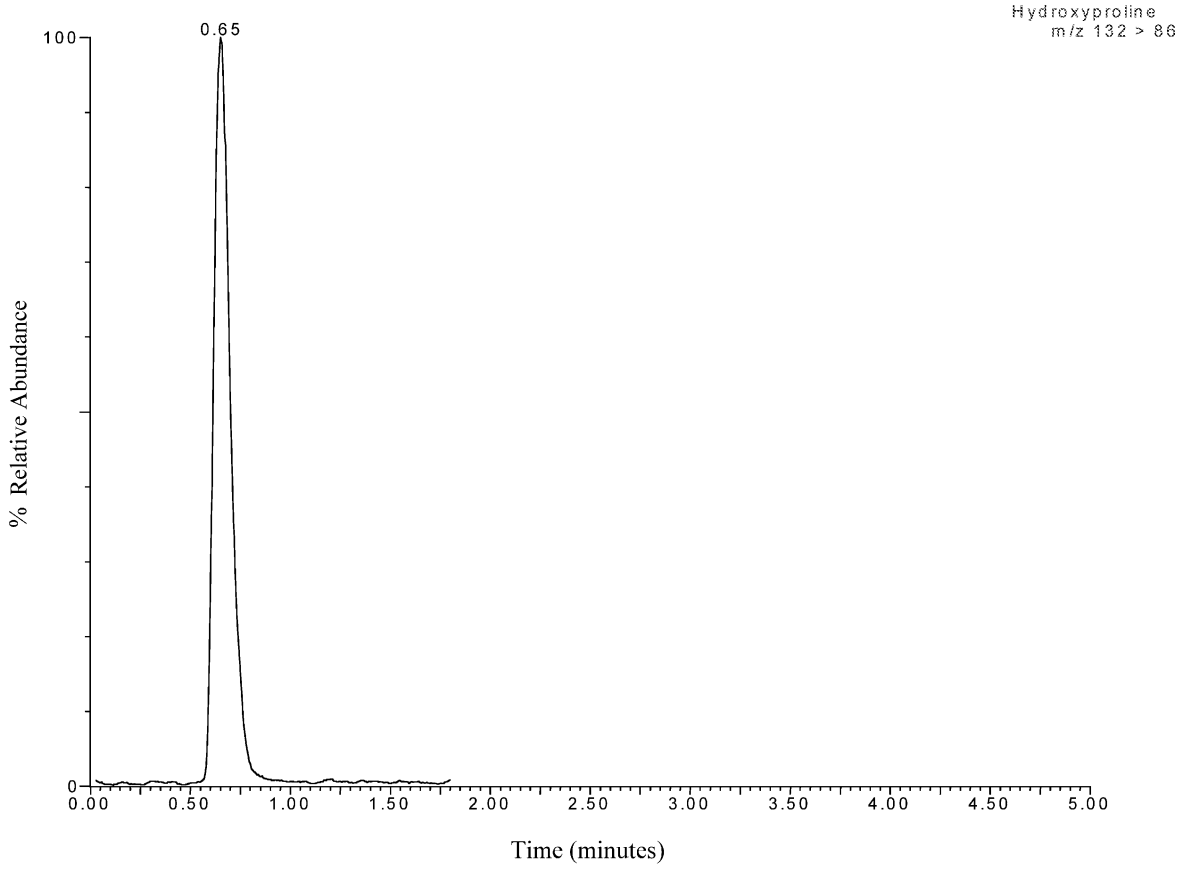


Fig. 4. MRM chromatogram of hydroxyproline obtained using a mobile phase composed of 0.1% formic acid:acetonitrile (50:50, v/v), a 50 × 2 mm, 3 μ C18 HPLC column, and monitoring a 132 → 86 m/z transition.

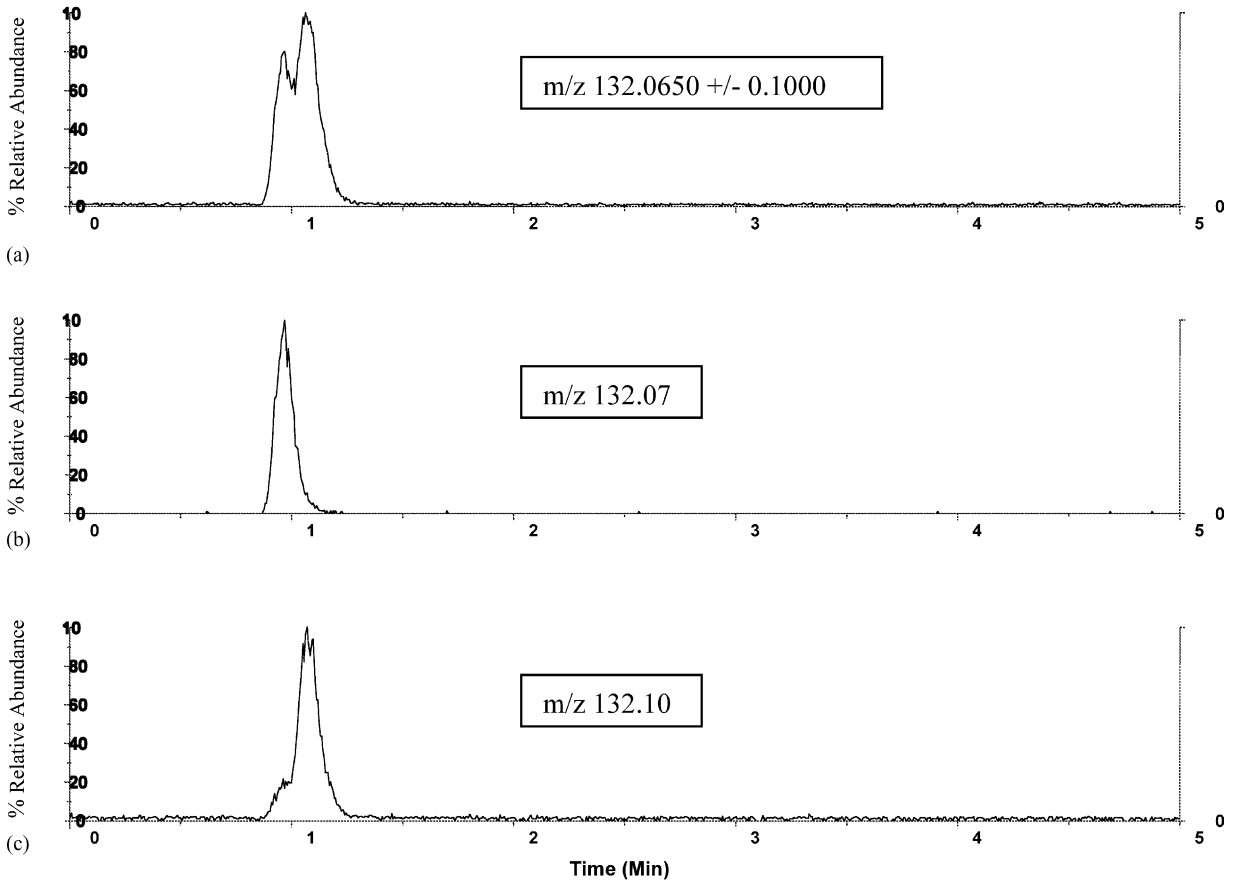


Fig. 5. Extracted chromatogram following the injection of both hydroxyproline and leucine at 5 μg/ml (a); extracted exact mass chromatogram of protonated hydroxyproline (b); extracted exact mass chromatogram of protonated leucine (c). The two $[MH]^+$ species have a theoretical mass difference of 0.0363 u.

contains embedded polar groups, this column isolated hydroxyproline from other detected sample constituents when acetonitrile comprised less than 20% of the mobile phase (Fig. 7).

Using accurate mass extraction on the LC/TOF, it was determined that the second peak to elute after hydroxyproline was leucine, while the third peak was not identified. Although the chromatographic separation of hydroxyproline from other interfering sample components continued to increase with smaller percentages of acetonitrile in the mobile phase, 10% was chosen because it gave the best peak shape and sensitivity.

As a final step to enhance the selectivity of the method, other product ions of hydroxyproline were evaluated on the mass spectrometer. It was observed that a product ion at m/z 68 could be produced in almost equal abundance to the m/z 86 fragment. Additionally, leucine did not generate this smaller product ion and subsequently was no longer detected in hydroxyproline chromatograms. As a result of this observation, the $132 \rightarrow 68$ m/z transition was monitored for all future assay sample analysis. However, despite cleaner chromatograms produced by this modification, the injection cycle time was kept at 8 min due to the appearance of unidentified late eluting peaks observed in hydrolysate sample chromatograms of some species.

Epinephrine was chosen as the internal standard for the assay because it is a relatively small molecule that demonstrated similar chromato-

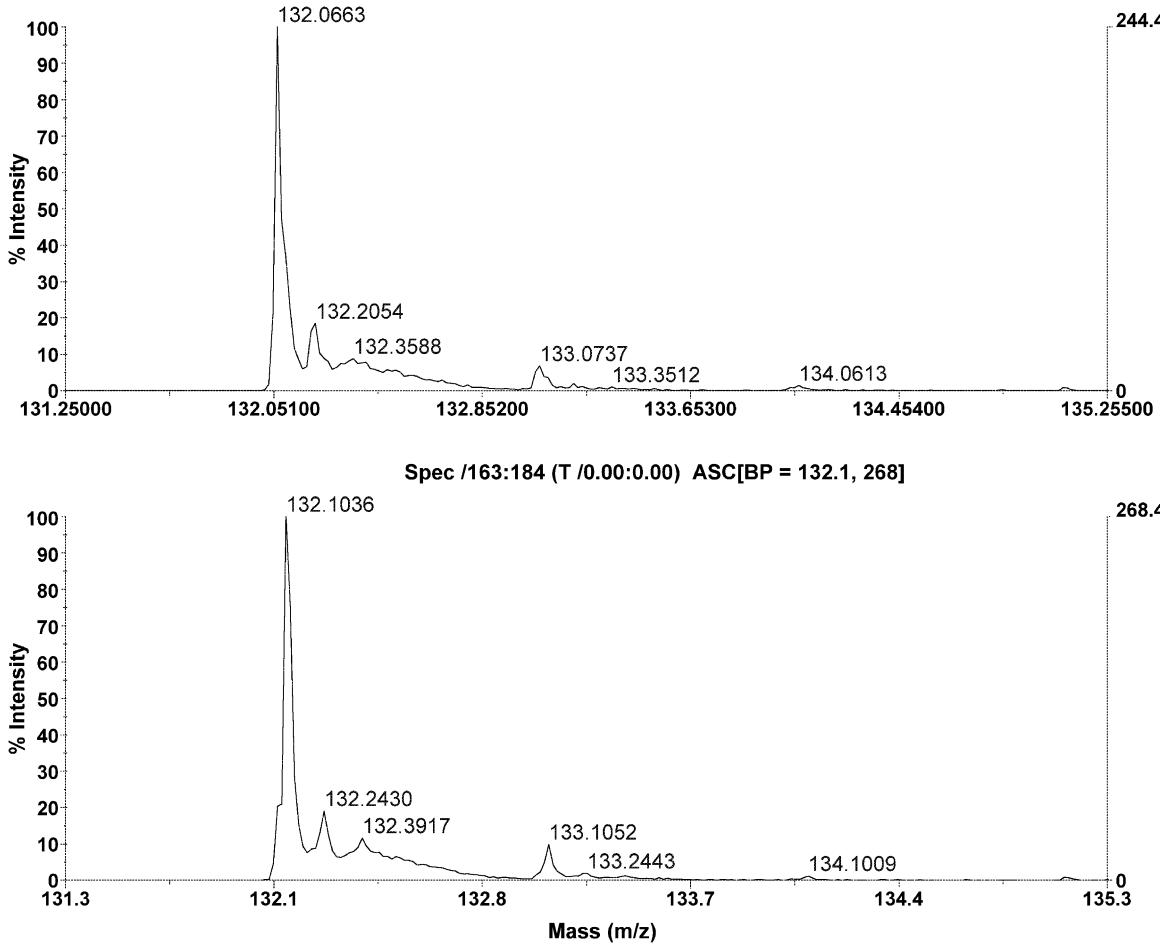


Fig. 6. External calibrated spectrum of hydroxyproline (top) within 2 ppm of exact mass and leucine (bottom) within 10 ppm of exact mass.

graphic characteristics as hydroxyproline, and was not detected in the sample matrices of interest. Although hydroxyproline and epinephrine appear to coelute in their SRM chromatograms (Fig. 2a), due to their distinct fragmentation reactions, no cross-channel interference was observed when each compound was injected individually while both SRM channels were monitored.

4. Conclusions

A method to determine hydroxyproline concentrations in plasma and tissue has been developed. Results demonstrate the described LC/MS/MS method maintained a high degree of analyte precision and accuracy, while sensitivity was increased to the low ng/ml range. SRM negates the necessity for elaborate HPLC conditions to resolve the peaks of interest from interfering components. Advantages over other common analytical techniques were also achieved by eliminating cumbersome sample derivatization steps. Selectivity and specificity were confirmed and optimized with the use of a polar end-capped reversed phase HPLC column, as well as high resolution mass determination on a TOF mass spectrometer. Hydroxyproline has been considered

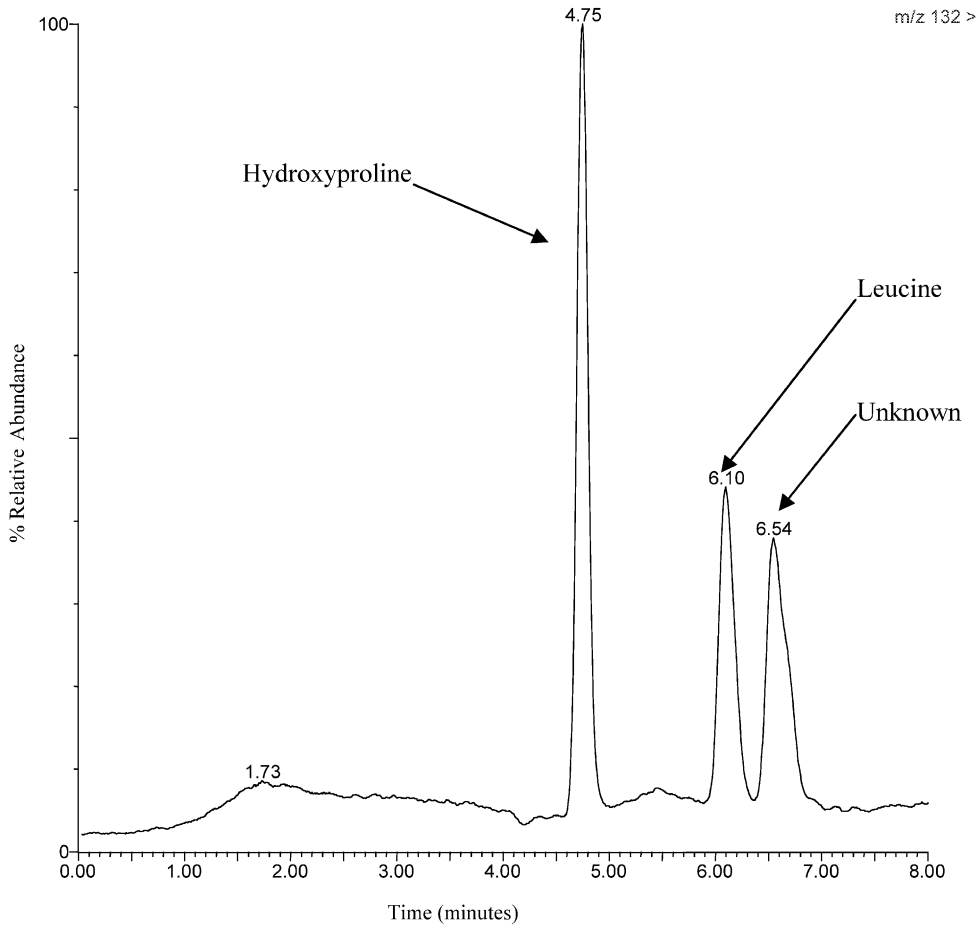


Fig. 7. Chromatogram of a rabbit tissue hydrolysate sample showing resolution of hydroxyproline from leucine and an unknown matrix component. LC/MS/MS conditions included a mobile phase composed of 0.1% formic acid:acetonitrile (90:10, v/v), a 150 × 4.6 mm, 3 μ MetaSil™ AQ C18 HPLC column, and monitoring a m/z ion transition of 132→86.

as a potential marker of collagen, and this assay could be a valuable tool for the development of therapeutic drugs that influence collagen content.

Acknowledgements

We thankfully acknowledge Dr Tom Peterson (Pfizer Research) for his investigations of collagen degradation. We also thank Dr Scott Fountain (Pfizer PDM) for providing his expertise in using the LC/TOF technology.

References

[1] M.A. Morleo, E. Musi, Boll. Chim. Farmaceutico. 132 (1993) 281–284.
[2] E. Kindt, D.T. Rossi, K. Gueneva-Boucheva, H. Hallak, Anal. Biochem. 283 (2000) 71–76.
[3] Y. Uji, A. Karmen, H. Okabe, K. Hata, M. Miura, K. Ozaki, M. Minamizaki, T. Shibata, S. Inayama, J. Clin. Lab. Anal. 8 (1994) 267–272.
[4] H. Inoue, Y. Date, K. Kohashi, H. Yoshitomi, Y. Tsuruta, Biol. Pharm. Bull. 19 (1996) 163–166.
[5] T. Soga, D.N. Heiger, Anal. Chem. 72 (2000) 1236–1241.
[6] P.D. Delmas, Am. J. Med. 95 (1993) 5A–16S.
[7] M. Saito, K. Marumo, K. Fujii, N. Ishioka, Anal. Biochem. 253 (1997) 26–32.

[8] Z. Gunja-Smith, A.R. Morales, R. Romanelli, J.F. Woessner, Jr, Am. J. Pathol. 148 (1996) 1639–1648.
[9] M.D. Rekhter, G.W. Hicks, D.W. Brammer, H. Hallak, E. Kindt, J. Chen, W.S. Rosebury, M.K. Anderson, P.J. Kuipers, M.J. Ryan, Circ. Res. 86 (2000) 101–108.
[10] M.D. Rekhter, G.W. Hicks, D.W. Brammer, C.W. Work, J.S. Kim, D. Gordon, J.A. Keiser, M.J. Ryan, Circ. Res. 83 (1998) 705–713.
[11] B.H. Strauss, R. Robinson, W.B. Batchelor, R.J. Chisholm, G. Ravi, M.K. Natarajan, R.A. Logan, S.R. Mehta, D.E. Levy, A.M. Ezrin, F.W. Keeley, Circ. Res. 79 (1996) 541–550.
[12] M.D. Rekhter, Cardiovasc. Res. 41 (1999) 376–384.
[13] M. Lange, M. Malyusz, Clin. Chem. 40 (1994) 1735–1738.
[14] Y. Tsuruta, H. Inoue, Anal. Biochem. 265 (1998) 15–21.
[15] N. Zhang, S.T. Fountain, H. Bi, D.T. Rossi, Anal. Chem. 72 (2000) 800–806.
[16] G.K. Svanberg, J. Periodontal Res. 22 (1987) 133–138.
[17] B.K. Matuszewski, M.L. Constanzer, C.M. Chaves-Eng, Anal. Chem. 70 (1998) 882–889.