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High-performance liquid chromatography with mass spectrometry detection for quantitating COL-3, a chemically modified tetracycline, in human plasma

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Abstract

COL-3, 6-deoxy-6-demethyl-4-dedimethylamino-tetracycline, is a matrix metalloproteinase inhibitor. A specific and sensitive analytical method was necessary to quantitate the analyte in human plasma. High-performance liquid chromatography with atmospheric pressure chemical ionization mass spectrometry detection was utilized to quantitate COL-3 from 30 to 10 000 ng/ml in two calibration curves: 30–1500 and 400–10000 ng/ml. The sample preparation consisted of acetonitrile precipitation for all plasma samples. COL-3 is separated on a Waters Symmetry® C-18 (2.1 × 150 mm) column with oxalic acid (0.01 M, pH 2.2)–acetonitrile mobile phase. The total run time was 23 min. Identification of COL-3 and the internal standard was through positive chemical ionization and selective ion monitoring. A quantifying and qualifying ion for COL-3 is used to verify the presence of COL-3 in patient samples. Inter- and intra-run mean percent errors for all of the quality controls were less than 18.3% and relative standard deviations were all less than 14.9%. Recovery of COL-3 and the internal standard was approximately 55 and 72%, respectively. Freeze–thaw stability of COL-3 was variable. This method is suitable for quantifying COL-3 in patient samples and to further characterize the clinical pharmacology of this compound.

Keywords: COL-3; Mass spectrometry; Chromatography; Tetracycline

1. Introduction

COL-3, 6-deoxy-6-demethyl-4-dedimethylamino-tetracycline (Fig. 1), is a non-antimicrobial tetracycline analog that was designed to inhibit matrix metalloproteinases (MMP), which

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are a class of enzymes involved in the turnover of the extracellular matrix in many physiologic processes. An imbalance between MMPs and the naturally occurring MMP inhibitors may cause an excess of extracellular matrix destruction, allowing cancer cells to invade surrounding tissues, metastasize and angiogenesis to occur [1]. COL-3 competitively inhibits MMP-2 and MMP-9 [2]. Cytotoxic activity against two human prostate cancer cell lines, DU145 and PC-3, and induction of apoptosis in Dunning MAT LyLu cells has been observed at COL-3 concentrations of approximately 5 μg/ml [3]. Lokeshwar et al. also demonstrated that COL-3 has anti-invasive properties by inhibiting the invasion of DU145 and PC-3 across Matrigel® at concentrations of approximately 2 μg/ml [3]. By demonstrating cytotoxic, anti-invasive and MMP inhibitory properties, COL-3 may have anti-angiogenic and antimetastatic activity.

Limited solubility tests have been conducted with COL-3. The solubility of COL-3 was 0.01 mg/ml in water at pH 4.3 and 3.2 mg/ml in ethanol (S Esmail Tabibi, personal communication, CTEP, Bethesda, MD). Marsh and Weiss analyzed the solubility of a number of tetracyclines in a variety of solutions [4]. Chlortetracycline sulfate butanolate, rolitetracycline, rolitetracycline nitrate, methacycline hydrochloride, methacycline base, demethylchlortetracycline base and calcium oxytetracycline were soluble in water at the following concentrations: ≥ 20, ≥ 20, ≥ 20, 18.368, 7.548, 1.515 and 0.260 mg/ml, respectively [4]. In ethanol, the solubilities were at least 20, 8.422, 8.260, 9.890, 7.118, 3.335, and 0.460 mg/ml, respectively [4]. From these data, it is observed that COL-3 is the most insoluble of the tetracyclines in water and has a similar solubility to demethylchlortetracycline base in ethanol.

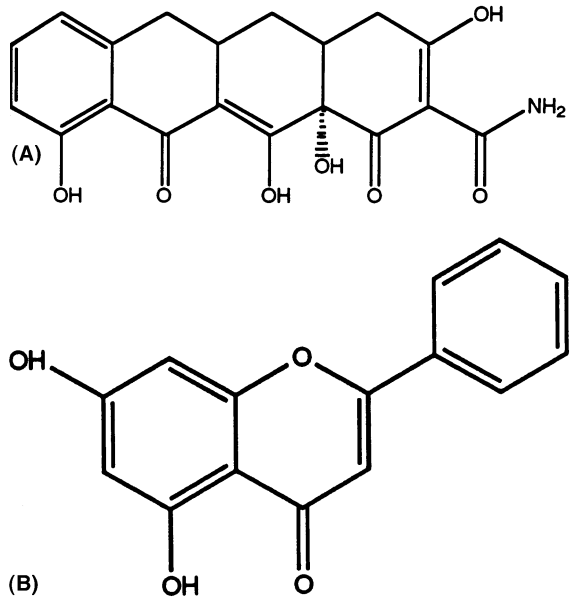


Fig. 1. Chemical structures of (A) COL-3 and (B) Chrysin [8].

COL-3 is currently in Phase I clinical trials in patients with refractory metastatic cancer. To characterize the clinical pharmacology, a method for the quantitation of COL-3 was necessary. The method discussed in this paper utilizes reverse-phase liquid chromatography with atmospheric pressure chemical ionization (APCI) mass spectrometry detection (LC/MS) to achieve a sensitive and specific assay method.

2. Experimental

2.1. Chemicals and reagents

COL-3 was a gift from CollaGenex Pharmaceuticals Inc. (Newtown, PA, USA). The internal standard, chrysin (see Fig. 1), was purchased from Sigma Chemical Company (St. Louis, MO, USA). Oxalic acid was purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Acetonitrile and methanol (high-performance liquid chromatography (HPLC) grade) were obtained from J.T. Baker (Phillipsburg, NJ, USA). Heparinized plasma was purchased from Biological Specialty Corporation (Lansdale, PA, USA). Deionized distilled water from a Hydro Reverse Osmosis system (Durham, NC, USA) in combination with a Milli-Q UV Plus filtration system (Marlborough, MA, USA) was used in this assay.

COL-3 was dissolved in absolute methanol at a concentration of 1000 ng/μl and stored at -20°C. Two separate stock solutions were prepared for COL-3: one for quality controls and one for standards. COL-3 stock solutions were prepared

Table 1
Gradient profile

Time (min)	% Solvent A[a]	% Solvent B[b]
0	55	45
7.5	55	45
9	10	90
16	10	90
17	55	45

[a] Oxalic acid (0.01 M, pH 2.2).
[b] Acetonitrile.

by serially diluting the 1000 ng/μl stock with absolute methanol to concentrations of 500, 250, 100, 25, 10 and 5 ng/μl. Chrysin was dissolved in absolute methanol at a concentration of 500 ng/μl and stored at −20°C. Chrysin was diluted to 0.5 ng/μl with acetonitrile just prior to use. Stability of stock solutions were assessed at 2 and 4 weeks.

2.2. Calibration curves and quality control samples

Microliter amounts of COL-3 stock solution (less than 1% of total plasma volume) were added into pooled plasma to prepare two calibration curves and quality controls. The standards were prepared at the following concentrations 30, 70, 150, 400, 700, 900, and 1500 ng/ml for the low standard curve, and 400, 700, 900, 1500, 3000, 6000 and 10 000 ng/ml for the high standard curve. The quality controls were prepared as 50, 500, and 1200 ng/ml for the low standard curve, and 500, 2500, and 8000 ng/ml for the high standard curve. All standard curves and quality controls were stored at −80°C. Freeze–thaw stability was assessed for three cycles at −80°C. For stability testing, new stock solutions were prepared and new calibration curves were prepared. The old quality controls were then assessed on the freshly prepared calibration curve.

The first validation run consisted of a duplicate standard curve, duplicate quality controls and six replicates of 30 and 400 ng/ml. The second validation run consisted of a duplicate standard curve, duplicate quality controls and three freeze–thaw cycles of the quality controls. The third validation run consisted of a duplicate standard curve and six replicates of the quality controls in an order to assess carryover. Blank plasma extracts, spiked to the theoretical amount of COL-3 and chrysin that would be injected in extracted samples assuming 100% extraction recovery, were also added to assess extraction efficiency through the standard curve. The fourth run consisted of a duplicate standard curve and duplicate quality controls. Five-week-old, and 2.5- and 4.5-month-old frozen quality controls were compared with a freshly prepared standard curve in the final run to assess long-term stability.

2.3. Sample preparation

A 250 μl plasma sample or standard was deproteinized using 50 μl of 0.5 ng/μl (25 ng) Chrysin solution and 1000 μl acetonitrile. The samples were vortexed for 30 s, allowed to sit at room temperature for at least 10 min and centrifuged ($9000 \times g$, 6 min, 4°C). One thousand microliters of supernatant were removed and evaporated to dryness under desiccated air (W.A. Hammond Drierite Company, Xenia, OH) at 40°C in a Zymark Turbo Vap® LV evaporator (Hopkinton, MA, USA). Four hundred microliters of methanol was added to the sample residue, and vortexed to wash down the sides of the tube and reconcentrate the sample. The sample was evaporated to dryness again. The samples were reconstituted with 100 μl acetonitrile–oxalic acid (0.01 M, pH 2.2) (45:55, v/v) and vortexed for 20 s. The reconstituted solution was transferred to an HPLC autosampler vial and 25 μl was injected into the system.

2.4. Chromatographic and mass spectroscopy conditions

The HPLC system consisted of a Hewlett Packard 1090 Series II Liquid Chromatograph equipped with a photodiode-array (2 nm resolution) and controlled by HP LC/MSD Chem Station software Rev A.06.01[403] run by a Hewlett Packard Vectra VL Pentium computer (Palo Alto, CA, USA). The mass spectrometer was a Hewlett Packard 1100 MSD. A gradient profile, as shown in Table 1, consisted of oxalic acid (0.01 M, pH

2.2) (Solvent A) and acetonitrile (Solvent B) and a flow rate of 0.3 ml/min was utilized with a total run time of 23 min. A needle wash of acetonitrile–water (90:10, v/v) increased the cycle time to 28 min. The chromatographic separation of COL-3 was accomplished with a Waters Symmetry® C-18 (2.1 × 150 mm) column with a 3.5 μm particle size and a Waters Symmetry® C-18 (2.1 × 10 mm) guard column with a 3.5 μm particle size (Millford, MA, USA).

Conditions for the mass spectrometer were optimized to maximize the signal at the quantitative ion for COL-3, 372.1 $m/z+$. APCI was operated in the positive mode. Selective ion monitoring (SIM) was utilized with a dwell time of 592 ms. The cycle time was 1.80 s/cycle and samples were analyzed in the high-resolution mode. To maximize the cleanliness of the source, the mass spectrometer was set for detection from 2 to 10 min of the total run time. Two of the ions ($m/z+$) monitored in SIM were 326.1 and 372.1, the qualifying and quantifying ions of COL-3, respectively. The third ion ($m/z+$) monitored was 255 for the internal standard, chrysin. The nebulizer pressure was 55 psig. Drying gas temperature was 170°C and flow was 5 l/min. The vaporizer temperature was set at 300°C. The V_{cap} and corona current were set at 3300 V and 4.0 μA, respectively. The fragmentor was set at 90 V and gain was set at 3.0. The source was cleaned after every run and calibrated at the same time. Corona needles were replaced approximately once a month only when the calibration failed or when the calibration had lower abundances than expected.

3. Results

3.1. Chromatography

An LC/MS method to determine COL-3 concentrations in human plasma was developed and validated. The method utilizes a quantifying and qualifying ion for COL-3, the analyte of interest. The qualifying ion (326.1 $m/z+$) was approximately 30% of the quantifying ion (372.1 $m/z+$). Fig. 2 represents typical selected ion chromatograms of 255, 326.1 and 372.1 $m/z+$. COL-3 and chrysin elute at 5.2 and 6.5 min, respectively. The mass spectra of COL-3 and chrysin are presented in Fig. 3. No interfering peaks were found in ten individual plasma lots. The calculated signal-to-noise ratio of the COL-3 peak detected on 372.1 $m/z+$ is approximately 15:1 at 30 ng/ml, the limit of quantitation. The signal-to-noise ratio for chrysin detected on 255 $m/z+$ is approximately 80:1 at 25 ng/ml.

Calibration curves for COL-3 standards were constructed from the peak area ratio of COL-3 quantifying ion (372.1 $m/z+$) to chrysin (255 $m/z+$). Heterocedasticity was observed using a linear standard curve over the entire range of 30–10 000 ng/ml. Therefore, the calibration curve was fit using a power fit (log–log–linear plot) in two separate ranges: 30–1500 and 400–10 000 ng/ml. The slope (1.044 ± 0.052 for the low calibration curve, 1.136 ± 0.048 for the high calibration curve (mean ± standard deviation); $n = 5$) and y-intercept (2.965 ± 0.153 for the low calibration curve, 3.221 ± 0.161 for the high calibration curve; $n = 5$) were calculated for each calibration curve. Correlation coefficients (0.997 ± 0.001 for low calibration curve, 0.997 ± 0.001 for high calibration curve; $n = 5$) were obtained for each calibration curve. Back-calculated errors were obtained for each point in the calibration curve and the quality controls.

Inter- and intra-run precision and accuracy was calculated for both calibrations curves and the quality controls. Precision was determined by the relative standard deviation (Eq. (1)), while accuracy was by the mean percent error (Eq. (2)).

$$\frac{\text{standard deviation of the back-calculated value}}{\text{mean}} \times 100\% \quad (1)$$

$$\frac{(\text{mean back-calculated value} - \text{theoretical value})}{\text{theoretical value}} \times 100\% \quad (2)$$

The data for quality controls are presented in Tables 2 and 3. The relative standard deviation ranged from 3.5 to 14.9% and the mean percent error ranged from −18.3 to 6.9% for the intra-run quality controls. The relative standard devia-

tion ranged from 5.8 to 13.4% and the mean percent error ranged from −18.2 to 3.1% for the inter-run quality controls. The intra-run relative standard deviation for 30 ng/ml, the lowest standard on the low standard curve, was 4.5% and the mean percent error was 2.4%. The intra-run relative standard deviation for 400 ng/ml, the lowest standard on the high standard curve, was 12.7%, with a mean percent error of −4.6%.

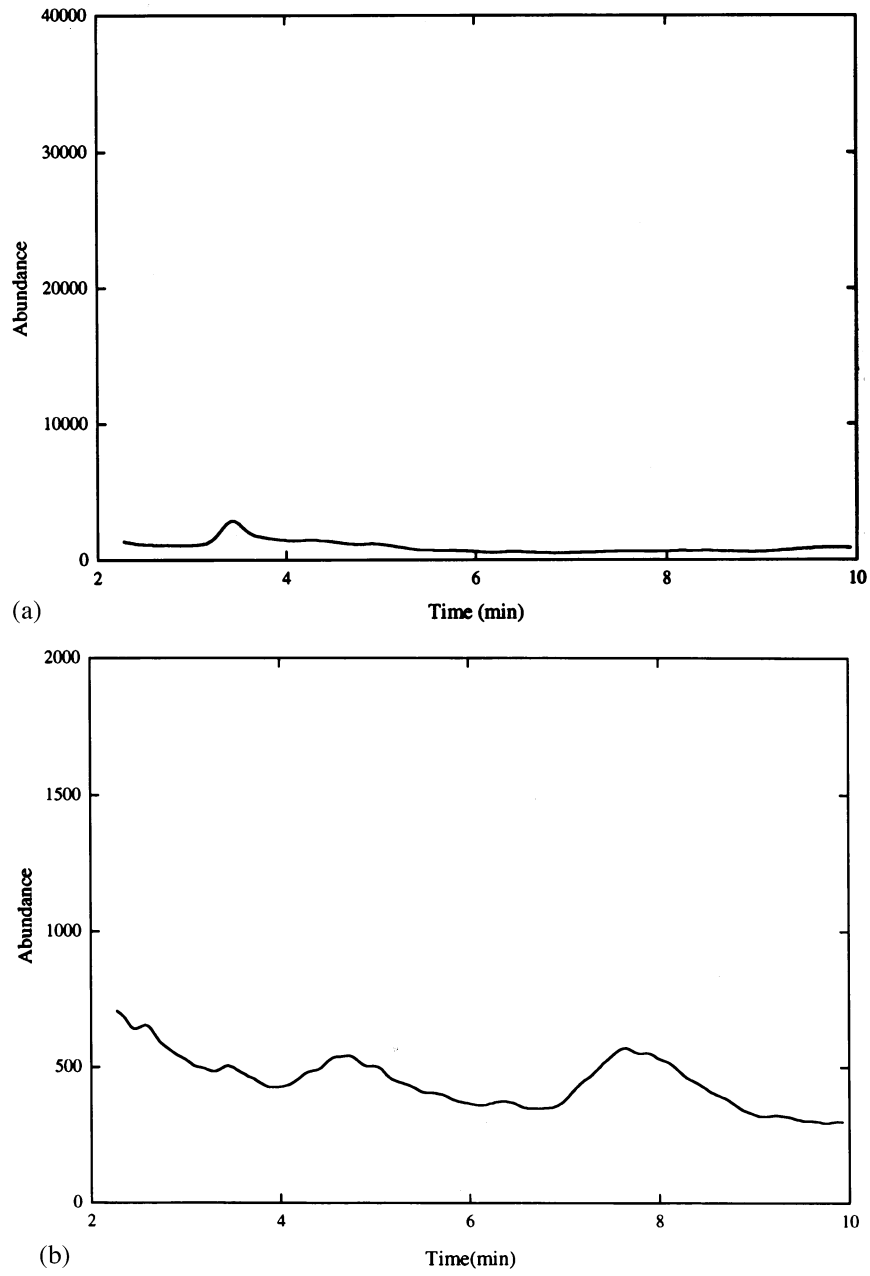


Fig. 2. Selected ion chromatograms of blank plasma (a, b, c) and plasma spiked with 30 ng/ml COL-3 (d, e, f). The following $m/z+$ were monitored 255 (a, d), 326.1 (b, e), and 372.1 (c, f).

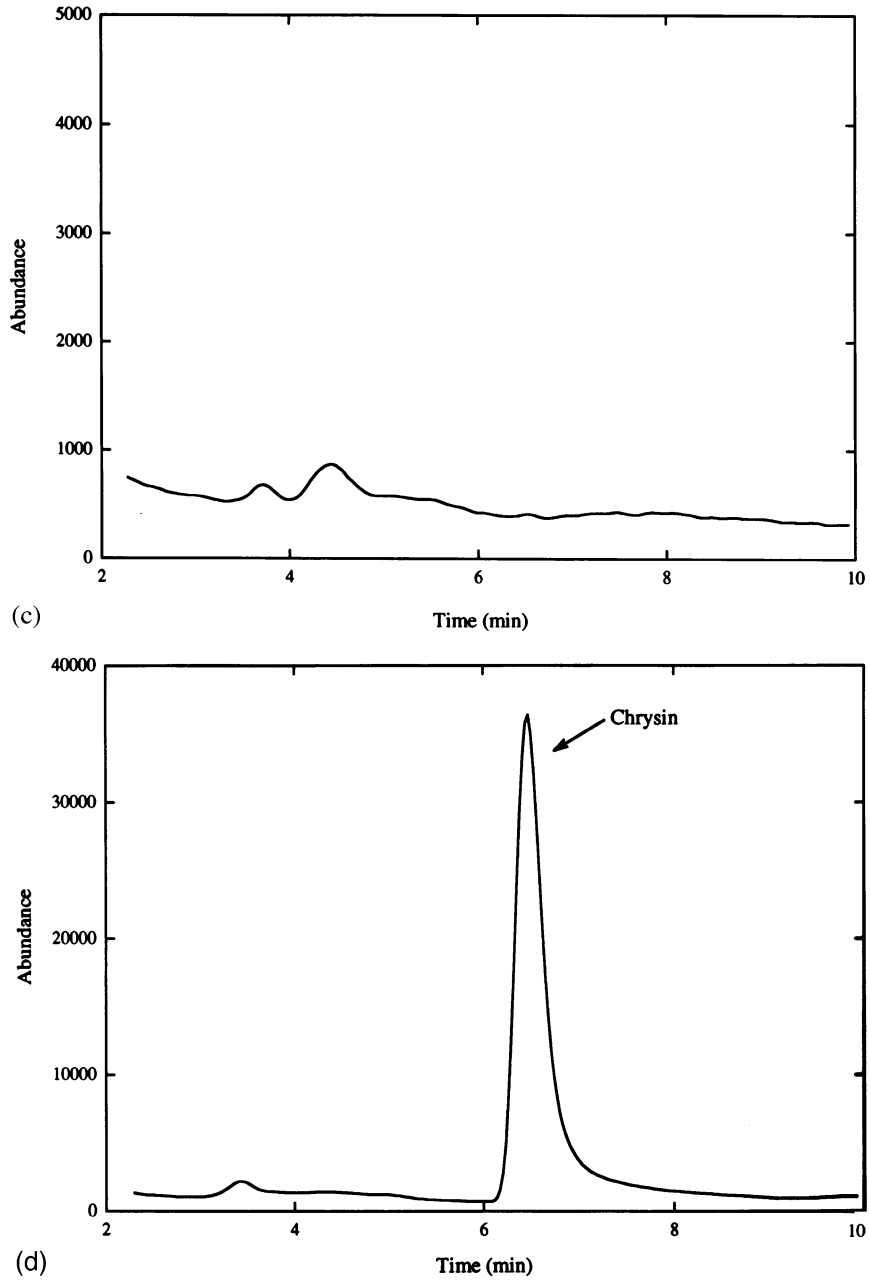


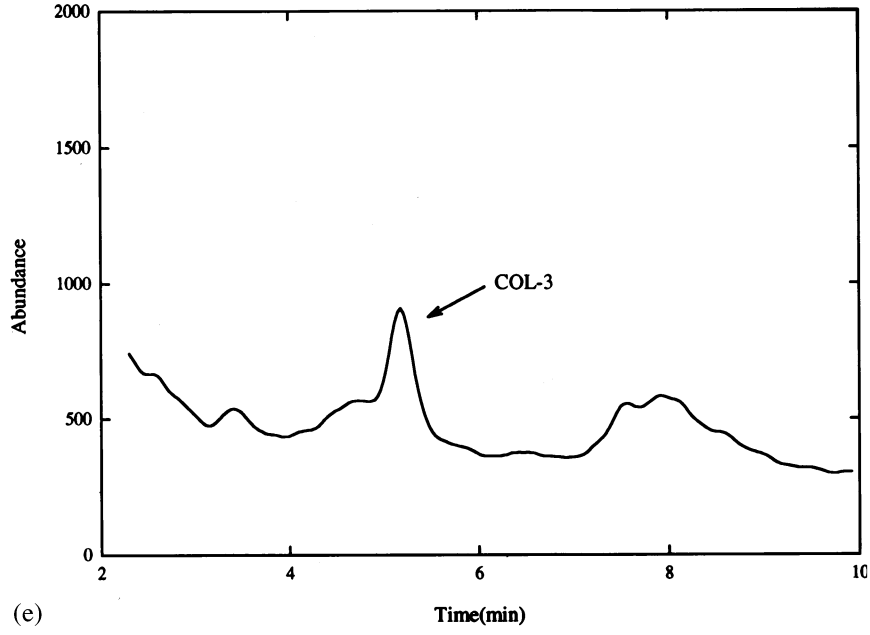
Fig. 2. (*Continued*)

3.2. Recovery

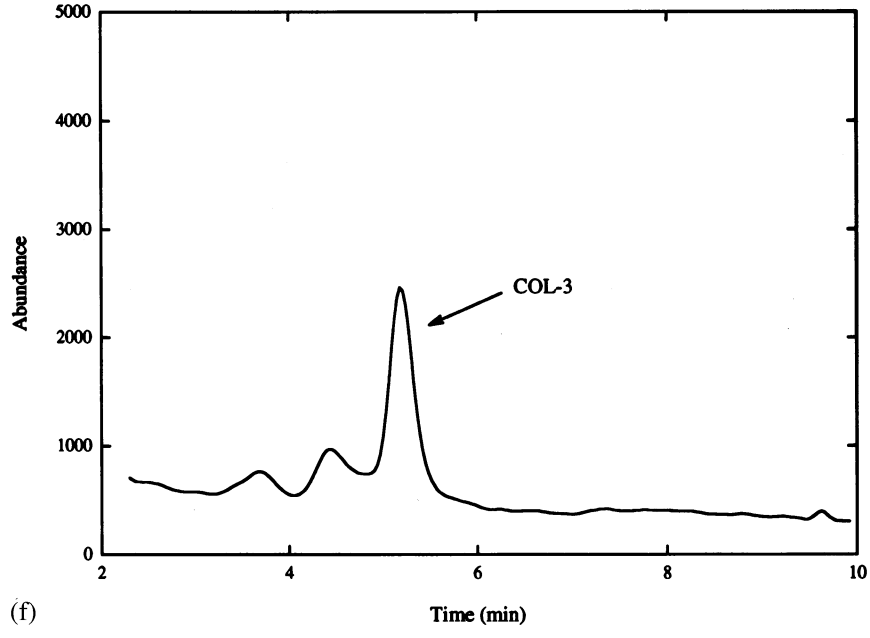
Recovery was calculated from raw area counts of 372.1 $m/z+$ for COL-3 and 255 $m/z+$ for chrysin from processed samples ($n=6$) to spiked-blank-plasma extracted samples ($n=3$). The recovery was 58.7, 52.1, 52.3 and 53.8% at 500, 1200, 2500, and 8000 ng/ml, while 25 ng/ml chrysin was recovered at 71.8%.

3.3. Storage and stability

COL-3 stock solution in methanol (1000 ng/μl) had a 1.9% loss at 2 weeks and 26.1% loss at 4 weeks when stored at −20°C and protected from the light with aluminum foil. Chrysin stock solution in methanol (500 ng/μl) had no loss at 2 weeks and 12.2% loss at 4 weeks when stored at −20°C and protected from the light with aluminum foil. COL-3 is stable in plasma in the



(e)



(f)

Fig. 2. (*Continued*)

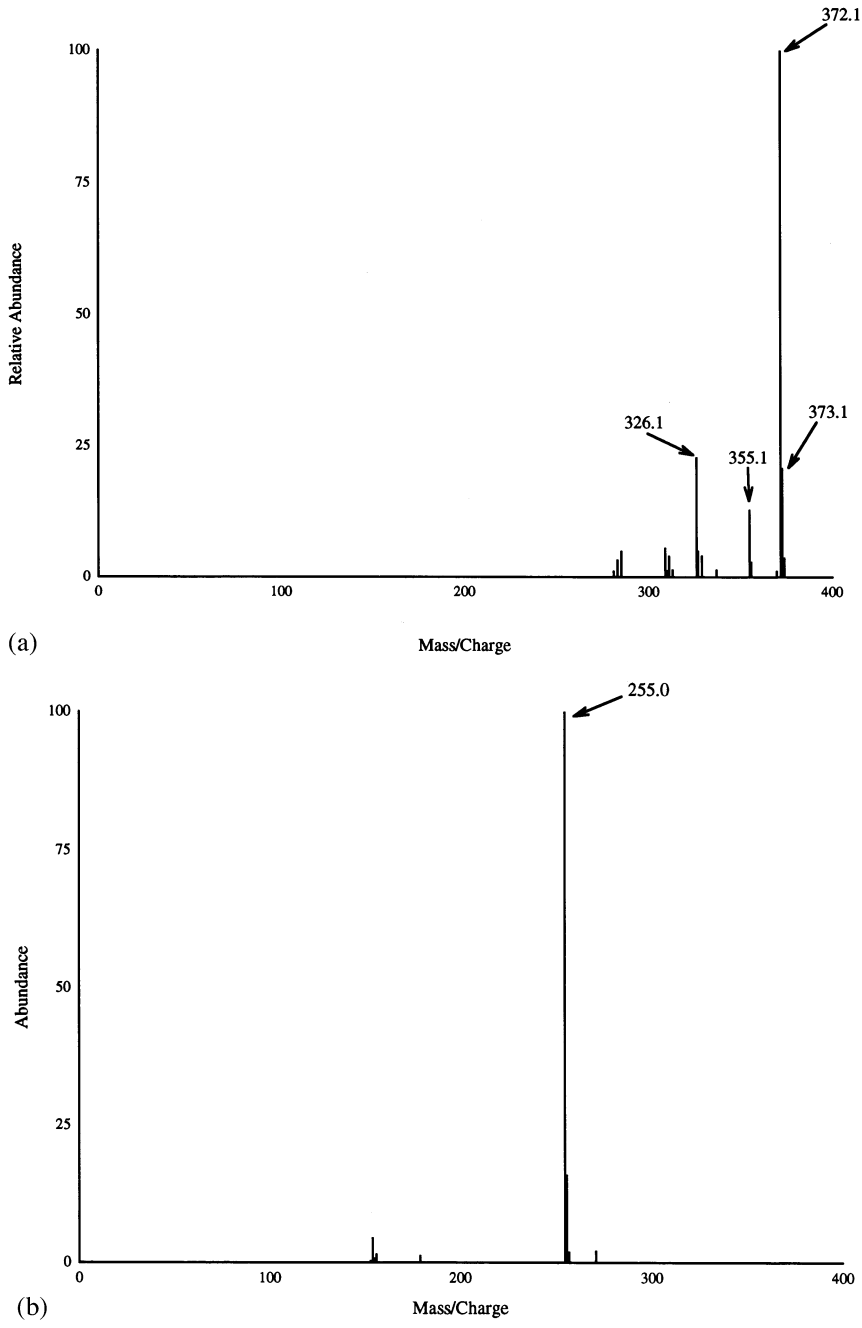


Fig. 3. Mass spectra of (A) COL-3 and (B) chrysin.

−80°C freezer for up to 4.5 months. All quality controls were within the acceptable limits except for one of the 50 ng/ml samples.

Freeze–thaw stability was assessed through three full cycles of freeze–thaws ($n = 2$) (Table 4). There was significant variability between replicates and between freeze–thaw cycles. In each quality control set, the percent recovery would

decrease then increase in the next freeze–thaw cycle. The differences were also greater than one would expect from experimental error.

4. Discussion

The goal of this process was to validate a

Table 2
Intra-run precision and accuracy of COL-3 in human plasma[a]

Low standard curve	50 ng/ml ($n=5$)	500 ng/ml ($n=6$)	1200 ng/ml ($n=6$)
Precision			
Mean	40.8	453.8	1141.4
S.D.	1.4	67.8	115.9
% R.S.D.	3.5	14.9	10.2
Accuracy			
Mean error	−18.3	−9.2	−4.9
S.D.	2.8	13.6	9.7
High standard curve	500 ng/ml ($n=6$)	2500 ng/ml ($n=6$)	8000 ng/ml ($n=6$)
Precision			
Mean	436.6	2525.3	8552.9
S.D.	61.6	335.0	608.0
% R.S.D.	14.1	13.3	7.1
Accuracy			
Mean error	−12.7	1.0	6.9
S.D.	12.3	13.4	7.6

[a] S.D., Standard deviation; R.S.D. (S.D./mean) × 100.

Table 3
Inter-run precision and accuracy of COL-3 in human plasma[a]

Low standard curve	50 ng/ml ($n=13$)	500 ng/ml ($n=14$)	1200 ng/ml ($n=13$)
Precision			
Mean	40.6	455.0	1236.6
S.D.	2.4	61.1	128.9
% R.S.D.	5.8	13.4	10.4
Accuracy			
Mean error	−18.2	−9.0	3.1
S.D.	4.7	12.2	10.7
High standard curve	500 ng/ml ($n=14$)	2500 ng/ml ($n=14$)	8000 ng/ml ($n=14$)
Precision			
Mean	456.0	2633.6	8068.6
S.D.	56.2	258.9	793.0
% R.S.D.	12.3	9.8	9.8
Accuracy			
Mean error	−8.8	−5.3	0.9
S.D.	11.2	10.4	9.9

[a] S.D., Standard deviation; R.S.D. (S.D./mean) × 100.

Table 4
Freeze–thaw stability of COL-3 in human plasma

COL-3 concentration (ng/ml)	Freeze–thaw cycle	Back-calculated concentration (ng/ml)[a]	% of zero freeze–thaw cycle (%)[b]
Low standard curve			
50	0	39.5	
	1	35.9	91.0
	2	38.4	97.2
	3	35.6	90.3
500	0	409.2	
	1	318.3	77.8
	2	440.9	107.7
	3	340.9	83.3
1200	0	1367.6	
	1	1022.5	74.8
	2	1450.3	106.1
	3	871.9	63.8
High standard curve			
500	0	433.3	
	1	348.7	80.5
	2	462.6	77.1
	3	369.9	86.0
2500	0	2853.9	
	1	2198.8	106.8
	2	2112.4	74.0
	3	2256.8	81.7
8000	0	7745.4	
	1	6659.2	85.4
	2	6326.5	79.1
	3	5353.4	69.1

[a] Mean ($n = 2$).

[b] Average of back-calculated average/average of original freeze–thaw sample × 100%. Mean ($n = 2$, except $n = 1$ for 2500 ng/ml second freeze–thaw cycle).

method to quantitate COL-3 in plasma samples. Since the samples would be obtained from a Phase I clinical trial, the concentration range necessary was wide and it was expected that concentration in the low nanograms per milliliter region would be necessary for the low dosing levels. A range from 30 to 10 000 ng/ml was expected to be able to quantitate the majority of the patient samples and could only be quantitated by separating the range into two standard curves. From preliminary pharmacokinetic data processed with a HPLC method with ultraviolet detection, it was expected that the majority of pharmacokinetic samples would be between 400 and 10 000 ng/ml.

An HPLC with ultraviolet detection method was originally developed for quantitation of COL-3. A Waters Nova-Pak® C-18 precolumn was used with a Waters Nova-Pak® Phenyl (3.9 × 150 mm) column with a 4 μm particle size for separation. A gradient method was used with sodium acetate (0.1 M, pH 4.5) and methanol. COL-3 had two UV maxima at approximately 264 and 350 nm; 264 nm had a greater intensity versus 350 nm. Positive interferences occurred at the same retention time as COL-3 with greater than 60% of plasma samples run on this method when the detection was set at 264 nm. A negative deflection was observed at 350 nm before COL-3 eluted, making hand integration a necessity for

every sample. Numerous chromatographic techniques and sample preparations were attempted to resolve the interferences at 264 nm with little or no success. Since the limit of quantitation with the UV method was only 750 ng/ml with the detection on 350 nm and there was 60% interference on 264 nm, development of the mass spectrometry method became necessary to quantitate low nanograms per milliliter concentrations.

Two published methods confirming the presence of tetracycline derivatives in samples with mass spectrometry incorporated oxalic acid into the mobile phase [5,6]. Blanchflower et al. utilized APCI techniques to obtain low nanogram quantitation, and determined that metal blocking agents such as ethylenediamine tetraacetic acid and oxalic acid may be used to improve peak shape and sensitivity [5]. In our assay, oxalic acid at a concentration of 0.01 M was determined to be sufficient enough to retain good peak shape without being detrimental to the source of the mass spectrometer. Kijak et al. determined that the combination of methanol–acetonitrile–oxalic acid demonstrated greater sensitivity but if the methanol was removed, better separation occurred between the tetracycline derivatives [6]. With the difficulties of separating the interferant with the UV method, it was felt that resolution would be more of an issue and therefore methanol was not included in the mobile phase.

The internal standard, chrysin, was chosen since it had baseline resolution from COL-3, good recovery and sufficient signal under the mass spectroscopy conditions. Flavonoids have been found to be present in parts of the tobacco flower, *Nicotiana tabacum* L. [7]. There was concern since chrysin is a flavonoid that there may be interference in patients who smoke heavily. Plasma samples from smokers who were on minimal medications were analyzed with this method to determine basal levels of chrysin in smokers. There were no peaks detected at the retention time of chrysin on $m/z+$ of 255 with plasma from smokers.

Even though COL-3 and chrysin elute within 8 min during the isocratic portion of the method, a gradient method was still necessary. Due to the crude sample preparation, a total run time of 60 min isocratically would be necessary to clean the column. The run time could not be shortened isocratically due to late eluting peaks that would co-elute around the COL-3 retention time in subsequent injections. Therefore, the addition of a 7 min wash in acetonitrile–oxalic acid (0.01 M, pH 2.2) (90:10, v/v) with re-equilibration of the column enabled the shortening of the cycle time by over 30 min. After assessing the carryover effects observed with this method, a needle wash utilizing acetonitrile–water (90:10, v/v) was added to minimize carryover. Several different sample preparation methods, liquid–liquid and solid phase extraction, had lower recovery and were less reproducible than the acetonitrile precipitation.

Plasma interferences were problematic with LC-UV and, therefore, a mass spectrometer was used to increase specificity. The fragmentation pattern was determined by increasing the fragmentor voltage. When the fragmentor voltage was increased, the abundance of $m/z+$ 372.1 decreased while 326.1 increased. At the fragmentor voltage of 90 V, the qualifying ion, $m/z+$ of 326.1, was approximately 30% of the quantifying ion, $m/z+$ of 372.1, and was sufficient to detect the qualifying ion throughout the range of the calibration curve. This ratio of the qualifying and quantifying ions were used to confirm COL-3 peaks in patient samples. The fragmentor voltage was mainly chosen to decrease background interferences. This will be useful in patient samples for confirming that any peak that elutes at 5.2 min on 372.1 $m/z+$ will have an increased probability of being pure COL-3 if it has a co-eluting peak at 326.1 $m/z+$.

The freeze–thaw stability of COL-3 was highly variable. The reason for this variability was not explored further. This method was not shown to be stability indicating, and thus the freeze–thaw results may indicate the presence of breakdown products that co-elute with COL-3. Since the results obtained from this experiment were variable, samples should not be frozen or thawed when analyzing patient samples.

This method may be applicable to other tetracycline derivatives, especially the various chemically-modified tetracyclines that are currently in

various stages of development. Tetracyclines have been noted as difficult compounds to analyze due to problems of solubility, peak shape and chelation with metal ions that could be impurities in a system. This report demonstrates that, in spite of the limited solubility and chemical and physical properties of COL-3, a method was developed which could reliably quantitate COL-3.

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