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Reversed-phase liquid chromatography method to determine COL-3, a matrix metalloproteinase inhibitor, in biological samples

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Abstract

A reversed-phase high-performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection was developed and validated for the quantification of 6-deoxy-6-demethyl-4-dedimethylamino-tetracycline (COL-3), a matrix metalloproteinase (MMPs) inhibitor, in rat serum. This simple, sensitive, rapid and reproducible assay involved a preliminary serum deproteinization by adding a mixture of acetonitrile–methanol–0.5 M oxalic acid (70:20:10 (v/v)), as the combined precipitant and metal blocking agent, into serum samples (2:1 (v/v)). An aliquot (20 μl) of the supernatant was injected into the HPLC system linked to a Waters XTerra™ RP_{18} column (150 mm × 4.6 mm i.d., particle size 5 μm). The compound was eluted by a mixture of acetonitrile–methanol–0.01 M oxalic acid (40:10:50 (v/v), pH 2.00), as the mobile phase, and detected at the wavelength of 350 nm. The total running time was 10 min. The low and high concentration calibration curves were linear in the range of 50–1200 ng/ml and 1200–12,000 ng/ml, respectively. The intra- and inter-day coefficients of variation at three quality control concentrations of 100, 1200, and 12,000 ng/ml were all less than 6%, while the percent error ranged from −2.5 to 6.6%. The limit of quantitation (LOQ) for COL-3 in serum was 50 ng/ml. This assay was successfully employed to study the serum concentration–time profiles of COL-3 after its intravenous and oral administration in rats. The method with some minor modifications in sample pretreatment was also applicable to the determination of the concentrations of COL-3 in rat bile, urine and feces.
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Keywords: COL-3; Metalloproteinase inhibitor

1. Introduction

6 - Deoxy - 6- demethyl- 4- dedimethylamino - tetracycline (COL-3), also named as Metastat® or CMT-3 (Fig. 1), is a new synthetic non-antimicrobial tetracycline analogue. It has been developed as a potential anti-tumor agent based on its inhibition of matrix metalloproteinases (MMPs), which belong to a family of Zn^{2+}-dependent proteinases that proteolytically degrade the extracellular matrix [1,2]. An imbalance between active MMPs and MMP inhibitors causes degradation of the basement membrane to allow angiogenesis, tumor growth, and metastasis [3–5]. COL-3 directly inhibits MT1-MMP (membrane-type-1 MMP) expression and activity and pro-MMP-2 expression in osteosarcoma cells [1]. In addition, COL-3 inhibits cell proliferation and induces apoptosis in a number of tumor cells [3,6]. In vivo studies have demonstrated that COL-3 has both anti-tumor and anti-metastasis activities [7,8].

COL-3 has undergone the Phase I clinical trial in the patients with refractory metastatic cancer [9] and AIDS-related Kaposi's sarcoma [10]. A reversed-phase high-performance liquid chromatography (HPLC) with atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) detection has been developed for quantification of COL-3 in human plasma [11]. COL-3 is separated on a Waters Symmetry® C_{18} column with oxalic acid (0.01 M, pH 2.2)–acetonitrile (55:45 (v/v)) mobile phase. This method is specific and sensitive, with the limit of quantitation (LOQ) at 30 ng/ml for a 25 μl injection of the reconstituted drug solution [11]. However, this assay method is hampered by the relatively large inter- and intra-day errors (mean, <18.3%) and coefficients of variation (mean, <14.9%) for all of the three quality control concentrations tested (50,

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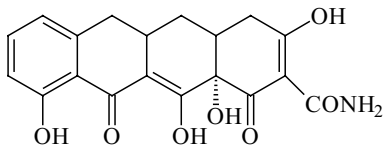

Fig. 1. Chemical structure of COL-3.

500, and 1200 ng/ml). In addition, this assay involves a complicated sample preparation procedure, which includes acetonitrile precipitation of plasma protein followed by evaporation of aqueous supernatant and reconstitution of sample residue with the mobile phase, and a time-consuming column clean-up after each run, using acetonitrile–oxalic acid (0.01 M, pH 2.2) (90:10 (v/v)) for 7 min, followed by re-equilibration of the column with the mobile phase for another 8 min in order to avoid the interferences of the late eluting peaks [11]. Moreover, this technique (HPLC-MS) is complex, and its use could be hampered by the high cost of the equipment.

It is thus desirable to have a simpler, faster, more reliable, and yet sensitive assay method for routine quantification of COL-3 in biological samples obtained from pre-clinical and clinical pharmacokinetic studies on COL-3. In the present study, an improved HPLC method with ultraviolet (UV) detection was developed and validated in rat serum. Separation was achieved on a new Waters XTerra™ RP_{18} (organic/inorganic hybrid particle-based, incorporated with methylsiloxane groups) column instead of a Waters Symmetry® C_{18} (silica-based) column, as there are reduced surface silanols available for the former compared to the latter. The effects of oxalic acid, employed as a metal blocking agent in the sample pretreatment, on the assay sensitivity, linearity, and precision was examined. This assay method was successfully employed to study the serum concentration–time profiles of COL-3, besides determining drug concentrations in rat bile, urine and feces, following its oral administration in rats.

2. Experimental

2.1. Chemicals and reagents

COL-3 (purity 98.7%, Fig. 1) was a gift from CollaGenex Pharmaceuticals (Newtown, PA, USA). Methanol, acetonitrile (both HPLC grade) were purchased from Mallinckrodt Baker (Paris, KY, USA). Anhydrous oxalic acid (extra pure reagent) was purchased from Nacalai Tesque (Kyoto, Japan). Carboxylmethyl cellulose sodium (CMC), polyethylene glycol 400 (PEG-400), Hanks' balanced salt solution (HBSS) (without phenol red and sodium bicarbonate), and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) were purchased from Sigma (St. Louis, MO, USA). Water was filtered and deionized with a Milli-Q-UF system (Millipore, Milford, MA, USA) and was used throughout the study.

2.2. HPLC instrumentation and conditions

Shimadzu HPLC system (Shimadzu, Kyoto, Japan) used was equipped with an SCL-10Avp system controller, LC-10ATvp pump, DGU-14A degasser, SIL-10ADvp autosampler and SPD-M10Avp UV/Vis photodiode array (PDA) detector. Chromatographic separation was conducted on a XTerra™ RP_{18} column (150 mm × 4.6 mm i.d., particle size 5 μm) with Sentry™ guard column (XTerra™ RP_{18}, 20 mm × 3.9 mm i.d., particle size 5 μm) (Waters, Milford, MA, USA). The mobile phase consisted of acetonitrile–methanol–oxalic acid (0.01 M, pH 2.0), a ratio of 40:10:50 (v/v) for biological samples and 40:20:40 (v/v) for HBSS samples. The mobile phase was degassed by ultrasonication and was delivered isocratically at a flow-rate of 1.0 ml/min. The column was maintained at 25 °C. The eluent was monitored at a wavelength of 350 nm. Peak recording and integration was performed using Shimadzu CLASS-VP data process system. The peak areas of COL-3 were used for quantitative computations. Calibration curves were constructed by least-squares linear regression of peak areas of COL-3 versus the concentrations of COL-3 spiked to drug-free biological samples.

2.3. Stock solutions and standards

Stock solutions of COL-3 were prepared in methanol at a concentration of 1 mg/ml, and 100, 10, 1 and 0.1 μg/ml. All solutions were stored at −20 °C.

The calibration standards were freshly prepared daily by spiking the corresponding blank matrices with appropriate amounts of stock solutions (less than 2% of total volume). Two sets of the standards were prepared according to the following concentrations: 50, 100, 200, 400, 800, and 1200 ng/ml for the low concentration standard curve, and 1200, 2000, 4000, 8000, and 12,000 ng/ml for the high concentration standard curve. Quality control (QC) samples for COL-3 were prepared at the concentrations of 100, 1200 and 12,000 ng/ml.

2.4. Sample preparation

Of acetonitrile–methanol–0.5 M oxalic acid (70:20:10 (v/v)), 200 μl was added to 100 μl of rat serum, while 100 μl was added to 100 μl of bile or urine sample. All the mixtures were vortex-mixed for 30 s, allowed to stand at ambient temperature (25 °C) for about 10 min, and centrifuged at 37,000 g, 4 °C for 8 min. The supernatant was collected and an aliquot of 20 μl was subject to the HPLC assay.

The feces sample (5 g) was extracted by adding 50 ml of acetonitrile–methanol–0.5 M oxalic acid (70:20:10 (v/v)) and homogenized manually. The homogenized feces was soaked in the extraction solution at 4 °C overnight and

ultrasonicated for 15 min. After centrifugation (37,000 × *g*, 4 °C, 8 min), the supernatant was collected and filtered through syringe filter (0.45 μm nylon membrane). An aliquot of 20 μl was injected into the HPLC system.

2.5. Peak purity of COL-3 in biological samples

The peak purity of COL-3 in biological samples was assessed by comparing the UV spectrum of COL-3 peak, obtained from the on-line scanning with SPD-M10Avp UV/Vis PDA detector, with that of COL-3 in methanol scanned with UV-1601 UV/Vis spectrophotometer.

2.6. Assay validation for COL-3 in rat serum

A validation run was performed on QC samples at three levels assayed in quintuplicate on three different days. The intra- and inter-day precision was assessed in terms of the coefficient of variation for the concentrations determined within- and between-day. The accuracy was evaluated as the percent error, which equals ((determined concentration/nominal concentration) − 1) × 100%. The limit of quantitation (LOQ) is defined as the lowest concentration level for which the intra- and inter-day coefficient of variation is no greater than 20% (ICH Harmonised Tripartite Guideline).

The stability of COL-3 in serum was studied at three concentrations of 100, 1200 and 12,000 ng/ml during three freeze–thawing cycles. The stability of COL-3 in the serum supernatant after deproteinized by acetonitrile–methanol–0.5 M oxalic acid (70:20:10) was studied at ambient temperature (25 °C) during 96 h. Duplicates were analyzed at 0, 24, 48, 72 and 96 h. The long-term stability of COL-3 in methanol at −20 °C was examined over a period of 6 months.

2.7. Role of a metal blocking agent in sample pretreatment

COL-3 was prepared at different concentrations in HBSS (pH 7.4) and rat serum. The test samples were prepared according to the following concentrations: 20, 50, 100, 200, 500, 1000, 2000, 5000, 10,000 and 20,000 ng/ml for COL-3 in HBSS and 100, 200, 500, 1000, 2000, 5000, 10,000 and 20,000 ng/ml for COL-3 in rat serum. Each test sample (100 μl) was subject to two sample pretreatments: one was added with acetonitrile–methanol–0.5 M oxalic acid (70:20:10 (v/v)) (100 μl used for HBSS sample and 200 μl used for serum sample) as the oxalic acid pretreatment group, while the other was added with acetonitrile–methanol–H_2O (70:20:10 (v/v)) as the control group. Of each group, 50 μl of the mixture was injected into the HPLC system. All test samples were prepared freshly. The HBSS samples were assayed daily in duplicate on three different days, while the serum samples were assayed in triplicate on the same day.

2.8. Applications

Male Sprague-Dawley (SD) rats (200–250 g) were given COL-3 in 2% CMC (30 mg/kg) orally or COL-3 solution in PEG-400/phosphate buffer (pH 7.4, 2:3 (v/v)) intravenously. Blood samples (about 0.5 ml) were collected at 0, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 30, 35 and 48 h after dosing. About 400 μl of blood obtained without anticoagulation was centrifuged (1100 × *g*, 37 °C, 10 min) to separate serum. The samples of urine and feces were collected at the interval of 0–24, and 24–48 h after dosing. The bile samples were collected during the entire 48 h study at an interval of 1 h from 0 to 12 h, and at the interval of 12–24, 24–35, and 35–48 h after oral administration of COL-3 to the bile-duct cannulated rat. All samples were stored at −20 °C until analysis.

3. Results

Fig. 2 shows the chromatograms of a blank rat serum sample, a serum sample spiked with 100 ng/ml COL-3, and a rat serum sample 30 h after intravenous injection of COL-3. The retention time of COL-3 under the chromatographic condition studied was 5.9 ± 0.5 min. The entire running time for one injection was established within 10 min. Fig. 3 demonstrates the UV spectrum of COL-3 peak of a serum sample scanned on-line with PDA detector compared to that of COL-3 in methanol scanned with UV/Vis spectrophotometer.

The assay was linear in the range of 50–1200 ng/ml for the low concentration calibration curve, and of 1200–12,000 ng/ml for the high concentration calibration curve (both $r^2 = 0.999$). Table 1 lists the intra- and inter-day precision and accuracy for COL-3 in rat serum at the three QC concentrations of 100, 1200, and 12,000 ng/ml. The intra- and inter-day coefficients of variation were all less than 6% at the three QC concentrations, while the accuracy in terms of percent error varied from −1.1 to 6.6, −2.5 to 0.18, and −0.5 to 1.5% for 100, 1200, and 12,000 ng/ml, respectively. The LOQ for COL-3 in serum was about 50 ng/ml, at which the intra-day coefficients of variation varied from 0.8 to 8.9%, while the accuracy in terms of percent error varied from −18.4 to 6.8%.

Freeze–thaw stability was assessed through three full cycles of freeze–thaws ($n = 3$). As shown in Table 2, the concentration changes between the freeze–thaw cycles varied from −14.5 to 12.5% (mean, −4%). Table 3 shows the stability of COL-3 in a supernatant after the serum sample deproteinization using an extraction solution of acetonitrile–methanol–0.5 M oxalic acid (70:20:10 (v/v)). COL-3 was found stable in the supernatant at ambient temperature for at least 96 h, which allows the assay to be performed continuously overnight for a large number of samples. Table 4 shows the stability of COL-3 in methanol, suggesting that the stock solution of COL-3, kept at −20 °C, could be used over a period of 6 months.

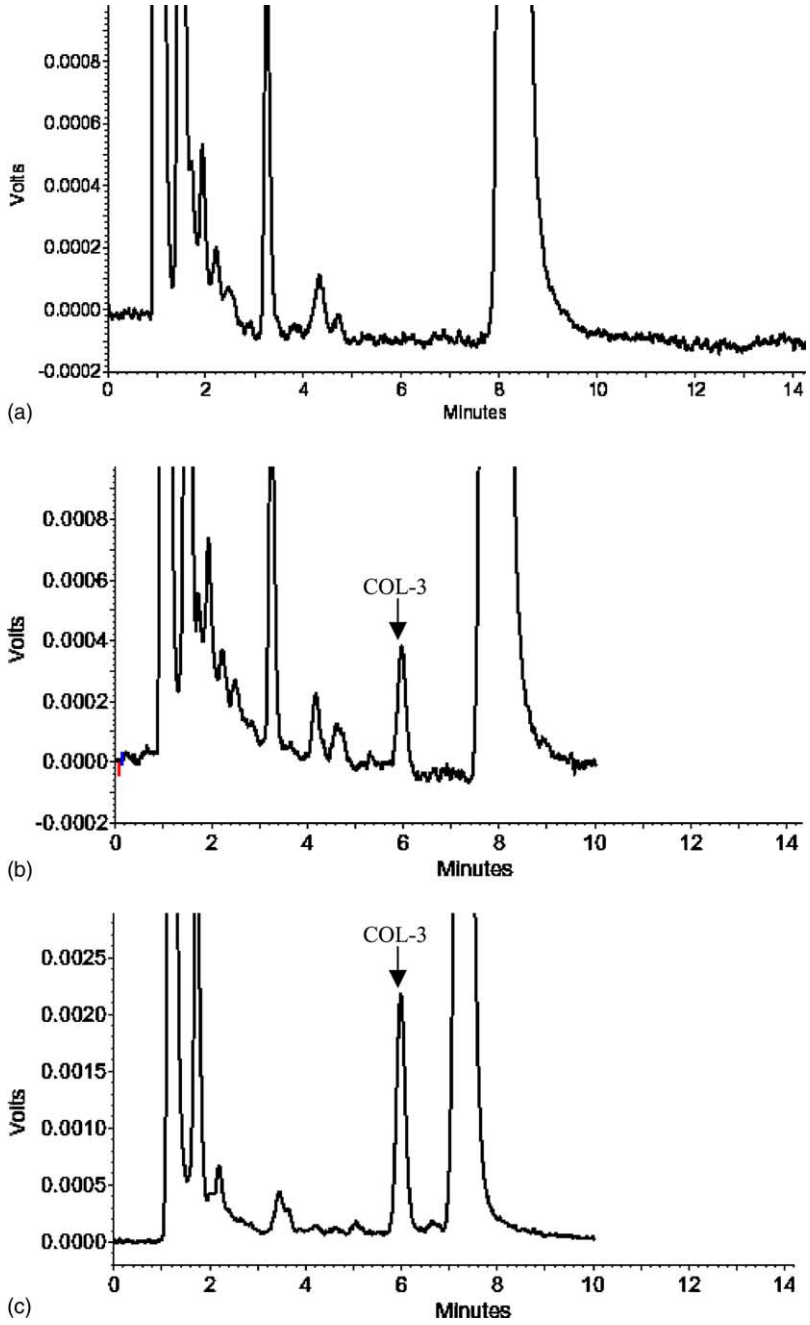

Fig. 2. Chromatograms of (a) blank rat serum, (b) blank rat serum spiked with 100 ng/ml COL-3, and (c) serum sample 30 h after intravenous injection of COL-3 (10 mg/kg) in rat.

This method was also suitable for the determination of COL-3 in rat bile, urine, and feces with some minor modifications in sample pretreatment. The chromatograms for bile, urine, and feces are demonstrated in Figs. 4–6, respectively. The recoveries of COL-3 in rat bile, urine, and feces within 48 h after dosing were determined (data reported elsewhere). The described HPLC method was successfully used to study the pharmacokinetics of COL-3 following its intravenous and oral administrations in rats. The serum concentration of COL-3 could be detected 48 h after dosing using this assay method [12]. Fig. 7 shows the typical serum concentration-time profiles of COL-3 obtained in rats.

Fig. 8 demonstrates the linearity between peak area and concentration prepared for the low, medium and high concentration range of COL-3 in HBSS with and without oxalic acid pretreatment. The respective slopes of the regression lines for the pretreatment groups (range, 115–122) were greater compared to those for the control groups (range, 44–48), suggesting a better assay sensitivity for the former. The correlations between peak

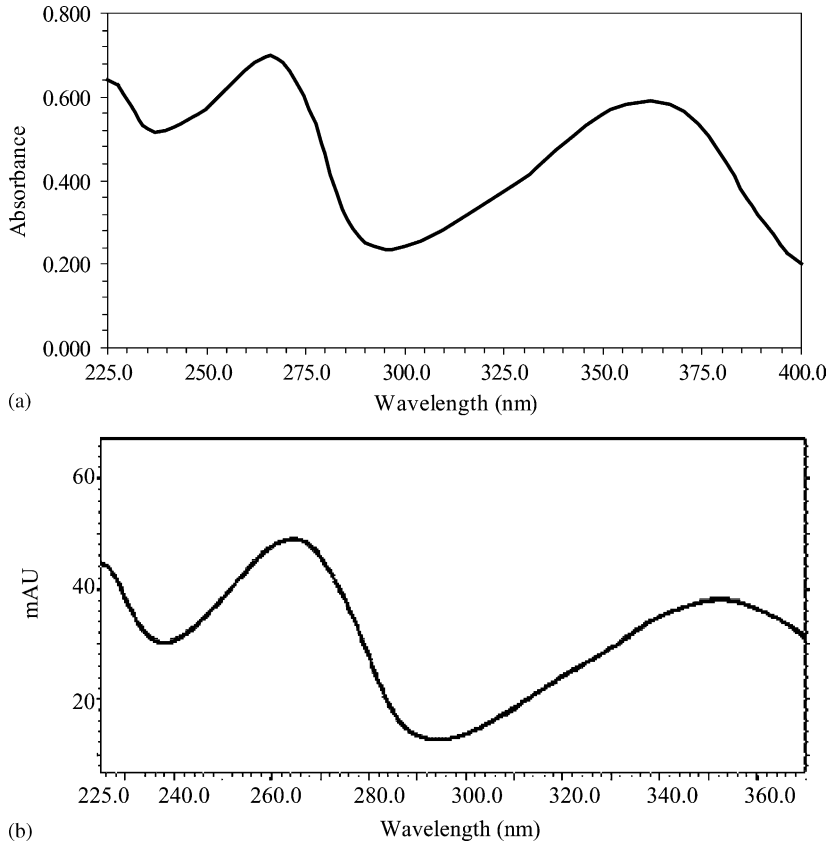

Fig. 3. Representative UV spectrum of (a) COL-3 methanol solution (10 μg/ml) scanned with UV-1601 UV/Vis spectrophotometer, and (b) COL-3 peak of rat serum sample, eluted from HPLC column, being on-line scanned with SPD-M10Avp UV/Vis photodiode array (PDA) detector.

Table 1
Intra- and inter-day precision[a] and accuracy[b] of the assay for the determination of COL-3 in rat serum

	Nominal concentration (ng/ml)			
	50[c]	100	1200	12,000
Day 1 ($n = 5$)				
Determined concentration (ng/ml)	53.3 ± 0.9	106.6 ± 3.2	1202.2 ± 33.8	12024.9 ± 113.4
Precision (CV%)	1.7	3.0	2.8	0.9
Accuracy (error%)	6.76 ± 1.7	6.6 ± 3.2	0.18 ± 2.8	0.2 ± 0.9
Day 2 ($n = 5$)				
Determined concentration (ng/ml)	40.8 ± 0.3	101.2 ± 4.6	1182.9 ± 49.8	12176.1 ± 229.0
Precision (CV%)	0.8	4.6	4.2	1.9
Accuracy (error%)	−18.4 ± 0.6	1.2 ± 4.6	−1.4 ± 4.2	1.5 ± 1.9
Day 3 ($n = 5$)				
Determined concentration (ng/ml)	46.6 ± 4.1	98.9 ± 5.9	1170.4 ± 51.8	11938.8 ± 164.8
Precision (CV%)	8.9	5.9	4.4	1.6
Accuracy (error%)	−6.7 ± 8.3	−1.1 ± 5.9	−2.5 ± 4.3	−0.5 ± 1.4
Overall ($n = 15$)				
Determined concentration (ng/ml)	47.5 ± 5.7	102.6 ± 5.9	1185.2 ± 44.6	12046.6 ± 191.6
Precision (CV%)	12.1	5.7	3.8	1.6
Accuracy (error%)	−5.1 ± 11.4	2.6 ± 5.9	−1.2 ± 3.7	0.4 ± 1.6

[a] Precision (CV%) $= \frac{\text{S.D.}}{\text{mean}} \times 100\%$.

[b] Accuracy (error%) $= \left(\frac{\text{determined concentration}}{\text{nominal concentration}} - 1\right) \times 100\%$.

[c] Estimated as the lower limit of quantitation (LOQ) (ICH Harmonised Tripartite Guideline).

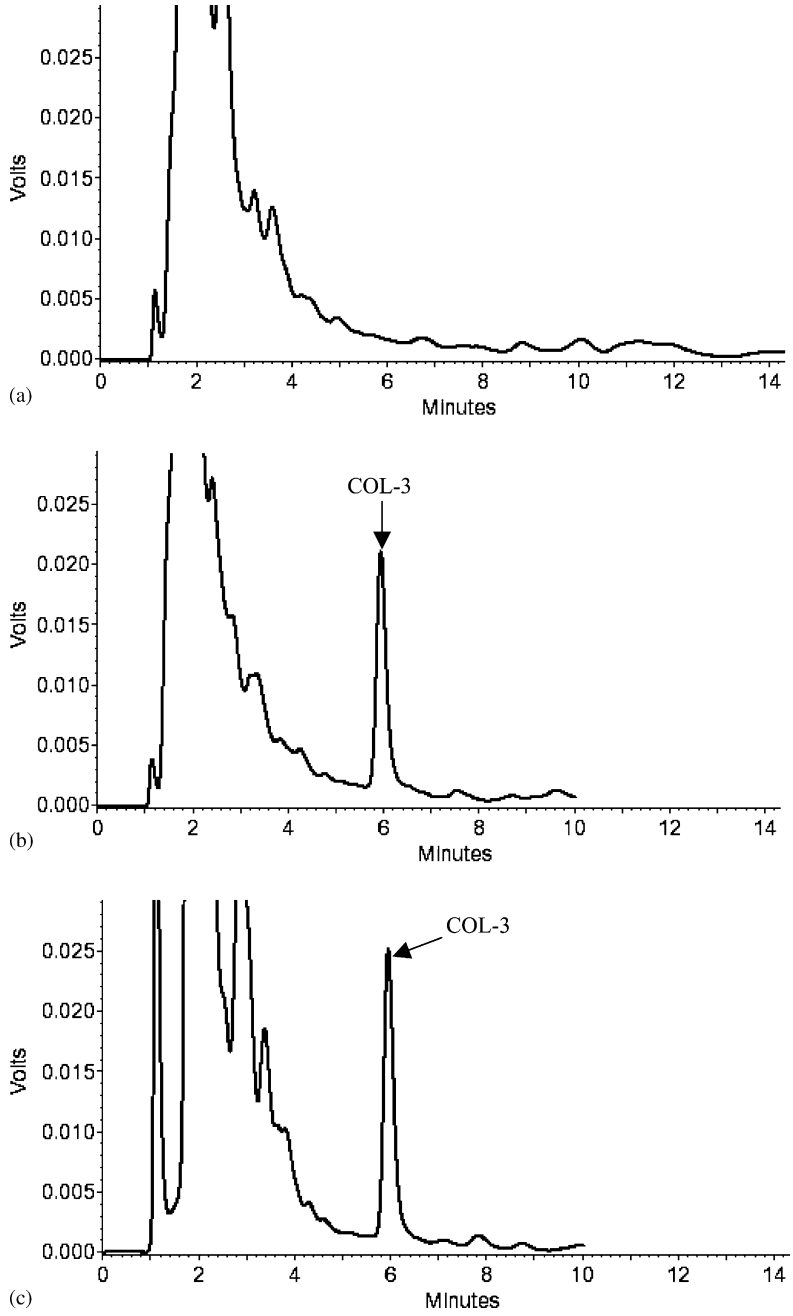

Fig. 4. Chromatograms of (a) blank rat bile, (b) blank rat bile spiked with 5 μg/ml COL-3, and (c) bile sample collected during 24–32 h after oral administration of COL-3 (30 mg/kg) in rat.

area and COL-3 concentration for the low, medium, and high calibration curves obtained with the pretreatment groups ($r^2 > 0.9995$) appeared to be slightly better than those obtained with the corresponding control group ($r^2 = 0.9827$–0.9974). All coefficients of variation and percent errors for the pretreatment group were smaller than those for the control group. A significant improvement in assay sensitivity was also demonstrated for COL-3 in rat serum with, compared to that without, oxalic acid pretreatment (Fig. 9).

4. Discussion

COL-3 is a yellow, odourless crystalline compound with a molecular weight of 371.35. It can be classified as the simplest tetracycline, structurally differing from tetracycline by the absence of the 4-dimethylaminl, 6-hydroxyl, and 6-methyl groups (Fig. 1). COL-3 possesses the first two of the acidic groupings of the tetracyclines, but due to the absence of the dimethyammonium functional group at C4, COL-3 not the third pK_a. Using a spectrophotometric

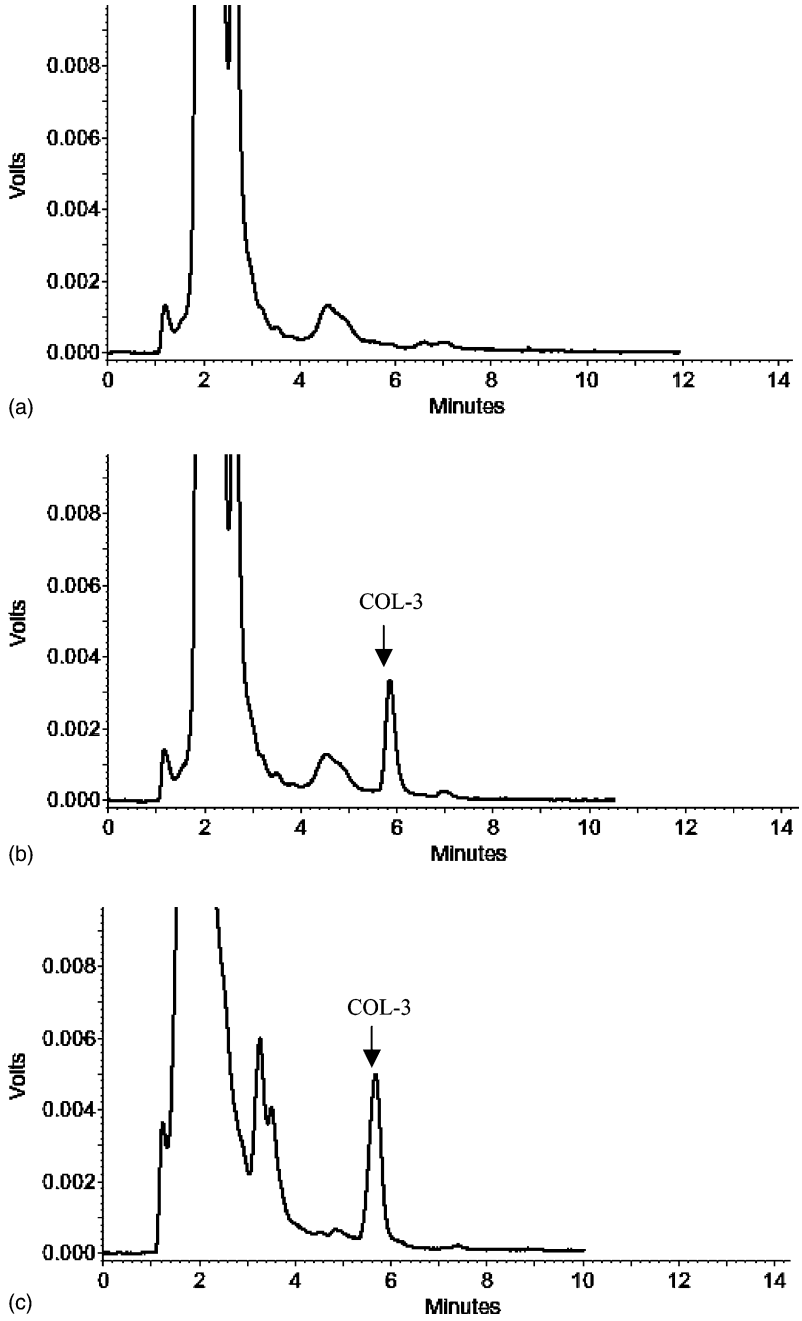

Fig. 5. Chromatograms of (a) blank rat urine, (b) blank rat urine spiked with 1 μg/ml COL-3, and (c) urine sample collected during 24–48 h after intravenous injection of COL-3 (10 mg/kg) in rat.

technique, the apparent pK_a values of COL-3, determined in 0.5% methanol aqueous media at 25 °C with a constant ionic strength of 0.2, are 5.64 (pK_{a1}) and 8.35 (pK_{a2}) [13]. In an aqueous solution, COL-3 thus exists, depending on the pH, as the unionized or/and ionized species (monovalent or/and divalent anion). COL-3 is practically insoluble in water (approximately 0.01 mg/ml). It is readily soluble in organic solvents such as methanol, polyethylene glycol, and benzyl alcohol. With increasing pH, the solubility of COL-3 in aqueous solution increases, but its stability decreases. The pH of maximum stability for COL-3 is below pH 4 [14].

Tetracyclines are well known to readily form chelate complexes with polyvalent metallic ions. Trace metal impurities may be present in HPLC columns and solid-phase extraction (SPE) cartridges. It has been reported that use of some manufacturers' SPE cartridges can lead to low and variable recoveries, probably due to the presence of trace metal impurities [15]. This can also be problematic with HPLC columns used for separation of tetracyclines. It is thus highly recommended to add a metal blocking agent such as oxalic acid or ethylenediamine tetraacetic acid (EDTA) to the mobile phase to reduce peak tailing and increase sensitivity. Such a

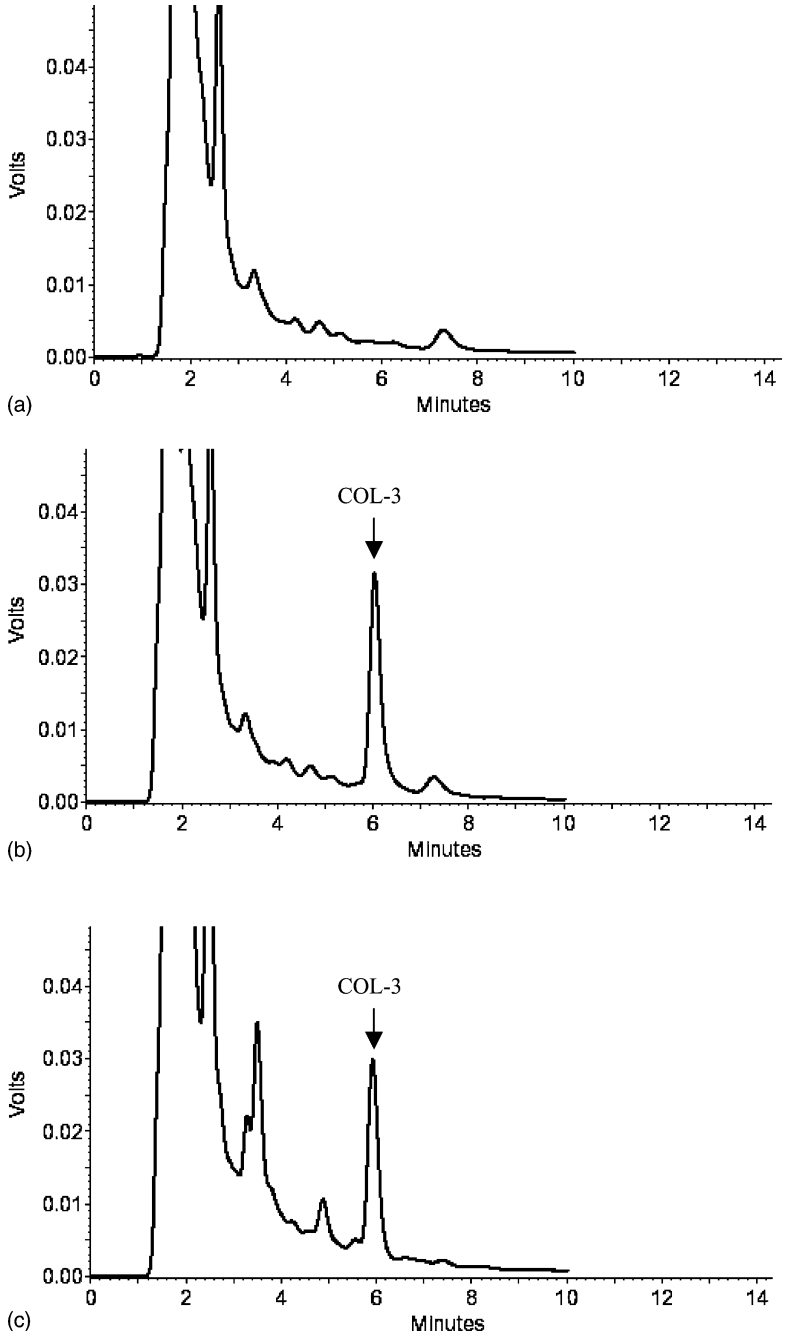

Fig. 6. Chromatograms of (a) blank rat feces, (b) blank rat feces spiked with 5 μg/ml COL-3, and (c) feces sample collected during 24–48 h after oral administration of COL-3 (30 mg/kg) in rat.

combination of organic solvents with oxalic acid as the mobile phase has been reported previously for COL-3 [11]. A concentration of 0.01 M oxalic acid (pH 2.00) in the mobile phase was adequate to retain good peak shape and sensitivity of COL-3 in the present study. However, such a low pH (less than or close to 2.00) of the mobile phase would lead to the decrease of column performance and shortening of column lifetime for the traditional silica-based column. The performance of a conventional C_{18} silica-based column used originally was deteriorated after appropriately 900 h in use (data not shown). The XTerra^{TM} RP_{18} hybrid particle-based column that allows us to work at a wide pH range of 1–12 was thus utilized in the present study. The peak of COL-3 obtained on the XTerra^{TM} RP_{18} column was sharp and symmetric (Figs. 2 and 4–6). In addition, the running time for each assay was shortened greatly compared to the conventional C_{18} column under the similar chromatographic condition. The retention time of COL-3 was 5.9 ± 0.5 min, and all the peaks (including endogenous peaks) of several biological samples (rat serum, bile, urine, and faces) were eluted

Table 2
Freeze–thaw stability of COL-3 in rat serum

Nominal concentration (ng/ml)	Freeze–thaw cycle	Determined concentration (ng/ml)[a]	Original concentration (%)[b]
100	0	109.4	
	1	123.1	112.5
	2	93.6	85.5
	3	97.3	88.9
1200	0	1212.4	
	1	1180.7	97.4
	2	1073.0	88.5
	3	1224.0	100.9
12,000	0	12098.3	
	1	11673.8	96.5
	2	12345.7	102.0
	3	11291.8	93.3

[a] Mean ($n = 2$).
[b] Mean of freeze–thaw concentrations/mean of original concentrations × 100% ($n = 2$).

Table 3
Stability of COL-3 in extraction supernatant obtained from rat serum deproteinization using acetonitrile–methanol–0.5 M oxalic acid (70:20:10 (v/v))

Nominal concentration (ng/ml)	Time (h)[a]	Determined concentration (ng/ml) (n = 5)	Original concentration (%)[b]
1200	0	1237.2 ± 52.0	
	24	1251.2 ± 55.0	101.1
	48	1218.3 ± 46.8	98.5
	72	1242.7 ± 38.2	100.4
	96	1303.1 ±51.5	105.3

[a] Time for which samples were placed at ambient temperature (25 °C).
[b] $\frac{\text{Mean of COL-3 concentrations at time } t}{\text{Mean of COL-3 concentrations at time 0}} \times 100\%$.

within 10 min. Thus, the total running time for each assay was 10 min. In contrast to the previous reported method using a Waters Symmetry® C_{18} column, the total running time for each assay was 23 min as the column needed to be washed and re-equilibrated after each run due to late eluting peaks that would co-elute around the COL-3 retention time in subsequent injections [11]. This carryover effect from previous injections was not encountered in the present study.

Table 4
Long-term stability of COL-3 stock solution in methanol at −20 °C

Nominal concentration (ng/ml)	Time[a] (month)	Determined concentration (ng/ml) (n = 3)	Original concentration (%)[b]
1200	0	1208.2 ± 31.2	100.7
	1	1247.0 ± 28.9	103.2
	3	1191.8 ± 34.5	98.6
	6	1256.3 ± 27.8	104.0

[a] Time for which samples were stored at −20 °C.
[b] $\frac{\text{Mean of COL-3 concentrations at time } t}{\text{Mean of COL-3 concentrations at time 0}} \times 100\%$.

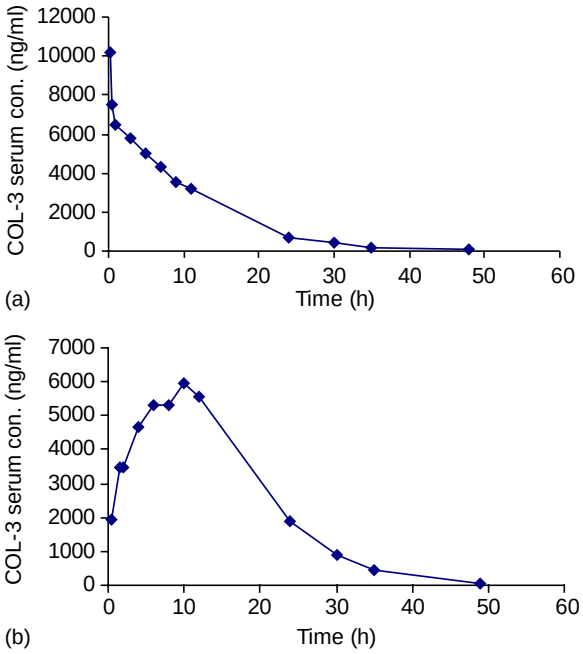

Fig. 7. Representative serum concentration–time profiles of COL-3 following its (a) intravenous injection (10 mg/kg) and (b) oral administration (30 mg/kg) in rats.

Polyvalent metallic ions, Ca^{2+} and Mg^{2+} in particular, are extensively present in almost all of the biological samples. A metal blocking agent may be necessary in sample pretreatment to release tetracyclines from their chelate complexes. However, the importance of a metal blocking agent during sample pretreatment is often overlooked [11]. In the present study, a mixture of acetonitrile–methanol–0.5 M oxalic acid (70:20:10 (v/v)) was used in the sample preparations. Oxalic acid employed here is not only functioning as a metal blocking agent to release COL-3 from its chelate complex, but also adjusts the pH to less than 2 at which COL-3 is quite stable and exists as the predominant un-ionized form so that it can be easily distributed into the organic phase from serum protein. The combination of acetonitrile with methanol acts as the effective protein precipitant to get rid of, and the extraction solvent to extract COL-3 from, serum protein. Furthermore, the importance of sample pretreatment with a metal blocking agent was demonstrated for COL-3 in HBSS and rat serum preparations without (as the control) and with oxalic acid pretreatment. HBSS, that mimics biological fluids (without protein constituents) containing Ca^{2+} and Mg^{2+}, was chosen to prepare COL-3 samples, in which COL-3 formed a chelate complex with the polyvalent metallic ions. It would be expected that COL-3 exists predominantly as the free form in the presence of oxalic acid. As shown in Fig. 8, the sample pretreatment with oxalic acid appeared to improve the assay sensitivity, linearity, and precision at all concentrations evaluated in HBSS preparations. Consistently, such an improvement in the assay sensitivity with oxalic acid pretreatment was also noted for COL-3 in rat serum preparations (Fig. 9). The overall findings of the present

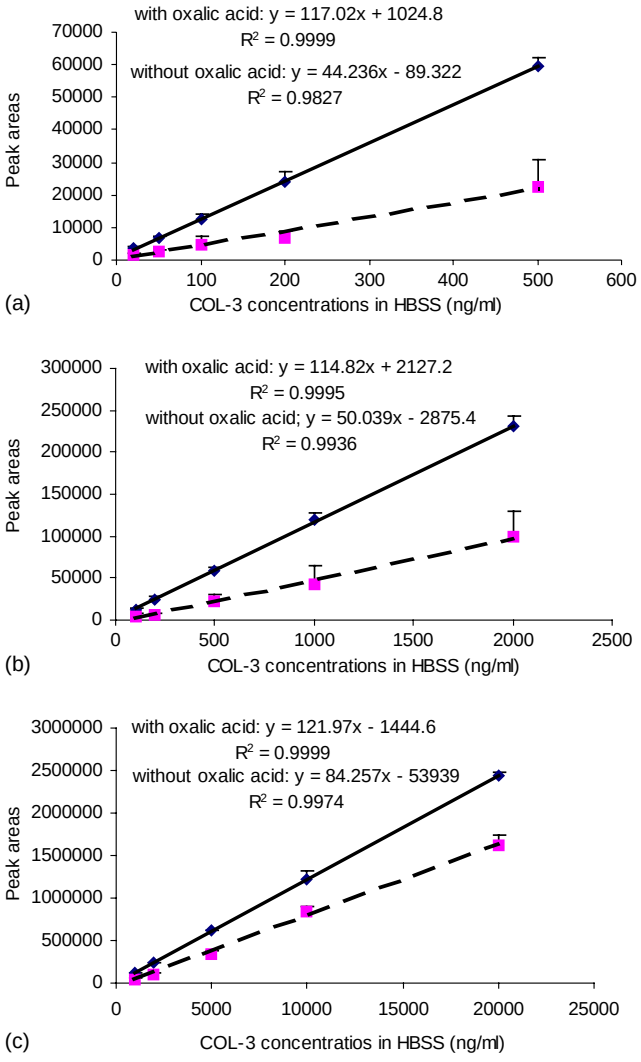

Fig. 8. Linearity between peak area and COL-3 concentration in HBSS for (a) low, (b) medium, and (c) high concentration ranges, with (—) and without (- - -) oxalic acid pretreatment. Determinations were made in duplicate on three different days. Each value was expressed as mean±S.D., and S.D. as error bar.

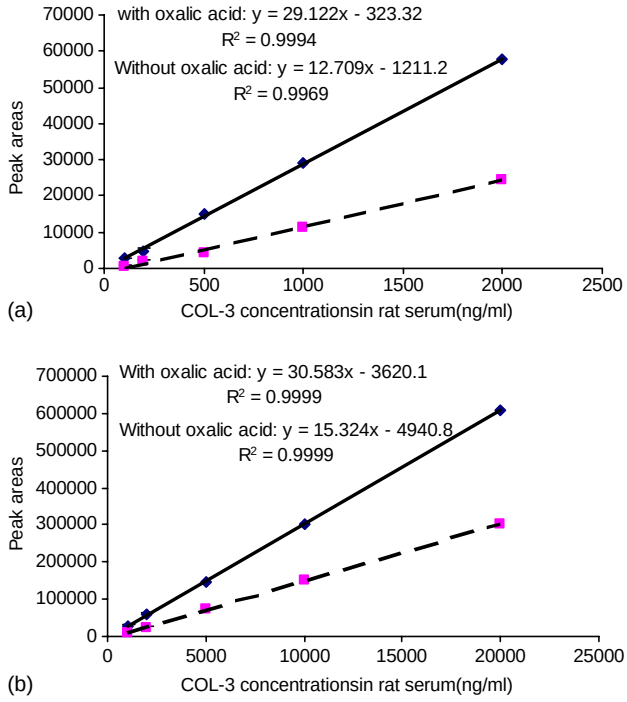

Fig. 9. Linearity between peak area and COL-3 concentration in rat serum for (a) low and (b) high concentration ranges, with (—) and without (- - -) oxalic acid pretreatment. Determinations were made in triplicate on the same day. Each value was expressed as mean±S.D. The error bars (S.D.) were overlaid by the data symbols.

study clearly indicate that sample pretreatment with a metal blocking agent is necessary for quantifying COL-3 in biological samples, especially for those with low concentrations of COL-3. Perhaps, this could explain at least partly, if not all, why the assay accuracy and precision of the present method are better compared to the previous reported method [11].

The present assay method is reliable. It was validated and was shown to have exceptional accuracy and intra- and inter-day precision in rat serum (Table 1). The method is also simple as it involves only a single-step sample pretreatment instead of the complicated sample preparation procedure as described previously [11]. The freeze–thaw stability of COL-3 obtained in rat serum (mean, −3.8%; range: −14.5 to 12.5%) was acceptable, suggesting that serum samples could be frozen or thawed when analyzing rat samples. This observation was in contrast to the previous report that the freeze–thaw stability of COL-3 assayed using the HPLC-MS method was highly variable in human plasma (mean, −14.9%; range: −36.2 to 7.7%) [11].

One of the potential problems associated with the crude sample pre-treatment is the interference from endogenous substances. However, as shown in Figs. 2–6, there is a good selectivity and specificity for COL-3 as demonstrated by its sharp and symmetric peak with no interference from endogenous substances in biological samples. The peak purity of COL-3 in the biological samples was confirmed by an identical UV spectrum for both COL-3 peak and COL-3 methanol solution. COL-3 exhibits two wavelengths of maximum UV absorbance at about 260 and 350 nm; the former gives greater absorbance than the latter. It appeared that an interfering peak co-eluted around the COL-3 retention time when the assay was performed at 260 nm. The present assay thus benefits at least partly, if not all, from the use of a longer detection wavelength at 350 nm instead of 260 nm. Another potential problem related to this single-step sample pretreatment might be a relatively low sensitivity due to the sample dilution without concentrating processes (involving evaporation and re-constitution). The LOQ value at 50 ng/ml obtained in this study for COL-3 in serum was slightly higher than that at 30 ng/ml reported using the HPLC-MS method [11]. Nevertheless, the LOQ of the present assay method should be adequately sensitive to perform pharmacokinetic studies in rats, as the rat serum concentration of COL-3 was detectable 48 h after its administration (Fig. 7).

In addition, this assay method, with only some minor modifications in sample pretreatment, was also applicable to the analysis of COL-3 in rat bile, urine, and feces samples.

In summary, a simple, sensitive, rapid and reproducible reversed-phase HPLC method with UV detection was developed and validated for the quantification of COL-3 in rat serum. The method was also applicable to the analysis of COL-3 in other rat biological samples.

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