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Journal of Chromatography B, 794 (2003) 77–88
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantification of 6-deoxy-6-demethyl-4-dedimethylaminotetracycline (COL-3) in human plasma using liquid chromatography coupled with electrospray ionization tandem mass spectrometry

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Received 4 June 2002; received in revised form 28 April 2003; accepted 13 May 2003

Abstract

An accurate and reliable liquid chromatographic–tandem mass spectrometric (LC–MS–MS) method has been developed and validated for the determination of 6-deoxy-6-demethyl-4-dedimethylaminotetracycline (COL-3) in human plasma. The assay used chrysin as an internal standard (I.S.). The analyte and the I.S. were extracted from acidified plasma by methyl-*t*-butyl ether. Separation was achieved on a YMCbasic column using acetonitrile–water–formic acid mobile phase. The MS–MS detection was by monitoring fragmentation 372.1→326.2 (m/z) for COL-3 and 255.1→153.1 (m/z) for the I.S. on a Sciex API 365 using a Turbo Ionspray in positive ion mode. The retention times were approximately 1.7 min for COL-3 and 1.8 min for the I.S. The validated dynamic range was 0.03–10.0 μg/ml using 0.25-ml plasma with correlation coefficients of ≥0.9985. The precision and accuracy for the calibration standards ($n=3$) were RSD≤5.3% and RE≤4.0%. The precision and accuracy for low-, mid- and high-concentration QC samples were RSD≤2.8% and RE≤5.1% for intra-batch ($n=6$) and RSD≤2.3% and RE≤3.4% for inter-batch ($n=18$), respectively. The extraction recoveries were 99% for COL-3 and 93% for I.S. The results showed that the quality control plasma samples were stable for at least 1 year if stored at approximately −70 °C. The presented method is simple, fast, specific and rugged. This method has been successfully used for supporting human pharmacokinetic studies.

Keywords: 6-Deoxy-6-demethyl-4-dedimethylaminotetracycline

1. Introduction

COL-3, i.e. 6-deoxy-6-demethyl-4-dedimethylaminotetracycline (Fig. 1), a chemically modified tetracycline, is a matrix metalloproteinase inhibitor and its anti-tumor function has been noticed [1,2]. Recent pharmacokinetic (PK) study in rats showed irregular absorption profiles after oral administration [3]. COL-3 is a weak acid with $pK_{a1}=5.64$ and $pK_{a2}=8.35$ [4]. Recently a liquid chromatographic–mass spectrometric (LC–MS) method was presented in which an atmospheric pressure chemical ionization (APCI) source was used and two separate curves were needed to cover the concentration range in clinical samples [5]. This method also required a

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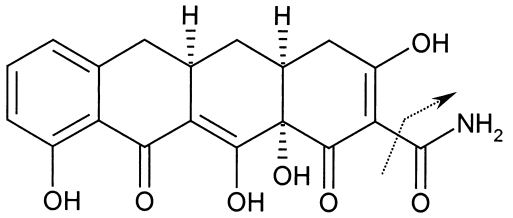
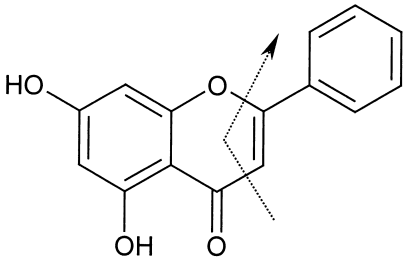
1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00419-7



(a) COL-3, m.w. 371.1



(b) Chrysin, m.w. 254.2

Fig. 1. Structures of COL-3 and chrysin (I.S.).

total cycle time of 28 min for each sample injection. Such a long cycle time for sample analysis plus use of two curves made the assay inconvenient in practice. In addition, highly variable validation data were observed, probably indicating poor ruggedness of the assay. The assay also had a relatively low recovery i.e. 55% for COL-3 and 72% for the internal standard (I.S.).

Evaluating a new drug candidate relies on accurate and reliable data obtained in each phase of the study and such data can be only generated using a specific, sensitive, and robust assay. Potentially numerous clinical samples need a fast analysis speed and data are needed to be delivered more efficiently. In this presentation we have developed a new LC–MS–MS assay. The fine-tuning of each stage of the procedure has significantly improved the assay recovery and sensitivity. More importantly this new assay method was robust and rapid with reduction of a cycle time to approximately 5.5 min. The assay has been successfully applied to the PK studies for clinical trials.

2. Experimental

2.1. Chemicals, materials and apparatus

COL-3 with a purity of >98% was from Colla-Genex (Newtown, PA, USA) and chrysin (I.S.) with a purity of 98% was purchased from Aldrich (Milwaukee, WI, USA). Formic acid (88%), ACS reagent, was from Aldrich. Methyl-*t*-butyl ether (MTBE), acetonitrile (ACN), methanol, and water were of HPLC grade, Fisher (Fair Lawn, New Jersey, USA). HCl was from Sigma (St. Louis, MO, USA). The blank human plasma with sodium-heparin anticoagulant was from Biochemed Pharmacologicals (Winchester, VA, USA).

A dry ice–acetone bath was used for freezing aqueous portion after the target compounds were extracted into the organic phase. A waterbath Turbo Vap evaporator from Zymark (Hopkinton, MA, USA) was used for evaporating organic solvent from extracts. The HPLC system consisting of solvent delivery LC10ADVP, autoinjector SIL10ADVP, Controller SCL10ADVP and column oven CT010ASVP was from Shimadzu (Kyoto, Japan). The analytical column used was YMCbasic 150×2.0 mm (5 μm) from Waters (Milford, MA, USA). The turbo electrospray ionization-triple quadrupole mass spectrometer API 365 made by Sciex (Concord, Canada) was used for detection. Sciex ANALYST software version 1.1 was used for data acquisition and analysis.

2.2. LC–MS–MS conditions

The mobile phase used in the experiments was ACN–water–formic acid (60:40:0.1, v/v). The flow-rate of the mobile phase was 0.4 ml/min. The column and autosampler were operated at room temperature. The injection volume was 5 μl. A solvent containing ACN–formic acid (100:0.1, v/v)

was used as the injector wash solution. The volume of both injector and sample loop was 50 μl. With this injector wash solution and rinse mode setting of 3 (arbitrary value) (rinsing injector both before and after each injection), no carryover was detected.

The electrospray ionization in positive mode was used to generate the molecular ions for mass spectrometric detection. The monitoring fragmentation and conditions for the analyte was optimized by infusing an approximately 2.5 μg/ml pure compound solution in methanol–water (1:1) containing 0.1% formic acid. The multiple-reaction mode (MRM) was used to acquire total ion counts at different time points. A high voltage of 5 kV was applied to the sprayer. The source temperature was 450 °C. The auxiliary gas flow was 8 l/min. The settings of nebulizer gas, curtain gas, and collision gas flow at instrument were 5, 10 and 2, (arbitrary scales), respectively. All of gas used in this experiment was high purity nitrogen (>99.99%) (AGA, Madison, WI, USA). Other optimal parameters included declustering potential (DP) 26 V, focusing potential (FP) 170 V, entrance potential (EP) −4 V, collision cell entrance potential (CEP) 12 V, collision energy (CE) 19 eV, collision cell exit potential (CXP) 24 V for COL-3, and DP 41 V, FP 190 V, EP −9.5 V, CEP 16 V, CE 37 V and CXP 10 V for the I.S., respectively. The MS–MS detection was by monitoring precursor fragment transitions at m/z 372.1$(M+H)^+ \rightarrow$326.2 for COL-3 and at m/z 255.1$(M+H)^+ \rightarrow$153.1 for the I.S. The fragmentation pathways for both COL-3 and chrysin are indicated in Fig. 1. The dwell time was 200 ms for both COL-3 and the I.S. In this assay, both Q1 and Q3 quadrupoles were set at unit resolution. For each injection, the total acquisition time was 3.5 min. An extra 1-min wash step for LC column clean-up was added to 2.4 min of each injection using 100% mobile phase B with a flow-rate of 0. 6 ml/min. Then the column was re-equilibriated with the mobile phase for a further 1 min. Thus the total cycle time for each injection was approximately 5.5 min.

The linear regression of the peak area ratios of analyte/I.S. vs. concentration using a weighted 1/concentration^2 was used to obtain calibration curve from the calibrators and the regression equation of the calibration curve was then used to calculate the plasma concentration.

2.3. Standard solutions and quality control (QC) samples

The stock standard solution of COL-3 was made at 1.00 mg/ml in methanol. The compound was dissolved by sonicating for 3 min and no degradation due to the sonication process was noticed. Two separate stock solutions were prepared. One was used to make spiking working standards, the other used to prepare the quality control (QC) samples. For validation, these two stock standard solutions must agree within 5% in their LC–MS–MS response. Eight spiking standard methanolic solutions were prepared at concentrations of 0.3, 0.6, 1, 4, 10, 40, 80 and 100 μg/ml. The I.S. working solution was 4.00 μg/ml in methanol directly diluted from the I.S. stock solution (1.00 mg/ml in methanol). All stock standard solutions and working standard solutions were stored in polypropylene vials in a refrigerator (2–8 °C) and protected from light with aluminum foil. The stock standard solution of COL-3 was confirmed to be stable for at least 3 weeks if stored refrigerated. After refrigeration (2–8 °C) for 12 months, the concentration of the stock solution dropped by 10% and the solution turned to brown from pale yellow. This observation was different from that in Ref. [5], where the authors noted a 26.1% loss after 4 weeks even when stored at −20 °C and protected from light.

All QC samples were made using standard solutions diluted from the second stock solution. The concentrations of COL-3 in plasma at three level regular QC samples were 0.09 (low), 3.6 (mid), and 7.2 (high) μg/ml. An over-the-curve QC at 25.0 μg/ml and a lower limit of quantitation (LLOQ) QC at 0.03 μg/ml in plasma were also prepared. Each of the plasma QC pools was aliquoted into prelabeled 0.5-ml polypropylene vials (approximately 0.35 ml sample per vial) and stored at −70 °C.

2.4. Sample preparation

For calibration standards, a 25.0-μl aliquot of each spiking standard solution was added into 0.25 ml of control blank plasma in polypropylene tube. For the QC samples and study samples, the same volume of methanol was added to make up the volume and composition of sample. Then 25.0 μl of the I.S.

working solution was added to each tube and all the samples were mixed briefly. The samples were acidified by mixing with 0.10 ml of 0.4 *M* HCl solution. A volume of 4 ml MTBE was utilized to extract by vortexing 2 min and centrifuging 2 min at 2500 rpm (1400×) and 20 °C. The aqueous portion was frozen in a dry ice–acetone bath and the organic layer was then transferred to a clean polypropylene tube. The organic phase was evaporated to complete dryness at 35 °C under a stream of nitrogen at 10 p.s.i. (1 p.s.i. = 6894.76 Pa) in a Turbo-Vap waterbath evaporator. The residue was reconstituted in 0.4 ml of the mobile phase by vortexing for 1.5 min and then the sample was transferred into a polypropylene HPLC injection vial. A 5-μl aliquot of each extracted sample was injected into the LC–MS–MS system.

2.5. Method validation

The validation protocol was established according to the food and drug administration (FDA) industry guidance [6]. During the validation, three validation batches on 3 separate days were processed and analyzed. Each batch included one set of calibration standards and six replicates of low-, mid- and high-concentration of QC samples. One of the validation batches also included the over-the-curve QC samples for the examination of sample dilution integrity. In such a batch, six replicates of over-the-curve QC samples and extra six replicates of high-QC samples were also processed. These samples used a partial volume of 0.05 ml diluted by control blank plasma by five-fold prior to extraction. In one of the batches, six replicates of LLOQ QC samples were also analyzed. The short-term stability of the plasma QC samples was tested in one validation batch. The short-term stability QC samples were obtained by allowing QC samples undergo three freeze–thaw cycles or sitting at room temperature (RT) for approximately 24 h. One batch of the extracted samples was stored at RT (~22 °C) for approximately 24 h then reinjected into the same system to check the storage and injector stability of the processed samples. The 6- and 12-month frozen matrix storage stability was evaluated using a calibration curve obtained from freshly prepared calibration standards, after the QC plasma samples were stored at approximately −70 °C for 6 and 12 months. The sample injection sequence was randomized throughout the run, but the batch always started and ended with a calibration standard.

3. Results and discussion

3.1. Extraction and sample handling

In a previous LC–MS assay [5], the authors found that liquid–liquid extraction (LLE) and solid-phase extraction (SPE) gave lower recoveries and less reproducible results than the ACN precipitation, thus a simple protein precipitation with ACN was recommended for sample preparation that showed 55% recovery for COL-3 and 72% recovery for chrysin. This implied that at least 45% COL-3 and 28% chrysin were still bound to protein or other species. A series of different extraction conditions were tested in our laboratory. Due to the fact that tetracyclines are known to chelate metal ions [7,8], trace metal impurities in SPE cartridges may lead to low and variable recovery [8]. The commonly used acidic condition of pH 2–4 [8] might not be strong enough to release the tetracyclines completely from the complexes. It was found that using 0.1 ml 0.4 *M* HCl to treat plasma samples allowed the drug to be released more effectively and then MTBE was used to extract the compounds. The results showed a 40–60% signal enhancement for COL-3 and at least a 15% enhancement for chrysin. This made a significant improvement in the extraction efficiency. The oxalic acid–MTBE extraction procedure was compared with the HCl–MTBE extraction procedure. The oxalic acid–MTBE system showed approximately 60% suppression but the HCl–MTBE system had no detectable suppression.

In addition, handling and processing samples with different wares produced substantial effects on the results. Compared to using polypropylene tubes in the entire process, using glass tubes showed 10–15% signal loss for COL-3 and 45–50% signal loss for the I.S.. This illustrated that both COL-3 and I.S., especially I.S., might tend to bind to the glass surface.

3.2. Chromatography

3.2.1. Comparison of different analytical columns

A series of analytical columns were investigated using an online-mixing mobile phase at a total flow-rate of 0.4 ml/min. This mobile phase consisted of 25% A and 75% B, where A was formic acid–water (0.1:100, v/v) and B was formic acid–ACN (0.1:100, v/v). The tested columns included a YMCbasic (150×2.0 mm, 5 μm), a Waters Symmetry C_{18} (150×2.1 mm, 3.5 μm), a Metasil basic (50×3.0 mm, 3 μm), a BDS Hypersil C_{18} (50×3 mm, 5 μm), a Zorbax XDB C_8 (50×2.1 mm, 3 μm), an Inertsil silica (50×3.0 mm, 5 μm), a Betasil Silica (50×4.6 mm, 5 μm), and a Hypersil Silica (50×4.6 mm, 5 μm). The different columns are compared in Table 1. The YMCbasic column showed the best peak shape, less tailing for both COL-3 and I.S., and had sufficient retention for separating the interference from the compound. Most of other tested columns either had very little retention or had poor peak shape. These data also demonstrated a typical reversed-phase retention behavior for both COL-3 and I.S. on the YMCbasic column because the retention increased as the ACN content in the mobile phase was decreased. Interference from the plasma blank, which was close to the analyte peak, was noticed. If the mobile phase containing 85% B was used and the flow-rate was 0.4 ml/min, COL-3 eluted at 1.22 min and the interference peak was at 1.17 min and they could not be separated from each other. This would give a significant contribution to the samples especially at the low concentration level. However, when the mobile phase containing 60% B

Table 1
Comparison of analytical columns

Column	Mobile phase[a] (A:B, v/v)	Retention time (min)	Other features
Waters Symmetry C_{18} (150×2.1 mm, 3.5 μm)	25:75	COL-3 1.15 I.S. 1.22	Peak slightly tailing
BDS Hypersil C_{18} (50×3.0 mm, 5 μm)	25:75	COL-3 0.98 I.S. 1.00	Not much retention
Zorbax XDB C_8 (50×2.1 mm, 3 μm)	25:75	COL-3 0.44 I.S. 0.44	No retention and peak tailing
Metasil basic (50×3.0 mm, 3 μm)	25:75	COL-3 0.86 I.S. 0.96	Not much retention
Inertsil silica (50×3.0 mm, 5 μm)	25:75	COL-3 0.81 I.S. 0.79	Not much retention
Betasil silica (50×4.6 mm, 5 μm)	25:75	COL-3 1.58 I.S. 1.53	Peak tailing
Hypersil silica (50×4.6 mm, 5 μm)	25:75	COL-3 1.59 I.S. 1.54	Peak tailing badly
YMCbasic (150×2.0 mm, 5 μm)	(1) 15:85	COL-3 1.22; I.S. 1.24 Interference 1.17	Good peak shape for both COL-3 and I.S., but the interference peak can only be well separated from COL-3 when using the condition (4).
	(2) 25:75	COL-3 1.32; I.S. 1.37 Interference 1.25	
	(3) 35:65	COL-3 1.53; I.S. 1.64 Interference 1.38	
	(4) 40:60	COL-3 1.68; I.S. 1.86 Interference 1.46	

[a] A = formic acid–water (0.1:100, v/v); B = formic acid–ACN (0.1:100, v/v); flow-rate = 0.4 ml/min.

was used at the same flow-rate, COL-3 had a retention time of 1.76 min and the interference had a retention of 1.47 min which reached a nearly baseline separation (Fig. 2c). The chromatograms of COL-3 and I.S. on the YMCbasic column under the optimized chromatographic conditions are shown in Fig. 2. Fig. 2a–d are representative mass chromatograms of the extracts from human plasma blank, and control 0, LLOQ (0.03 μg/ml) sample, and upper limit of quantitation (ULOQ) (10 μg/ml) sample.

3.2.2. Effects of different mobile phases

The mobile phase containing a certain amount of oxalic acid was recommended for LC methods to prevent the formation of metal–tetracyclines complexes [5,7,8]. However, the oxalic acid mobile phase was not friendly to LC–MS–MS because oxalic acid is nonvolatile and would significantly suppress the efficiency of electrospray ionization process. The poor ruggedness of the previous LC–MS assay [5] was probably, at least partially, caused by using an oxalic acid mobile phase. The same mobile phase as in Ref. [5], i.e. 0.01 *M* oxalic acid in the mobile phase A, was also tested in our laboratory and an unstable signal response was observed because the sampling orifice of the mass spectrometer was readily blocked by the accumulation of white solid oxalic acid. Other mobile phases containing different additives such as 0.01 *M* EDTA, 0.01 *M* citric acid, 0.1% formic acid, and 0.01% trifluoro-

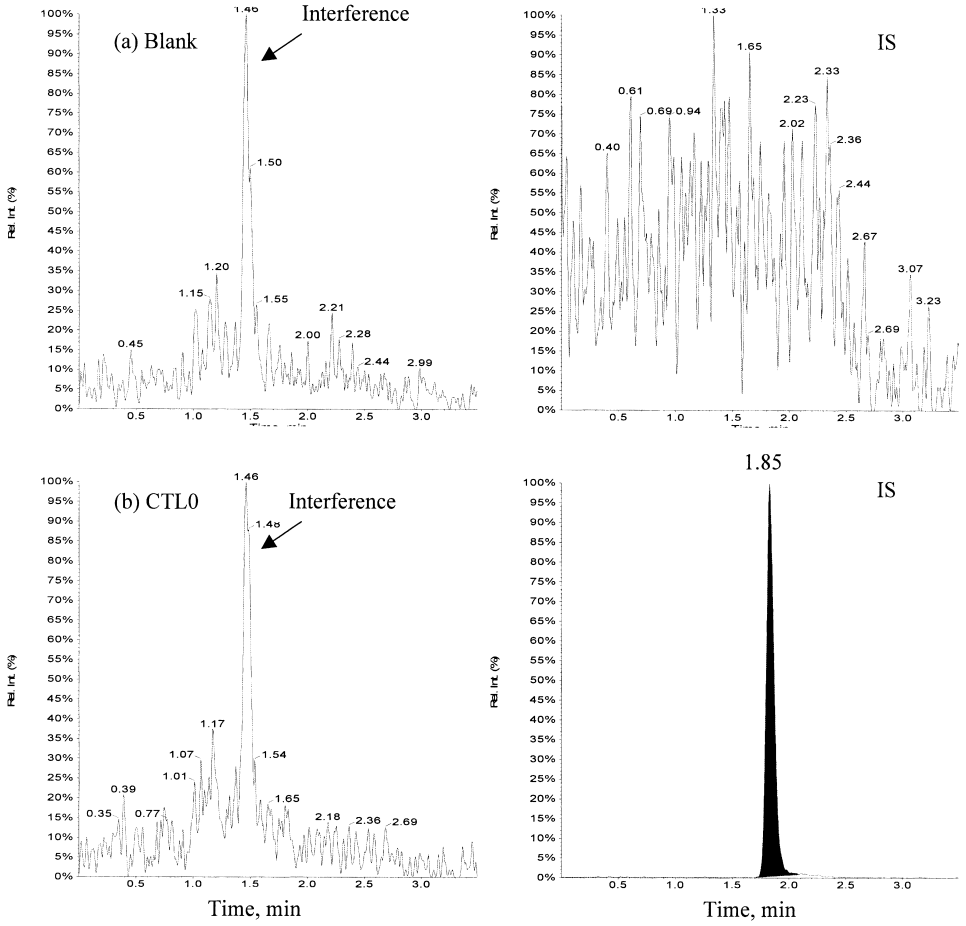


Fig. 2. Mass chromatograms of (a) plasma blank extract, (b) control 0 (plasma blank spiked with I.S.), (c) LLOQ sample (0.03 μg/ml), and (d) ULOQ sample (10 μg/ml).

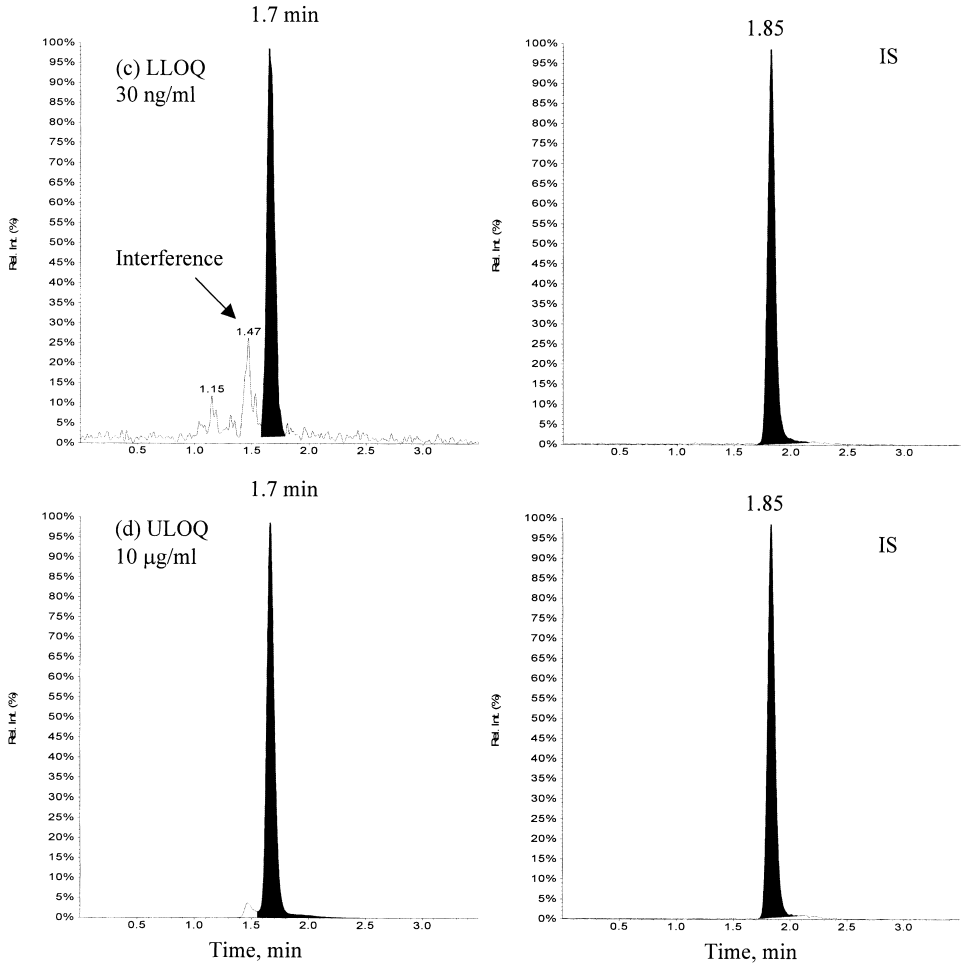


Fig. 2. (*continued*)

acetic acid (TFA) were also compared. The EDTA mobile phase showed a very similar pattern to the oxalic acid mobile phase. The citric acid and TFA mobile phases also produced significant signal suppression. The 0.1% formic acid mobile phase gave the best peak shape and had no suppression.

3.2.3. Carryover

Overcoming carryover was another challenge. COL-3 is considered as a very ''sticky'' compound. It was necessary to flush the analytical column with a solvent stronger than the mobile phase for up to 60 min after each injection to eliminate the carryover from the system if an isocratic mobile phase was used [5]. Using a column clean-up procedure with a solution stronger than the mobile phase was considered but it still required a 28 min of cycle time using a Waters Symmetry C_{18} (150×2.1 mm, 3.5 μm) column [5]. In the present method, a mobile phase of ACN–water–formic acid (60:40:0.1) was used to elute the compound and the I.S. from the YMCbasic column. If there was no extra flushing step, after an ULOQ sample was injected, a carryover of approximately 30–40% response of the LLOQ sample was detected, which was not acceptable by the FDA guidelines for bioanalytical laboratories [6]. However, this carryover was eliminated by adding a 1-min flushing step immediately after the compound was eluted. This flushing used 100% mobile phase B, i.e. formic acid–ACN (0.1:100) at a flow-rate of 0.6

ml/min. The column then was re-equilibriated with the regular mobile phase for another 1 min before processing the next injection. This flushing step plus re-equilibrium only added 2 min to each cycle but effectively resolved the carryover issue. A comparison of the chromatograms obtained with and without the extra flushing step is shown in Fig. 3. Fig. 3a is the chromatogram of an ULOQ sample that was used for the carryover test. Fig. 3b is the chromatogram of the reconstitution solvent injected immediately after the ULOQ sample using the chromatographic conditions without an extra flushing, which showed a carryover of approximately 40% response of LLOQ sample (Fig. 3d). Fig. 3c was obtained using the same procedures as in Fig. 3b but with an additional 1-min flushing step. The carryover was completely eliminated. If this flushing step was added after a full cycle of data acquisition was completed, a significantly longer and more aggressive flushing was required.

3.3. Specificity

Six lots of blank plasma were tested for matrix effects and the assay selectivity. For each lot of plasma, a plasma blank (free of both analyte and I.S.), control 0 (plasma blank spiked with I.S. only), control 0.09 (spiked with 0.09 μg/ml COL-3), and control 7.2 (spiked with 7.2 μg/ml COL-3) were used to check interference and lot-to-lot matrix variation. The measured values and statistics for the spiked samples in the individual lot of plasma are given in Table 2. For all of six lots plasma, the regions of the analyte and the I.S. peaks were found to be free of interference. An interference peak, eluting close to but well separated from the COL-3 peak (Fig. 2), was detected from most of the tested blank plasma lots. When the blank samples were spiked with COL-3 at 0.09 μg/ml, the measured mean, relative standard deviation (RSD) and relative error (RE) were 0.0893 μg/ml, 2.7%, and −0.8%, respectively. For the blank samples spiked with 7.20 μg/ml of COL-3, the measured mean, precision and accuracy were 7.04 μg/ml, 4.8% RSD and −2.2% RE, respectively. The results demonstrated that these six lots plasma had no significant lot-to-lot matrix variation.

3.4. Sensitivity, linearity, matrix effect, and recovery

The calibration curve range was 0.03–10 μg/ml of COL-3 using 0.25-ml plasma. This validated curve had an LLOQ of 30 ng/ml in plasma, which gave a signal-to-noise (S/N) ratio of approximately 100 (Fig. 2c). Since only ~1.25% of sample (5 μl out of 400 μl) was injected into the system, a much lower than 30 ng/ml of detection limit could be expected. For instance, assuming reconstituting residue in 100 μl solution and injecting 20 μl to the column, an ~0.2 ng/ml concentration in the plasma sample would be able to achieve a S/N ratio of ~10. This has not been further evaluated experimentally yet. Due to a relatively high dosage, a quantitation limit of 30 ng/ml was sufficient for clinical trials. Thus the validated curve range was 0.03–10 μg/ml. Over this dynamic range, the linear correlation coefficients were $\geqslant$0.9985. No matrix effect was detected with the current extraction procedure and LC–MS–MS conditions. The equation obtained from the experimental data is given below.

$$y = 2.22 \times 10^{-3}x + 4.43 \cdot 10^{-3}$$

where x is the concentration of COL-3 in plasma with unit of ng/ml and y is the peak area ratio of COL-3 to I.S.

The recovery was determined by comparing the absolute peak areas of the analyte and I.S. extracted from plasma with those of postextraction spiked plasma blanks at corresponding concentrations within the same batch. The recovery data are summarized in Table 3. The average recoveries were 96.7–100.3% for COL-3 at three different concentrations over the calibration curve range and 92.6% for the I.S. These recoveries are a significant improvement on the previous protein precipitation procedure [5].

3.5. Precision and accuracy

As summarized in Table 4, for all three validation curves, the back-calculation results for all calibration standards showed $\leq$5.3% RSD and −3.6–4.0% RE. The precision and accuracy for the QC samples are given in Table 5. For the low-concentration (0.09 μg/ml) level QCs, the precision and accuracy were 2.8% RSD and 5.1% RE for intra-batch assays (n =

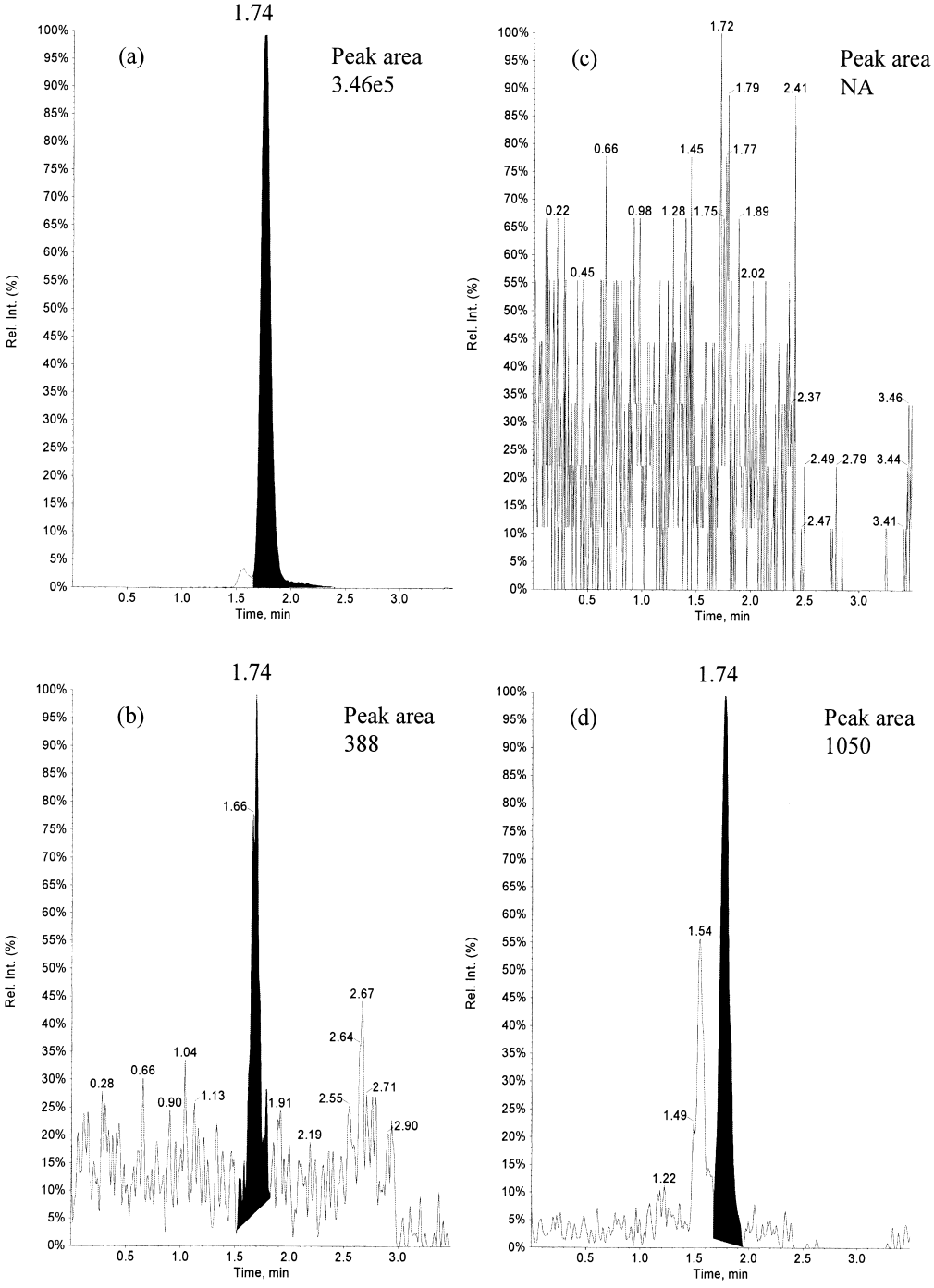


Fig. 3. Comparison of the carryover with and without an extra flushing step. For this test, the injection volume was 2 μl. (a) Mass chromatogram of an ULOQ (10 μg/ml) sample; (b) Mass chromatogram of a reconstitution solvent injected right after an ULOQ sample without an extra flushing; (c) Mass chromatogram of a reconstitution solvent injected right after an ULOQ sample but with an extra 1-min flushing by 100% of mobile phase B at 0.6 ml/min; (d) mass chromatogram of an LLOQ (0.03 μg/ml) sample.

Table 2
Measured COL-3 concentrations in spiked individual lot plasma

Lot no.	Measured plasma concentration (μg/ml)	
	Spiked 0.09 μg/ml	Spiked 7.2 μg/ml
1	0.0894	7.03
2	0.0879	6.64
3	0.0909	7.46
4	0.0863	6.77
5	0.0881	6.91
6	0.0931	7.43
Mean (μg/ml)	0.0893	7.04
RSD (%)	2.7	4.8
RE (%)	−0.8	−2.2

Table 3
Recovery data

Compound	Recovery (%) (mean±SD)
COL-3 ($n=6$)	
0.09 μg/ml	99.8±3.3
3.6 μg/ml	100.3±4.1
7.2 μg/ml	96.7±0.9
Chrysin ($n=18$)	
0.25 μg/ml	92.6±2.7

6), and 2.3% RSD and 3.4% RE for inter-batch assays ($n=18$), respectively. For the medium-concentration level QCs (3.6 μg/ml), the precision and accuracy were 0.8% RSD and 2.5% RE for intra-batch assays ($n=6$), and 2.0% RSD and 0.6% RE for inter-batch assays ($n=18$), respectively. For the high-concentration level QCs i.e 7.2 μg/ml, the precision and accuracy were 1.2% RSD and 0.0% RE for intra-batch assays ($n=6$), and 2.1% RSD and −1.1% RE for inter-batch assays ($n=18$), respectively. The intra-batch precision and accuracy were 2.9% RSD and −1.7% RE for of LLOQ QCs. These results demonstrated excellent precision and accuracy even at the lower end of the quantitation dynamic range.

3.6. Dilution integrity, stability and robustness

A five-fold dilution for over-the-curve QC sample and high concentration QC sample by matrix blank prior to extraction was used to evaluate sample dilution integrity. Six replicates of partial volume of over-the-curve QC and high QC samples were processed in one of the validation batches. These data are also included in Table 5. The results indicated that taking partial volume and diluting with matrix blank did not produce significant deviation from their nominal values.

The stability experiments were designed for testing all conditions that the plasma samples might experience during sample shipping, pre- and post-processing such as a few freeze–thaw cycles and a short storage at room temperature (RT) and during analysis such as extracted samples sitting in sample tray or refrigerator. These were performed as described in the Section 2.5. All stability results are summarized in Table 6. The QC samples undergoing three freeze–thaw cycles gave ≤2.9% RSD and the accuracy of 99.2–102.3%. The samples stored at RT for 24 h gave ≤1.8% RSD and the accuracy of 98.7–102.1%. Keeping the extracts at RT for approximately 24 h prior to injection had ≤3.2% RSD and the accuracy of 97.1–103.9%.

The 6- and 12-month storage stabilities of the QC

Table 4
Calibration curve data for COL-3 in plasma ($n=3$)

Theoretical (μg/ml)	COL-3 in plasma (μg/ml)								r^2
	0.03	0.06	0.1	0.4	1	4	8	10	
Batch 1	0.0299	0.0601	0.101	0.397	1.01	3.96	8.13	9.88	0.9999
Batch 2	0.0287	0.0617	0.110	0.400	1.01	3.89	7.90	9.49	0.9985
Batch 3	0.0298	0.0604	0.100	0.407	1.01	4.07	7.95	9.56	0.9997
Mean	0.0295	0.0607	0.104	0.400	1.01	0.397	7.99	9.64	–
RSD (%)	2.3	1.4	5.3	1.9	0.0	2.3	1.5	2.2	–
RE (%)	−1.7	1.2	4.0	0.0	1.0	−0.8	−0.1	−3.6	–

Table 5
Intra- and inter-batch precision and accuracy of QC samples

Compound	COL-3 in plasma (μg/ml)					
	LLOQ	Low	Medium	High	High*	Over-the-curve*
QC sample	0.03	0.09	3.6	7.2	7.2	25
Intra-batch ($n=6$)						
Mean (μg/ml)	0.0295	0.0946	3.69	7.20	7.26	25.0
RSD (%)	2.9	2.8	0.8	1.2	2.3	2.9
RE (%)	−1.7	5.1	2.5	0.0	0.8	0.0
Inter-batch ($n=18$)						
Mean (μg/ml)	–	0.0931	3.62	7.12	–	–
RSD (%)	–	2.3	2.0	2.1	–	–
RE (%)	–	3.4	0.6	−1.1	–	–

*, Five-fold dilution of QC samples.

samples stored at −70 °C were also tested and the data are included in Table 6. These data illustrate that the COL-3 plasma samples were stable for at least 12 months if stored frozen at approximately −70 °C. This was different from the conclusion drawn by Rudek et al. [5].

Table 6
Short-term and long-term stability data

Compound	COL-3 in plasma		
Nominal concentration (μg/ml)	0.09	3.6	7.2
Three freeze–thaw cycles ($n=3$)			
Mean (μg/ml)	0.0921	3.65	7.14
RSD (%)	2.9	2.2	2.4
Accuracy (%)	102.3	101.4	99.2
Bench-top ambient ~24 h ($n=3$)			
Mean (μg/ml)	0.0919	3.60	7.11
RSD (%)	0.6	1.8	1.6
Accuracy (%)	102.1	100.0	98.7
Extracts stored ambient ~24 h ($n=6$)			
Mean (μg/ml)	0.0935	3.62	6.99
RSD (%)	3.2	0.3	0.7
Accuracy (%)	103.9	100.7	97.1
6-month storage at −70 °C ($n=3$)			
Mean (μg/ml)	0.0873	3.15	7.63
RSD (%)	5.3	4.5	1.4
Accuracy (%)	93.0	87.5	106.0
12-month storage at −70 °C ($n=5$)			
Mean (μg/ml)	0.0855	3.56	7.05
RSD (%)	11	13.7	4.1
Accuracy (%)	95.0	98.9	97.9

4. Conclusion

A simple, rapid and robust LC–MS–MS method for the determination of COL-3 in human plasma has been developed and validated. This method used a simple LLE and a YMCbasic column coupled with MS–MS for separation and detection. The results showed excellent precision and accuracy and the frozen plasma stable for at least 12 months. The validated assay used a 0.25-ml plasma sample and the standard curve range was 0.03–10 μg/ml in human plasma, which covered the concentration range in real clinical samples. Due to the high sensitivity of MS–MS detection, this method can be modified to be suitable for microsample analysis or lower concentration levels. The method has already been used to support the PK studies.

Acknowledgements

The authors would like to thank Sandra Thompson for searching the literature.

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