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A sensitive method for determination of COL-3, a chemically modified tetracycline, in human plasma using high-performance liquid chromatography and ultraviolet detection

Michelle A. Rudek[a,*], Carol Hartke[b], Yelena Zabelina[b], Ming Zhao[b], Pamela New[c,1], Sharyn D. Baker[b]

[a] *Division of Medical Oncology, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Bunting-Blaustein Cancer Research Building, 1650 Orleans Street, Room 1M90, Baltimore, MD 21231, USA*
[b] *Division of Experimental Therapeutics, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD 21231, USA*
[c] *Department of Medicine/Neurology, University of Texas Health Center, San Antonio, TX, USA*

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Abstract

COL-3, 6-deoxy-6-desmethyl-4-desdimethylamino-tetracycline, is a matrix metalloproteinase inhibitor currently in clinical development. A HPLC–UV method to quantitate COL-3 in human plasma was developed. COL-3 was extracted from plasma using solid-phase extraction cartridges. COL-3 is separated on a Waters Symmetry Shield RP8 (3.9 mm × 150 mm, 5 μm) column with EDTA (0.001 M) in sodium acetate (0.01 M, pH 3.5)–acetonitrile mobile phase using a gradient profile at a flow rate of 1 ml/min for 22 min. Carryover was eliminated by using an extended needle wash of methanol:acetonitrile:dichloromethane (1:1:1, v/v/v). Detection of COL-3 and the internal standard, chrysin, was observed at 350 nm. COL-3 and chrysin elute at 8.9 and 9.9 min, respectively. The lower limit of quantitation in human plasma of COL-3 was 75 ng/ml, linearity was observed from 75 to 10,000 ng/ml. A 30,000 ng/ml sample that was diluted 1:50 with plasma was accurately quantitated. This method is rapid, widely applicable, and suitable for quantifying COL-3 in patient samples enabling further clinical pharmacology characterization of COL-3.

Keywords: COL-3; Tetracycline; Matrix metalloproteinase inhibitor; HPLC; Pharmacokinetics

1. Introduction

COL-3, 6-deoxy-6-desmethyl-4-desdimethylaminotetracycline, is a non-antimicrobial, chemically modified tetracycline with documented anti-tumor effect [1]. COL-3 is being tested in clinical trials in patients with refractory advanced cancers, increasing the need to develop a rapid, accurate method for the detection of COL-3 [1–4].

Two published methods quantitated COL-3 in the concentration range of 30–10,000 ng/ml [5,6]. The first method utilized a HPLC/MS and two standard curves [5], while the second method utilized a HPLC/MS/MS and one standard curve [6]. The concentration range of both assays was sufficient to characterize the pharmacokinetics of COL-3 for up to 1 week and weekly pre-treatment trough levels after administration of doses of 36–98 mg/m^2 in a phase I clinical trial [7,8].

In another phase I clinical trial, COL-3 was being administered at escalating doses starting at 25 mg/m^2 on a continuous daily dosing schedule, only pre-treatment trough concentrations being obtained [9]. In this trial, several patients received a drug-metabolizing enzyme-inducing anticonvulsant

* Corresponding author. Tel.: +1 410 502 7149; fax: +1 410 614 9006.
E-mail address: mrudek2@jhmi.edu (M.A. Rudek).
[1] Present address: Department of Neurology and Oncology, Baylor College of Medicine, Houston, TX, USA.

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agent, which could result in increased COL-3 metabolism and low plasma concentrations. The challenge was to develop an assay with suitable sensitivity allowing quantitation over a broad concentration range, and a method that did not use mass spectrometric detection since many laboratories do not have that capability. As a result, we have developed a high-performance liquid chromatography method using ultraviolet detection (HPLC/UV) that quantitates COL-3 in plasma in the range of 75–10,000 ng/ml.

2. Experimental

2.1. Materials

COL-3 (97.0% pure) was a gift from CollaGenex Pharmaceuticals, Inc. (Newtown, PA, USA). Chrysin (96.0% pure) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Heparinized human plasma was purchased from Pittsburgh Blood, Inc. (Pittsburgh, PA, USA). All other reagents were of the highest grade commercially available.

2.2. Preparation of stock solutions, calibration standards, and quality controls

COL-3 at a concentration of 1 mg/ml (corrected for purity) was dissolved in Tween 80/ethanol (1:1, v/v) in Sigmacote®-treated glassware. The solution was sonicated for 20 min and then allowed to sit at room temperature for at least 1 h prior to use. COL-3 was stable for at least 1 month when stored at −80 °C and protected from light. The stock solution was serially diluted in Tween 80/ethanol (1:1, v/v) on each day of analysis to prepare working standards of 250, 100, and 25 μg/ml. Microliter amounts of COL-3 stock solution were added into pooled human plasma to prepare a calibration curve and quality controls (QC). The standards were prepared at 75, 200, 350, 600, 900, 1250, 2500, 5000 and 10,000 ng/ml, and the QCs at 150, 2000, and 8000 ng/ml. An additional dilutional QC was prepared at 30,000 ng/ml. The dilutional QC was diluted 1:50 with blank pooled human plasma prior to sample preparation to ensure patient samples could be diluted. All standards and quality controls were prepared fresh daily. For long-term and freeze-thaw stability, quality controls were stored at −80 °C.

Chrysin was dissolved at a concentration of 250 μg/ml in absolute methanol. Chrysin was stable for 2 weeks when stored at −20 °C and protected from light [5].

2.3. Assay procedure

An amount of 5 μl of chrysin (250 ng/μl) was added to 500 μl plasma sample on ice. The sample was acidified using 20 μl of 50% phosphoric acid and vortex-mixed for 30 s. An amount of 500 μl of the acidified sample was transferred onto an activated OASIS HLB 1 cc (30 mg) extraction cartridge (Waters Corporation, Milford, MA, USA) that was conditioned and equilibrated by washing with 1 ml of methanol (100, v/v) and 1 ml of Milli-Q water. The cartridge was washed twice with 1 ml of methanol/water (5/95, v/v). Analytes were eluted with 1 ml of methanol (100, v/v). After evaporation, the samples were reconstituted with glacial acetic acid (1/99, v/v) in acetonitrile/0.01 M sodium acetate, pH 3.5 (30/70, v/v) and 20 μl of the resulting solution was injected into the HPLC system.

Table 1
Gradient profile

Time (min)	% Solvent A[a]	% Solvent B[b]
0	70	30
4	70	30
9	20	80
10.5	20	80
11	30	70
16	30	70
17.5	70	30
22	70	30

[a] EDTA (0.001 M) in sodium acetate (0.01 M, pH 3.5).
[b] Acetonitrile.

2.4. Chromatographic conditions

Chromatographic analysis was performed using a Waters Model 2690 separations system (Milford, MA, USA) equipped with a Waters Model 2487 UV detector. Separation of the analyte was achieved at ambient temperature using Waters Symmetry Shield C_8 (3.9 mm × 150 mm, 5 μm particle) column protected by a Waters C_{18} guard column (Milford, MA, USA). A gradient profile, as shown in Table 1, consisted of EDTA (0.001 M) in sodium acetate (pH 3.5, 0.01 M)-acetonitrile with a flow rate of 1 ml/min and a total run time of 22 min. An extended needle wash was utilized and consisted of a mixture of methanol–acetonitrile–dichloromethane (1:1:1, v/v/v) [10]. Column eluant was monitored at 350 nm. The instrument was controlled by ChromPerfect Spirit software version 4.4.23 (Justice Laboratory Software, Denville, NJ, USA).

2.5. Validation procedures

COL-3 working standards were added to human plasma to prepare calibration samples over the range of 75–10,000 ng/ml. Calibration curves were computed using the ratio of the peak area of COL-3 and internal standard by using a weighted (1/[nominal COL-3 concentration]) linear regression analysis. The assay lower limit of quantitation (LLOQ) was determined to be 75 ng/ml for COL-3. The LLOQ was determined by meeting the following two criteria: a signal to noise ratio of the peak areas larger than 10 and the values for precision and accuracy less than 20%.

Method validation runs were performed on 9 days. Each analytical run consisted of a calibration curve using single samples with duplicate samples at the lower limit of quantiation (LOQ) and upper limit of quantiation (ULQ) and QC

samples in triplicate. QC samples were prepared independently in blank plasma at COL-3 concentrations of 150, 2000, 8000, and 30,000 ng/ml. The 30,000 ng/ml dilutional QC was assayed once it was diluted 1:50 (v/v) with blank plasma prior to sample preparation. The dilutional QC was used to determine the accuracy and precision of diluted patient samples and was performed on 3 days. The accuracy and precision of the assay was assessed by the mean relative percentage deviation (DEV) from the nominal concentrations and the within-run and between-run precision, respectively. The accuracy for each tested concentration was calculated as:

$$\text{DEV(COL-3)} = 100 \times \frac{\text{[COL-3]mean} - \text{[COL-3]nominal}}{\text{[COL-3]nominal}}$$

Estimates of the between-run precision were obtained by one-way analysis of variance (ANOVA) using the run day as the classification variable. The between-groups mean square (MSbet), the within-groups mean square (MSwit), and the grand mean (GM) of the observed concentrations across runs were calculated using the JMP™ statistical discovery software version 4 (SAS Institute, Cary, NC, USA). The between-run precision (BRP), expressed as a percentage relative standard deviation, was defined as:

$$\text{BRP} = 100 \times \frac{\sqrt{(\text{MSbet} - \text{MSwit})/n}}{\text{GM}}$$

where n represents the number of replicate observations within each run. For each concentration, the estimate of the within-run precision (WRP) was calculated as:

$$\text{WRP} = 100 \times \frac{\sqrt{\text{MSwit}}}{\text{GM}}$$

The mean and standard deviation were calculated on the slope and intercept of the linear regression line. The correlation coefficients were reported. The specificity of the method was tested by visual inspection of chromatograms of extracted human plasma samples from six different donors for the presence of endogenous or exogenous interfering peaks. The extraction efficiency of the assay was measured by comparison of extracted plasma samples and aqueous samples of COL-3 in quadruplicate at concentrations of 150, 2000, and 8000 ng/ml. The mean values of the triplicate samples were compared.

2.6. Patient samples

A phase I study of oral COL-3 was conducted with dose escalation commencing at 25 mg/m^2 daily [9]. The protocol was conducted by the NABTT and was approved at each site by the Institutional Review Board. All patients were provided written informed consent.

Blood samples were collected in heparin-containing tubes before drug administration at several time points. Samples were processed immediately by centrifugation at 1000 g for 10 min at 4 °C, and the plasma supernatant was collected and initially frozen at −20 °C or below until analysis. Runs were considered acceptable if at least 75% of the standards were within 20% of the nominal concentration with at least one of the lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) replicates passing, and if greater than 66% of the quality control samples were within 20% of the expected value. Samples that were above the ULOQ were re-assayed and diluted 1:50 with control blank plasma.

3. Results and discussion

3.1. Analytical conditions

A HPLC–UV method to determine COL-3 concentrations in human plasma was developed and an in-study validation was performed. The chromatographic conditions described yielded retention times of 8.9 (k' = 11.5) and 9.9 min (k' = 13.0) for COL-3 and chrysin, respectively. Utilizing the Waters Alliance HPLC, it was necessary to incorporate an extended needle wash and a gradient run (22 min) was necessary to eliminate carryover. The cycle time is 1.25 times shorter than the initial HPLC/MS method but 4 times longer than the LC/MS/MS method [5,6]. Chromatograms representing blank plasma (Fig. 1A), plasma spiked with COL-3 2000 ng/ml and chrysin 1.25 μg/sample (Fig. 1B), and a sample from a patient receiving 50 mg/m^2 after 1 month on treatment (Fig. 1C). No interfering peaks were found in blank plasma samples from 12 separate plasma lots. The calculated signal to noise ratio of the COL-3 peak is approximately 12:1 at 75 ng/ml, the limit of quantitation. The sensitivity of this method is 2.5 times less than both the HPLC/MS and HPLC/MS/MS methods but utilizes the more readily available HPLC/UV technique [5,6]. The signal to noise ratio for chrysin is approximately 180:1.

Calibration curves for COL-3 standards were constructed from the peak area ratio of COL-3 to chrysin. The calibration curve was fitted using a weighted (1/[nominal COL-3 concentration]) linear regression analysis. The slope ($6.00 \times 10^{-4} \pm 0.15 \times 10^{-4}$, mean ± S.D., $n = 3$), and y-intercept ($-1.55 \times 10^{-2} \pm 1.87 \times 10^{-2}$, mean ± S.D., $n = 3$) were calculated. The correlation coefficients for the calibration curves of ≥0.99 were observed. The within- and between-day precisions were less than 17.0 and 2.0%, respectively, and the accuracy was within 13.9% (Table 2).

Table 2
Validation characteristics of COL-3 in human plasma

Nominal concentration (ng/ml)	150	2000	8000	30000
Accuracy (%)	109.5	113.9	110.8	103.7
Precision (%)				
Within-day	16.9	10.4	12.2	5.5
Between-day	*	*	*	1.3
Extraction recovery (%)	61.2	65.3	63.8	ND
Autosampler stability (36 h)	ND	ND	−6.4%	ND

(*) No significant additional variation was observed as a result of performing the assay in different runs. ND: not done.

3.2. Recovery in plasma

Sample clean-up steps were sufficient to remove interfering peaks from the samples while utilizing UV detection. The absolute recovery of COL-3 ranged from 61.2–65.3% (Table 2), while 1.25 μg/sample of chrysin was recovered at 86.0%. A 20% increase in recovery was achieved when compared to the HPLC/MS method which used 0.25 ml plasma with acetonitrile precipitation [5]. However, the HPLC/MS/MS method, which used 0.25 ml plasma with acidification and liquid–liquid extraction, had a 35–40% greater recovery when compared with this HPLC/UV method [6].

3.3. Storage and stability

Contrary to the previously published methods, COL-3 with a purity equal to or less than 97% was not soluble at

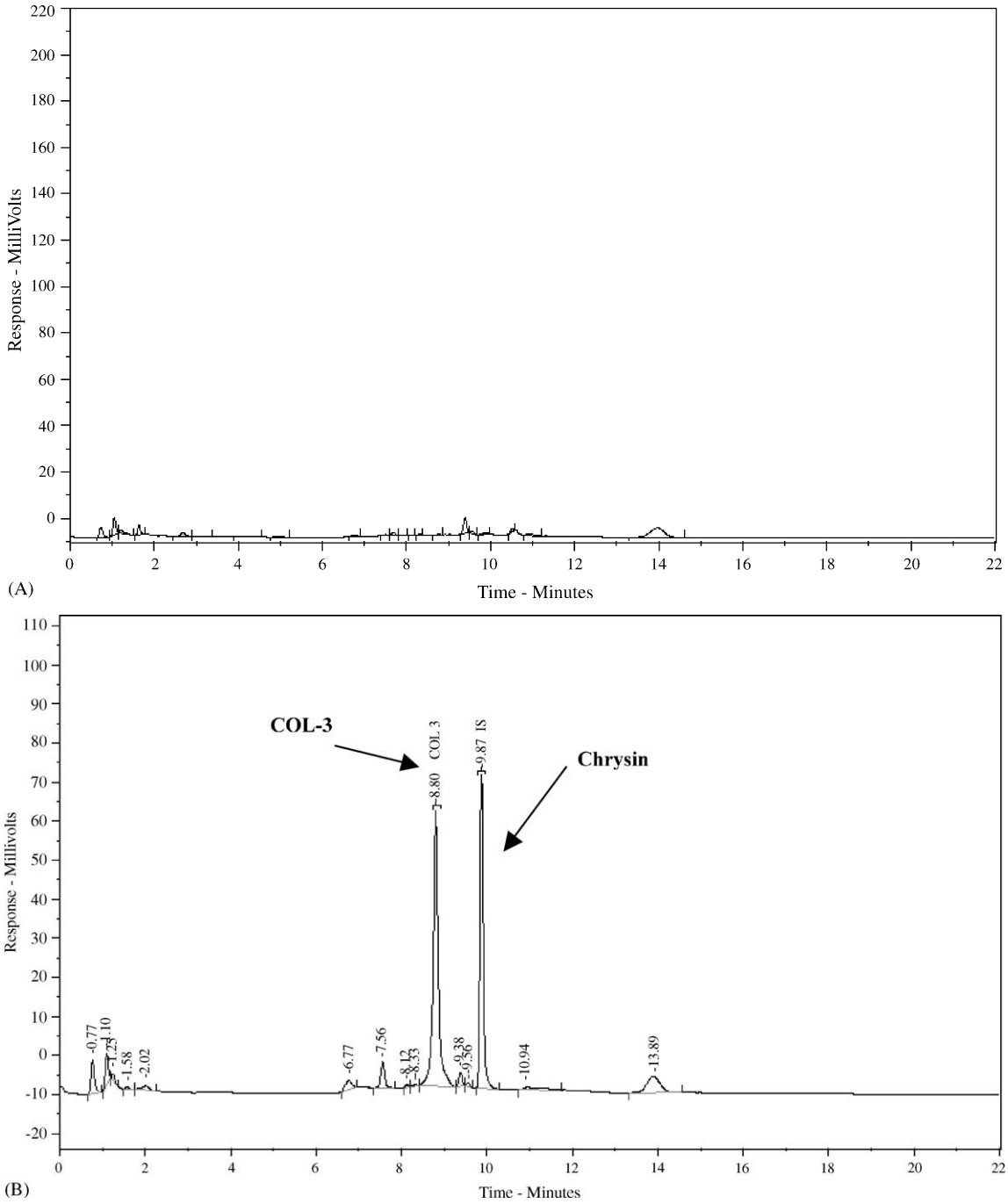

Fig. 1. Chromatogram of (A) blank plasma, (B) plasma spiked with COL-32,000 ng/ml and chrysin 1.25 μg/sample, and (C) a sample from a patient receiving 50 mg/m^2 after 1 month on treatment. Absorbance wavelength is 350 nm.

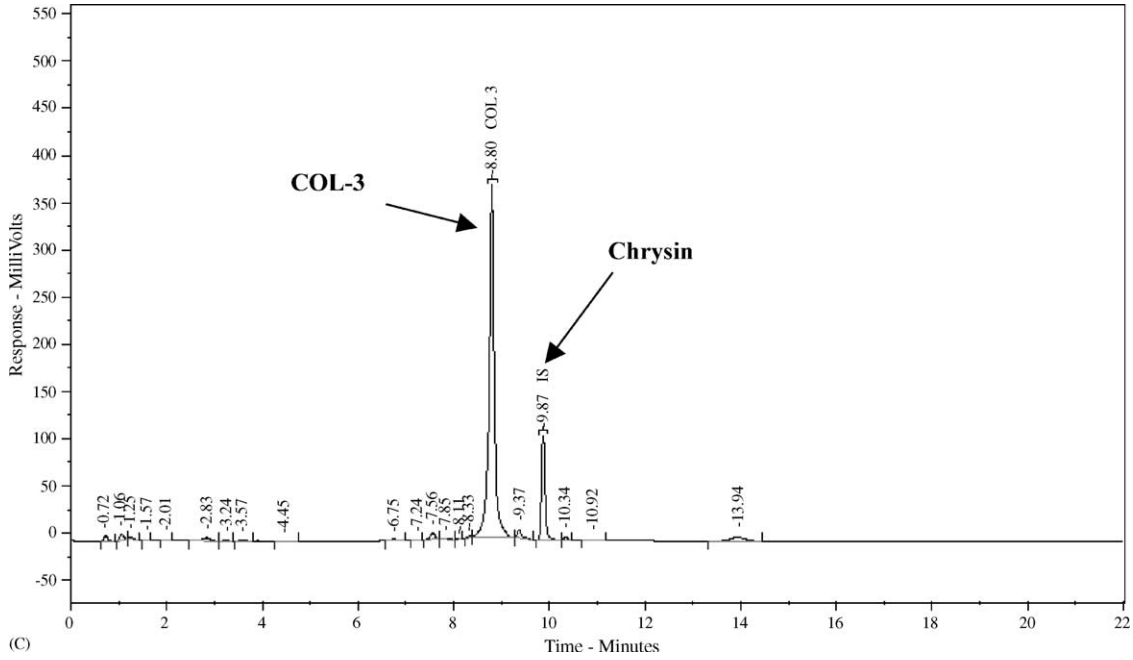

Fig. 1. (*Continued*).

1 mg/ml in methanol [5,6]. Experimenting with various solvents, we determined a combination of Sigmacote®-treated type A glassware and Tween 80/ethanol (1/1, v/v) was necessary to dissolve COL-3 and resulted in less than 5% variance. COL-3 was stable for at least 1 month when stored at −80 °C and protected from light.

Consistent with a previous assay, the addition of 1% glacial acetic acid to the reconstitution solution and the use of a refrigerated autosampler were necessary to stabilize COL-3 prior to injection onto the HPLC/UV [5]. The extended autosampler stability of 36 h allowed for up to 94 samples to be analyzed in one run (Table 2).

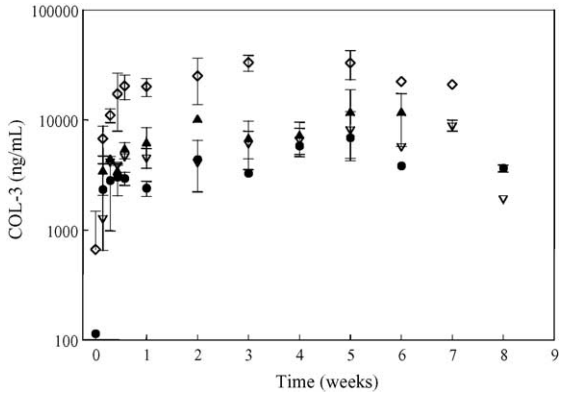

Fig. 2. Mean ± S.D. plasma concentration–time profile following administration of COL-3 25 mg/m^2 (●), 50 mg/m^2 (▽), 75 mg/m^2 (▲), and 100 mg/m^2 (◊) plotted on a log–linear scale. Each sample utilized in this figure is a documented plasma trough concentration.

3.4. Plasma concentration–time profile

The suitability of the developed method for clinical use was demonstrated by the determination of COL-3 in patient's plasma samples. A total of 15 patients received COL-3 at doses of 25 ($n = 3$), 50 ($n = 3$), 75 ($n = 3$), and 100 mg/m^2/day ($n = 6$). The pre-treatment trough plasma concentrations measured over an 8-week period ranged from approximately 100 ng/ml after a single dose at the 25 mg/m^2 dose to 40,000 ng/ml after multiple dose administration at the 100 mg/m^2 dose level (Fig. 2). No patient samples were less than 75 ng/ml, the limit of quantitaiton. The data obtained on these patients is consistent with previously published data from other COL-3 trials [7,8,11].

4. Conclusion

In conclusion, we have developed and validated a novel assay for the quantitation of COL-3 in human plasma. The described method is sufficiently sensitive to allow the measurement of COL-3 in pre-treatment trough samples obtained from patients receiving COL-3 25 to 100 mg/m^2 on a daily continuous schedule. This method is as dynamic and comparable in sensitivity when compared with the previously published HPLC/MS and HPLC/MS/MS methods [5,6].

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