—Regular Articles—

Determination of Colchicine in Mouse Plasma by High Performance Liquid-chromatographic Method with UV Detection and Its Application to Pharmacokinetic Studies

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A simple and sensitive high performance liquid chromatography method with UV detection was described for the determination of colchicine (COL) in mouse plasma. After single-step deproteinization by acetonitrile using berberine hydrochloride as an internal standard (I.S.), solutes were separated on a Diamonsil C_{18} column (250 mm×4.6 mm I.D., 5 μm particle size) (Dikma), using acetonitrile–0.15% phosphoric acid solution (27 : 73, v/v) as mobile phase (flow-rate 1.0 ml/min); wavelength of the UV detector was set at 350 nm. No interference from any endogenous substances was observed during the elution of COL and internal standard (I.S., berberine hydrochloride). The retention times for COL and I.S. were 11.23 min and 8.82 min, respectively. The limit of quantification was evaluated to be 1.5 ng/ml and the limit of detection was 0.5 ng/ml. The method was used in the study of pharmacokinetics of COL after intravenous injection (*i.v.*) and intraperitoneal injection (*i.p.*). The result indicated that COL disappears from the plasma according to a three compartment open model.

Key words——Colchicine; high performance liquid chromatography; pharmacokinetics; berberine hydrochloride; plasma

INTRODUCTION

COL is the main alkaloid obtained from the bulb and seeds of colchicum. It is used chiefly in the treatment of gout but is also valuable for other diseases such as familial Mediterranean fever (FMF), primary biliary cirrhosis and breast cancer.[1,2] In clinical use, channels to ingest COL are mainly oral administration and *i.v.* In addition, COL was used intraperitoneally against the animal liver toxicity of recombinant human tumor necrosis factor-α(TNF-α) and renal interstitial fibrosis.[3,4] The dosage for a human being is 0.5–1 mg/1–2 h and the most common side effect is gastrointestinal disturbance when oral administered. In *i.v.* administration, COL often produces toxic effects affecting multiple organs, so it is necessary to develop new preparations for topical use in treating gout and other diseases. To evaluate a new preparation, a simple and sensitive analytical method is required to assay the COL concentration in the biological specimens. The molecular structure of COL is illustrated in Fig. 1.

There are many methods developed to assay COL in plasma, including liquid chromatographic tandem mass spectrometry (LC－MS／MS),[5] DAD (HPLC–DAD),[6] high-performance liquid chromatography coupled to ion spray mass spectrometry (HPLC–ISP–MS),[7] or variable wavelength UV detector (HPLC–UV).[8,9] Liquid chromatography-mass spectrometric (LC–MS) and gas chromatography-mass spectrometric (GC–MS) methods has been used for the determination of COL in human plasma.[10] MS detection not only is expensive, but also requires highly trained persons, so the device may not be available in many pharmaceutical laboratories. Several HPLC methods using UV detection have been reported for analysis of COL, however, there are noticeable shortcomings of the methods described in the literature, especially the selection of the I.S. For example, the analysis time is too long and

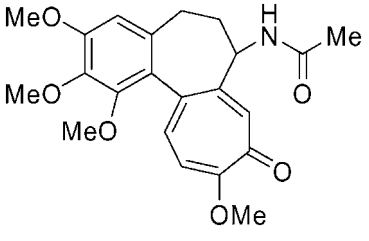

Fig. 1. Chemical structure of COL

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the resolution between COL and endogenous substances was less than 1.5. In new preparation studies, the proposed method should be simple, accurate and reproducible for the quantification of the tested drug in biological fluids.

In this paper, a new HPLC–UVmethod is established to assay COL in mouse plasma with berberine hydrochloride as I.S., and *in vivo* pharmacokinetics of COL in mouse is studied using this method. In literature research, COL for the first time has been assayed with berberine hydrochloride as internal standard and has been determined using a UV detector. This method was fully validated for its specificity, accuracy, precision and sensitivity. The LOQ and LOD of the assay is in the low ng/ml range. Good separation, high sensitivity and simple approach, which does not compromise the specificity, are the main advantages of such a technique. At the same time, the method was efficient in analyzing large quantities of plasma obtained for pharmacokinetic study after 10 mg/kg doses of COL to mice.

MATERIALS AND METHODS

Materials and Reagents Colchicine (MW＝399.44) was a gift from Yunnan Baiyao Group Co., Ltd. The purity of Col was calculated as 99.2%, using the area normalization method. I.S., berberine hydrochloride (MW＝407.85) was purchased from the National Institution for the control of pharmaceutical and biological products of China. Methanols (Hanbon, Jiangsu, China), and acetonitrile (Fisher) were HPLC grade. Orthophosphoric acid (H_3PO_4) was analytical grade and purchased from Chengdu Fangzhou Sci. and Tech. Co., Ltd. All chemical and solvents were analytical or HPLC grade. All standard solutions and mobile phases were prepared using doubly distilled water.

Preparation of Calibration Standard and Quality Controls (QC) Samples Stock standard solutions of COL and berberine hydrochloride were prepared by dissolving accurately weighed amounts of both in methanol to yield final concentrations of 11.94 μg/ml and 2.06 μg/ml. The stock solutions were stored at −20℃ in the dark and brought to room temperature before use. Due to the light-sensitivity of COL, working solutions were prepared before use by appropriate methanolic dilutions ranging from 0.04 to 11.94 μg/ml. Calibration curves were prepared within the COL concentration range of 2–900 ng/ml. In disposable glass tubes, 70 μl of each working standard COL solution was mixed with 200 μl mouse blank plasma for 5 min on a vortex mixer (Jiangxi Medical Instrument Group, China). Quality control (QC) samples were prepared at low (3 ng/ml), medium (120 ng/ml) and high (900 ng/ml) concentrations in the same way as the plasma samples for calibration. The samples were extracted and then divided into 100-μl aliquots in tightly closed microtubes and kept frozen at −20℃ in the dark.

Sample Preparation To disposable plastic tubes each containing a 200 μl plasma sample, 20 μl of the I.S. (berberine hydrochloride, 1.031 μg/ml) was added except for a blank plasma sample. Then 600 μl of acetonitrile was added to each sample. The obtained solution was vortexed for 5 min on a vortex mixer at room temperature. The mixture was centrifuged at 12000×g for 20 min to remove any protein and the supernatant was removed to 2 ml glass tubes where evaporation was completed to dryness under nitrogen at 30℃. After adding 100 μl of the mobile phase, vortexing (2 min) and centrifuging (12000 g for 20 min), a volume of 20 μl of the supernatant was injected into the HPLC for analysis.

High-performance Liquid Chromatographic Apparatus and Chromatographic Conditions The HPLC system consisted of an Agilent chemistation Rev. A. 08.03 system (HP1100, Agilent, America) equipped with a G1311A quaternary pump, a fixed injection-loop of 50 μl, and a G1314A variable wavelength detector operated at a wavelength of 350 nm. The analytical column employed was a Diamonsil C_{18} column (250 mm×4.6 mm I.D., 5 μm particle size) (Dikma) and protected by a ODS guard column (10 mm×4.0 mm I.D., 5 μm particle size). The mobile phase comprised of acetonitrile–0.15% phosphoric acid solution (27 : 73, v/v), was filtered through a 0.45 μm cellulose membrane filter (Auto Science, Tianjin, China), then pumped through the system at a rate of 1.0 ml/min. Twenty microliters of sample solution was then injected onto the column. The chromatograph run required 15 min for completion. Separation was achieved at 30℃. All chromatograms obtained were evaluated by the peak area ratio of COL to the I.S. The calibration curve was plotted with the peak area ratio of COL to the I.S. against the plasma concentration of COL. The data demonstrated a linear function for COL according to the equation:

$$Y = a + b\,X,$$

where Y is the peak area ratio of COL to the I.S., X is plasma concentration of COL (ng/ml), a is the intercept and b is the slope.

Validation of the Method For method validation and linearity studies, blank plasma samples obtained from Kunming male mouse (19–22 g) were used. The specificity of the assay was evaluated by comparing the mouse blank plasma sample and mouse plasma sample spiked with COL and the I.S. The efficiency of the extraction procedure was observed at low (3 ng/ml), medium (120 ng/ml) and high (900 ng/ml) concentrations. Recovery was calculated by comparing the respective peak areas of the chromatograms of the extracted samples relative to the untreated standards containing an equivalent amount of the compounds in methanol. Calibration curves were constructed by linear least-squares regression analysis plotting of peak-area ratio (COL/I.S.) versus the drug concentrations. The lower limit of quantification (LOQ) was defined as the lowest concentration with a coefficient of variation (CV) of less than 20% and accuracy of 80–120%. The limit of detection value (LOD) in mouse plasma was calculated as the amount of the injected sample, which resulted in a signal-to-noise ratio of 3. The accuracy and precision of the method were evaluated with QC samples at concentrations of 3, 120, 900 ng/ml on three consecutive days, accompanied by a standard calibration curve on each analytical run.

The quality control (QC) samples were assayed under five different conditions to assess the stability of COL in mouse plasma. One set of QC samples was stored at room temperature (approximately 22–25℃) for 24 h in a volumetric flask made opaque and covered with black cloth. The stability of the sample at room temperature was evaluated by comparing the assay results of the stored QC samples with that of the freshly thawed QC samples. Another set of QC samples was subjected to three freeze-thaw cycles and was then assayed to evaluate freeze-thaw stability of COL in mouse plasma. Long-term stability was studied by assaying samples that had been stored at −20℃ for a certain period of time (0, 5, 10 and 30 days). COL was considered stable under storage conditions if the assay percent recovery was found to be 90–110% of the nominal initial concentration.

Application of the Method Kunming male mice (19–22 g) were administered COL intravenously or by *i.p.* injection at a dose of 10 mg/kg (~0.2 ml). The mice were then sacrificed at 5, 10, 30 min, 1, 2, 4, 6, 8 and 10 h after injection. Blood samples were rapidly collected and the plasma was separated by centrifugation and stored at −20℃ until required. The COL plasma levels in the samples were determined using the above-mentioned HPLC method. The concentration data at each time point represented the mean±standard deviation obtained from five mice. Pharmacokinetic parameters were calculated using the DAS software.

RESULTS AND DISCUSSION

Specificity No endogenous interference was found at the retention times of COL or the I.S. Representative chromatograms for COL standard and I.S. standard, mouse blank plasma, mouse plasma spiked with berberine hydrochloride (I.S.) and mouse plasma spiked with COL (300 ng/ml) and the I.S. (120 ng/ml) are shown in Fig. 2 (A), (B), (C) and (D), respectively. COL and the I.S. were well resolved with respective retention times of 8.82 min and 11.23 min. Figures 2 (E) and 2 (F) represent the chromatograms of plasma sample obtained at 0.5 h and 8 h from Kunming mice after the *i.v.* treatment with 10 mg/kg of COL.

Linearity, Limit of Detection, Precision and Accuracy The standard calibration curves were linear over the concentration ranges of 2–900 ng/ml for COL with a correlation coefficient of 0.993. The linear regression equation for COL mouse plasma concentration was: $Y = (0.0094)\ X - (0.0011)$ ($r^2 = 0.993$), where Y was the peak area ratio of COL to the I.S. and X, the plasma concentration of COL (Fig. 3). The limit of quantification (LOQ) in mouse plasma for COL was 1.5 ng/ml. The limit of detection (LOD) in mouse plasma for COL was 0.5 ng/ml, determined as the concentration with signal-to-noise ratio of 3.

Intra- and inter-day precision (as relative standard deviation (RSD)) and accuracy (as relative error) were based on assay of the QC samples at concentrations of 3, 120, 900 ng/ml. The result of intra-day and inter-day precision and accuracy of the QC samples for COL in mouse plasma are presented in Table 1.

Recovery To determine the recovery of COL in mouse plasma, an aliquot of blank mouse plasma was spiked with COL to achieve a final concentration of

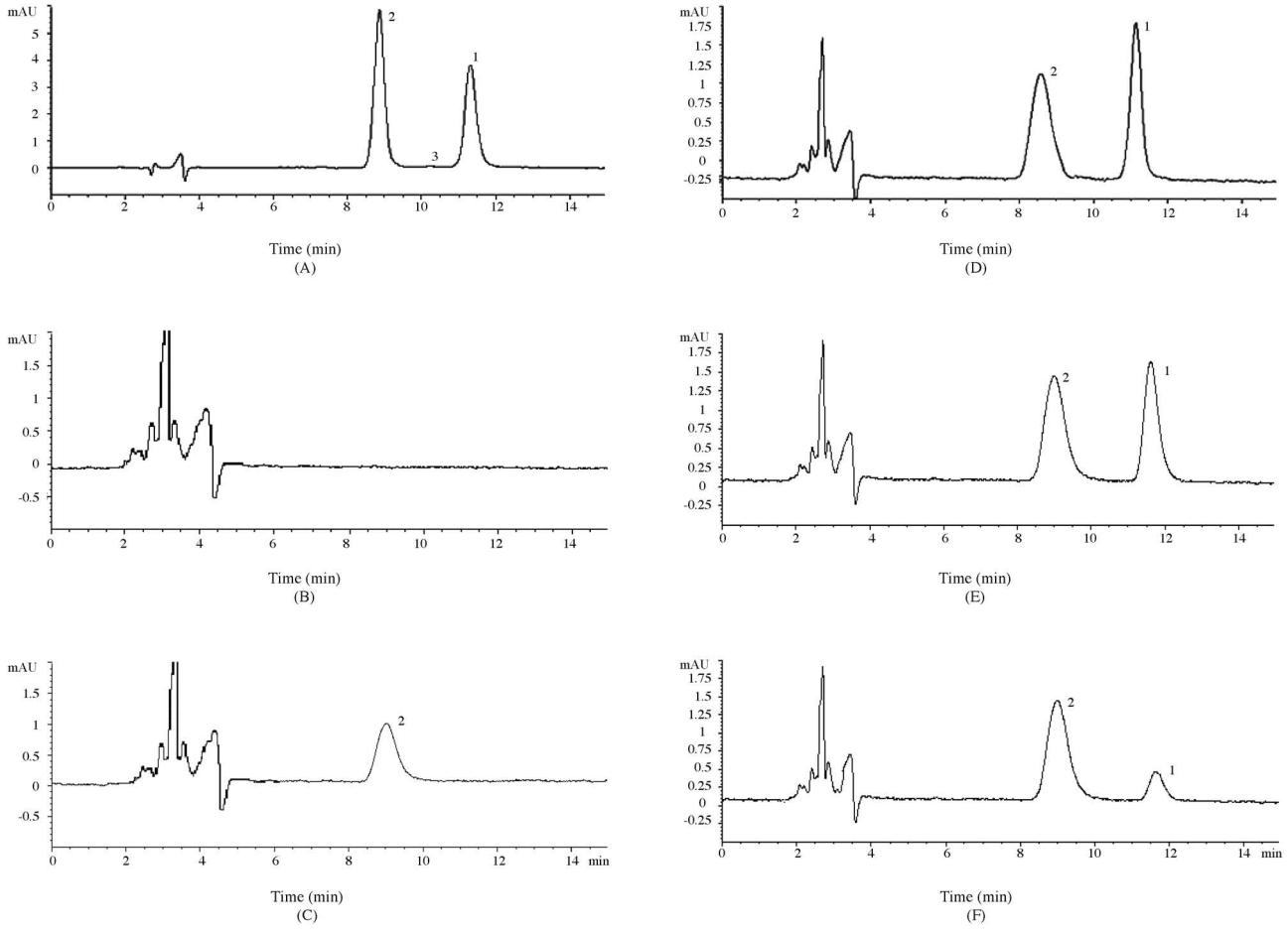

Fig. 2. Representative Chromatograms of COL Standard and I.S. Standard in Methanol (A), Blank Pllasma (B), Plasma Spiked Berberine Hydrochloride (I.S.) (C), Plasma Spiked with COL (300 ng/ml) and I.S. (Berberine Hydrochloride, 120 ng/ml) (D), Plasma Samples Obtained at 0.5 h (E) and 8 h (F) after a Single *i.v.* Treatment with 10 mg/kg of COL from Mice
Peak 1: COL, Peak 2: berberine hydrochloride and Peak 3: impurity in COL, Wavelength=350 nm.

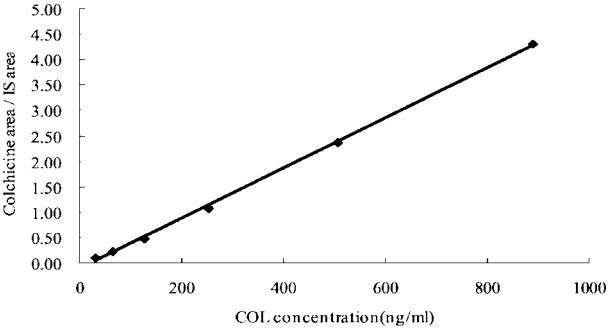

Fig. 3. Calibration Curve for COL Quantification Using Berberine Hydrochloride as Internal Standard
$y=0.0049x-0.0011$, $r^2=0.993$.

Table 1. Assay Precision and Accuracy of COL in Mouse Plasma

COL nominal concentration (ng/ml)	COL measured concentration (ng/ml)	R.S.D. (%) *a)*	Accuracy (%)	*n*
Inter-day assay				
3	2.785±0.116	4.16	92.83	6
120	115.91±4.52	3.90	96.59	6
900	867.52±21.69	2.50	96.39	6
Intra-day assay				
3	2.765±0.108	3.90	92.17	6
120	113.56±4.77	4.20	94.63	6
900	880.64±20.25	2.29	97.85	6

a) R.S.D=relative standard deviation.

3120 and 900 ng/ml. The plasma samples were then extracted with acetonitrile. Nine samples were analyzed for each concentration. The absolute recoveries of COL were determined by comparing peak areas of extracted QC samples with those of corresponding concentration standard solutions. The analysis was performed on 3 consecutive days, each day the analysis was performed for three replicates at the concen-

tration levels mentioned above. The mean recoveries of COL from mouse plasma at concentrations of 3, 120 and 900 ng/ml were 92.8%, 94.6% and 96.0%. Using the same method, the recovery of the I.S. was obtained and was 92.1%.

Sample Stability Stock solution of COL in methanol was found to be stable for at least one month when stored at 4℃. COL in QC samples had a long-term stability, and COL was also stable in a plasma sample for at least 30 days when stored frozen at −20℃ in the dark. (R.E.<−3.89%). Extracted plasma samples were found to be stable for at least 10 h when the samples were kept at room temperature (22–25℃) in the dark and the final concentrations were 93.4–98.1% of the initial values. COL was also stable following three freeze-thaw cycles in the dark. Extracted from mouse plasma samples in the mobile phase, it was stable (with a relative errors less than −5.2%) for at least 10 h at room temperature in the dark.

Application of the Method The pharmacokinetic profile of COL in the plasma of Kunming mice following *i.v.* and *i.p.* administration is shown in Fig. 4. After *i.v.* administration, the drug disappears from the plasma according to a three compartment open model with $t_{1/2(\alpha)}$, $t_{1/2(\beta)}$ and $t_{1/2(\gamma)}$ being 8 min, 0.62 h and 3.90 h. After *i.p.* administration, the elimination of COL is also according to a three compartment open model and $t_{1/2(\alpha)}$, $t_{1/2(\beta)}$ and $t_{1/2(\gamma)}$ are 0.44 h, 2.00 h and 7.93 h. The $t_{1/2(\alpha)}$ and $t_{1/2(\beta)}$ values were similar to those reported in the literature, but the $t_{1/2(\gamma)}$ is shorter.[5] COL concentration in plasma exhibited a secondary peak within 6 h of *i.p.* administration, possibly in relation to a second absorption site or enterohepatic recirculation. This second absorption process was significantly longer than the first one; this was similar to the result reported elsewhere.[11]

Robustness of Method The result regarding the robustness of this method showed that the chromatographic patterns were not significantly changed when different solvent sources and a different HPLC system (Shimadzu, Japan) were used. Identical chromatograms were achieved when different analytical columns (Kromasil C_{18} column and Diamonsil C_{18} column) were employed. In addition, the method allowed variation in analytical parameters such as column temperature adjusted to 20–40℃.

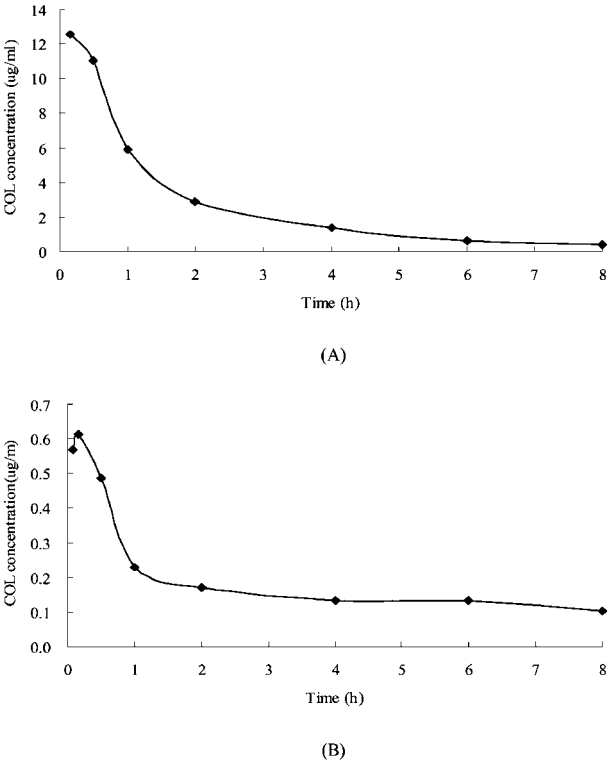

Fig. 4. COL Plasma Concentration vs. Time Profile after an *i.v.* (A) and an *i.p.* (B) Aadministration of 10 mg/kg of Drug

Each point represents the mean of the concentration obtained from five mice.

DISCUSSION

Some authors employ quinidine as an I.S., since both this drug and COL exhibit marked absorbance in the UV region from 330 to 350 nm.[8] But the resolution of COL and an endogenous substance using the mobile phase was less than 1.5. An original solution was proposed by Lhermitte et al.[6] using morpholinopropylcolchicamide, a home-made, synthetic analogue of COL, however, this latter technique is tedious and requires the operator to be experienced in organic synthesis. Marc Deveaux used prazepam as an I.S. of COL. The separation column was C8, and the mobile pH must be adjusted to 3.5; in addition, prazepam is not an alkaloid.[9] COL analogues such as colchiceine and desmethylcolchicine cannot be employed since they are naturally present as congener alkaloids in C. automnale preparations and/or appear as steps of COL metabolic pathways.[6] Finally, we chose berberine hydrochloride for the I.S. with a structure related to COL. Both berberine hydrochloride and COL are alkaloids and exhibit absorbance in the UV region 330 to 350 nm. COL presents a close

retention time, and is well co-extracted (recovery > 90% at the concentration used) and separated with good resolution towards COL. Plasma levels are relatively low after single dose administration of COL; thus, for analysis of the drug using HPLC with UV detection, high efficient extraction procedures should be developed and the drug must be separated chromatographically without any interfering or coeluting peaks. Extraction efficacy of several organic solvents including methanol, diethyl ether, acetonitrile, trifluoroacetic acid : acetonitrile (1 : 99), hexane: dichloro- methane: isopropanol (300 : 150 : 15, v/v/v), and dichloromethane was investigated for extraction of COL from plasma. More efficient extract was obtained with acetonitrile and no endogenous interference was found at the retention times of COL and the I.S. in chromatograms. The resolution between COL and endogenous substances was more than 1.5, which met the requirements for quantification analysis. COL, I.S. and an impurity in COL could be separated satisfactorily under these conditions.

CONCLUSION

In conclusion, a simple, accurate and sensitive reversed-phase HPLC method using UV detection has been described for the determination of COL in plasma with berberine hydrochloride as I.S. The method was capable of accurately determining COL down to 2 ng/ml. This report described the procedure to determine COL in mouse plasma. The method has a high degree of sensitivity, precision and accuracy and good separation. Due to the HPLC instrument, easily obtained I.S., ng/ml, LOQ and simple extraction procedure, the method is relatively practical. The method has been applied successfully for the first time in the pharmacokinetic studies of COL. Therefore, it could also be used to assay COL in clinical samples and other biological fluid samples following appropriate adjustment.

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Note: All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Sichuan University, Chengdu, China, and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals