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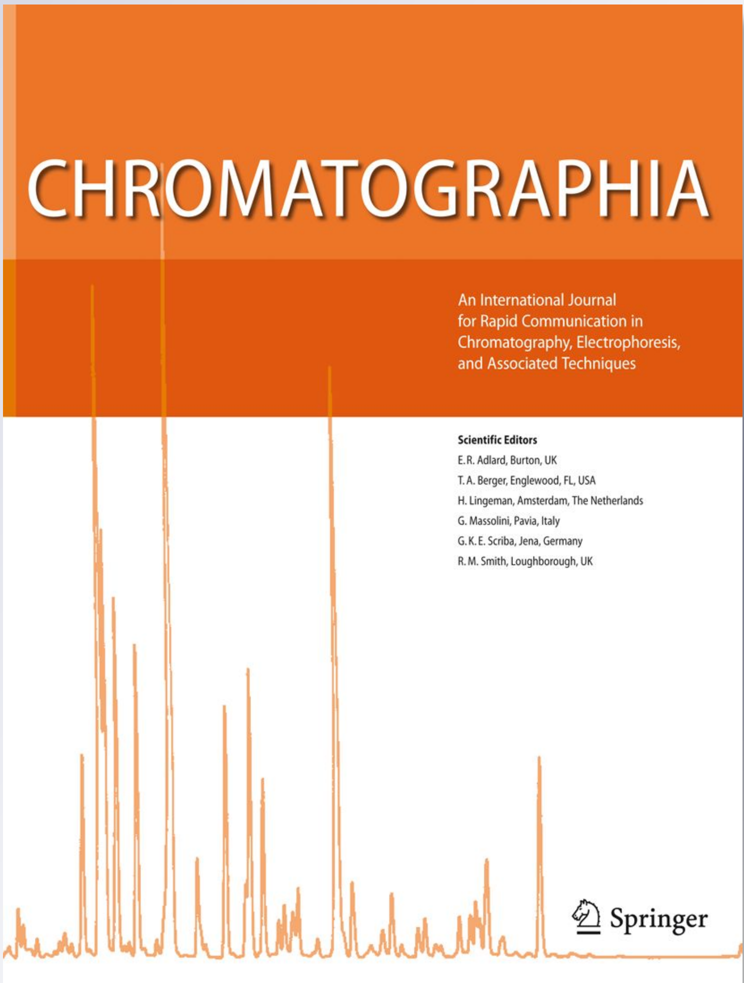

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Quantitative Analysis of Fudosteine in Human Plasma Using a Simple Precipitation Step Followed by LC–MS–MS

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Abstract Fudosteine [(-)-(*R*)-2-amino-3-(3-hydroxypropylthio) propionic acid] is a synthetic derivative of L-cysteine as a novel expectorant. To facilitate pharmacokinetics studies of fudosteine in man, a sensitive and specific LC–MS–MS method for the quantitative detection of fudosteine in human plasma was developed and validated. The method involved the addition of fosfomycin as internal standards, protein-precipitation, HPLC separation, and quantification by MS/MS system using negative electrospray ionization in the multiple reaction monitoring mode. The precursor → product ion transitions were monitored at 177.6 → 90.9 and 136.8 → 78.9 for fudosteine and the IS, respectively. The lower limit of quantitation (LLOQ) was 5 ng mL^{-1}. The calibration curves for fudosteine was linear over a concentration range of 0.005–10 μg mL^{-1}. The intra- and inter-day analyses of QC samples at 0.01, 1, 10 μg mL^{-1} indicated good precision %RSD between 92.00 and 101.02%. The fudosteine was stable in human plasma stored at room temperature for at least 12 h, 4 °C for at least 12 h, −20 °C for at least 20 days, and at least three freeze–thaw cycles procedures. This accurate and highly specific assay provides a useful method for evaluating the pharmacokinetics of fudosteine in human.

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Introduction

Fudosteine [(-)-(*R*)-2-amino-3-(3-hydroxypropylthio) propionic acid] is a synthetic derivative of L-cysteine as a novel expectorant. Fudosteine decreased the number of goblet cells increased by lipopolysaccharide or β-stimulant in airway epithelium, and increased serous secretion in broncho-alveolar lavage fluid (BALF) of rats. It also has already reported that fudosteine inhibits MUC5AC mucin hypersecretion by reducing MUC5AC gene expression and the effects of fudosteine are associated with the inhibition of extracellular signal-related kinase and p38 mitogen-activated protein kinase in vivo and extracellular signal-related kinase in vitro [1, 2].

However, the high polarity of fudosteine leads to little retention on the ODS column. Only a few methods have been reported so far for the determination of fudosteine in serum or plasma, several methods require prior derivatization of fudosteine to achieve a suitable chromatographic behavior and detection sensitivity such as high performance liquid chromatography with fluorescence detection [3, 4] and liquid chromatography-electrospray ionization mass spectrometry [5]. These methods have limitations such as low sensitivity, time-consuming derivatization steps and long chromatographic run time. Wen et al. [6] described a liquid chromatography with tandem mass spectrometry (LC–MS–MS) method which achieved better sensitivity. The assay was found to be linear in the range 0.02–10 μg mL^{-1} with a LLOQ of 0.02 μg mL^{-1}. However, it used the 96-well plate format to carry out sample preparation, which is not widely used in all laboratories. It was needed that a simple and general method to quantitation fudosteine in biofluids. The method described in this paper, using a simple precipitation step followed by LC–MS–MS with an LLOQ of 0.005 μg mL^{-1}, can fully meet the requirement to assess the pharmaceutics parameters of fudosteine in healthy subjects.

Materials and Methods

Chemicals and Materials

Fudosteine (Batch No. 0350-20001) and fosfomycin (Batch No. 100920-200801, internal standard, IS) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purities of all chemicals were above 99.9%. Methanol, gradient grade for liquid chromatography, was obtained from Merck KGaA (Darmstadt, Germany). Ammonium acetate, HPLC grade, was purchased from DIMA Technology Inc. All other chemicals and solvents used were obtained from standard vendors, and were of the highest quality available. Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA).

Drug administration and Plasma Sample Collection

In a single-dose pharmacokinetic study, six healthy volunteers each received an oral 200 mg of fudosteine. Venous blood samples (3 mL aliquots) were collected into heparinized tubes via an indwelling catheter pre-dose and at the following times: 0.17, 0.33, 0.75, 1, 1.5, 2, 3, 4, 6, 8 and 10 h post-dose.

LC–MS–MS Conditions

The LC–MS–MS system consisted of a high-throughput and an API4000 mass spectrometer (Applied Biosystems, Singapore, DIV. of MDS INC.). An Ultimate® XB-CN column (150 × 4.6 mm, 5 μm, Welch Materials, Ellicott, MD, USA) with a 4 mm × 3.0 mm i.d. Security Guard C18 guard column (Phenomenex, Torrance, CA, USA) was used as the analytical column. A mobile phase composed of methanol–10 mM ammonium acetate (20:80, v/v) at a flow rate of 0.5 mL/min was used to obtain the baseline separation of all analytes. The column was maintained at 24 °C. The chromatographic run time for one sample was 5.5 min. For the first 2.3 min the eluent was diverted to waste. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of *m/z* 177.6 → 90.9 for fudosteine, *m/z* 136.8 → 78.9 for IS, respectively. The electrospray ionization (ESI) source was set in negative ionization mode. The optimal MS parameters were as follows: the turbo-gas temperature was set at 400 °C and the ion spray needle voltage was adjusted to −4,500 V; GS1, GS2, curtain gas and collision gas, were set at 50, 60, 20 and 10 psi, respectively; interface heater (ihe) on. The mass spectrometer was operated at unit resolution for Q1 and low resolution for Q3, with a dwell time of 100 ms per MRM channel. The collision energy were set at −16.00 and −25.00, declustering potential (DP) −43.48 and −40.30, entrance potential (EP) −9.03 and −9.09, collision cell exit potential (CXP) −3.00 and −12.00, for fudosteine and IS, respectively. Data acquisition was performed with analyst software (Version1.5).

Preparation of Calibration and Quality Control (QC) Samples for LC–MS–MS Analysis

The standard stock solutions of fudosteine (10 mg mL^{-1}) was prepared in deionized water, while fosfomycin (1 mg mL^{-1}) in methanol. Appropriate serial dilutions of the stock solution were made in deionized water for spiking blank biometrics. Internal standard working solution was prepared by diluting internal standard stock solution with methanol. All solutions were stored at 4 °C.

Calibration standards ranging from 0.005–10 μg mL^{-1} at seven concentration levels were prepared by spiking fudosteine and fosfomycin standards into human plasma blanks. The standards were prepared using the sample preparation procedure given below. QC samples at concentration levels of 0.01, 1, 10 μg mL^{-1} were prepared by spiking both fudosteine and fosfomycin standards into human plasma blanks. These QC samples (80 μL) were transferred into separate vials on the day of sample preparation for later use.

Sample Preparation for LC–MS–MS Analysis

Each collected blood sample was immediately centrifuged at 4,000 rpm for 5 min and plasma was transferred into a clean Eppendorf tube. All plasma samples were stored at −20 °C until analysis. An aliquot (100 μL) of the plasma, spiked with internal standard working solution (5 μL mL^{-1}, 20 μL), was vortex-mixed for 3 min and then deproteinized with 500 μL of methanol. The precipitate was removed by centrifugation at 15,000 rpm at 4 °C for 10 min (Micromax RF, Thermo Electron Corporation, USA). The supernatant (80 μL) was pipetted to an autosampler vial, and 10 μL was injected onto column for analysis.

Method Validation

The method was validated according to FDA guidelines on specificity, sensitivity, precision, recovery and stability.

To evaluate the assay specificity, six blank plasma samples obtained from six volunteers were tested to demonstrate that there were no interfering components.

Calibration curves were prepared by assaying standard plasma samples at seven concentrations of fudosteine ranging 0.005–10 μg mL^{-1} ($n = 5$). The linearity of each calibration curve was determined by plotting the peak area ratio (y) of fudosteine to fosfomycin (IS) versus the nominal concentration (x) of fudosteine. The calibration curves were constructed by weighted ($1/x^2$) least square linear regression.

The lower limit of quantification (LLOQ) was defined as the lowest concentration of the calibration curve at which the signal-to-noise ratio (S/N) was much larger than 10.

Intra-day precision (each $n = 6$) was tested by analysis of the QC samples at three concentrations (0.01, 1, 10 μg mL^{-1}) at different times during the same day. Inter-day precision was determined by analysis of the QC samples over three consecutive days. Precision was determined by relative standard deviation (%RSD) of peak areas.

The extraction recovery of fudosteine was determined by calculating the peak areas obtained from blank plasma samples spiked with analyte before extraction with those from blank plasma samples, to which analyte was added after extraction. According to the guidance of FDA [7], recovery experiments should be performed at three concentrations (low, medium, and high). So this procedure was repeated for six replicates at three concentrations of 0.01, 1 and 10 μg mL^{-1}.

The matrix effect was estimated by spiking QC neat solutions into extracted blank plasma samples with the concentration the same as normal QC samples (0.01, 1, 10 μg mL^{-1}, $n = 6$). Results were calculated by comparing the mean peak areas of fudosteine base in these post-spiked samples (A) with those in corresponding neat solutions (B). The ratio (A/B × 100)% was used to evaluate the matrix effect. The matrix effect of internal standard was also evaluated using the same method.

Freeze-and-thaw stability was determined by freezing (−20 °C)and thawing (37 °C) the low-, middle and high-QC samples for three cycles before analysis ($n = 6$), with at least a 12 h interval between each cycle. Long and Short-term stabilities were measured by leaving QC samples kept at −20 °C or at ambient temperature (24 °C) for a certain time period (20 days or 12 h, respectively), before extraction. Post-preparation stability of fudosteine was determined for 12 h at 4 °C. To test the stock solution stability of fudosteine and the IS, five aliquots of stock standard 10 mg mL^{-1} for fudosteine) and the IS (1 mg mL^{-1} for fosfomycin) solution were left at 4 °C for 35 days. Then, the concentrations were analyzed and compared with the fresh stock solution.

Pharmacokinetic Analysis

Plasma concentrations equal to or above LLOQ were used for pharmacokinetics analysis using DAS software (Ver 2.0, Medical College of Wannan, China).

Results

An LC–MS–MS method was developed to determine the concentrations of fudosteine human plasma. In this method, fudosteine and IS were deproteined by methanol from human plasma using one-step protein-precipitation procedure. The final extract was analyzed by LC–MS–MS to quantify the levels of fudosteine.

Optimization of the Preparation Procedure and LC–MS–MS Condition

Protein precipitation is a common preparation method for substance with high polarity; furthermore, it can reduce sample processing time. The selected protein precipitant was methanol because of its satisfactory efficiency and less extent ion suppression compared to acetonitrile.

The chromatographic conditions, especially the composition of mobile phase and types of column were tested. After carefully comparison of many columns, an UltimateTM XB-CN column was adopted in the experiment.

Among the mobile phase additives investigated (formic acid, ammonium formate, acetic acid and ammonium acetate), ammonium acetate gave the best response. So the percentage of ammonium acetate (2, 5, 10 mM) was optimized to maintain symmetric peak shape while being consistent with good ionization as far as possible in mass spectrometer. Eventually, a mixture of methanol–water (20:80, v/v) containing 10 mM ammonium acetate was adopted to achieve an efficient chromatographic separation of the analyte and the endogenous plasma components for reducing the matrix effects and shorten running time.

Fudosteine gave a strong mass response in negative ESI mode, the analyte and IS formed predominately deprotonated molecular ions $[M–H]^-$ at m/z 177.6 and m/z 136.8 in full scan mass spectra, respectively. The dominant product ion is m/z 90.9 for fudosteine and m/z 78.9 for IS using −16 and −25 eV collision energy, respectively.

Separation, Selectivity, and Sensitivity

Analysis of fudosteine blanks showed no interference in the final extract, although there several endogenous peaks that exhibited the same m/z transitions at different retention times. Typical chromatograms of fudosteine blank extracts

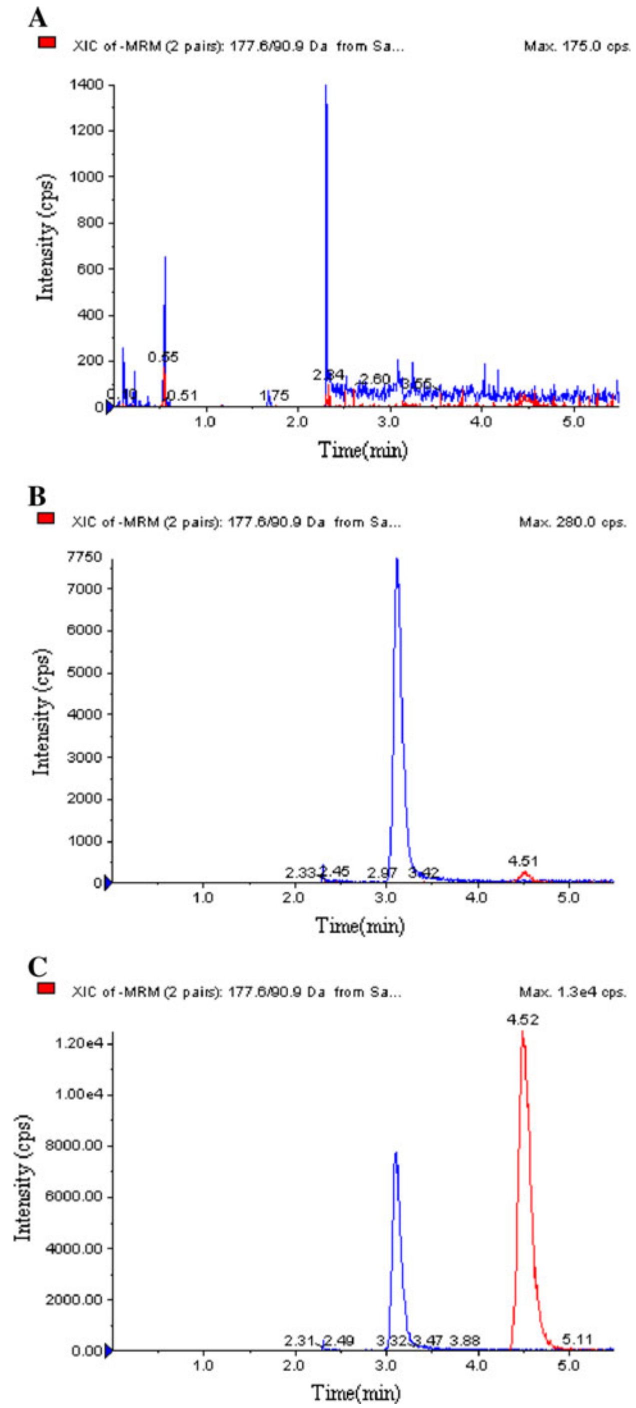

Fig. 1 Representative MRM chromatograms for fudosteine (1) and fosfomycin (2, IS) from **a** a blank plasma sample; **b** a blank plasma sample spiked with fudosteine at the LLOQ of 5 ng mL^{-1} and IS standards (1 μg mL^{-1}); **c** a plasma sample from a volunteer 4 h after oral administration of fudosteine (200 mg)

are presented in Fig. 1a. The results indicate that the method provides adequate separation and selectivity through HPLC separation and LC–MS–MS detection. The method provides acceptable sensitivity for the compound of interest. Typical LLOQ has a signal-to-noise ratio greater than ten in the validation. Typical chromatograms for the LLOQ sample and volunteer plasma sample are presented in Fig. 1b and c, respectively.

Recovery and Matrix Effect

The recovery of fudosteine was determined by comparing the peak area ratio of extracted samples with samples spiked after extraction. The recovery were 110.02 ± 4.01, 95.74 ± 7.23, and 107.83 ± 9.75, at three concentrations (0.01, 1, 10 μg mL^{-1}, $n = 6$). The overall matrix effect is above 94.99% for fudosteine base on three levels (Data not shown). The results suggested negligible matrix effect occurred in this method.

Standard Curve Linearity

Best fit for the calibration curve could be achieved by a linear equation of $y = 0.0020x + 0.0005$, with $1/x^2$ weighting factor (where, y is the peak–area ratio and x the concentration). The correlation coefficient (r^2, mean value, $n = 5$) for fudosteine was 0.9979.

Accuracy and Precision

QC samples were analyzed in six replicates at three concentrations to determine the accuracy and precision of this method. The intra-day %RSD data were 3.98, 5.47 and 9.77%, while the inter-day were 4.91, 2.75, 5.15%, respectively. These results indicated that this method has a satisfactory precision and reproducibility.

Stability

The fudosteine in plasma was found to be stable at room temperature for 12 h (remaining 90.58–108.22%), at 4 °C for 12 h (remaining 97.13–99.55%), at the −20 °C for 20 days (remaining 91.66–93.27%), at freeze and thaw stability (remaining 91.24–98.14%). The stock solutions were stable for at least 35 days. The difference values between the fresh samples and the test solution in stock solution stability were <5% for fudosteine and fosfomycin, respectively (Data not shown).

The results from all stability tests presented in demonstrated a good stability of fudosteine over all steps of the determination.

Pharmacokinetics Studies

This method was applied to the analysis of human plasma from healthy subjects after a single oral dose of 200 mg kg^{-1} of fudosteine. The mean plasma concentration–time curves

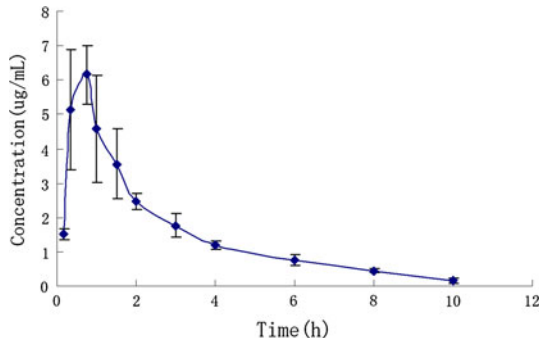

Fig. 2 Mean plasma concentration–time curve of fudosteine in six volunteers after a single oral dose (200 mg) of fudosteine

for fudosteine are shown in Fig. 2. Due to the excellent sensitivity of assay, with an LLOQ of 5 ng mL^{-1}, plasma concentrations were determined up to 10 h.

The concentration–time data were analyzed using DAS software (Ver 2.0) non-compartmental method and the mean values of pharmacokinetic parameters are C_{max} 6.16 ± 1.85 μg mL^{-1}, T_{max} 0.75 ± 0.34 h, $t_{1/2}$ 3.77 ± 1.12 h, MRT 4.55 ± 1.67 h, AUC$_{0-10}$ 14.67 ± 1.57 μg h mL^{-1}, AUC$_{0-\infty}$ 14.95 ± 1.81 μg h mL^{-1}. The pharmacokinetic parameters are similar with Zhou et al. [4] reported the pharmacokinetic parameters of fudosteine in 12 healthy Chinese volunteers after single-dose administration of 200 mg. Except that the T_{max} was 0.75 ± 0.34 h meanwhile the T_{max} was 0.40 ± 0.21 h in their study. The discrepancies of T_{max} from other studies seemed to be linked to the between-study difference (for example, different in analyzing software, sampling time, interpolation mode, study population, study conduct, study formulation and inter-individual variability). Due to small sample size ($n = 6$) we did not find obviously difference between genders.

Conclusion

An easy and general LC–MS–MS assay for the measurement of fudosteine in human plasma has been established. The method is specific, sensitive, and accurate over a concentration range of 0.005–10 μg mL^{-1}. The precipitation step used in sample preparation, proved to be simple, rapid and successful with an average recovery rate within 95.74–110.02% under all tested concentrations. All of these guaranteed its future widespread application in other laboratories. The method has been used to quantify levels of fudosteine in human plasma, following an oral administration of fudosteine with a single dose 200 mg kg^{-1}.

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