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Sensitive determination of erdosteine in human plasma by use of automated 96-well solid-phase extraction and LC–MS/MS

Hohyun Kim [a,b,*], Kyu Young Chang [a], Hee Joo Lee [a,b], Sang Beom Han [c], Kyung Ryul Lee [a,b]

[a] *Department of Pharmacokinetics, Seoul Medical Science Institute, Seoul Clinical Laboratories (SCL), 7-14 Dongbinggo-Dong, Yongsan-Gu, Seoul 140-809, South Korea*
[b] *Department of Drug Development Service, BioCore Co. Ltd., Seoul 137-130, South Korea*
[c] *College of Pharmacy, ChungAng University, Seoul 156-756, South Korea*

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Abstract

A sensitive and selective method for quantitation of erdosteine in human plasma was established by use of 96-well solid-phase extraction (SPE) and liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI/MS/MS). Plasma samples were transferred into 96-well OASIS HLB extraction plate using an automated sample handling system and the drugs were eluted with methanol. The eluents were then evaporated and reconstituted with mobile phase. All sample transfer and SPE was automated through the application of both the Perkin-Elmer MultiPROBE II HT and TOMTEC Quadra 96 workstation. Compounds were separated on a C_{18} column with 1 mM ammonium acetate–acetonitrile (80:20, pH 3.2), as mobile phase at a flow rate of 0.3 ml/min. The limit of quantitation (LOQ) was 0.2 ng/ml, using a sample volume of 0.2 ml for the analysis. The reproducibility of the method was evaluated by analyzing three at 14 quality control (QC) levels over the nominal concentration range from 0.2 to 5000 ng/ml. The intraday accuracy was found to range from 99.6 to 105.0% with precision (% RSD) of less than 4.76% at five QC levels. The interday accuracy was found to range from 95.0 to 100.5% with precision of less than 5.26% at five QC levels. Erdosteine produced a protonated precursor ion ($[M+H]^+$) at *m/z* 250, and a corresponding product ion at *m/z* 204. Internal standard (letosteine) produced a protonated precursor ion ($[M+H]^+$) at *m/z* 280 and a corresponding product ion at *m/z* 160. The high sample throughput of the method has been successfully applied to a pharmacokinetic study of erdosteine in human plasma.

Keywords: Tandem mass spectrometry; Liquid chromatography; 96-Well solid-phase extraction; Erdosteine; Letosteine

1. Introduction

Erdosteine, (±)-{[2-oxo-2[(tetrahydro-2-oxo-3-thienyl)amino]ethyl]thio}acetic acid, is a mucolytic drug. It can increase the concentration of antibiotics in sputum by enhancing the penetration of the antibiotics through the bronchial mucosa and clear the free radicals produced by imflammatory cells. Also, it is used in the treatment of oxidative lung injury caused by smoking [1].

Two different methods have been reported for the analysis of erdosteine in biological samples;

* Corresponding author. Tel.: +82-2-571-4431; fax: +82-2-3461-0590.
E-mail address: novakims@yahoo.com (H. Kim).

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high-performance liquid chromatography with fluorescence detection (HPLC-FLD) [2] and liquid chromatography with mass spectrometry (LC–MS) [3]. Muramatsu et al. [2] reported enantio-selective determination of erdosteine and its metabolite by HPLC-FLD. And Savu et al. [3] reported determination of erdosteine by use of solid-phase extraction (SPE) and derivatization step after protein precipitation and LC–MS with selective ion monitoring (SIM). Unfortunately, these methods were not easily adapted to the pharmacokinetics work, because of complicated sample preparation, low sensitivity (LOQ: 200 ng/ml), and long chromatographic run times.

Currently, solid-phase extraction has been a widely utilized technique for biological sample preparation, not only because of its ease for automation but also because of its ability to selectively remove interfering matrix components. Recent development and commercialization of 96-channel robotic liquid handling workstations as well as a wide selection of 96-well SPE sorbents afford the rapid development and automation of SPE methods to eliminate traditional time-consuming and labor-intensive biological sample preparation steps for plasma [4–6] and urine [7] samples. SPE automation in a 96-well format significantly reduces sample preparation time, increases sample throughput, and improves assay reproducibility.

This paper reports a novel high-throughput analytical method for the determination of erdosteine in human plasma by use of automated 96-well SPE and LC–MS/MS. Validation experiments of the method have shown that the assay has good precision and accuracy over a wide concentration range and stability, matrix effect, recovery, and reproducibility were evaluated. This simple, rapid, and sensitive LC–MS/MS method has been successfully applied to a pharmacokinetic study of erdosteine in human plasma.

2. Experimental

2.1. Chemicals and solutions

Erdosteine and letosteine (internal standard) were obtained from CKD pharmaceutical Co. Ltd. (Seoul, South Korea) and Handok Pharmaceutical Co. Ltd. (Seoul, South Korea). HPLC grade methanol, acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA), and ammonium acetate and formic acid from Sigma–Aldrich (St. Louis, MO, USA). Fig. 1 shows the structures of erdosteine and letosteine.

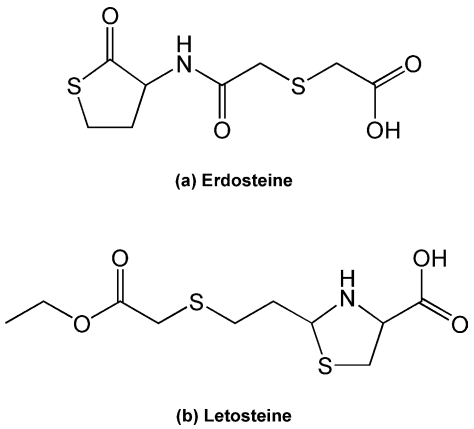

Fig. 1. Structures of (a) erdosteine and (b) letosteine (internal standard).

To prepare standard stock solutions, appropriate amount of erdosteine and letosteine were weighted accurately and dissolved separately in 50 ml water. From these stock solutions, working standard solutions containing from 20 to 500 μg/ml erdosteine were prepared by sequential dilutions with water.

2.2. Instrumentation and LC–MS/MS conditions

Tandem mass spectrometry (MS/MS) was performed with a Quattro Ultima Pt triple quadruple mass spectrometer (Micromass Co., Manchester, UK) equipped with an electrospray ionization (ESI) source. Sample (20 μl) was delivered into the ESI source by LC (liquid chromatograph and autosampler, Model Waters HT 2795, Waters Co., Milford, USA) with C_{18} Capcell Pak column (2.0 mm × 150 mm, 5.0 μm particle). The mobile phase was 1 mM ammonium acetate–acetonitrile (80:20, pH 3.2 with formic acid) was used after degassing. The flow rate was 0.3 ml/min and the total run time was 4.0 min.

The electrospray interface was maintained at 300 °C. Nitrogen nebulization was performed with a nitrogen flow of 1100 l/h. Argon was used as collision gas. Erdosteine and internal standard were detected by the multiple reaction monitoring (MRM) scan mode with positive ion detection, the parameter

settings used were: capillary voltage at 3.3 kV, cone voltage at 35 V, RF lens one voltage at 20 V, aperture at 0 V, RF lens 2 at 0.1 V, collision cell entrance potential at 1.0 V, collision energy at 11 eV, collision cell exit potential at 0 V, collision cell gas pressure at $3.85e^{-3}$ mbar, multiplier at 650 V, and dwell time of 0.50 s.

Mass calibration was performed by infusion of a 10^{-4} M polyethylene glycol 1000 (PEG 1000) solution into the ionspray source. The peak widths of precursor and product ions were maintained at ~0.7 mass unit at half-height in the MRM mode.

2.3. Sample preparation

Plasma specimens (0.2 ml) were transferred into 96-deep well plate by an automated sample handling system (Perkin-Elmer life Science, MultiPROBE II HT, Perkin-Elmer life Science Inc., Boston, USA), and spiked with 0.1 ml of 100 ng/ml internal standard solution and 0.1 ml of 100 mM ammonium acetate (pH 3.2) solution by TOMTEC Quadra 96 workstation (Tomtec Inc., Hamden, USA). The mixture was vortexed for 10 s and loaded onto 96-well OASIS HLB extraction plate (10 mg, Waters Co., Milford, USA), which has been conditioned by washing with methanol followed by water. The 96-well OASIS HLB extraction plate was drained by vacuum manifold system, and washed with water. The drugs were eluted with methanol (1 ml) into a 96-deep well plate and completely evaporated at 40 °C under a stream of nitrogen. The dry residue was reconstituted with 0.2 ml mobile phase. The 96-deep well plate was loaded onto the autosampler and then 20 μl of the reconstituted sample was injected into the LC–MS/MS system.

2.4. Validation procedures and calibration curves

To assess the intraday precision and accuracy of the method, five replicate analyses were performed on plasma standards containing five different concentrations (0.2, 2, 20, 500, and 5000 ng/ml) of erdosteine. Five replicate analyses of the same five samples were also performed to determine the initial interday precision and accuracy. The accuracy was expressed as (mean observed concentration)/(spiked concentration) × 100%, with the precision expressed as relative standard deviation (RSD).

For the plasma QC samples the appropriate QC working solution (0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200 and 500 μg/ml; 400 μl) was added to 50 ml polypropylene tubes containing 39.6 ml pooled human blank plasma to yield QC concentration of 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/ml. The QC samples were used to construct the calibration curve. The calibration curves ($y = mx + b$) were generated by a weighted linear least-squares regression of the peak area ratios (y) of the analytes to their internal standards versus the concentrations (x) of the calibration standards. Concentrations of analytes in QC samples were calculated using the resulting peak area ratios and the regression equations of the calibration curves. The bulk QC plasma samples were then vortex mixed, and 1.5 ml aliquots were transferred to 2 ml microcentrifuge tubes and capped, and stored at −70 °C.

2.5. Pharmacokinetic assay

For the human assay, triple 300 mg dose of erdosteine was administered orally to 24 volunteers who were advised about the nature and purpose of the study. The volunteers possessed good health and had not taken any medication for at least 2 weeks before the study. The group consisted of healthy males with a mean age of 23.6 ± 1.6, mean weight of 69.1 ± 7.2 kg, and mean height of 174.8 ± 5.0 cm. Blood samples were taken, by use of heparin vacutainer collection tubes, 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3–6 and 7 h after ingestion. Human plasma was obtained by centrifugation at 2000 × g for 10 min. Plasma specimens were then stored at −70 °C before analysis.

3. Results and discussion

We attempted to develop a rapid and sensitive method for detecting erdosteine in human plasma by use of automated 96-well SPE and LC–MS/MS for pharmacokinetic studies.

3.1. LC–MS/MS conditions

Under electrospray ionization conditions, erdosteine and letosteine (internal standard) exhibit a fairly high sensitivity in positive ion detection mode

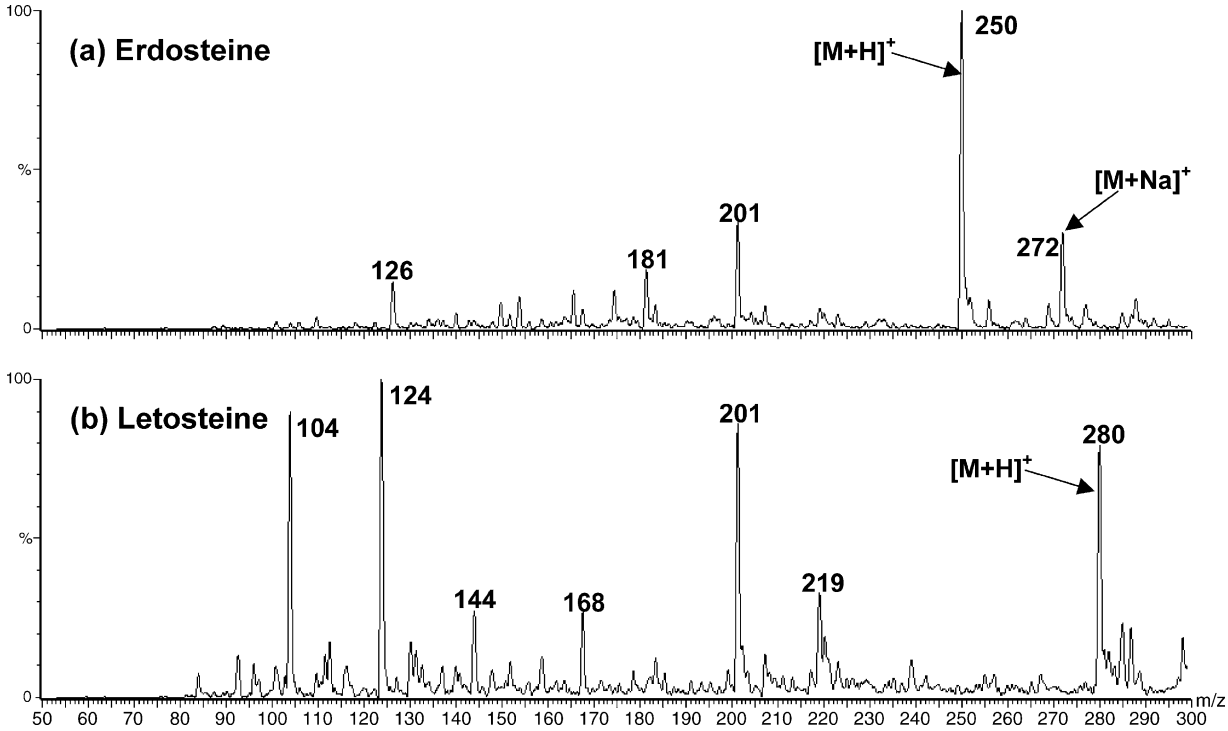

Fig. 2. Full scan first quadrupole spectrum of (a) erdosteine and (b) letosteine (internal standard).

rather than negative ion detection mode. Fig. 2a and b shows the full scan first quadrupole positive ion spectrum of erdosteine and internal standard. These formed protonated precursor ion $[M + H]^+$ as major ion peaks. Erdosteine produced a protonated precursor ion ($[M+H]^+$) at *m/z* 250 with a major product ion at *m/z* 204 and letosteine (internal standard) produced a protonated precursor ion ($[M + H]^+$) at *m/z* 280 with a major product ion at *m/z* 160. The product ion mass spectrum, and their postulated rationalization in terms of major fragmentation patterns, of erdosteine and letosteine are illustrated in Fig. 3a and b. The most abundant product ions (*m/z* 204 for erdosteine and *m/z* 160 for letosteine) were selected for MRM analysis.

Liquid chromatography-tandem mass spectrometry is still limited to conditions that are suitable for mass spectrometry operations. There are restrictions on pH, solvent choice, solvent additives, and flow rates for LC in order to achieve optimal ESI/MS/MS sensitivity. For the chromatographic analysis and electrospray ionization of erdosteine and letosteine, we initially attempted to develop a reversed phase chromatographic method with methanol or acetonitrile as mobile phase. Acetonitrile was used instead of methanol, because acetonitrile affords better sensitivity and resolution in the analysis of erdosteine and internal standard. The amount of acetonitrile in mobile phase was optimized at 20%. Ammonium acetate buffer was used because it was easily miscible with organic solvents and led to improved peak symmetry and ionization. It was found that much higher ion intensities were observed by the use of an ammonium acetate (1 mM) aqueous phase adjusted to a pH of 3.2 by formic acid. Under these conditions, we have obtained good chromatograms (Fig. 4) and the chromatographic detection of erdosteine and internal standard was highly sensitive. (Fig. 5a).

3.2. Automated 96-well solid-phase extraction

In addition to a reduction of labor, it was found that the use of an automated 96-well SPE method for the analysis of erdosteine reduces sample preparation time, increased sample throughput, labor-intensive,

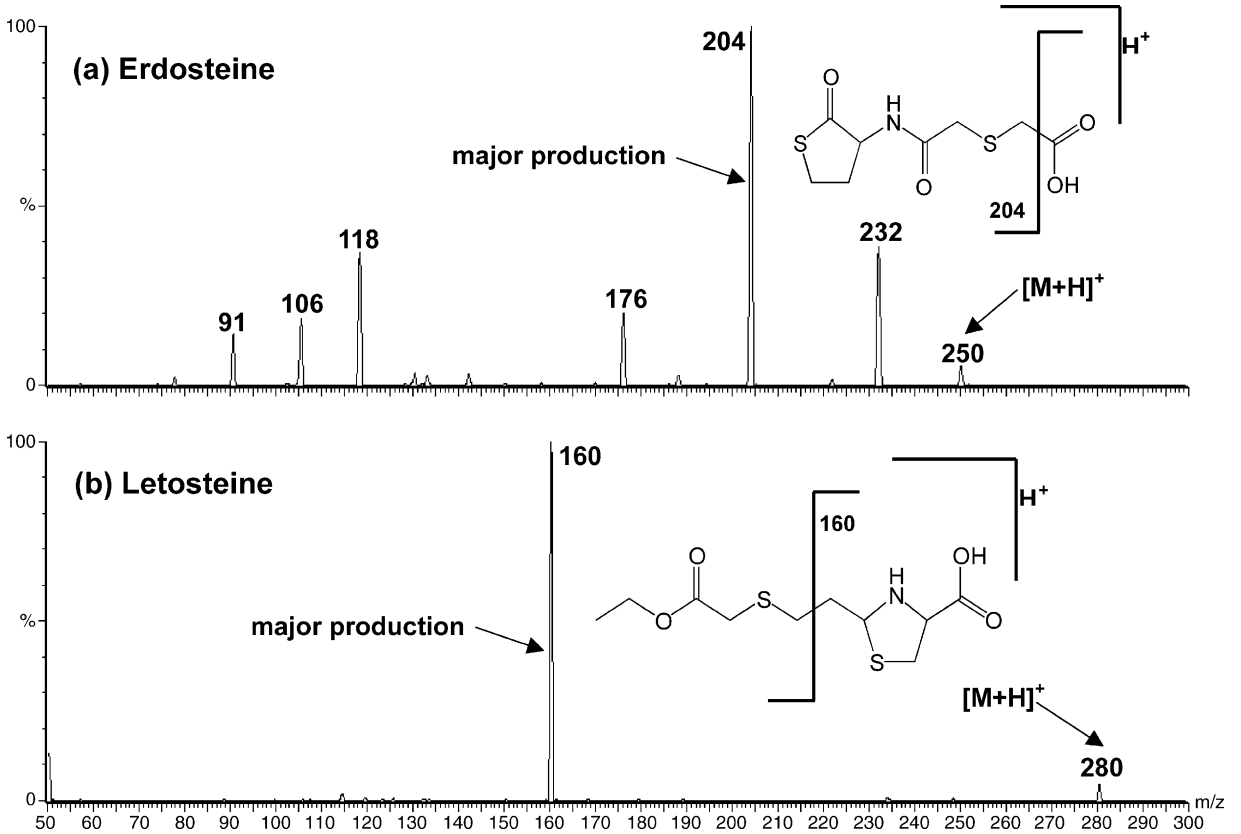

Fig. 3. Product ion spectrum of (a) erdosteine and (b) letosteine (internal standard).

and improved assay reproducibility over traditional SPE methods. Under the conditions described, four 96-well plates could be prepared in less than 6 h. With a run time of 4.0 min per sample, the four plates (384 samples) could be prepared and analysis completed within 32 h.

3.3. Analyte stability and limit of quantitation

The stability of erdosteine and internal standard was evaluated in the dissolution solvent and in human plasma. It was found that erdosteine and internal standard were stable for the entire duration of the experiment.

On the basis of a signal-to-noise ratio (S/N) of 10, the limit of quantitation (LOQ) erdosteine was found to be 0.2 ng/ml on injection of 20 μl of sample into the LC–MS/MS system (Fig. 5a).

3.4. Interference and matrix effect

The specificity and selectivity of the method was investigated by preparing and analyzing blank plasma from five different batches of pooled human blank plasma. Both blank plasma and blank plasma with internal standard samples were analyzed on each validation day. No interference was observed in either the blank plasma or blank plasma with internal standard. Fig. 5b shows LC–MS/MS chromatogram of human blank plasma and Fig. 5c shows LC–MS/MS chromatogram of human blank plasma spiked with internal standard.

Since chromatographic conditions may cause co-elution of a number of endogenous compounds that are undetected by the MS/MS but which may affect the ionization efficiency, the effect of matrix on the response of the analyte was also evaluated. To

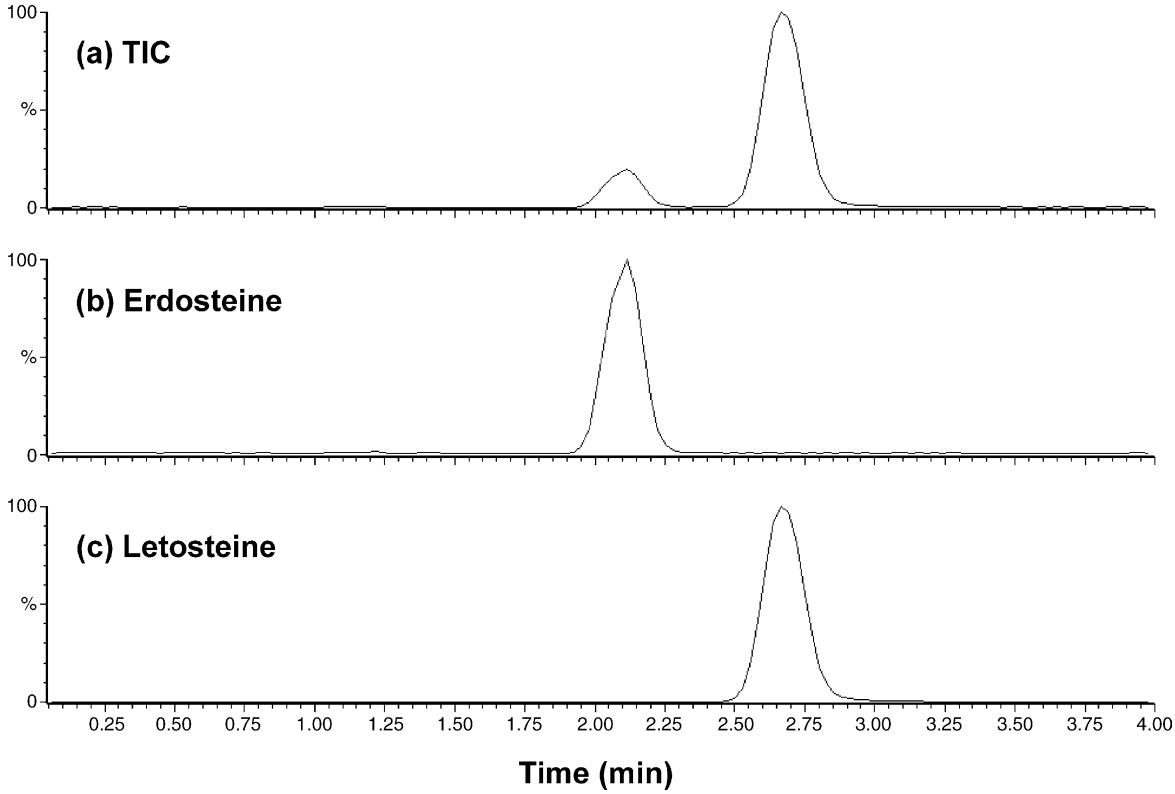

Fig. 4. LC–MS/MS chromatograms of (a) TIC, (b) erdosteine (MRM: 250 → 204, 20 ng/ml) and (c) letosteine (internal standard, MRM: 280 → 160) in human plasma.

determine the possible influence of the matrix on the analysis, the response of five extracted spike matrix blank samples were compared to those of five analytical standards. No matrix effect was detected in the study.

3.5. Recovery

Analyte recovery from a sample matrix (also called extraction efficiency) is a comparison of the analytical response from an amount of analyte added to and extracted from the sample matrix (pre-extraction spike) with that from a post-extraction spike. The recovery (recovery (%) = (response of pre-extraction spike of analyte/post-extraction spike of analyte) × 100) was measured as 81.9–91.1% for 0.2, 2, 20, 500, and 5000 ng/ml standard concentrations, with five replicates at each concentration level (Table 1).

3.6. Validation

The intraday precision expressed as % RSD was measured as 1.04–4.76% for 0.2, 2, 20, 500, and 5000 ng/ml standard concentrations, by performing five replicate analysis at each concentration level. The intraday accuracy expressed as a percentage of nominal values was measured as 99.6–105.0% for five

Table 1
Recovery of erdosteine in human plasma

Standard concentration in plasma (ng/ml)	Recovery[a] (%)
0.20	91.1
2.0	85.3
20	88.2
500	86.7
5000	81.9

[a] Five replicates at each concentration level ($n = 5$).

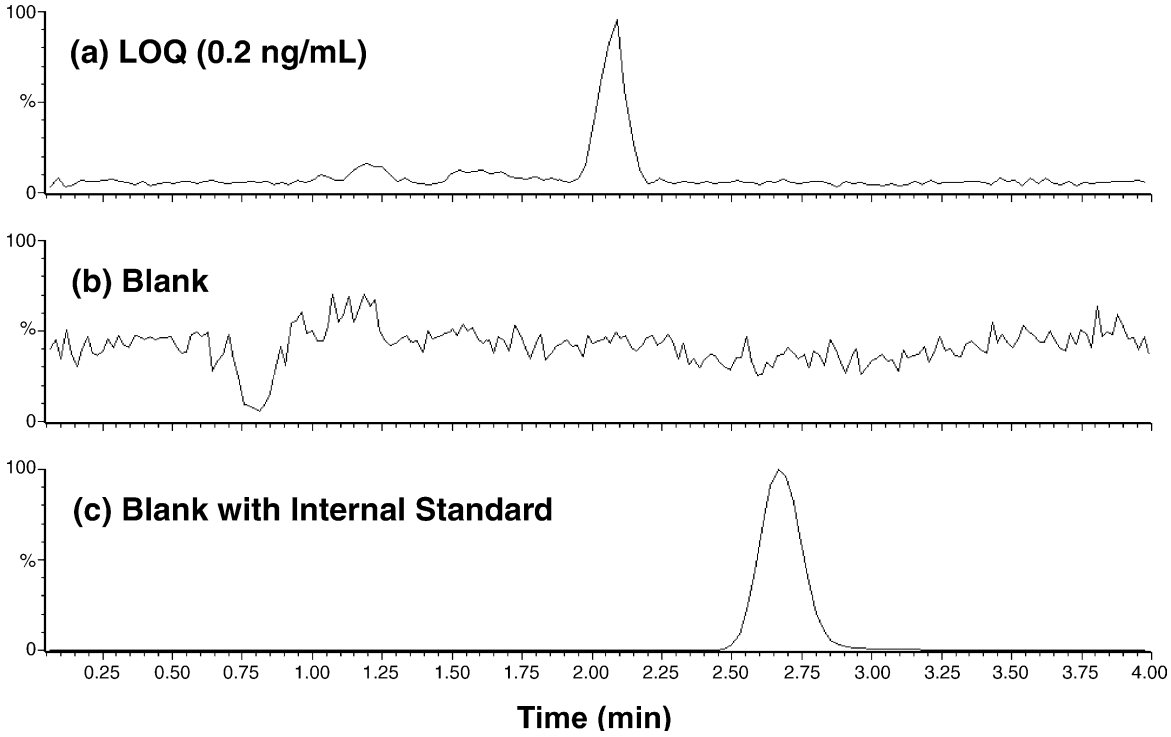

Fig. 5. LC–MS/MS chromatograms of (a) limit of quantitation of erdosteine (MRM: 250 → 204), (b) blank plasma and (c) blank plasma spiked with internal standard (letosteine, MRM: 280 → 160).

standard concentrations, by performing five replicate analysis at each concentration level. Table 2 shows intraday precision and accuracy of measurement of erdosteine in human plasma. The interday precision was measured as 1.18–5.26% for five standard concentrations, by performing five replicate analysis at each concentration level. The interday accuracy was measured as 95.0–100.5% for five standard concentrations, by performing five replicate analysis at each concentration level. Table 3 shows interday precision and accuracy of measurement of erdosteine in human plasma.

3.7. Calibration curve (reproducibility)

Standard calibration curves were constructed on different working days (3 days) using the QC samples. The response was linear throughout the concentration range of the study, with the correlation coefficient of determination (R^2) always greater than 0.9997 in all

Table 2
Intraday precision and accuracy of measurement of erdosteine in human plasma

Erdosteine nominal concentration (ng/ml)	Erdosteine calculated concentration [mean ± S.D.][a] (ng/ml)	Accuracy (%)	Precision (% RSD)
0.2	0.21 ± 0.01	105.0	4.76
2	2.02 ± 0.06	101.0	2.97
20	20.1 ± 0.7	100.5	3.48
500	498 ± 10	99.6	2.01
5000	4985 ± 52	99.7	1.04

[a] Five replicates at each concentration level ($n = 5$).

Table 3
Interday precision and accuracy of measurement of erdosteine in human plasma

Erdosteine nominal concentration (ng/ml)	Erdosteine calculated concentration [mean ± S.D.][a] (ng/ml)	Accuracy (%)	Precision (% RSD)
0.2	0.19 ± 0.01	95.0	5.26
2	2.01 ± 0.07	100.5	3.48
20	19.8 ± 0.6	99.0	3.03
500	501 ± 14	100.2	2.79
5000	4995 ± 59	99.9	1.18

[a] Five replicates at each concentration level ($n = 5$).

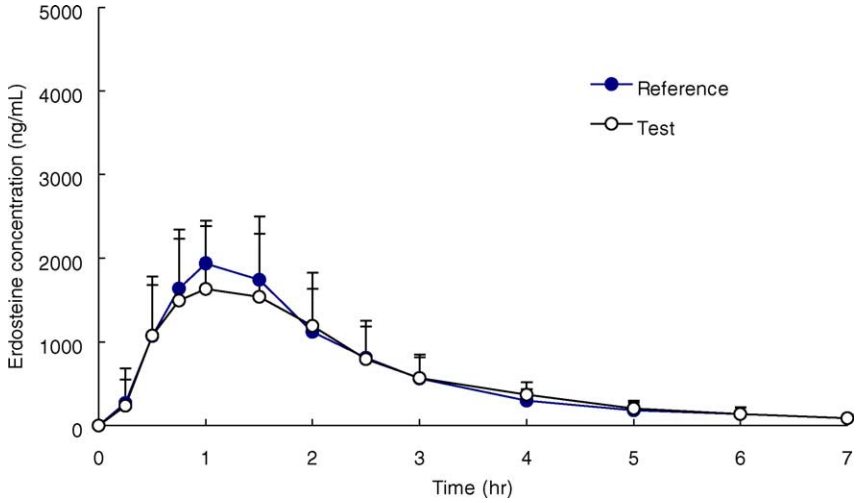

Fig. 6. Plasma concentration of erdosteine in human plasma vs. time curve.

cases. The equations were $y = 0.00457 + 0.0000422$ ($R^2 = 0.9997$), $y = 0.00447 + 0.0000158$ ($R^2 = 0.9999$), and $y = 0.00452 - 0.0000257$ ($R^2 = 0.9999$) in human plasma. Calibration curves of the method have shown that the assay has good reproducibility over a wide concentration range (0.2–5000 ng/ml).

Table 4
Pharmacokinetic parameters of reference erdosteine and test erdosteine

No.	AUC[a] (ng/ml/h)	C_{max}[b] (ng/ml)	T_{max}[c] (h)
Reference	4334 ± 1227	2220 ± 632	1.15 ± 0.47
Test	4209 ± 1148	2210 ± 639	1.23 ± 0.52

The values are expressed as mean ± S.D.
[a] AUC: area under the plasma concentration vs. time curve extrapolated to infinity.
[b] C_{max}: peak plasma concentration.
[c] T_{max}: time to reach C_{max}.

3.8. Pharmacokinetic assay

Determining the concentration of erdosteine in human plasma was applied to pharmacokinetic studies by use of 96-well SPE and LC–ESI/MS/MS. Fig. 6 shows the concentration of erdosteine in human plasma versus time curve after administration of triple 300 mg dose of erdosteine. Table 4 shows the pharmacokinetic parameters of reference erdosteine and test erdosteine. Fig. 6 and Table 4 indicate that the proposed method is suitable for pharmacokinetic studies to determine the concentration of erdosteine in human plasma.

4. Conclusion

A high throughput method for the determination of erdosteine using automated 96-well SPE and

LC–MS/MS has been developed and validated, a lower quantitation limit of down to 0.2 ng/ml (1000 times sensitive than LC–MS method), which is better than attainable by HPLC-FLD and LC–MS. Validation experiments have shown that the assay has good precision and accuracy over a wide concentration range (0.2–5000 ng/ml), and no interferences caused by endogenous compounds were observed by matrix effect test. The automated 96-well SPE method significantly reduces sample preparation time, increases sample throughput, labor-intensive, and improves assay reproducibility. This simple, rapid and robust assay will enable the complete processing of a large number of samples for pharmacokinetic studies of erdosteine in human plasma.

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