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# High performance liquid chromatographic determination of 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-ethane (BBSKE), a novel organoselenium compound, in dog plasma using pre-column derivatization and its application in pharmacokinetic study

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**Abstract**

A novel HPLC–UV method with pre-column derivatization by using 2-mercaptoethanol was established for determination of 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-ethane (BBSKE) in dog plasma. The derivatives were identified by mass spectrometry. The method had a good linear range of 0.05–2 μg/ml ($r^2 = 0.9995$). The lower limit of quantification (LOQ) was 0.05 μg/ml. The precision and accuracy were less than 7%. After dosing of BBSKE (30 mg/kg, p.o. and 0.79 mg/kg, i.v.) in dogs, $AUC_{0-t}$ were $5.72 \pm 2.42$ and $1.35 \pm 0.41$ μg h/ml; $t_{1/2}$ were $4.6 \pm 2.1$ and $1.7 \pm 0.6$ h, respectively. The method was successfully applied to the pharmacokinetic study in dogs.
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*Keywords:* 1,2-[bis(1,2-Benzisoselenazolone-3(2H)-ketone)]-ethane (BBSKE); 2-Mercaptoethanol; Pre-column derivatization; High performance liquid chromatography (HPLC)

## 1. Introduction

1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-ethane (BBSKE) is a novel antitumor agent and it belongs to heterocyclic organoselenium compound [1,2] (Fig. 1). Different from its lead compound, ebselen, a glutathione peroxidase mimic [3,4], BBSKE has been reported to have antitumor effect. As a thioredoxin reductase (TrxR) inhibitor, BBSKE can induce thioredoxin mediated mitochondria-dependent apoptosis [5], S phase cell cycle arrest [1], and inhibit proliferation of tumor cells in human carcinoma cell lines including human pulmonary adenocarcinoma, hepatocarcinoma and cervical adenocarcinoma cells, etc. [6]. BBSKE is deserved for further research as a prospective antitumor candidate.

In previous researches, it was demonstrated that ebselen could covalently bound to plasma protein by selenium–sulfur bond [7,8], and glutathione can catalyze an exchange of this covalently bound ebselen forming the selenosulfide adduct [9,10]. The process contributed to the equilibrium between plasma and target protein [9]. In our preliminary experiments, a high binding rate of BBSKE with plasma protein was observed and the tight binding made it very difficult for the samples to be processed. Since BBSKE and ebselen have similar chemical structure, it was assumed that BBSKE also could react with thiols, and then firmly bound to plasma protein. Therefore, a new method is needed to be developed for pharmacokinetic study.

The purpose of the present study was to establish a new HPLC method with pre-column derivatization for measuring the plasma concentration of BBSKE. In this method, 2-mercaptoethanol reacted with BBSKE to form a new adduct. The adduct could be extracted by acetic ether and expediently measured by HPLC with ultraviolet detection. The new product has been identified by mass spectrometry. The method has

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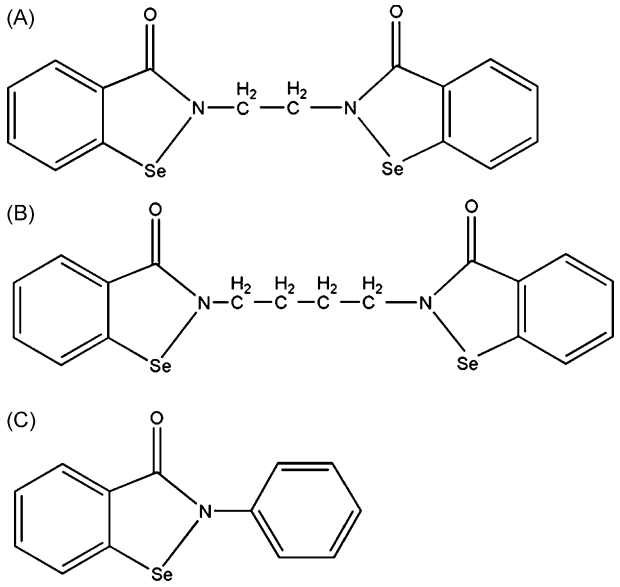


Fig. 1. Structures of 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-ethane (BBSKE) (A); 1,4-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-butane (internal standard) (B) and 2-phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen) (C).

been fully validated and can be applied to the pharmacokinetic research of BBSKE in dogs.

## 2. Materials and methods

### 2.1. Chemicals and reagents

BBSKE (purity > 98.6%) and internal standard (IS: 1,4-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-butane, BBSKB, purity > 98.6%), were prepared by School of Pharmaceutical Science, Peking University (Beijing, China). 2-Mercaptoethanol (2-ME) was purchased from Merck schuchardt OHG (Hohenbrunn, Germany). HPLC grade acetonitrile and methanol were obtained from J.T. Baker Company (Phillipsburg, USA). Acetic acid, tetrahydrofuran, dimethyl sulfoxide and potassium phosphate monobasic and dibasic salts (all chemical grade) were purchased from Beijing Chemical Reagents Company (Beijing, China). Distilled water was prepared by our group.

### 2.2. Animal handling and plasma sample collection

Three adult male beagle dogs (12 months age, $10 \pm 1$ kg weight) were obtained from Institute of Jing Feng Medical Animal Center (Beijing, China). The dogs were housed in a temperature controlled room (25 °C) and maintained in a reverse 12 h light/dark cycle with free access to food and water. The animal studies were approved by the Animal Ethics Committee of Beijing University, and carried out in accordance with the requirements of China national legislation.

After overnight fasting, dogs were orally administered a single dose of BBSKE tablets (30 mg/kg). Blood samples were collected from vein in fore leg at the predose, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 15, 20, 24, 48 and 96 h post-dosing, respectively. After a washout period of 10 days duration, the dogs were intravenously injected a single dose of BBSKE (0.79 mg/kg) into peripheral vein in the fore leg. Blood samples were collected from vein in another fore leg at the predose, 0.08, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 16, 20, 24, 48 and 96 h post-dosing, respectively. The blood samples collected at each time point were anticoagulated with heparin (20–30 IU/ml), centrifuged at 11,700 × *g* for 5 min and the plasma samples were kept in −20 °C until HPLC analysis.

### 2.3. HPLC instrumentation and conditions

HPLC system (Agilent Technologies, Palo Alto, CA) consisted of a quaternary pump, an autosampler, a thermostatted column compartment and an ultraviolet detector. The chromatograph was equipped with a reversed-phase Kromasil $C_{18}$ column (4.6 mm × 150 mm, 5 μm, Eka chemicals AB, Sweden). The mobile phase was water–acetonitrile–tetrahydrofuran (70:20:10, v/v/v) and pH was adjusted to 3 by addition of acetic acid (3 ml/l). The variable wavelength UV detector operated at 320 nm. The flow-rate was at 1.0 ml/min and the column temperature was controlled at 25 °C.

### 2.4. Mass spectrometry analysis

Ion trap-based mass spectrometer was performed on a Finnigan LCQ Advantage (Thermo Finnigan, USA) equipped with an electrospray ionization interface. After the derivatized adducts from plasma spiked with BBSKE (5 μg/ml) were separated by HPLC, the elution were collected and the MS conditions were optimized by directly infusing the eluted solutions into the mass spectrometer at 200 μl/min using infusion pump. Electrospray was accomplished using the following settings: ion transfer capillary temperature of 200 °C, spray voltage of 4.5 kV, sheath gas pressure of 20 arbitrary units, auxiliary gas pressure of 0 arbitrary units, capillary voltage of 15 V, and tube lens offset of 40 V, respectively. The mass spectrometer was operated in positive ion mode with a scan range from *m/z* 100–1000. Data were collected and analyzed by the Navigator software (version 1.2, Thermo Electron).

### 2.5. HPLC pre-column derivatization and sample preparation

The derivatized sample preparation of BBSKE and IS using 2-ME was performed by reported methods [8] with modification. Aliquot of 190 μl of plasma sample was added to 10 μl dimethyl sulfoxide containing internal standard (20 μg/ml) and mixed slightly. Then 17.5 μl of 1 mol/l 2-ME and 183 μl of 10 mmol/l potassium hydrogen phosphate buffer (pH 7.5) were added later. The mixture was vortexed and incubated in water bath at 37 °C for 2 h. The derivatized samples were extracted twice with 600 μl of ethyl acetate by vortex-mixing for 2 min. After centrifugation at 11,700 × *g* for 5 min, the supernatant was transferred and evaporated to dryness under nitrogen stream at room temperature. The residue was dissolved in 50 μl of methanol, vortexed and centrifuged. An aliquot (20 μl) was injected into HPLC for analysis.

### 2.6. Preparation of calibration curve and quality control samples

Stock solutions of BBSKE and internal standard (both 400 μg/ml) were prepared in dimethyl sulfoxide and stored at room temperature. A series of working standard solutions (1, 2, 4, 10, 20, 40 μg/ml) was obtained by further dilution the stock solution of BBSKE with dimethyl sulfoxide. Internal standard working solution (20 μg/ml) was also prepared by diluting the stock solution with dimethyl sulfoxide. A six-point calibration curve was made by spiked blank plasma with the working standard solutions to cover a series of concentrations which is 0.05, 0.1, 0.2, 0.5, 1, 2 μg/ml. Quality control (QC) samples (0.05, 0.2 and 1 μg/ml) were prepared in a similar way.

### 2.7. HPLC method validation

The method was fully validated by its specificity, sensitivity, stability, precision and accuracy. The sensitivity was expressed by the lower limit of quantification (LLOQ) with peak height to base line signal ratio of 10:1. The limit of detection (LOD) was determined as the concentration with signal-to-noise (S/N) ratio of 3. The intra- and inter-precision was determined by analyzing five spiked plasma sample at each QC level (0.05, 0.2 and 1 μg/ml) on 3 separate validation days. Accuracy was assessed by the percentage deviation of mean observed from spiked values and expressed as relative error (RE). Precision was expressed by the relative standard deviation. In the stability experiments, the samples stored in −20 °C for 1 week and three freeze–thaw cycles (freezing at −20 °C for 24 h and thawing at room temperature) were investigated. The effect of derivatized samples kept in autosampler vial at room temperature for 24 h was also evaluated.

### 2.8. Pharmacokinetic parameters and statistic analysis

Pharmacokinetic parameters were calculated by non-compartmental method. The plasma peak concentration ($C_{max}$) and the time to reach $C_{max}$ ($T_{max}$) were obtained directly from the experimental data. The initial concentration ($C_0$) after intravenous injection was determined by logarithmic back-extrapolation of the first two data points to the time of administration ($t_0$). The area under the curve to the last measurable concentration ($AUC_{0-t}$) was calculated by the linear trapezoidal rule. The elimination rate constant ($K_e$) was obtained from the least-square fitted terminal log-linear portion of the plasma concentration–time profile. The terminal elimination half-life ($t_{1/2}$) was calculated by $0.693/K_e$. All data were presented as mean ± SD.

## 3. Results and discussion

### 3.1. Derivatization

In our preliminary experiment, the degree of plasma protein binding at three levels of concentration (0.05, 0.2, 1 μg/ml) of BBSKE in three different species (dog, rat and human) were evaluated by ultrafiltration. The result indicated that the rate of plasma protein binding of BBSKE were higher than 99.6% (dog), 99.7% (rat) and 99.9% (human), respectively. Similarly, the free BBSKE could not be detected in samples after oral and intravenous administration of BBSKE in dogs. These results suggested that BBSKE existed mainly in bound form in plasma. Moreover, in the present experiment, the excess amount of 2-mercaptoethanol was applied to perform the reaction of derivatization. Therefore, it was considered that the standards and samples were almost equivalent in derivatization yields.

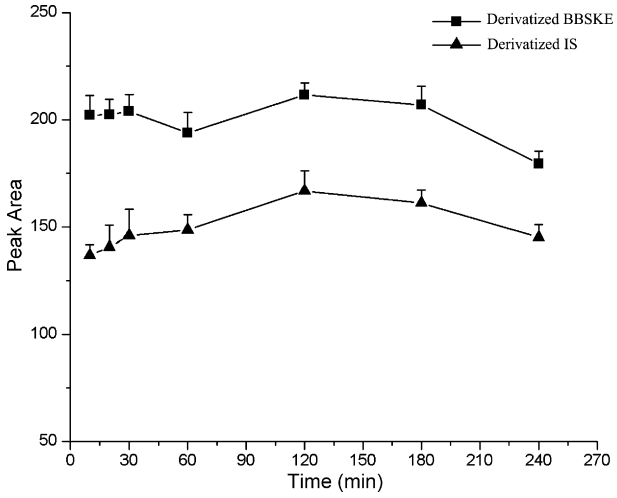


Fig. 2. Formation of 2-mercaptoethanol-1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-ethane (2-ME-BBSKE) and 2-mercaptoethanol-1,4-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-butane (2-ME-IS) as a function of incubation time. Peak areas are reported as mean ± SD ($n = 3$).

#### 3.1.1. Investigation of derivatization conditions

The reaction time was evaluated by measuring the peak areas of derivatives at different time. As shown in Fig. 2, 2.4 pmol BBSKE and IS in dimethyl sulfoxide were added into 0.19 ml blank plasma and then allowed to react with 1 mol/l 2-ME for 10, 20, 30, 60, 120, 180 and 240 min. The results indicated that the maximum adducts were obtained at 120 min incubation time. In addition, the concentration of 2-ME was investigated. From 1 to 14.3 mol/l, the increment of 2-ME had no effect on the quantity of adducts which indicated that 1 mol/l of 2-ME was enough to react with BBSKE and IS. Therefore, the 120 min incubation time and 1 mol/l 2-ME were selected for assay development and validation of the method.

#### 3.1.2. Identification of derivatized adducts

Both BBSKE and IS are kinds of benzisoselenazolones which can react with thiols group of protein [10,11]. After being added into the dog plasma sample, 2-ME promoted an exchange of the BBSKE bound to protein forming a new 2-ME derivatized adduct. The ultraviolet spectra of 2-ME-BBSKE, 2-ME-IS, BBSKE and IS are shown in Fig. 3. Compared with the characteristic absorption wavelength of parent compounds at 320 nm, the minor hypsochromic shift of 10 nm in the two derivatives was observed, suggesting that it might be due to the structural change of compound. In addition, the spectra showed the similar

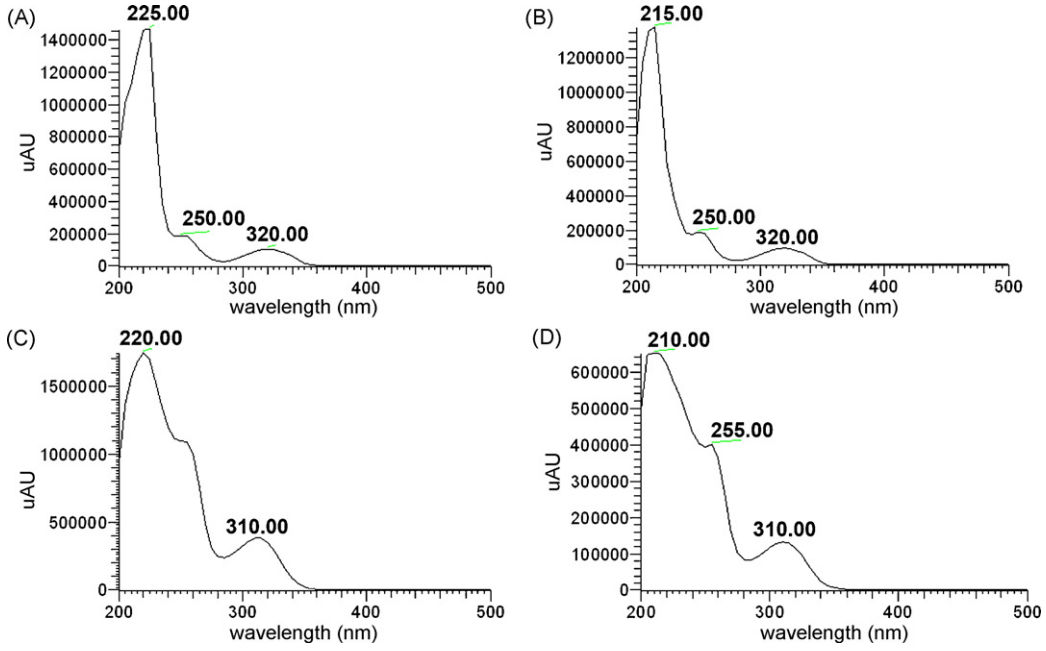


Fig. 3. Ultraviolet spectra of 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-ethane (BBSKE) (A), 1,4-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-butane (IS) (B), 2-mercaptoethanol-BBSKE (C), 2-mercaptoethanol-IS (D). The spectra were collected by Surveyor photodiode array detector.

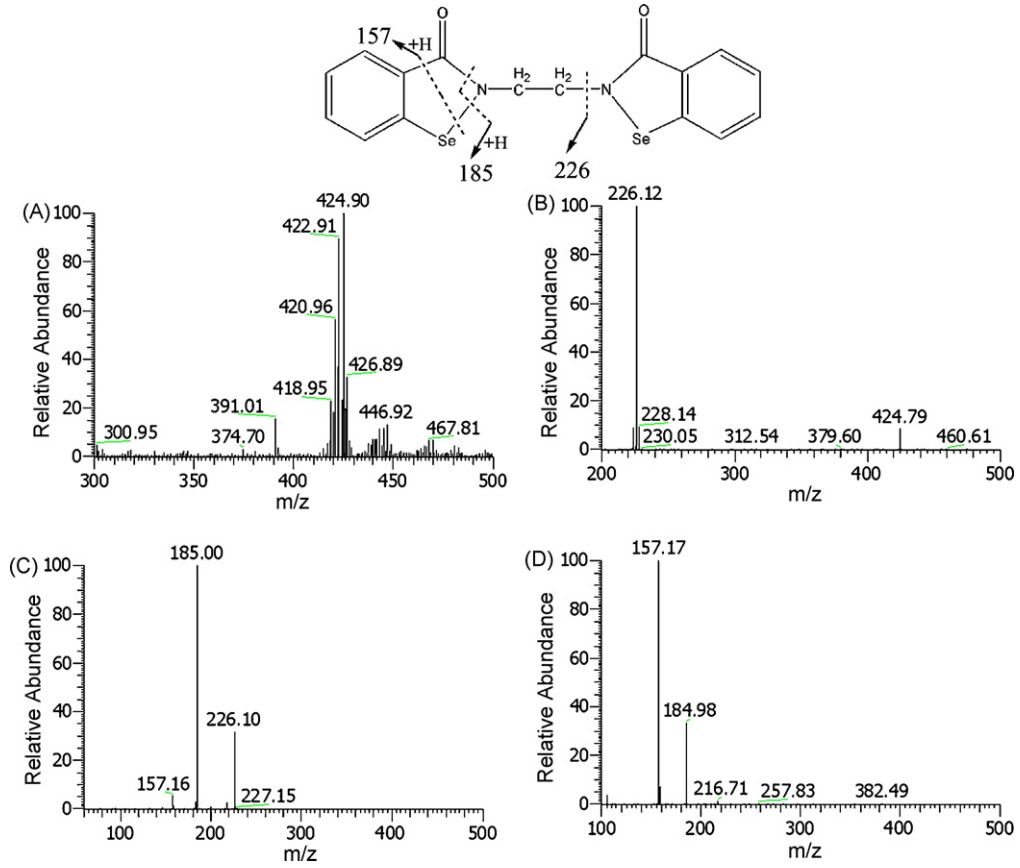


Fig. 4. The multi-stage mass spectra of 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-ethane (BBSKE) standard in positive mode using electrospray ionization and in-source collisionally induced dissociation. (A) $MS^1$ *m/z* 425, (B) $MS^2$ *m/z* 226, (C) $MS^3$ *m/z* 185 and (D) $MS^4$ *m/z* 157.

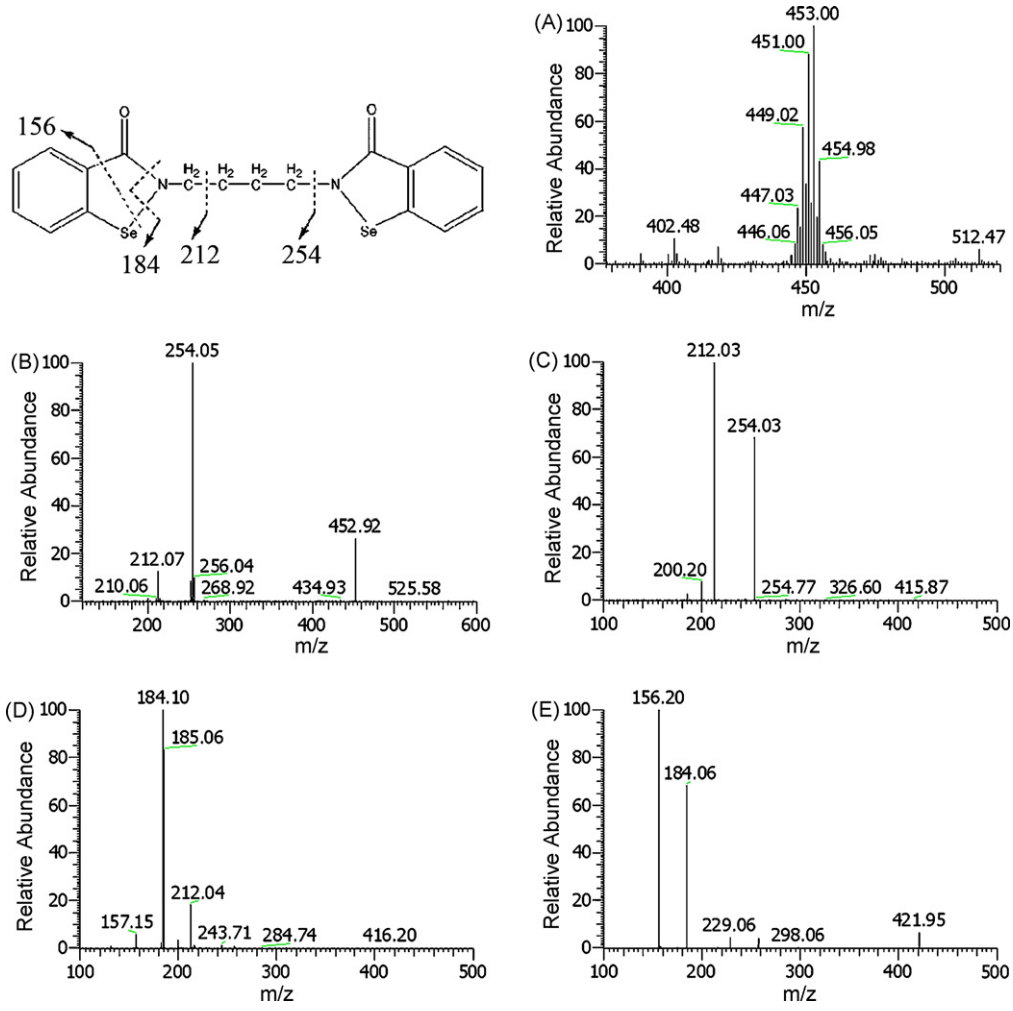


Fig. 5. The multi-stage mass spectra of 1,4-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-butane (internal standard) in positive mode using electrospray ionization and in-source collisionally induced dissociation. (A) $MS^1$ *m/z* 453, (B) $MS^2$ *m/z* 254, (C) $MS^3$ *m/z* 212, (D) $MS^4$ *m/z* 184 and (E) $MS^5$ *m/z* 156.

absorption characteristic between parent compounds and 2-ME derivatized adducts. In order to confirm the above assumption, the mass spectrometry was applied. The proposed structures were shown and interpreted as follows.

Firstly, the mass spectrum of BBSKE and IS were obtained. The Se-containing compound showed the expected pattern of the selenium isotopes. Single-stage full-scan mass spectrum of BBSKE showed a molecular ion $[M+H]^+$ at *m/z* 425 which have three characteristic fragment ions at *m/z* 226, 185 and 157 (Fig. 4). In the same way, the single-stage full-scan mass spectrum of IS gave a molecular ion $[M+H]^+$ at *m/z* 453 which can produce four characteristic fragment ions at *m/z* 254, 212, 184 and 156 (Fig. 5).

Secondly, after being purified by HPLC, the derivatized adducts were injected into mass spectrometer through infusion

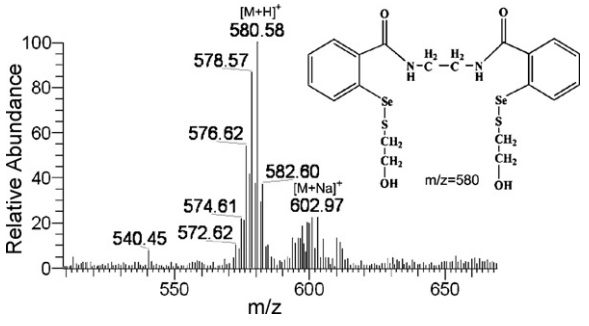


Fig. 6. Proposed structure and full scan molecular ion spectra of 2-mercaptoethanol-1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-ethane (2-ME-BBSKE) derivative.

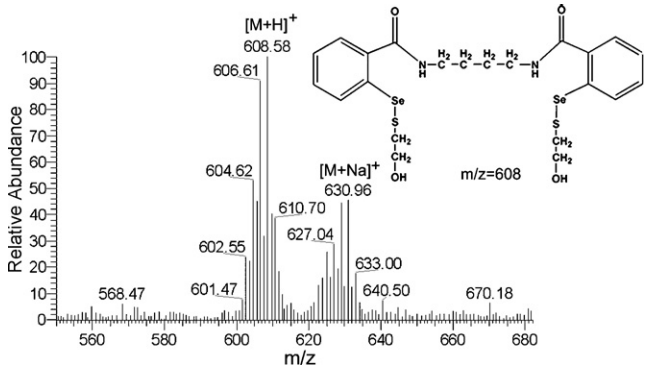


Fig. 7. Proposed structure and full scan molecular ion spectra of 2-mercaptoethanol-1,4-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-butane (2-ME-IS) derivative.

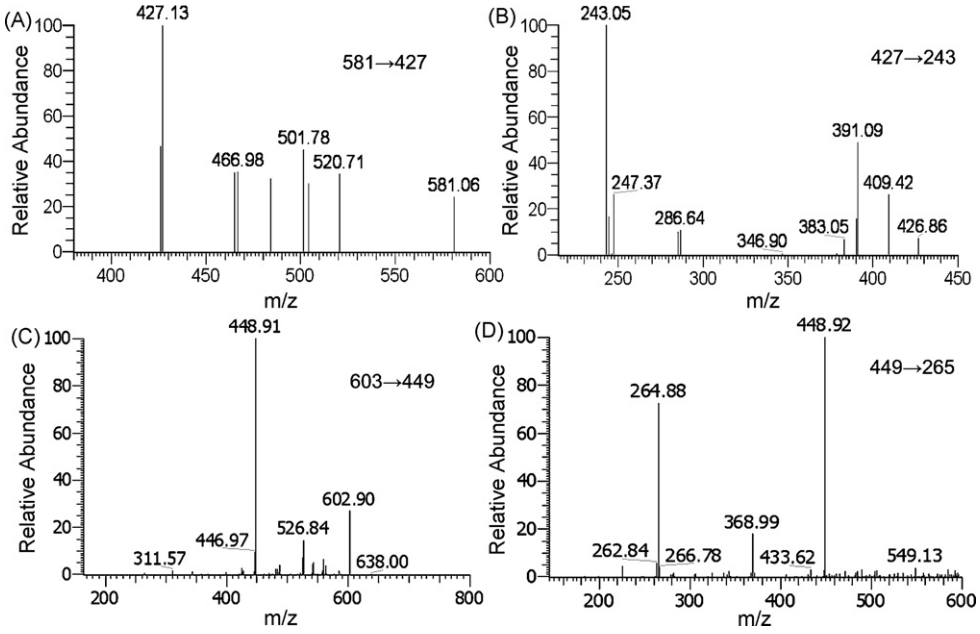


Fig. 8. The multi-stage mass spectra of 2-mercaptoethanol-1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-ethane (2-ME-BBSKE) in positive mode using electrospray ionization and in-source collisionally induced dissociation. (A) and (B) $MS^2$ and $MS^3$ spectra of the *m/z* 581 $[M+H]^+$ ion and (C) and (D) $MS^2$ and $MS^3$ spectra of the *m/z* 603 $[M+Na]^+$ ion.

pump. The mass spectrum of 2-ME-BBSKE derivative showed protonated molecules $[M+H]^+$ *m/z* 581 and $[M+Na]^+$ *m/z* 603 (Fig. 6). The mass spectra of 2-ME-IS derivative gave protonated molecules $[M+H]^+$ *m/z* 609 and $[M+Na]^+$ *m/z* 631 (Fig. 7). As shown in Fig. 8A and B, the $MS^2$ and $MS^3$ spectrum of *m/z* 581 ($[M+H]^+$) produced the fragment ions at *m/z* 427 and 243, respectively. Fig. 8C and D showed the fragment ions at *m/z* 449 and 265 from the *m/z* 603 ($[M+Na]^+$). In Fig. 9, the molecular ion $[M+H]^+$ *m/z* 609 produced the fragment ions at *m/z* 455 and 256, and the $[M+Na]^+$ *m/z* 631 gave the product ions at *m/z* 477 and 293.

Obviously, the protonated molecule $[M+H]^+$ (*m/z* 581) of 2-ME-BBSKE derivative was 156 Da higher than that of BBSKE (*m/z* 425), and it is the same with IS, the molecular ion $[M+H]^+$ (*m/z* 609) was 156 Da higher than that of IS *m/z* 453. The molecular mass of 2-ME is 78 Da, and then 156 Da may be two units of 2-ME. Compared with $[M+H]^+$ ion of parent compound from both BBSKE and IS, the (156 + 22) Da higher units of $[M+Na]^+$

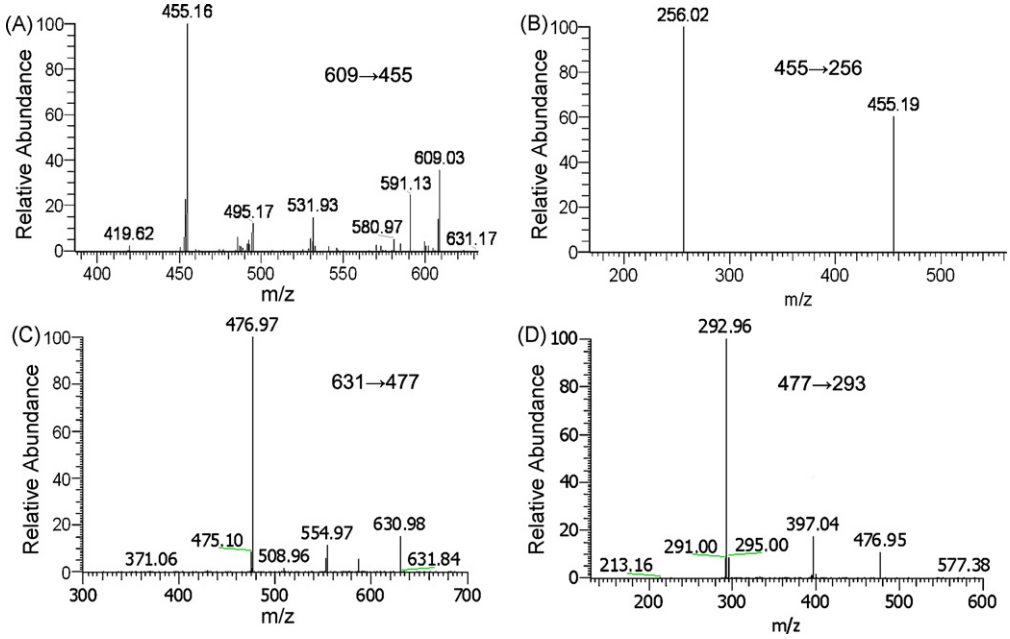


Fig. 9. The multi-stage mass spectra of 2-mercaptoethanol-1,4-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-butane (2-ME-IS) derivative in positive mode using electrospray ionization and in-source collisionally induced dissociation. (A) and (B) $MS^2$ and $MS^3$ spectra of the *m/z* 609 $[M+H]^+$ ion and (C) and (D) $MS^2$ and $MS^3$ spectra of the *m/z* 631 $[M+Na]^+$ ion.

Table 1
Mass spectrometry ($MS^n$) data of parent compounds and derivatives of 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-ethane (BBSKE) and internal standard (IS) using 2-mercaptoethanol (2-ME)

| Compound | Molecular mass | $MS^2$ | $MS^3$ | $MS^4$ | $MS^5$ |
|---|---|---|---|---|---|
| BBSKE | $[M+H]^+$ 425 | 226 | 185 | 157 | |
| 2-ME-derivatized BBSKE | $[M+H]^+$ 581 | 427 | 243 | | |
| | $[M+Na]^+$ 603 | 449 | 265 | | |
| Internal standard | $[M+H]^+$ 453 | 254 | 212 | 184 | 156 |
| 2-ME-derivatized IS | $[M+H]^+$ 609 | 455 | 256 | | |
| | $[M+Na]^+$ 631 | 477 | 293 | | |

ion were observed in above two derivatives. The fragment ions of derivatives showed the consistent behavior with those of parent compounds. As shown in Table 1, the loss of 184 Da in the $[M+H]^+$ ion of 2-ME-BBSKE ($m/z$ 427 → 243) and the $[M+Na]^+$ ion of 2-ME-BBSKE ($m/z$ 449 → 265) showed the characteristic fragment pattern of BBSKE. Similarly, the loss of 184 Da in the $[M+Na]^+$ ion of 2-ME-IS ($m/z$ 477 → 293) also indicated the characteristic fragment pattern of IS. Moreover, the loss of 199 Da in $[M+H]^+$ ion of 2-ME-IS ($m/z$ 455 → 256) was consistent with that in molecular ion of IS ($m/z$ 453 → 254). Therefore, it was presumed that the derivatized adducts might be produced through the formation of selenosulphides.

### 3.2. Method validation

#### 3.2.1. Specificity

Representative chromatograms of blank plasma, plasma spiked compounds and dog samples are shown in Fig. 10. BBSKE and internal standard were eluted with retention time 22 and 31 min, respectively. No chromatographic peaks in blank plasma at the retention times of analytes were observed, indicating that endogenous substances in plasma did not interfere with the HPLC analysis.

#### 3.2.2. Calibration curve and sensitivity

Calibration curve in plasma for BBSKE was linear in the concentration range of 0.05–2 μg/ml. The average regression equation was $y = -0.0131x + 0.0011$ ($r^2 = 0.9995$, $n = 5$). The lower limit of quantification (LOQ) and the limit of detection (LOD) of BBSKE were found to be 0.05 and 0.02 μg/ml, respectively.

#### 3.2.3. Precision, accuracy and stability

Intra- and inter-day precision and accuracy of BBSKE are shown in Table 2. The precision for intra-day ranged from 3.2 to 6.3% and for inter-day ranged from 5.2 to 6.4%, respectively. The accuracy expressed as relative error (% error) was less than 6.5% at all three QC concentrations. These results indicated that the method had good precision and accuracy.

The stability of BBSKE in different conditions was investigated. The data from the samples stored in −20 °C for 1 week and the analysis after three freeze–thaw cycles showed that BBSKE is stable under these conditions. The derivatized samples kept in autosampler vials can remain stable at room temperature for 24 h.

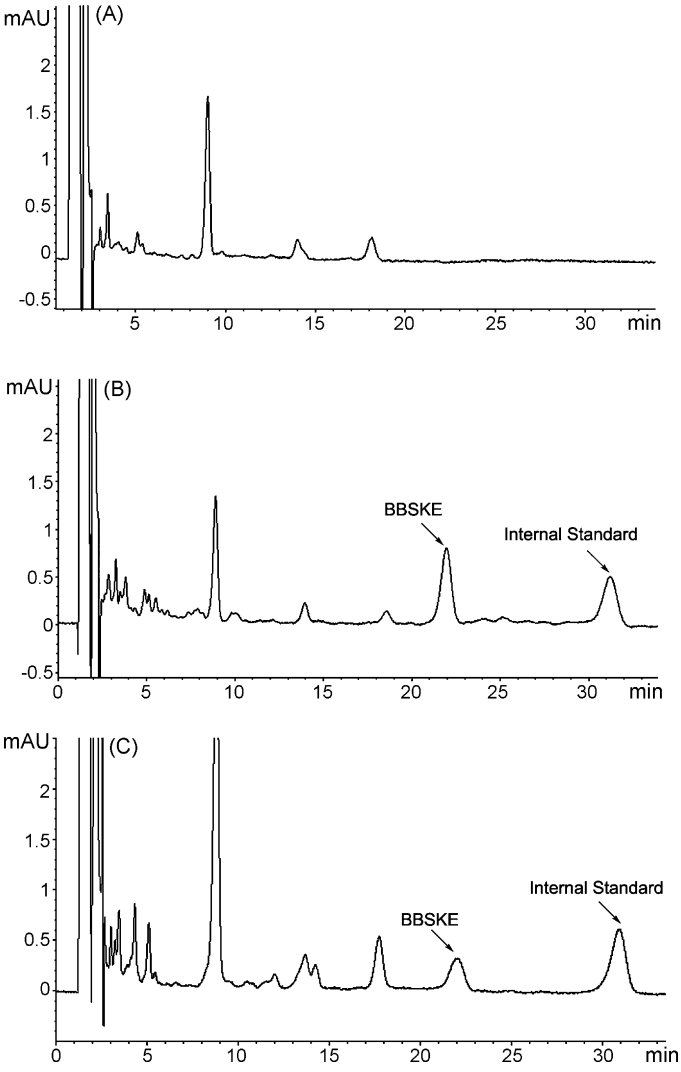


Fig. 10. Representative HPLC–UV chromatograms of (A) blank dog plasma, (B) blank plasma spiked with 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-ethane (BBSKE) at a concentration of 1 μg/ml and (C) a dog plasma sample 30 min after intravenous administration of BBSKE (0.79 mg/kg).

### 3.3. Application to the pharmacokinetic research in beagle dogs

The validated method was applied to pharmacokinetic research in beagle dogs. The mean plasma concentration versus time curve after intravenous (0.79 mg/kg) and oral (30 mg/kg)

Table 2
Precision and accuracy data for 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-ethane (BBSKE) measured by HPLC in plasma (data is based on assay of five replicates on three separate days. Mean ± SD)

| Actual concentration (μg/ml) | Observed concentration (μg/ml) | Intra-day precision (RSD, %) | Inter-day precision (RSD, %) | Accuracy (error, %) |
|---|---|---|---|---|
| 0.05 | 0.053 ± 0.004 | 6.3 | 6.4 | 6.5 |
| 0.2 | 0.194 ± 0.012 | 5.7 | 5.2 | −2.8 |
| 1.0 | 0.971 ± 0.040 | 3.2 | 5.5 | −2.9 |

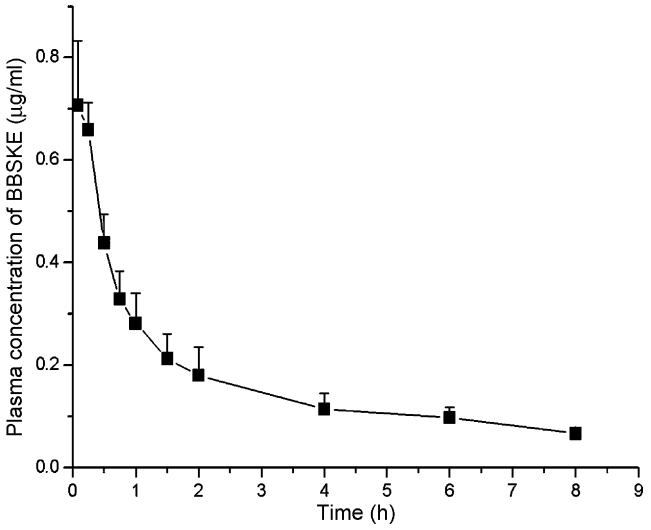


Fig. 11. The plasma concentration-time curve of 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-ethane (BBSKE) after a single dose (0.79 mg/kg) of intravenous administration in beagle dogs (mean ± SD, $n = 3$).

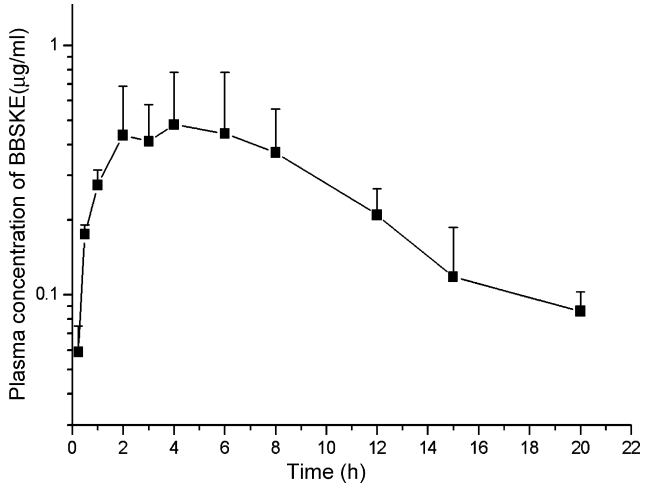


Fig. 12. The plasma concentration-time curve of 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]-ethane (BBSKE) after a single dose (30 mg/kg) of oral administration in beagle dogs (mean ± SD, $n = 3$).

administration are shown in Figs. 11 and 12, respectively. After oral administration of BBSKE, the $C_{max}$ of BBSKE detected in dogs was 0.51 ± 0.28 μg/ml and $T_{max}$ was 6.0 ± 2.0 h. The corresponding values for $t_{1/2}$ after oral and intravenous administrations were 4.6 ± 2.1 and 1.7 ± 0.6 h; the $AUC_{0-t}$ were 5.72 ± 2.42 and 1.35 ± 0.41 μg h/ml, respectively.

## 4. Conclusion

A new HPLC method has been developed for determining BBSKE in dog plasma with 2-ME as a derivatizing agent. Using this method with pre-column derivatization, BBSKE could be separated from plasma protein by 2-ME, which make it easy for BBSKE to be detected in plasma samples. The derivatives were identified by mass spectrometry. The accuracy, precision and sensitivity of HPLC method were satisfactory which was successfully applied for the evaluation of pharmacokinetic profiles of BBSKE in dogs.

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