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Validation of a sensitive gas chromatographic–mass spectrometric method for the simultaneous determination of β-elemene and β-elemenal in human plasma

Zhihang Chen[a,b], Yanxia Song[a,b], Jinjing Che[a,b], Xiaolei Liu[a,b], Yu Ning[a,b], Chengqi Shan[a,b], Yunan Hou[a,b], Yunlong Liu[a,b], Xiaohong Miao[a,b], Yuanguo Cheng[a,b,*]

[a] State Key Laboratory of Pathogen and Biosecurity, Beijing, PR China
[b] Laboratory of Drug Metabolism and Pharmacokinetics, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences (AMMS), No. 20, Dongda Street, Fengtai District, Beijing, PR China

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ABSTRACT

A sensitive gas chromatographic–mass spectrometric assay was described for determination of β-elemene and β-elemenal in human plasma, which has been successfully applied in clinical trial. After liquid–liquid extraction and gas chromatographic separation, the analytes were identified and quantitated. Calibration curves were linear in range from 31.25 to 8000 ng mL^{-1} and the limit of quantification for both was 31.25 ng mL^{-1}. Intra- and inter-day precision at three concentrations were 2.3–8.3% with accuracy of −8.5 to 6.1% for elemene and 3.0–9.9% with accuracy of −2.3 to 5.9% for elemenal. The method was validated to be suitable for further pharmacokinetic study.

1. Introduction

Elemene, a novel antineoplastic agent isolated from the Chinese medicinal herb Rhizoma Zedoariae, has been used to treat patients with malignant tumor in China, such as lung cancer and breast cancer [1]. Experimental and clinical data indicate that it has higher specificity and fewer side effects compared with those cytotoxic chemotherapeutic agents, especially no myelosuppression. Elemene exists as a mixture of α-, β-, γ- and δ-isomers. It has been demonstrated that the main antitumor active component of elemene is the β-isomer [2].

Elemene has been shown to exert broad and obvious antitumor activity *in vitro* and *in vivo* [3–5]. The cytotoxicity of elemene has been determined in various cell lines, which appears that the growth inhibitory effect of elemene on tumor cells is much stronger than on normal cells. Moreover it was also reported to be effective in alleviating the pain, decreasing the side effects of chemotherapy and improving the quality of life of patients in some clinical studies [6]. But until now, the mechanism of its action has not been systematically revealed. Some results indicate that it involves direct cytotoxic activities [7,8], such as the induction of cell cycle arrest and apoptotic cell death, the inhibition of the mitosis of tumor cells, the increasing of SOD activity and the inhibition of oxygen free radical formation, etc. In addition, the indirect immunostimulatory effects may also contribute to the antitumor activity of elemene.

The preliminary studies on absorption, distribution and excretion of elemene have been performed in animals using [^{3}H]-labeled method, gas chromatography [9], high-performance liquid chromatography [10,11]. Recently, the gas chromatography–mass spectrometry (GC/MS) method for determination of β-elemene in zedoary turmeric oil was also developed [12,13]. But the pharmacokinetic data of β-elemene in human has not been reported so far. The quantification limit of existing method for analysis of β-elemene was only about 100–200 ng mL^{-1}.

It was reported that there was a primary metabolite in the bile of rat after β-elemene intravenous administration, which was then analyzed, by mass spectrometry, nuclear magnetic resonance, infrared spectrometry and ultraviolet spectrometry [14]. It was confirmed that 4-isopropenyl of β-elemene was oxidized to be acraldehyde in rat bile metabolite and the molecular weight of the metabolite was added to 218. The structure of the metabolite in bile of rat was then identified to be 2-[(1-ethenyl-1-methyl-2-isopropenyl)-cyclohexyl]-acraldehyde, which was nominated as elemenal in this article. The chemical structure of β-elemene and β-elemenal was shown in Fig. 1. Our further work indicated that the latter seemed to be more effective than β-elemene in antitumor activity and may be developed to be another potent drug.

In this study, a prospective, open phase I clinical trial was conducted to evaluate the safety, tolerance and pharmacokinetics of β-elemene emulsion injection when it was administered to patients with lung cancer and brain metastases. So, we established a GC/MS method for simultaneous quantitation of β-elemene and

* Corresponding author. Tel.: +86 10 66948493; fax: +86 10 66948441.
E-mail address: chenzhihang@nic.bmi.ac.cn (Y. Cheng).

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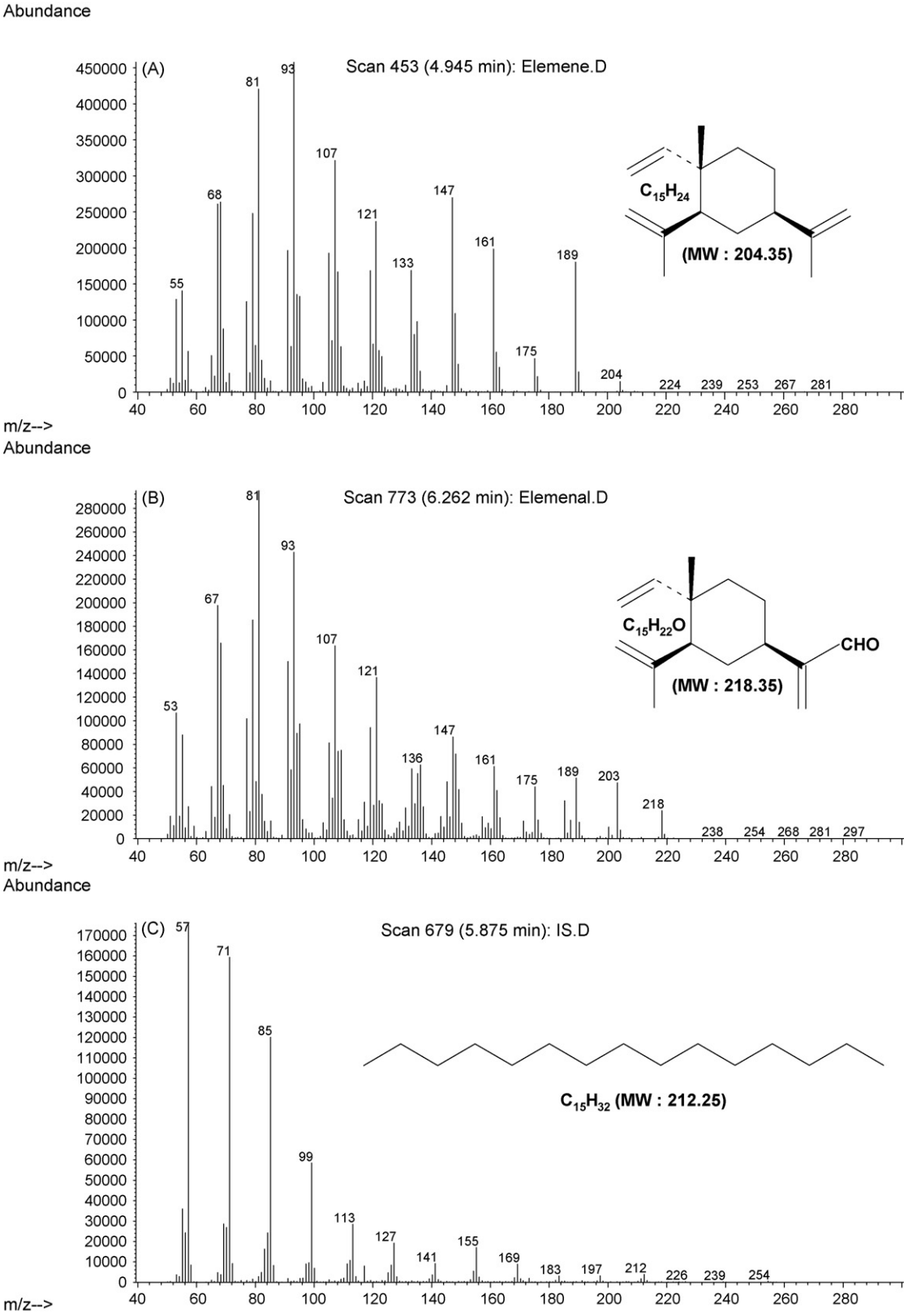

Fig. 1. EI mass spectra and structures for identification of β-elemene, β-elemenal and the internal standard *n*-pentadecane in SCAN mode. (A) β-Elemene; (B) β-Elemenal; (C) *n*-pentadecane (IS).

β-elemenal for the first time to reveal whether β-elemenal also existed in blood samples of human as an intermediate metabolite and assess the pharmacokinetic properties after β-elemene administration.

2. Experimental

2.1. Chemicals and reagents

Certified reference compounds of β-elemene and β-elemenal were provided by the YuanDa Pharmacy Inc. (Dalian, China), the purity was 99.2% (for elemene) and 95% (for elemenal); *n*-pentadecane (internal standard, IS), analytical-reagent grade (Beijing Chemical Reagent Company, China), the purity was 99.5%; *n*-hexane, analytical-reagent grade (Beijing Chemical Reagent Company, China).

2.2. Biological samples

Pooled blank human plasma samples were obtained from healthy volunteers in the ward of the Hospital of No. 307, PLA, China. Authentic plasma samples from cases of clinical trial had been submitted to our laboratory for analysis.

2.3. Sample preparation

The following description was applied to test samples as well as calibrators and QC samples. Firstly, a 20-μL volume of the IS working solution (equivalent to 10 μg IS) was added to the 1000-μL volume plasma in a 10-mL glass tube and then 5 drops of saturated NaCl solution were added into it. After standing still for up to 5 min, a 2-mL of *n*-hexane was added. The mixture was vortexed for 3 min and centrifuged at 4000 × *g* for 20 min. After the organic phase was transferred into a clean tube, the plasma sample was extracted again with a 2-mL of *n*-hexane. The organic phase was removed and evaporated under a gentle stream of nitrogen at room temperature (BF-2000 M, BFC Technologies Inc., Beijing, China). The residue was reconstituted in hexane (final volume was 500 μL). After vortexing for 30 s, a 1-μL aliquot of this solution was injected into the GC system.

2.4. Apparatus and operating conditions

An Agilent 6890N gas chromatograph with autosampler, split–splitless injector 7683 and mass-selective detector MSD 5973i was used and connected to a personal computer with Enhanced ChemStation software for parameter control, raw data acquisition and analysis (Agilent Technologies, USA).

2.4.1. Gas chromatographic conditions

The GC conditions were as follows: column, HP-1ms capillary column of 15 m × 0.25 mm ID (No. 19091S-931, Agilent), crosslinked 100% dimethylsilicone, 0.25 μm film thickness; splitless injection mode; injection port temperature, 250 °C; injection volume, 1 μL; carrier gas, helium; flow-rate, 1.0 mL min^{-1}; temperature program, initial temperature 70 °C, raised to 130 °C at 50 °C min^{-1}, then to 170 °C at 10 °C min^{-1}, finally and quickly up to 300 °C at 50 °C min^{-1}, hold time 1.0 min; solvent delay, 1.2 min. With a total run time of approximately 9.0 min, the retention time of β-elemene, β-elemenal and IS was equal to 2.93, 4.18 and 3.75 min, respectively.

2.4.2. Mass spectrometric conditions

The mass-selective detector was set to the selected ion monitoring (SIM) mode for quantitative analysis with *m*/*z* 93 (for β-elemene and β-elemenal) and *m*/*z* 212 (for IS). The others were as follows: electron ionization (EI) mode; ionization energy, 70 eV; transferline temperature, 280 °C.

2.5. Data acquisition and analysis

Data acquisition was performed using Enhanced ChemStation software (Agilent Technologies, USA). Calibration curves were constructed using the peak area ratio of analyte to IS by weighted least square linear regression. Test samples and quality control samples were then interpolated from the calibration curves to obtain the concentrations of the respective analytes.

2.6. Assay validation

The GC–MS assay was validated for the quantification of β-elemene and β-elemenal according to the criteria established by Lindner and Wainer [15].

2.6.1. GC–MS identification and optimization

Prepared working solution with certified reference standard compounds in *n*-hexane which contained β-elemene, β-elemenal and *n*-pentadecane (IS), respectively, were operated as 'Apparatus and operating conditions' (cf. Section 2.4) except for the following: full scan mode (SCAN), *m*/*z* 40–300; temperature program, initial temperature 70 °C, raised to 170 °C at 15 °C min^{-1}, then to 300 °C at 50 °C min^{-1}, hold time 3.0 min; solvent delay, 3.0 min.

2.6.2. Calibration and quality control (QC) samples

Stock solutions were prepared with certified reference standard compounds in *n*-hexane, enveloped and stored at −70 °C. The working solutions which simultaneously contained equal amounts of β-elemene and β-elemenal were prepared by serially diluted of the stock solutions with *n*-hexane to obtain final drug concentrations of 80, 40, 20, 10, 5, 2.5, 1.25, 0.62, and 0.31 μg mL^{-1}, separately. The stock solution of the IS was prepared in hexane and diluted to 0.05%.

Calibrators and QC samples were prepared by spiking equal volumes (100 μL) of the above working solutions of β-elemene and β-elemenal into 900-μL blank plasma to yield the final plasma concentrations of 8000, 4000, 2000, 1000, 500, 250, 125, 62.5, and 31.25 ng mL^{-1} (for calibrators) or 2000, 500 and 62.5 ng mL^{-1} (for QC samples). The following procedures were the same as described above (cf. Section 2.3).

2.6.3. Plasma samples assay

The mixed blank plasma from five separated persons were analyzed for peaks interfering with the detection of the analytes or the IS. Additionally, cross-interference was investigated by analyzing β-elemenal in samples spiked with β-elemene and *vice versa*. Calibration standards with concentrations ranging from 31.25 to 8000 ng mL^{-1} were assayed for *linearity of calibration*. For determination of the limit of detection (LOD, signal-to-noise ratio greater than 3:1), quality control samples with 7.8–15.6 ng mL^{-1} (n = 3) of β-elemene and β-elemenal were assayed. The criteria for the limit of quantification (LOQ, signal-to-nose ratio greater than 5 and reproducibly with the precision and accuracy of ±20%) were fulfilled by the lowest point of the calibration curve (31.25 ng mL^{-1}). Quality control samples in the low (2000 ng mL^{-1}), medium (500 ng mL^{-1}) and high (62.5 ng mL^{-1}) concentration ranges of β-elemene and β-elemenal were isolated and analyzed. Each sample was injected five times within a single sequence and during the course of five consecutive sequences alternately to evaluate *repeatability*. Quality control samples (n = 5) at three concentrations of β-elemene and β-elemenal (as above) were assayed against a calibration curve to determine the *intra-day accuracy*. The concentrations of the analytes were calculated by using a weighted

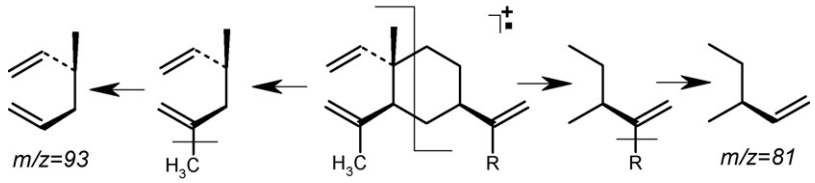

Fig. 2. The fragmentation pathway from the molecular ions. elemene (R = CH_3); elemenal (R = CHO).

least square linear regression model and then compared to the actual concentrations. The calculated values at each concentration were averaged and the percentage bias was calculated to estimate accuracy. The relative standard deviation (RSD) was calculated as a criterion of precision. The *inter-day accuracy and precision* of the method was assessed from the comparison of the analysis of control samples ($n = 5$) on each of five consecutive days in the above-mentioned manner. *Analyte stability* for long-term storage was tested by analyzing spiked samples ($n = 5$) before and after storage for 6 months at −70 °C. Stability for short-term (12 h) at room temperature, 48 h refrigeration at 4 °C and three freeze–thaw cycles at −70 °C were also determined. The samples were analyzed together with a freshly prepared calibration curve. *Matrix effects* for analytes were evaluated by comparing the peak areas of analyte in extracted samples of blank plasma spiked with known concentrations with the corresponding peak areas obtained by direct injection of standard solutions. Matrix effects for the IS and the interference between β-elemene and β-elemenal were also observed. The *extraction recoveries* of β-elemene and β-elemenal at three QC levels ($n = 5$) were determined by comparing peak area of the analytes obtained from plasma samples with the analytes spiked before extraction to those spiked after extraction.

2.7. Application in pharmacokinetic study of phase I clinical trial

Plasma samples from authentic cases of phase I clinical trial were assayed with the described method.

3. Results and discussion

3.1. Sample preparation

Due to the complex nature of plasma, a sample pre-treatment was often needed to remove potential interferences prior to GC/MS analysis. Currently, the most widely employed biological sample preparation methodologies are protein precipitation (PPT) [16,17], solid phase extraction (SPE) [18,19], and liquid–liquid extraction (LLE) [20,21]. Comparatively speaking, the PPT procedure has the advantage of simplicity but more background impurities left which may reduce the sensitivity, and the SPE has the perfect extractive advantage but be much more time- and money-consuming which is not suitable for masses of sample detection in clinical trials. Hence our approach of developing an assay for β-elemene and β-elemenal in plasma finally proceeded based on LLE with organic solvents. Aether was the primary solvent for extraction. However, its evaporability was too strong to control and resulted in losses of our analytes. Moreover, the residue after concentration always had something, which may cause interference and be difficult to remove. Diethyl ether, ethyl acetate, *n*-hexane and some solvent combinations in use were tested, finally *n*-hexane was adopted because of its high extraction efficiency and less interference. Saturated NaCl solution was added into the plasma samples before extraction to reduce emulsification and accelerate separation. Since both analytes were high evaporable materials like perfume, the process of solvent volatilization needed to be operated carefully to reduce losses. In fact, losses during evaporation may be compensated by the IS as indicated by the good linearity, accuracy and precision (see blow). To avoid adherence of plastic tubes, a sample preparation method of repeated liquid–liquid extraction in glass tubes was used and it was found with satisfactory results.

3.2. EI spectra of standard compounds and quantitative optimization

Prepared working solutions contained β-elemene, β-elemenal or *n*-pentadecane (IS), respectively, were operated as 'Assay Validation' (cf. Section 2.6.). With a total time of approximately 12.3 min, the retention time of β-elemene, β-elemenal and IS were equal to approximately 4.94, 6.26 and 5.87 min, respectively. The EI mass spectra of the relative compounds were listed in Fig. 1(A–C). The full scan spectra revealed that β-elemene and β-elemenal had similar major fragment ions, which were observed at m/z 81, 93 and 107. The most abundant ion m/z 93 was then chosen for simultaneous quantification of β-elemene and β-elemenal to improve the sensitivity considering their different retention time values. The fragmentation pathway of the main daughter ion from the molecular ion was shown in Fig. 2 and the process of β-elemenal was similar to that of β-elemene after the aldehyde group broke down from the parent molecule. *n*-Pentadecane was used as internal standard here for lack of other available and more suitable commercially compound and our further experiment approach showed that it had similar extraction characteristics and better chromatographic resolution with a retention time between those of the β-elemene and β-elemenal. Since the higher abundant ion m/z of IS was rather small and there may be higher background noise if chosen to be monitored, the molecular ion m/z 212 was used for qualification of IS in the SIM mode then.

3.3. Validation data

3.3.1. Peak purity and selectivity

Mass chromatograms of mixed blank plasma sample are presented in Fig. 3(A). It was shown that there was no significant interference at the retention time of β-elemene, β-elemenal or IS in blank matrix. No significant matrix effect for analytes (β-elemene RSD = 2.7%, β-elemenal RSD = 6.1%) and IS (RSD = 5.0%) was observed for five different plasma pools which indicated that no undetected co-eluting compounds that could influence the detection of the analytes. In addition, the β-elemenal did not interfere with β-elemene and *vice versa*.

3.3.2. Calibration curves and limit of quantification (LOQ)

A calibration curve was consist of the blank sample (plasma matrix sample processed without internal standard), the zero sample (matrix sample with internal standard), and replicates of 9 non-zero samples covering the expected range. Chromatographic raw data were automatically analyzed by the MSD Enhanced Chemstation software. Using internal standardization, calibration curves were constructed by plotting relative response ratio of analyte to IS *versus* amount ratio of analyte to IS and fitting linear equations using $1/a^2$-weighting. It was linear from 31.25 to 8000 ng mL^{-1}

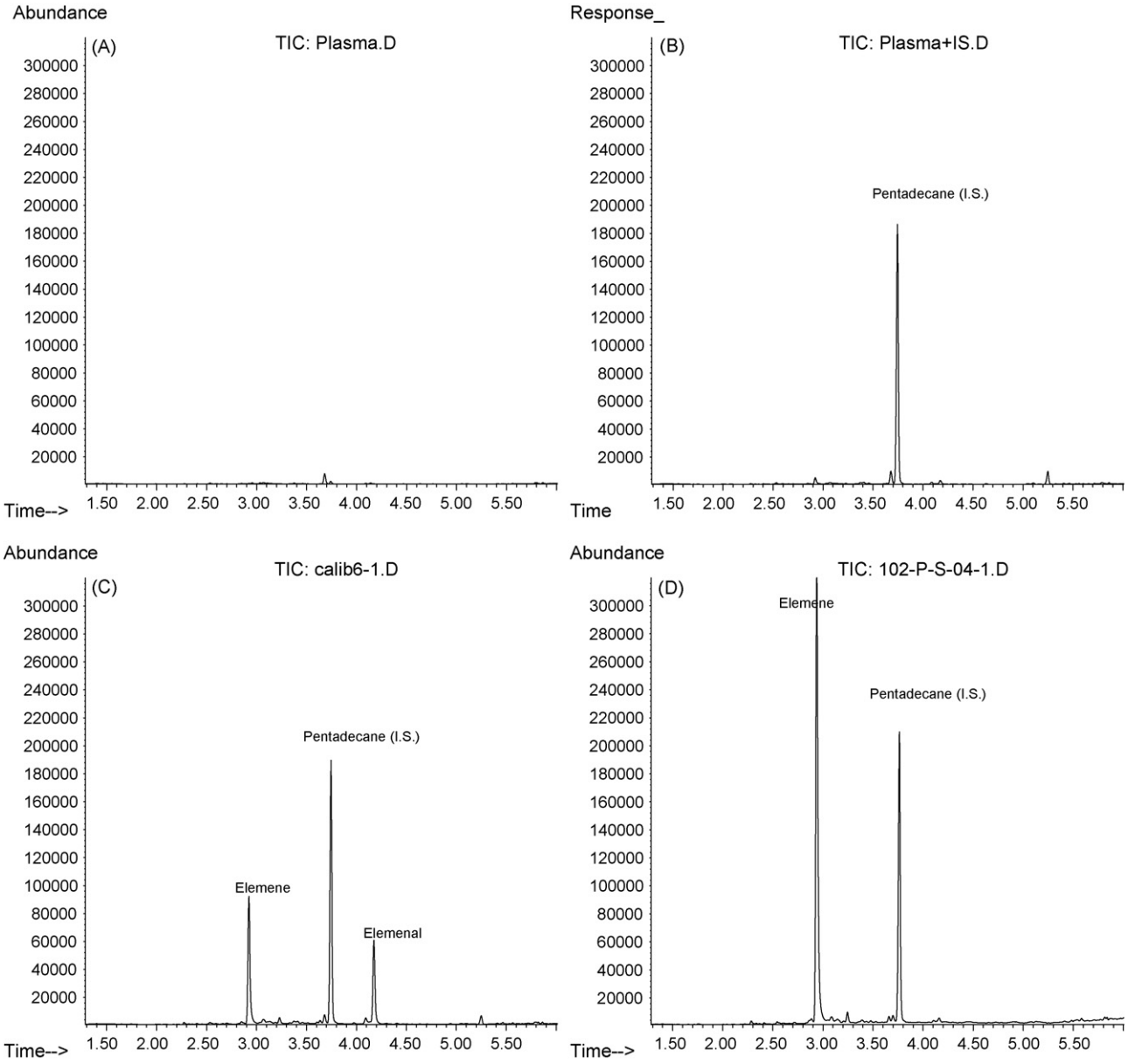

Fig. 3. Merged mass chromatograms with the ions *m/z* 93 (β-elemene and β-elemenal) and *m/z* 212 (IS) in SIM mode. (A) Blank plasma sample; (B) zero plasma sample (only IS added); (C) calibrator sample (contained 1000 ng mL^{-1} of β-elemene, β-elemenal and equivalent IS as above); (D) unknown sample (at 3 h after administered with β-elemene of 5 mg kg^{-1} d^{-1} in No. 102 patient).

for β-elemene ($Y = 5.56 \pm 0.04A + 0.0069 \pm 0.004$) and β-elemenal ($Y = 3.63 \pm 0.07A + 0.0028 \pm 0.001$) with correlation coefficient (r^2) values of 0.997 and 0.993, respectively.

LOQ is determined by 5–7 replicates of samples at lowest amounts of calibrators (31.25 ng mL^{-1}) on three consequent days. If the following conditions were met, the lowest amount of calibrators should be accepted as LOQ: (i) S/N (signal-to-noise ratio) of analytes should be at least 5; (ii) the analytes response should be reproducible with a precision and an accuracy of maximum ±20%. With the accuracy and precision of ±7 and ±14%, respectively, 31.25 ng mL^{-1} was defined as LOQ of the assay for determination of β-elemene and β-elemenal. The LOD was 7.8 ng mL^{-1} for β-elemene and 15.6 ng mL^{-1} for β-elemenal with the S/N at least 3.

3.3.3. Repeatability, precision and accuracy

Quality control samples in the low and high concentration ranges of β-elemene and β-elemenal were isolated and examined in the indicated manner to evaluate repeatability (cf. Section 2.6.3). RSD values for β-elemene and β-elemenal were 2.0 and 3.8% for the low QC samples and 1.6 and 2.7% for the high QC samples.

The precision and accuracy validation of each assay comprised the analysis of five replicates of spiked matrix samples per concentration level along with blank matrix samples per day. Usually 3 levels in the working range were concurrently investigated and the validation experiment was repeated on five consequent days. The precision and the accuracy should not exceed ±15%.

The intra- and inter-day precision and accuracy data for the assay were shown in Tables 1 and 2. The intra-day precision and accuracy were 2.3–6.8% and −8.5 to 4.5% for β-elemene, with those of β-elemenal ranged from 3.0 to 9.9% and from −1.0 to 5.9%, respectively. The inter-day precision and accuracy were 7.7–8.3% and −(1.8–6.1)% for β-elemenal, with those of β-elemenal ranged from 6.5 to 9.5% and −2.3 to 2.1%, respectively.

Table 1
Intra-day precision and accuracy of the determination of β-elemene and β-elemenal in plasma.

Intra-day ($n=5$)	Actual concentration (ng mL^{-1})	Mean calculated concentration (ng mL^{-1})		Precision[a] (%)		Accuracy[b] (%)	
		β-Elemene	β-Elemenal	β-Elemene	β-Elemenal	β-Elemene	β-Elemenal
Low QC	62.5	57.2	63.2	2.9	6.4	−8.5	1.0
Medium QC	500	522.7	495.2	6.8	9.9	4.5	−1.0
High QC	2000	2015	2118	2.3	3.0	0.8	5.9

[a] Precision = (SD/mean) × 100.
[b] Accuracy = [(mean calculated concentration − actual concentration)/actual concentration] × 100.

Table 2
Inter-day precision and accuracy of the determination of β-elemene and β-elemenal in plasma.

Inter-day ($n=25$) 5 days	Actual concentration (ng mL^{-1})	Mean calculated concentration (ng mL^{-1})		Precision[a] (%)		Accuracy[b] (%)	
		β-Elemene	β-Elemenal	β-Elemene	β-Elemenal	β-Elemene	β-Elemenal
Low QC	62.5	58.7	63.8	8.3	6.5	−6.1	2.1
Medium QC	500	491.0	488.7	7.9	7.9	−1.8	−2.3
High QC	2000	1924	1970	7.7	9.5	−3.8	−1.5

[a] Precision = (SD/mean) × 100.
[b] Accuracy = [(mean calculated concentration − actual concentration)/actual concentration] × 100.

3.3.4. Recovery

The extraction recovery was determined by spiking analytes (to reach three QC levels) and IS into blank plasma matrix before (a) and after (b) extraction procedure, respectively, and calculating the quotient of signal intensities *a*:*b* in %. The mean recoveries of the LLE procedure were 78.8 ± 4.1 and 84.9 ± 7.2% for β-elemene and β-elemenal, respectively. To IS, the mean extraction recovery was 77.1 ± 5.0%.

3.3.5. Stability

The storage stability of the analytes in the plasma matrix was assessed at intended storage temperatures for different periods (6 months at −70 °C, 48 h refrigeration at 4 °C and 12 h at room temperature) and the influence of freeze–thaw cycles was studied at −70 °C for at least 3 cycles. The results showed no significant differences under these conditions, which were summarized in Table 3.

3.4. Application in pharmacokinetic study

Authentic plasma samples from a prospective, open, phase I clinical trial was conducted using the method described above. In accordance with the declaration of Helsinki, 26 patients (16 males and 10 females, aged 33–60) were recruited in this dose-ascending study. The subjects were randomly assigned to six sequential dose groups (10, 15, 20, 25, 30 and 35 mg kg^{-1} d^{-1} β-elemene) administered intravenously once daily on days 1–5 with 4 weeks as one cycle. Initially β-elemene was given in a 3-h period via a continuous infusion pump for 10, 15 and 20 mg kg^{-1} groups. The results indicated that the observed peak plasma concentration (C_{max}) profiles were 7.6 ± 4.7, 9.6 ± 0.9 and 10.6 ± 3.2 μg mL^{-1}, respectively, with a peak time (T_{max}) of 3 h postadministration. After that the plasma concentration decreased quickly with a elimination half time ($T_{1/2\beta}$) between 1.91 and 2.41 h and a systemic clearance rate (*Cl*) of 0.54–0.68 L kg^{-1} h^{-1}. The peak plasma concentration (C_{max}) and area under curve (AUC) were prone to increase in proportion to the dose in all groups, but there were no significant differences among *Cl* values which showed linear pharmacokinetic properties of β-elemene in the range of dosages. The duration with higher plasma levels beyond 1 μg mL^{-1} (minimum effective concentration *in vitro*) was no more than 5 h which was insufficient to some extent for analytes to exert antitumor activity. Therefore, subsequent groups of patients may receive a longer infusion period (8 h) to improve efficacy. The plasma concentration

Table 3
Stability of β-elemene and β-elemenal in plasma ($n=5$).

	Actual concentration (ng mL^{-1})	Mean calculated concentration (ng mL^{-1})		Precision[a] (%)		Accuracy[b] (%)	
		β-Elemene	β-Elemenal	β-Elemene	β-Elemenal	β-Elemene	β-Elemenal
Long-term (6 months)							
Low QC	62.5	61.1	54.6	3.8	5.6	−2.2	−13
Medium QC	500	544.4	555.7	6.3	6.6	8.9	11
High QC	2000	2077	2207	4.8	4.7	3.9	10
Short-term (48 h)							
Low QC	62.5	58.4	61.8	2.4	5.6	−5.8	−1.1
Medium QC	500	534.8	550.1	1.5	2.9	7.0	10
High QC	2000	1923	2000	10	8.8	−3.8	0.0
RT (12 h)							
Low QC	62.5	59.0	63.8	6.8	5.4	−5.5	2.1
Medium QC	500	491.3	527.1	10	11	−1.7	5.4
High QC	2000	2108	2255	14	14	5.4	13
Freeze–thaw (3 cycles)							
Low QC	62.5	65.8	65.1	4.3	9.4	5.3	4.2
Medium QC	500	507.6	536.9	8.9	7.4	1.5	7.4
High QC	2000	2068	2127	4.8	3.9	3.4	6.3

[a] Precision = (SD/mean) × 100.
[b] Accuracy = [(mean calculated concentration − actual concentration)/actual concentration] × 100.

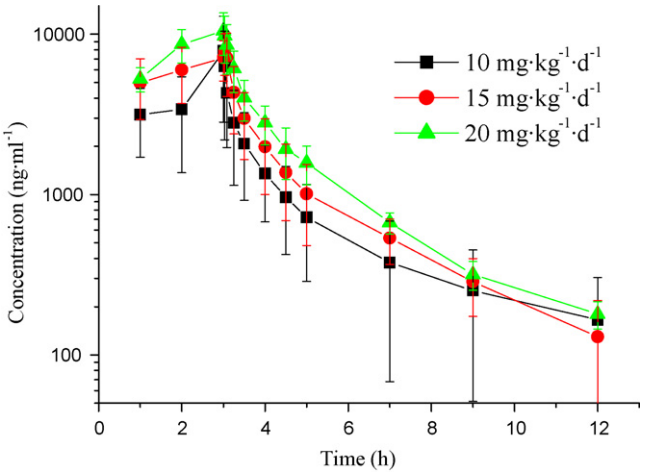

Fig. 4. Concentration–time profiles for β-elemene administered during 3 h on day 1.

time profile for β-elemene was presented in Fig. 4 (partial data only).

It was shown that β-elemenal was not detected in plasma. Although some evidences indicated its existence in rat bile after β-elemene administration, β-elemenal was surely not an intermediate metabolite in human plasma and urine (data not shown). Moreover, further work may be needed to determine the process of β-elemene *in vivo*.

4. Conclusion

The GC–MS assay reported here allowed the precise and sensitive identification and quantification of β-elemene and β-elemenal in human plasma, which involved a sample preparation, by LLE with satisfactory recovery. The method fulfilled the requirements for a validated assay. The assay has proved to be efficient in pharmacokinetic study of phase I clinical trial and provided some useful information for the adjustment of clinical regimen. No detectable β-elemenal was found in plasma in our study and there is no other obvious homologous fragments else after careful search, which indicated that β-elemene may be chiefly decomposed into some small hydrophilic metabolites in human body. In addition, the possibility that β-elemenal exists in human bile as metabolite should be further confirmed, because this may be valuable for therapy of certain diseases such as carcinoma of gallbladder.

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