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# Determination of alpha-bisabolol in human blood by micro-HPLC–ion trap MS and head space-GC–MS methods

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**Abstract**

Alpha-bisabolol is a compound present in some essential oils, widely distributed in several plants, including camomile. Two different methods for analysing an essential oil, such as alpha-bisabolol in human blood are reported: the first uses micro-liquid chromatography–electrospray ionisation-mass spectrometry (μHPLC–ESI-MS), whereas the second is based on "head space" injection coupled to gas chromatography–mass spectrometry (HS-GC–MS). For LC–ESI-MS, human blood samples, spiked with alpha-bisabolol, were extracted with hexane and evaporated to dryness under air stream. The residue was then reconstituted with methanol and injected into a C18 column, connected to an ion trap mass spectrometer equipped with an ESI source. Spectra were recorded in the positive ion, selected ion monitoring mode. The detection limit of alpha-bisabolol in blood was 0.125 μmol/l. The preparation of samples for the analysis in HS-GC–MS was limited to blood dilution with water (0.5 ml blood + 1 ml water). Head space vials were heated at 125 °C for 1 h before automatic injection. The HS-GC–MS detection limit (0.13 μmol/l) was similar to that achieved with the μHPLC–ESI-MS method. Successful tests were performed to verify if alpha-bisabolol could be directly measured by the HS-GC–MS method in different biological samples (blood, urine, faeces, homogenate tissues) from rats treated with the camomile essential oil.


*Keywords:* Alpha-bisabolol; Blood; μHPLC–ESI-MS method; HS-GC–MS method

## 1. Introduction

Alpha-bisabolol is an essential oil naturally present in several plants, including *Chamomilla recutita* and *Achillea millefolium* L., with structure of unsaturated monocyclic sesquiterpene alcohol (mw 222.37 Da, density 0.929 g/ml, boiling point 153 °C at 12 Torr), which has found wide application in cosmetic products. Fig. 1 shows the molecular structure of alpha-bisabolol and those of its oxides isolated in camomile, as it was proposed by Isaac [1]. Terpens are a large family of more than 7500 molecules produced in the plant metabolism, all deriving from the mevalonic acid molecule. In medicine and pharmacology monoterpens, which include alpha-bisabolol, are the most important group of terpens extractable from several species of plants, fruits and seeds and often used as traditional natural remedies [2]. As a matter of fact, antimicrobic, antiphlogistic and anticancer activities of monoterpens have been reported "in vitro" [3–6]. According to Elegbede et al. [3] the addition of D-limonene to the diet of rats could modify the process of mammary tumour induction exerted by 7,12-dimethylbenz[*a*] anthracene; a similar effect was observed for tumours induced by nitrosomethylurea [4]. In addition, the growth suppression of P388 leukaemia cells [5] and of cultured tumour cells [6] by geraniol, an acyclic monoterpenoid alcohol, was reported in the literature.

Also, beside known antiphlogistic and antipeptic actions [7–11], alpha-bisabolol has recently been investigated in view of a possible anticancer activity [12]. In the frame of this

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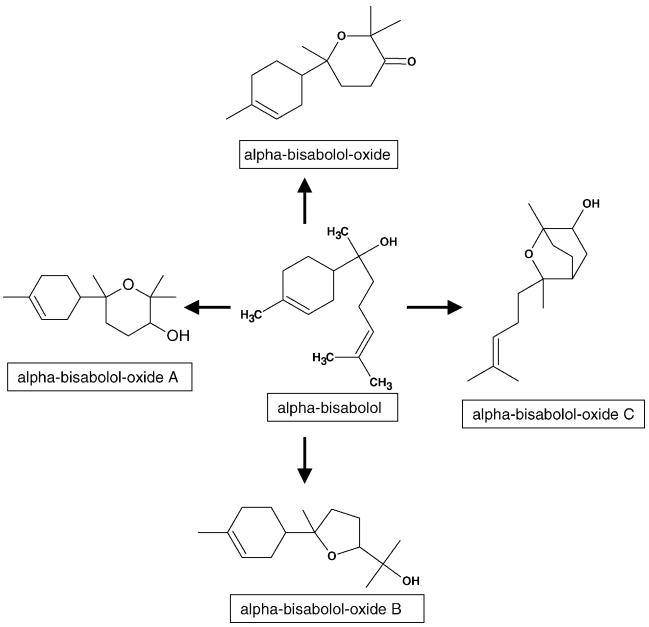


Fig. 1. Molecular structure of alpha-bisabolol and some of oxidation products isolated from camomile [1].

research, alpha-bisabolol accurate and precise determination in blood and in other biological fluids and tissues was needed. Quite surprisingly, to the best of our knowledge, no instrumental methods for alpha-bisabolol analysis in biological samples are reported in the literature.

Aim of the present work was the development and validation of methods suitable for facing the different analytical problems, which are encountered in the analysis of a natural product, such as alpha-bisabolol, in different biological samples. In particular, head space-gas chromatography (HS-GC) coupled with mass spectrometry (MS) was chosen due to the ability of this technique to deal with volatile molecules in complex matrices. On the other hand, because of a higher compatibility with delicate biological molecules, also micro-HPLC coupled with ion-trap MS was used, particularly in view of a possible application to the determination of alpha-bisabolol metabolites.

## 2. Experimental

### 2.1. Reagents and standards

Alpha-bisabolol was purchased from Sigma (St. Louis, MO, USA). All the organic solvents (methanol, acetonitrile, formic acid and hexane) were purchased from Carlo Erba (Milan, Italy) and were of HPLC reagent grade or better. A stock solution of alpha-bisabolol was prepared in methanol (1 mg/ml corresponding to 4.5 mmol/l) and stored in a refrigerator at +4 °C. Calibration curves in blank blood (seven points) were prepared from this solution in the concentration range from 0.25 to 10.00 μmol/l.

Whole blood (anticoagulant:heparin) collected from two healthy researchers from our laboratory were used for the preparation of the calibration curves and for the quality control specimens, which were spiked with adequate amounts of standards. Samples were maintained at −20 °C until analysis, which was performed within 7 days after collection.

### 2.2. Instrumentation

(a) *μHPLC–ESI-MS*: All experiments were performed on a Series 1100 liquid chromatograph–mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The system was composed of a model 1100 gradient micro-HPLC with a model SL ion trap mass spectrometer with an electrospray ionisation (ESI) source. The HPLC was equipped with a reversed phase column packed with Zorbax SB $C_{18}$ (150 mm × 0.5 mm i.d., 5 μm, Agilent).

(b) *HS-GC–MS*: All experiments were performed with a model 7694E head space autosampler (Hewlett-Packard, Palo Alto, CA, USA) connected to a model 6890 gas chromatograph (Hewlett-Packard), interfaced with a model 5973 mass detector operating in the electron impact (EI) mode. The gas chromatograph was equipped with a SPB1 column (30 m × 0.32 mm i.d., 1 μm film thickness, Supelco, Bellefonte, PA, USA). Rubber septa lined with PTFE (150 °C proof) were obtained from Analytical Technology (Milan, Italy).

### 2.3. Sample preparation and analysis

(a) *μHPLC–ESI-MS*: Five hundred microlitres of human blood diluted with 1 ml of water was added with 3500 μl of hexane in polypropylene tubes. After manual mixing for 5 min and centrifugation at 3500 rpm for 10 min, the organic phase (2800 μl) was collected and evaporated to dryness under air stream. The residue was finally reconstituted with 500 μl of methanol, 0.3 μl of which was injected.

The isocratic chromatographic conditions were as follows: mobile phase, 20 mmol/l formic acid; pH 3, methanol (85:15); flow rate, 20 μl/min; temperature, 25 °C.

The MS conditions were: nebulizer gas, $N_2$ (15 psi); drying gas $N_2$ (4 ml/min); source temperature 325 °C; capillary voltage, 5000 V; fragmentator voltage, 40 V; ionisation mode, positive; mass scan range, *m/z* 50–300. The MS was operated in selected ion monitoring mode (SIM).

(b) *HS-GC–MS*: 500 μl of human blood was added with 1 ml of distilled water and, after gentle mixing, the mixture was transferred into glass “head space” vials (12.5 ml volume), which were closed with 20 mm rubber PTFE lined septa (150 °C proof) and crimped with perforated aluminium seals. After 60 min equilibration time on a rotating shaker at room temperature (22–23 °C), samples were placed in the auto-sampler where they were heated at 125 °C and shaken for 60 min before the head space

was withdrawn. The head space auto-sampler was provided with a 1 ml loop kept at 180 °C; the transfer line, heated at 200 °C, was connected with the injector of the GC (split 1:20) via an interface heated at 250 °C. The oven temperature was kept at 100 °C during the injection (1.5 min) then the temperature was increased at 30 °C/min up to 170 °C. After 1 min, the temperature was risen to 240 °C at a rate of 25 °C/min and kept at 240 °C for 2 min. Helium was used as the carrier gas at the flow rate of 2 ml/min. Under these conditions, the retention time of alpha-bisabolol was 8.02 min. The mass detector ion source operating in electron impact mode was kept at 280 °C. The solvent delay time was 4 min and the dwell time 50 ms. The mass spectrometer was operated in selected ion monitoring mode on ions at *m*/*z* 109, 119 and 204. The quantification was based on the peak areas of the ion at *m*/*z* 119.

### 2.4. Calibration, detection limit and repeatability

Eight blood samples spiked with alpha-bisabolol to obtain concentrations of 0, 0.25, 0.5, 1, 2, 4, 8 and 10 μmol/l were used for the preparation of the calibration curves and for calculating the detection limits (LOD) [13]. The repeatability of the assays, expressed as coefficient of variation (CV), was estimated by repeated analysis of blood samples spiked with 1 and 4 μmol/l of alpha-bisabolol. Five determinations per day of each concentration were performed in three non-consecutive days. Accuracy was estimated by the closeness of agreement between measured concentration of alpha-bisabolol and its theoretical concentration in spiked blood samples (1 and 4 μmol/l).

## 3. Results and discussion

In the early part of the present research, it was clearly found that alpha-bisabolol was mainly present in the red blood cells, whereas less than 10% was in plasma. On the basis of these data, whole blood was used after haemolysis by dilution 1:3 with bidistilled water.

### 3.1. μHPLC–ESI-MS

Preliminary experiments by direct infusion of alpha-bisabolol diluted in the mobile phase (1 μg/ml) allowed the optimization of the ionisation/ion trap conditions. Fig. 2 shows the mass spectrum of alpha-bisabolol (50 ng/ml) and its postulated fragmentation. The most abundant ion was at *m*/*z* 205, clearly resulting in the source from the $M - H^+$ (*m*/*z* 223) species by loss of a water molecule. This ion was used for quantification. A daughter fragment ion at *m*/*z* 149, generated by loss of water was used for confirmation. Other smaller fragments (*m*/*z* 135, 121, 109, 95) were of non-clarified origin.

The isocratic, reversed phase μHPLC provided rapid (less than 3 min), although not highly efficient, separation

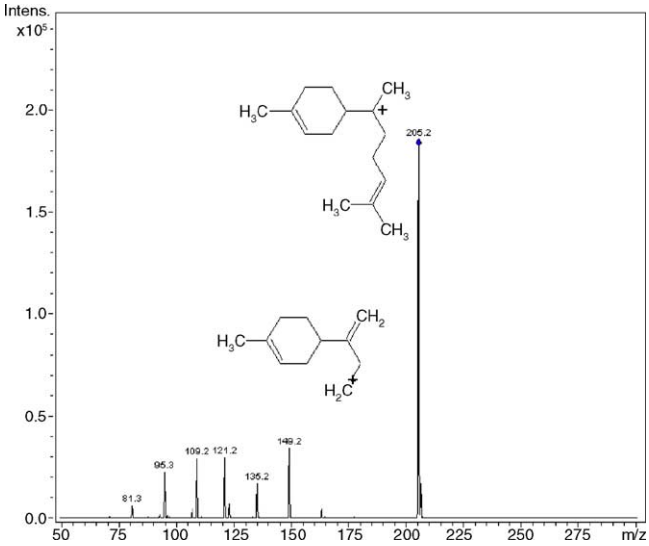


Fig. 2. Mass spectrum in ESI–ion trap MS of alpha-bisabolol and its postulated fragmentation.

of alpha-bisabolol. Unfortunately, the constrains in mobile phase composition dictated by the requirements of ESI-MS, did not allow the optimization of the chromatographic performances. However, the selectivity of MS detection produced clean chromatograms, without interferences from endogenous compounds. Fig. 3 shows the chromatograms of (A) extract ions at *m*/*z* 205 (corresponding to alpha-bisabolol) in blank human blood and (B) the same ions from a blood sample spiked with 2 μmol/l alpha-bisabolol standard.

The mean recovery from blood was about 45% using hexane. Methylene chloride gave worse results in terms of either recovery and cleanliness of extracts.

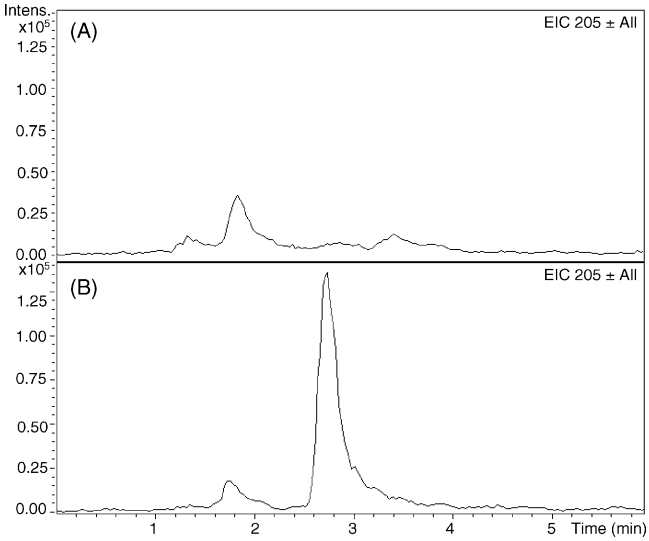


Fig. 3. (A) Single ion mass chromatogram corresponding to a blank blood sample. (B) Single ion mass chromatogram corresponding to a blood sample added with 2 μmol/l of alpha-bisabolol. The alpha-bisabolol (*m*/*z* 205) has a retention time of about 3 min.

Table 1
μHPLC–ESI-MS: intra-day and day-to-day precision (CV) and accuracy (recovery, %) calculated on concentrations of 1 and 4 μmol/l of alpha-bisabolol in human blood (each measure is the mean of five samples)

| | Alpha-bisabolol (1 μmol/l) | | Alpha-bisabolol (4 μmol/l) | |
|---|---|---|---|---|
| | CV | Recovery (%) | CV | Recovery (%) |
| Day 1 | 3.9 | 97.8 | 2.0 | 95.9 |
| Day 2 | 2.0 | 102.5 | 2.9 | 100.9 |
| Day 3 | 3.4 | 99.7 | 2.2 | 100.7 |
| Overall | 2.0 | 100 | 2.8 | 99.17 |

Accuracy and precision data of the method are detailed in Table 1. Overall, the CVs were less than 4%. The LOD, calculated at a signal-to-noise ratio ≅3, was 0.12 μmol/l.

### 3.2. HS-GC–MS analysis

Fig. 4 shows the electron impact ionisation mass spectrum of alpha-bisabolol. In comparison to the ESI spectrum, electron impact shows a much higher fragmentation of difficult interpretation, with dominant ions at *m*/*z* 109, 119 and 205. Fig. 5 shows the single ion chromatogram at *m*/*z* 119 of a blank human blood sample and of the same sample added with 2 μmol/l of the alpha-bisabolol. The analyte is univocally characterised on the basis on its retention time and mass-to-charge ratio. The calibration curves of alpha-bisabolol in blood were linear in the range investigated; the LOD was 0.13 μmol/l.

Calibration curves with points at 0, 0.3, 0.5, 1, 2, 4, 6, 8 and 10 μmol/l determined in three non-consecutive working days yielded comparable results with slopes ranging between 19.53 and 20.34 and correlation coefficients between 0.9992 and 0.9998.

Table 2 summarizes the intra- and inter-day precision (CVs) and accuracy (recovery, %) data calculated by analysis of human blood on three different days during 2 weeks. The concentration of alpha-bisabolol was 1 and 5 μmol/l in blood, respectively.

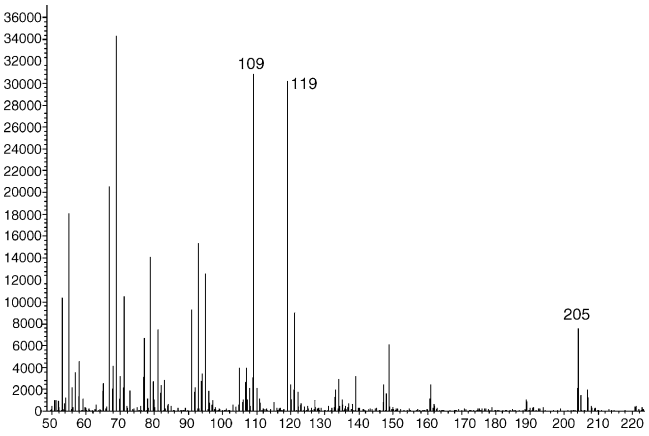


Fig. 4. Electron impact mass spectrum of alpha-bisabolol obtained with a GC–MS analysis.

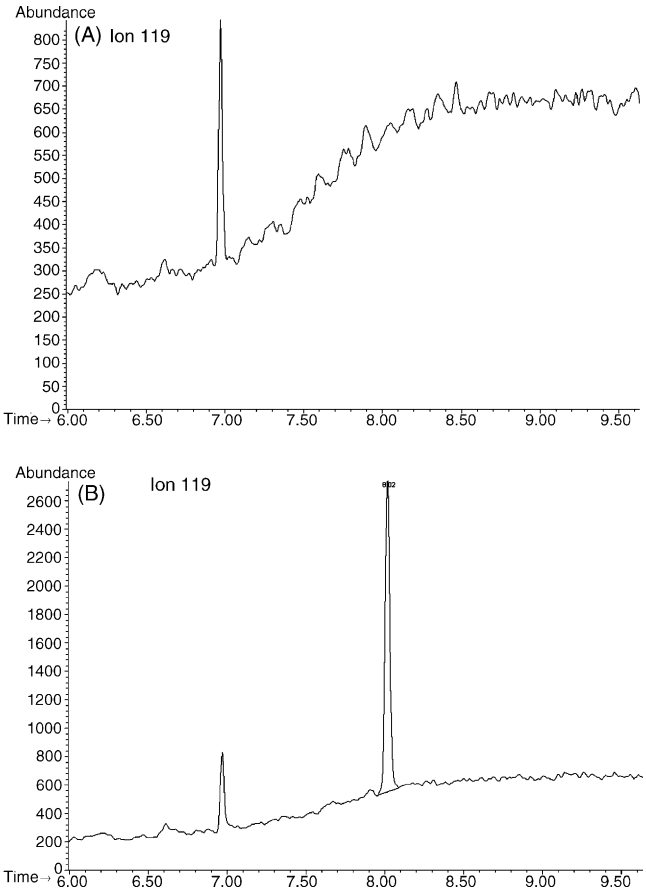


Fig. 5. Single ion mass chromatogram (*m*/*z* 119) corresponding to a blank blood sample (A) and the corresponding chromatogram after addition of alpha-bisabolol (*m*/*z* 119) (2 μmol/l) (B). For conditions, see text.

In conclusion, alpha-bisabolol analysis by μHPLC–ESI-MS, because of the need of delicate sample extraction, proved more complicated and time consuming than the HS-GC–MS method. We tried to use different analogues as internal standard, but the results were not reliable enough for their adoption because of too high differences in extraction recovery; it is likely that only the use of deuterated alpha-bisabolol, currently not available on the market, would be required to overcome completely this problem. On the other hand, μHPLC–ESI-MS has proved able to provide rapid and accurate analyses of this natural product.

Table 2
HS-GC–MS: intra-day and day-to-day precision (CV) and accuracy (recovery, %) calculated on concentrations of 1 and 5 μmol/l of alpha-bisabolol in human blood (each measure is the mean of five samples)

| | Alpha-bisabolol (1 μmol/l) | | Alpha-bisabolol (5 μmol/l) | |
|---|---|---|---|---|
| | CV | Recovery (%) | CV | Recovery (%) |
| Day 1 | 5.1 | 91.5 | 5.3 | 102.9 |
| Day 2 | 3.1 | 96.1 | 3.6 | 107.8 |
| Day 3 | 2.7 | 96.4 | 5.1 | 100.7 |
| Overall | 2.9 | 94.7 | 3.5 | 103.6 |

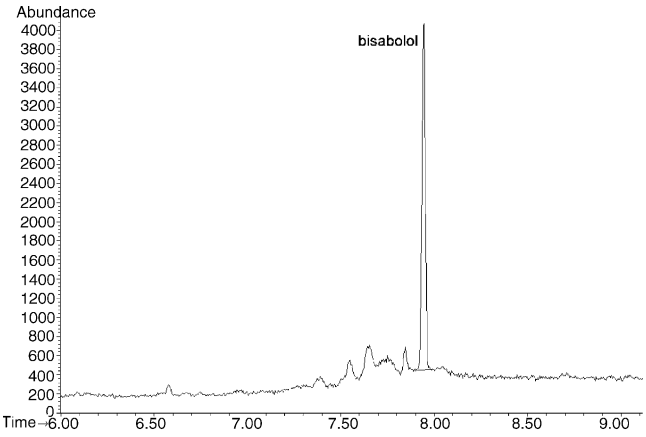


Fig. 6. Single ion mass chromatogram (*m*/*z* 119) corresponding to a faecal sample obtained from a rat treated with alpha-bisabolol. For conditions, see text.

The analysis of alpha-bisabolol with the "head space" technique, coupled with a gas chromatograph and a mass spectrometry proved rapid and highly satisfactory. The preparation phase is extremely short. After having adjusted the conditions for heating the samples (temperature and equilibration time) the analyses yielded reliable results. The automatic injection of the "head space" make unnecessary the use of an internal standard.

The reported conditions were also used to analyse biological samples (blood, urine, faeces and different tissues) obtained from rats treated with alpha-bisabolol. Results were highly encouraging, since no potentially interfering compounds were found in biological media. The high selectivity of head space sampling allowed the analysis of alpha-bisabolol in particularly complex samples. Fig. 6 shows a chromatogram from rat faeces.

In conclusion the two methods for measuring alpha-bisabolol in human blood, here reported, proved reliable and sensitive enough for being used in pharmacokinetic studies. The HS-GC–MS method is easier and more rapid than the μHPLC–ESI-MS method; on the other hand, HPLC, being not dependent on the volatility of the analyte, is potentially more suitable for studying alpha-bisabolol together with its more polar metabolites in metabolism studies.

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