Original Research Article

High Performance Liquid Chromatography Method for the Determination of Anethole in Rat Plasma

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Abstract

Purpose: *To identify and quantify anethole in the essential oil of fruits of Illicium verum Hook (star anise) and in vivo in rat plasma using reverse-phase liquid chromatography.*
Methods: *Anethole was identified in the essential oil of the fruits of Star anise and determined by gas chromatography-tandem mass spectrometry (GC-MS), nuclear magnetic resonance (NMR), ultraviolet visible spectrophotometry (UV-VIS). A simple, sensitive and validated high performance liquid chromatography (HPLC) technique with UV-VIS detection method was developed for the determination of the compound in rat plasma using: methanol-water (85:15, v/v) as mobile phase at a flow rate of 0.2 ml/min Hypersil ODS Thermo (150 mm × 2.1 mm × 3.0 μM) as column with wavelength detection at 259 nm.*
Results: *GC determination showed that anethole in the essential oil of star anise exhibited a retention time of 21.02 min. The validation results for anethole in plasma were satisfactory, with coefficient of determination (R^2) of 0.9945 and relative standard deviation of < 3 %. HPLC run time of 4 min with a retention time of 2.73 min was the faster method to determine anethole when compared to a previously reported method which had a run time of 15 min.*
Conclusion: *Anethole in the essential oil of Illicium verum Hook can be identified and determined by GC-MS, NMR and UV-VIS, and a superior HPLC method has been developed for the determination of the compound in rat plasma.*

Keywords: *Anethole, High performance liguid chromatography, Star anise, Essential oil, Rat plasma, Illicium verum Hook.*

INTRODUCTION

Anethole or trans-anethole (Trans-1-methoxy-4-[prop-1-enil] benzene) [1,2] is present as a major component in many essential oils, such as those obtained from star anise (*Illicium verum Hook*) [1,3], *Pimpinella anisum* [4], and *Foeniculum vulgare* [5]. Plants that contain anethole are used in cooking as a condiment and flavoring in alcoholic drinks [2]. Anethole has been utilized in folk medicine because of its soothing and antispasmodic properties [3]. Antiviral activity has

been demonstrated for herpes simplex virus type 1 [6], in addition to antimicrobial [4,5] and antifungal [4] activity. The identification and quantification of essential oils are usually performed by gas chromatography (GC) [2,5,7] combined with other techniques [5,7,8] because of the chemical characteristics of these compounds.

In contrast to the majority of papers in the literature that have reported pharmacokinetic profiles obtained using high-performance liquid

chromatography (HPLC) [9,10], pharmacokinetic studies of 1-8 cineole [11] in the plasma of humans, a compound that is present in many essential oils, such as rosemary [11] and ginger [8], have been conducted using GC. Methodological analysis of substances in plasma are extremely important, and the best method for this type of quantification is HPLC because GC is very time-consuming.

In the scientific literature, several analytical methods for anethole have been described [1, 2, 12]. For this compound, chromatographic identification, quantification, and analysis, even by HPLC, are time-consuming, making bioavailability studies impractical when the number of samples is large and the total analysis time is prolonged. They are also unviable because of the high use of solvents and high costs.

EXPERIMENTAL

Acquisition of anethole

Fruits of star anise (*Illicium verum Hook*) were collected from the herbarium of medicinal plants of State university of Maringa, Maringa, Brazil, identified and authenticated, by Dr. Maria Serti, a botanist of botanical department of State University of Maringa. A voucher specimen was deposited in the herbarium of State University of Maringa (no. 11612) They were dried and used to obtain the essential oil in which Anethole is present. These fruits were placed in a distillation flask with two outputs in the presence of drag steam distillation using the Clevenger method was then performed. After the essential oil was obtained, it was subjected to steam distillation, with the purpose of obtaining Anethole at the highest purity possible.

Chemicals

For the HPLC analysis and sample extraction, we used HPLC-grade methanol as the reagent (JT Baker, Phillipsburg, NJ, USA). Deionized water was purified using a Milli-Q water purification system (Millipore, Billerica, MA, USA).

Analysis by NMR and GC-MS

The chemical composition of the oil was determined by analyzing the nuclear magnetic resonance (NMR) spectra, model Mercury plus BB, (Varian Inc, USA) of carbon (^{13}C; 75.5 MHz) and hydrogen (^{1}H; 300 MHz). The solvent used for the dissolution of the samples was deuterated chloroform ($CDCl_3$). The chemical shifts are expressed in parts per million, and the coupling constants (J) are expressed in Hertz (Hz). Together the NMR, to determine the purity of Anethole, gas chromatography coupled with mass spectrometry (GC-MS) was performed (Thermo Electron Corporation Focus GC model).

GC-MS analysis were performed using a quadrupole mass spectrometer detector, with an impact electron ionization (EI) source operating at 70 eV. The identification of the compounds was determined by analyzing and comparing the retention index observed in the chromatogram with the indices described in the literature. The chromatograms of the samples were recorded under the following conditions: DB-5 capillary column (30 m x 0.32 mm I.D., 0.50 μm), initial column temperature of 250 °C, an initial time of 1 min at 60 °C, ramp heating to 180 ºC at 3 ºC/min, injector temperature of 220 °C, and helium as the carrier gas at constant flow rate of 1.0 ml/min. The injected volume was 1 μl of the sample dissolved in acetone (1:10).

UV-VIS spectrum from photodiode array detector

The HPLC equipment, model Pro Star (Varian Inc, USA), coupled with a photodiode array detector (PDA) was used to determine the optimal wavelength for the analysis of Anethole. This test used the following parameters: methanol-water (86:14) mobile phase at a flow rate of 1.2 ml/min, reverse-phase C18 column (SGE; 150 mm × 4.6 mm × 5 μm), and a scanning spectrum of 210 - 400 nm. The volume of the injection was 15 μl.

Standard solution

The standard solution was prepared by dissolving the Anethole standard in HPLC-grade methanol to obtain a solution with a 1000 μg/ml concentration and stored at 4°C.

Animals

The experiments were performed using the plasma of rats that weighed 220-280 g, were fasted from food and water, and were obtained from the central vivarium of the State University of Maringa. The rats were housed at 22 ± 2 °C under a 12 h/12 h light/dark cycle with artificial light. The experimental protocol was approved by the Ethics Committee on Animal Experimentation of the State University of Maringa (CEAE/UEM 066/2010) and was conducted in accordance with the internationally accepted laboratory animal use, care and Guidelines of Institute for Laboratory Animal Research (ILAR) [13]. The blood of the rats was collected in a syringe that contained heparin and centrifuged at 10,000

rotations per minute (rpm) for 10 min at room temperature to obtain the plasma.

Sample preparation

The samples were prepared by spiking blank rat plasma with a standard solution of anethole. Plasma proteins were precipitated by adding 0.3 ml of cold HPLC-grade methanol. The sample was vigorously homogenized for 2 min and centrifuged at 10,000 rpm for 10 min at 4 °C.

Instruments and chromatographic conditions

The chromatographic system was composed of a high-performance liquid chromatograph, model 2695, with autosampler, (Waters Corp, Milford, MA, USA), UV- VIS detector, model 2487 (Waters Corp., Milford, MA, USA), and Empower One software. In the development of an analytical method for Anethole using HPLC, different mobile phases were used to obtain optimal results. The best method developed used the following parameters: methanol-water (85:15, v/v) mobile phase, 0.2 ml/min flow rate, Hypersil ODS THERMO column (150 mm × 2.1 mm × 3.0 µM), 259 nm wavelength, and 25 µl injection volume.

Validation of method for the analysis of anethole in plasma

The validation of the method used several tests recommended by the Brazilian National Agency of Sanitary Surveillance (ANVISA) and U.S. Food and Drug Administration (FDA) [14,15], including specificity, selectivity, linearity, precision, and accuracy. To evaluate the stability of the sample, we performed post-processing, short-term temperature, and freeze-thaw stability tests [14,15].

To evaluate specificity and selectivity, a chromatographic comparison of the standard anethole solution in methanol and contaminant-free rat plasma was conducted. A detectable interference in the chromatogram in the rat plasma blank at the same retention time of Anethole, could not be > 20 % when compared to that peak area of the lower limit of quantification (LLQ) of anethole.

The calibration curves were constructed using quantification with an external standard and in relation to linear regression based on the peak area of anethole in plasma. Seven different concentrations were used (0.2 - 3.0 µg/ml). The determinations of the linearity of the calibration curves were performed in triplicate and obtained from the peak area in relation to plasma concentrations. Using the linear regression as model, in which a value of R^2 greater than 0.99 was expected.

The precision and accuracy of the method were performed at three different analysis. In each one analysis, three concentration of control samples were evaluated (low, medium, and high concentration of anethole in plasma), in quintuplicate, according to ANVISA and FDA recommendations [14,15]. The data of precision and accuracy were evaluated by intra-batch and inter-batch. A low quality control (QCL) must be three-fold more than or equal to the LLQ, which has a concentration of 0.2 µg/ml. Therefore, a concentration of 0.6 µg/ml was used as the QCL. The medium quality control (QCM) is the average between the low and high quality controls and used a concentration of 1.7 µg/ml. The rules define that high quality control (QCH) needs to be between 75 and 90 % of the concentration of the highest point on the calibration curve; therefore, a concentration of 2.7 µg/ml was used.

Precision was expressed as the relative standard deviation (%RSD), which should be < 15 %. Accuracy (%) was determined as the ratio between the average of the determined concentrations and nominal concentration, with a deviation < 15 %.

The extracted samples recoveries were established by comparing the peak area of plasma samples with those direct injections of a standard solution of anethole in methanol at the same concentrations and conditions.

The test of short-term stability, in which the sample remained for 6 h at room temperature. Post-preparative stability tests were then performed, in which the sample was extracted and injected into the HPLC column 2, 4, 6, 8, 10, 12, and 24 h after extraction. Finally, a freeze-thaw stability test was performed after a first melting cycle, in which a sample curve calibration is prepared and analyzed and another aliquot is frozen and thawed 24 h later. The results were compared to determine the stability test results.

Plasma anethole determination

The rats (*n* = 8) were treated by gavage with anethole at 250 mg/kg. Plasma (300 µl) was collected from the animal 0 min, 15 min, 30 min, and 1, 2, 4, 8, 12 and 24 h after administration. The samples were prepared for subsequent HPLC analysis.

Statistical analysis

Statistical analysis, including linear regression, was carried out by ANOVA. To prove the significance of the regression, F- test was employed. The level of significance used was $p < 0$ or $p < 05$ while the software used was Microsoft Office Excel, version 2010.

RESULTS

GC-MS and NMR spectra

The chromatogram of the essential oil from star anise (Fig 1) obtained by GC-MS showed that anethole has a retention time of 21.02 min. Identification was made using a single mass spectrum, and the mass found for anethole was 148 mz (Fig 2).

^{1}H and ^{13}C NMR spectra also showed identiified anethole, and just like GC-MS, NMR indicated the presence of some contaminants, but these did not interfere with the identification of the main compound.

NMR [1]H (300 MHz, $CDCl_3$): 7.27 (dt, 2H, H-3 and H-5, $^3J_{H_3,H_2}$ and $^3J_{H_5,H_6}$ = 8.7 Hz, $^4J_{H_3,H_5}$ and $^4J_{H_5,H_3}$ = 3.0 Hz), 6.84 (dt, 2H, H-2 and H-6, $^3J_{H_2,H_3}$ and $^3J_{H_6,H_5}$ = 8.7 Hz, $^4J_{H_2,H_6}$ and $^4J_{H_6,H_2}$ = 3.0 Hz), 6.35 (dq, 1H, H_a, $^3J_{H_a,H_b}$ = 15.7 Hz, $^4J_{H_a,H_c}$ = 1.6 Hz), 6.10 (dq, 1H, H_b, $^3J_{H_b,H_a}$ = 15.7 Hz, $^3J_{H_b,H_c}$ = 6.6 Hz), 3.79 (s, 3H, H_d), 1.86 (dd, 3H, H_c, $^3J_{H_c,H_b}$ = 6.5 Hz, $^4J_{H_c,H_a}$ = 1.5 Hz).
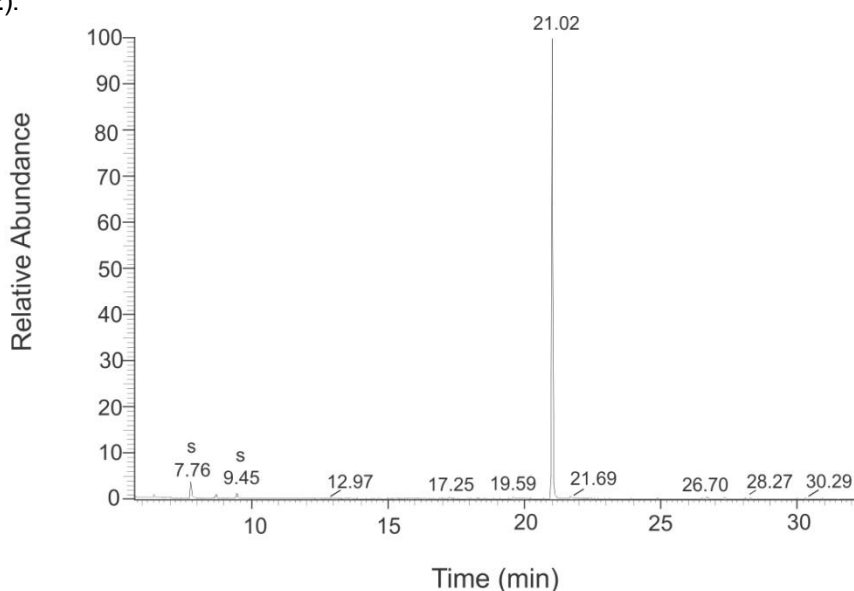

Fig 1: Chromatogram of the essential oil of *Illicium verum Hook* obtained by GC-MS; retention time of anethole = 21.02 min
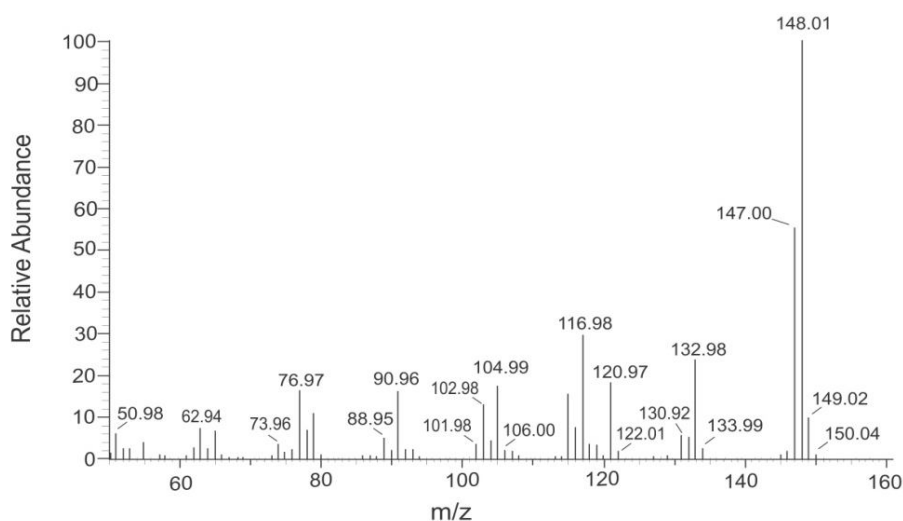

Fig 2: Identification by mass spectra; the mass found for anethole was 148 mz

Table 1: Precision and accuracy data

Parameter	Found value (mean ± SD, µg/ml)	Nominal value (µg/ml)	Accuracy (%)	RSD (%)
QCL	0.544±0.005	0.60	90.74	0.926
QCM	1.741±0.047	1.70	102.44	2.715
QCH	2.786±0.013	2.70	103.21	0.486

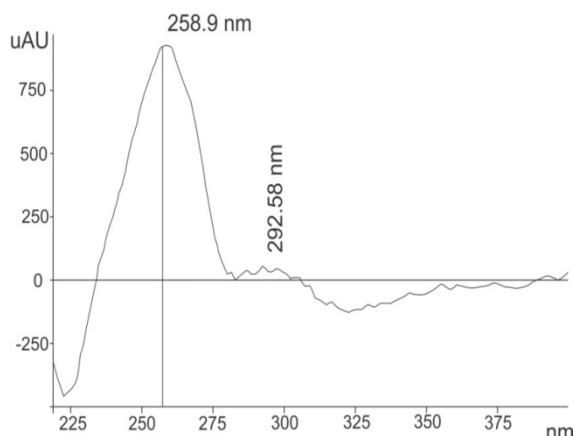*LQC = low quality control; **MQC = medium quality control; ***HQC = High quality control.

Fig 3: UV-VIS spectrum showing maximum wavelength of absorption for anethole is 259 nm.

NMR ^{13}C (75 MHz, CDCl$_3$): 158.7 (C-1), 131.0 (C-4), 130.5 (C-8), 127.0 (C-3 and C-5), 123.6 (C-9), 114.0 (C-2 and C-6), 55.4 (C-7), 18.6 (C-10).

The results of GC-MS and NRM show that anethole was the major compound in the essential oil, and the content was approximately 9 8%.

UV-VIS spectra

The results show that the optimum wavelength for the analysis of this compound was 259 nm (Fig 3).

Chromatograms

The wavelength utilized was 259 nm [1]. HPLC run time using these parameters was 4 min, with a retention time of 2.73 min.

Extraction efficiency

The best method for extracting this type of substance is the protein precipitation method, which was very effective and exhibited good performance, with high sample recovery of approximately 96 %.

Validation data for anethole in plasma

No significant interference was found in rat plasma at the same retention time of anethole, proving that endogenous substances did not interfere with the high specificity and selectivity of the method. The calibration curve was linear over the seven points used within the range of 200 to 3000 ng/ml in plasma. Each point on the curve was obtained from three replicates. The regression equation obtained was Y = 5.379x + 108.300, with regression coefficient (R^2) = 0.9945. R^2 value was > 0.98, which is the minimum recommended value for linearity [14,15].

Precision (inter-batch) determination using %RSD indicate a value < 3 % for three levels of quality control (Table 1); accuracy (inter-batch) was in the range of 90.74 to 103.21 % (Table 1). Precision was consistent with reports that assume a maximum deviation of 15 % and standards established for the validation method. Accuracy was also met the standard specifications [14,15].

The test of 6 h exposure to room temperature showed that the samples showed stability under these conditions, indicating that they could be kept at room temperature during extraction. The post-processing stability of the sample at the initial time (time zero) and 16 h after extraction showed no no important variations ($p < 0, 05$). However, this did not occur 24 h after extraction when the calibration curve showed no linearity. The samples were considered unstable because significant variation was observed compared with the sample analysis results at time zero and the test of Freeze-thaw stability was performed only once because the sample did not need to be reused during the validation. The results indicated no loss of stability during this cycle.

Anethole profile *in vivo*

The results showed that the C_{max} of anethole was approximately 1 h after administration of the substance, and peak concentration was a mean of 2.1 µg/ml of rat plasma (Fig 4). Anethole was
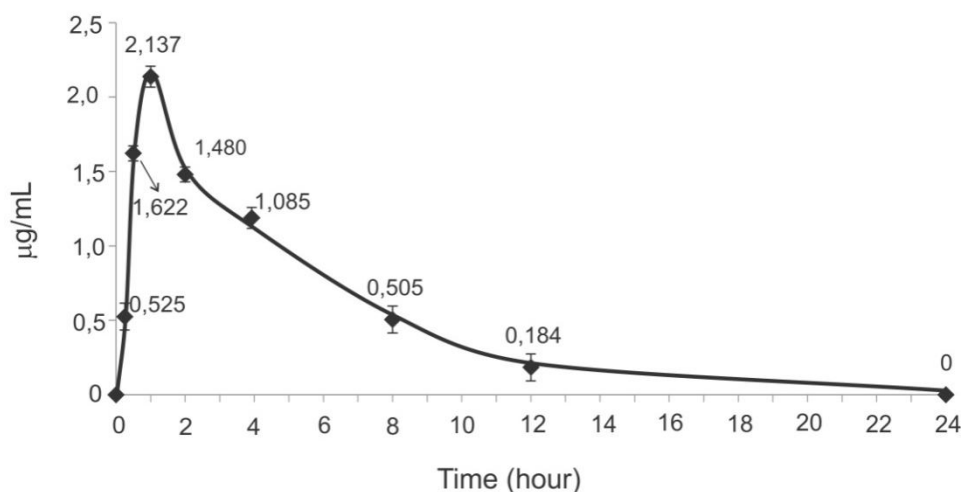
Fig 4: Anethole profile in rat plasma

not detectable in plasma 24 h after administration.

DISCUSSION

The mass spectrum of 148 mz was obtained by GC-MS for anethole, and the results of GC-MS and NMR show that anethole is the major compound in the *Illicium verum Hook* (star anise) star anise oil, approximately 98 %, and is consistent with the literature for this substance [16]

A UV-VIS analysis was performed to determine the best wavelength for the analysis and validation of the anethole quantification method. The result found is consistent with literature data [1,3].

The high proportion of methanol in the mobile phase was justified by the fact that Anethole is practically insoluble in water. Therefore, this analytical method for Anethole was faster than the LC method [1,4]. In cases of validating large quantities of biological samples, the HPLC method is viable and usually presents very fast analysis time for single substances, in contrast to GC method, which is extremely slow.
The extraction method proposed in this study involved precipitation of the proteins with methanol as the precipitant. Several substances, such as methanol, ethanol, acetonitrile, and perchloric acid, have been shown to be good precipitatants of endogenous substances that can interfere with chromatographic analysis [17-18].

Another methodology for the extraction of samples is liquid-liquid extraction using, for example, dichloromethane as the solvent. However, this extraction method is not effective because chromatography with reverse-phase

C18 column was used in the present study, which requires the sample to be dry and re-suspended in methanol. Indeed, this method was not feasible in our experiments because anethole is an essential oil and therefore volatile, and would be evaporated when the sample is dried.

An internal standard is desirable, but it was not essential in the present method because the external standard could effectively evaluate the samples prepared in plasma. The present results were considered satisfactory in accordance to references [14,15], without need of an internal standard, similar to another quantitative method in the literature [19,20].

Altogether, the present results indicate that the test had remarkable reproducibility with acceptable accuracy and precision for the parameters determined, as stipulated by FDA FDA [15].

The results obtained from the stability tests indicate that the method is robust and stable. Post-processing stability test of the samples showed that the samples were stable for a long time after extraction and thus could be extracted and stored in the autosampler for long periods of time. Therefore, performing serial extractions and leaving the samples in standby mode in the HPLC are feasible. The test after the first melting cycle was performed according to FDA and ANVISA regulations [14,15]. This test becomes important in cases of thawing a sample several times to remove some portions, which may reduce sample stability. Therefore, this test has great importance for stability.

The proposed method using HPLC was effective to determine the pharmacokinetic profile of anethole. Indeed, we can assert that this method

would be suitable for the analysis of similar drugs, and even in clinical studies of anethole.

CONCLUSION

The present results indicate that anethole can be identified and determined by GC-MS, NMR, and UV-VIS, and that methanol is a suitable solvent for anethole extraction in plasma. Thus, a simple and sensitive HPLC method has been successfully developed and validated for the determination of anethole in rat plasma.

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