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Original Research Article

One Step Extraction Method, Sample Preparation Procedure for HPLC/MS Analysis of Altrenogest Sesame Oil Solutions [Extraction of altrenogest from sesame oil with acetonitrile, HPLC/MS]

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Abstract: The extraction of altrenogest with acetonitrile from sesame oil is not an easy task due to the formation of a stable emulsion. The problem of extracting more than 99% of altrenogest from sesame oil with acetonitrile was solved. The optimal ratio of extractant to extractor was established. This approach allowed for a quantitative analysis of altrenogest in sesame oil solutions by HPLC/MS. A generalization of the proposed approach to cases of altrenogest extraction from stable emulsions was discussed.

Keywords: Sesame oil emulsion, extraction, altrenogest, HPLC/MS.

Abbreviations:
ACN – Acetonitrile
DAD -Diode array detector
ESI - Electrospray ionization
FDA - Food and Drug Administration
MSD - Mass selective detector
MW- Molecular weight
RP- Reversed-phase
RSD- Relative standard deviation
SD – Standard deviation
SQ - Single quadrupole
UV-VIS - Ultraviolet-Visible.

INTRODUCTION

Altrenogest (**$C_{21}H_{26}O_2$. MW** 310.4 $g \cdot mol^{-1}$), also known as allyltrenbolone, is a steroidal progestin that is widely used in veterinary medicine to suppress estrus in animals (*Riviere J. et al., (2013);* Heriberto R., (2010); Philip K., (2015). Altrenogest is sparingly soluble in aqueous buffers, but is soluble in organic solvents such as ethanol, DMSO, and dimethyl formamide (Cayman chemicals, (2019)). The solubility of altrenogest in these solvents is approximately 20, 0.1, and 2 mg/ml, respectively. Sesame oil solutions of altrenogest from various manufacturers (Altren, Ovamed, SwineMate etc.) are widely represented on the market. Sesame oil is soluble in mineral oil, slightly soluble in alcohol and insoluble in water (JEEN, (2003)). For HPLC analysis, altrenogest must first be isolated from the oil solution; for this purpose, a solid-phase extraction is performed (Philip K., (2015). There is data on the extraction of veterinary drugs by dissolving them in a mixture of acetonitrile and water (Multi-residue Method, (2018). According to our preliminary data, the best chromatograms of altrenogest are obtained in acetonitrile solutions; therefore we focused on extracting altrenogest from sesame oil using acetonitrile. The thermodynamic law for the partition of a solute between two immiscible or partially miscible solvents (E.g., sesame oil/ acetonitrile) is:

$$A = K*a'$$

A and ***a'*** are the activities in the two phases, ***K*** is a constant (MCKAY H. *et al.*, (1963). Based on this law, we derived a formula and proposed a method for determining the minimum volume of an extractor needed to achieve the required degree of extraction.

MATERIALS AND METHODS

Chemicals - Water and Acetonitrile were purchased from Agilent. Altrenogest USP standard was purchased from Sigma, Altrenogest sesame oil 150 g/L solution was obtained from Wedgewood pharmacy. All the solvents used were of HPLC grade.

The Instrument - Agilent Single Quadrupole LC/MS 2019 instrument includes the following components: OpenLAB CDS Version 2.2. software; Diode Array Detector , single quadrupole mass selective detector with electrospray ionization with 150 V fragmentor, gas flow: 7 L/min, gas temperature 300°C, capillary 4000 V and nebulizer 15 psi; Column is Poroshell 120 EC-C18, 50x4.6mm, particles size 2.7 µm, with Guard precolumn; quaternary pump with flow: 1.0 mL/min, high pressure limit: 600 bar. Isocratic elution was performed with mobile phase: 0.1% formic acid/ACN solution.

A qualitative Analysis - of Altrenogest was made by value of mass/charge (m/z+). Altrenogest exhibits a predominant signal of 311 m/z+ corresponding to the cation Altrenogest-H+.

A quantitative Analysis - was made using the DAD at 342 nm. The amount of altrenogest in the sample was determined using a standard calibration curve.

Preparation of Standard Stock Solution – 50 mg of Altrenogest USP standard was dissolved in 50 mL ACN and filtrated. Five calibration solutions in range from 0.1 – 1.0 g/L was prepared from a 1.0 g/L standard solution.

Calibration Curve and Coefficient of Correlation - The set of five dilutions made from the standard stock solutions ranging from 0.1 g/L to 1.0 g/L were tested. The correlation coefficient (r) of peak areas with the corresponding concentrations was calculated by least square method and a calibration curve was plotted. Each point of the calibration curve was an average of five measurements. Calculations were done automatically by OpenLAB CDS. The coefficient of correlation was greater than or equal to 0.999 (CDER, 1994) with a working range from 0.1 to 1.0 g/L.

Sample Preparation - 2 mL of sesame oil solution was mixed, vortexed, and sonicated for 10 minutes in a corresponding volume of acetonitrile. After 2 hours or 2 days of incubation at room temperature (20° C – 25° C), the solution was then filtrated through a 0.45 µm syringe filter.

Sample Analysis- The amount of altrenogest in the extract was determined by HPLC/MS. The residual amount of altrenogest in the oil was calculated as the difference between the total and extracted amounts.

STATISTICAL ANALYSIS

The variation of data is expressed in terms of the standard deviation (SD) and Relative Standard Deviation (RSD), along with the number of observations (n). The results with $p<0.05$ were considered statistically significant. To construct the calibration curve, a regression analysis based on the least-squares method was used. The correlation coefficient (r) and the determination coefficient (r^2) were calculated automatically by OpenLAB CDS during analysis.

RESULTS AND DISCUSSION

According to the Multi-residue Method (2018) and our research for the analysis of altrenogest using HPLC, the best mobile phase is a mixture of acetonitrile and water in a ratio of 80:20. In most cases, samples were prepared in an acetonitrile medium. However, solutions of altrenogest in sesame oil have been problematic. Sesame oil does not dissolve in acetonitrile. Multi-step extraction with acetonitrile in the usual way using a separatory funnel is also impossible due to the formation of a stable emulsion. The task was to determine the extractant/extractor volume ratio at which more than 99% of altrenogest is extracted. The sesame oil/acetonitrile extract was analyzed chromatographically (Figure 1 A, B). The amount of altrenogest extracted into the acetonitrile solution was calculated based on the measured concentration of altrenogest and the volume of the extractor. The total amount of altrenogest (N) is known from the pharmaceutical formula. The prepared acetonitrile extracts were analyzed after 2 hours and after 2 days of incubation at room temperature (20° C – 25° C).

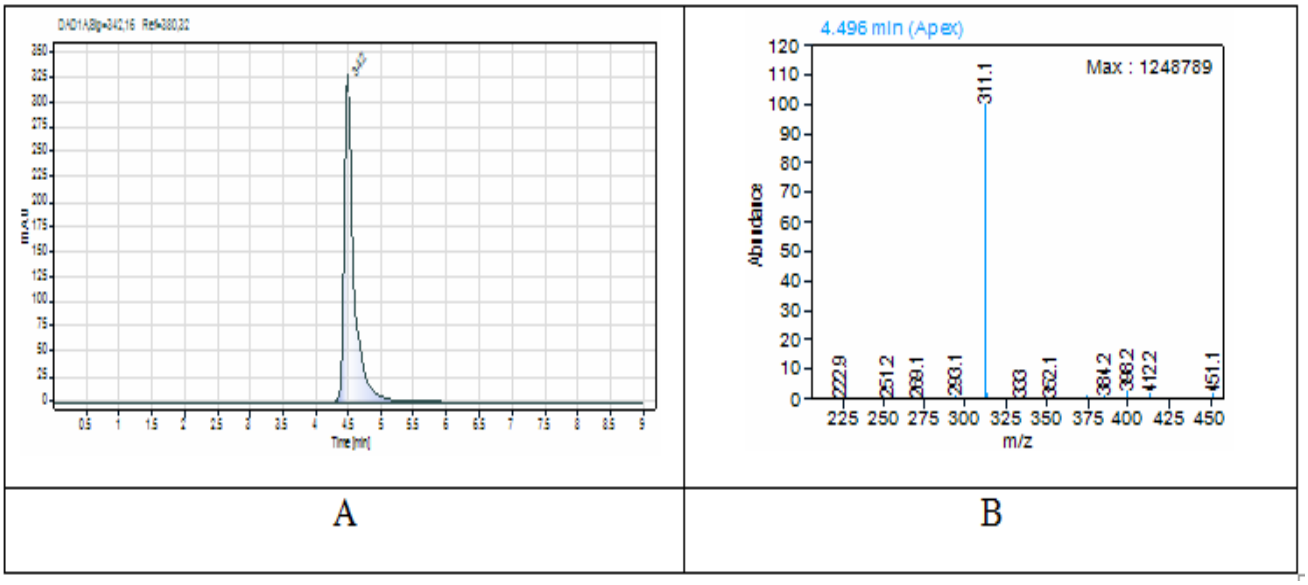

Fig-1: A, B. Representative chromatogram (A) and mass spectrum (B) of an acetonitrile extract of altrenogest in sesame oil solution

The theoretical dependence of the proportion of altrenogest in the oil (a/N) on the volume of the extractor (V) can be deduced (Appendix 1) from distribution law (MCKAY H. *et al.*, (1963)). The dependence is exponential:

$a/N = e^{(-kV)}$	[1]

It is much more convenient to use a linear relationship:

$\ln(a/N) = -kV$	[2]

The experimental results are consistent with the theoretical model. Experimental points and linear regression (dashed line) are shown in Figure 2 A, B.

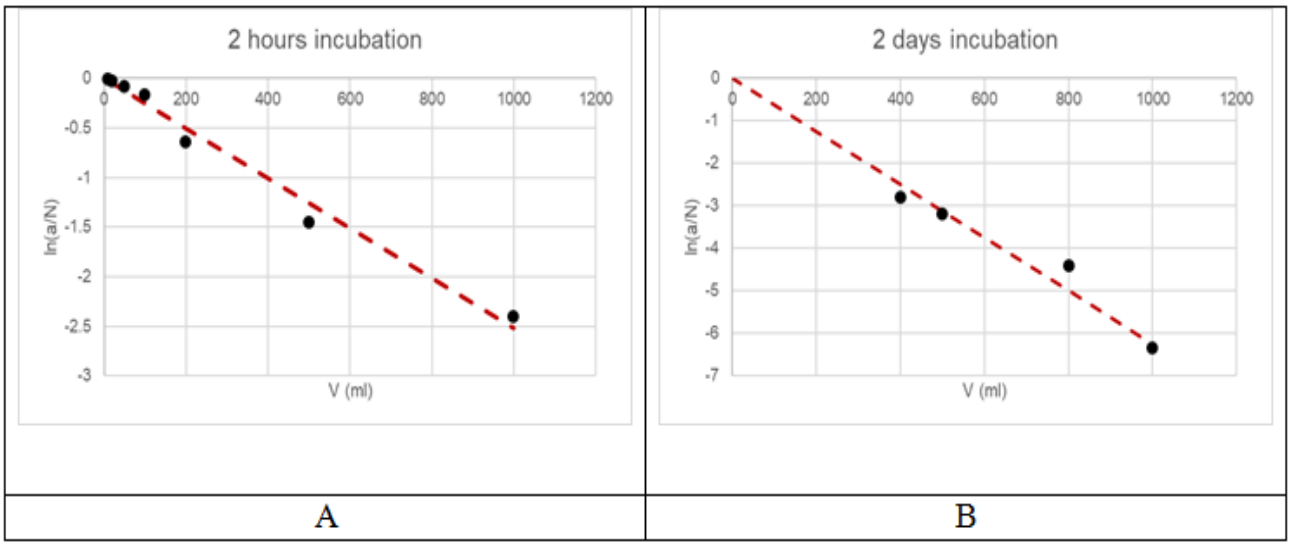

Fig-2: A, B. Dependence of the logarithm of the relative content of altrenogest in oil on the volume of the extractor (acetonitrile). Altrenogest 150 g/L was extracted with acetonitrile from 2 mL of sesame oil. Data represent the mean of five independent determinations (n = 5), maximum RSD = 10%. Experimental points are black circles, linear regression is dashed lines.

299.4 mg of altrenogest was extracted from 2 mL of oil to 1000 ml of acetonitrile, which corresponds to 99.8% (300 mg – total amount). The recovery rates of Altrenogest for all tested volumes are presented in Table 1.

Table-1: Percentage of one step extraction of Altrenogest 150 g/L from 2 mL sesame oil with acetonitrile. The data presents an average value of five independent determinations (n = 5) ± 2*SD, p<0.05

Acetonitrile Vol. (mL)	2 hours incubation Extraction %	2 days incubation Extraction %
100	22.0 ± 0.2	-
200	40.0 ± 0.4	71.0 ± 0.3
400	-	92.0 ± 0.3
500	72.0 ± 0.4	96.0 ± 0.2
800	-	99.0 ± 0.4
1000	92.0 ± 0.4	99.8 ± 0.4

A satisfactory degree of extraction (99.8%) was achieved with a volume ratio of sesame oil and acetonitrile of 1/500. Subsequently, for reasons of economy, we took 0.5 mL of sesame oil and 250 mL of acetonitrile. The concentration of altrenogest in the oil was high, and its concentration in the extract, was high enough (0.3 g/L) for HPLC/MS analysis. In other cases, the concentration of the extract can be increased by evaporation in a rotary evaporator. The incubation time (2 days) can likely be shortened if the solution is constantly or periodically stirred.

CONCLUSION

The proposed method can be used to quantify the degree of extraction of any acetonitrile soluble substance in sesame oil. The method can also be applied to the extraction of substances that form stable emulsions.

In general, the procedure consists of the following steps: 1) Preparation of a series of emulsions with different ratios of extracted solution and the extractor. 2) Separation of dispersed systems by filtration or centrifugation. 3) Determination of the content of the extractable substance in the extractor and calculation of the degree of extraction. 4) Plotting the dependence logarithm of the residual amount of extractable substance in the extractable solution on the volume of the extractor. 5) Linearization of the obtained experimental dependence by the Least Squares Method. 6) Determination of the ratio of volumes between the extracted liquid and the extractor for a predetermined criterion, i.e., extraction of more than 99%.

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Appendix 1.

Derivation of the dependence of the fraction of the remainder of the extracted component on the volume of the extractor.

The distribution of component **a** between two liquid phases obeys the Law of Distribution (MCKAY H. *et al.*, (1963)). Replacing activity with concentration, we arrive at formula (1):

a / v = k * (N-a) / V	[1]

or

a = K * (N-a)/V	[2]

Where **N** is the total content of the component, **a** - the current content of the component in the first phase of the volume **v**, and at the initial moment **a = a0 = N**, **k** and **K = k * v** are constants, **V** is the current, variable volume of the second phase (extractor). An increment in V (dV) causes an increment in the value of **a**: **-da = (N-a)**. Considering the assumptions made, we transform formula (2) into (3)

da / a = -K * dV	[3]

or

d(ln(a)) = - K * dV	[4]

After integration, considering **a0 = N** and **V0 = 0**, we obtain (5)

ln(a / N) = - K * V	[5]

and

a / N = exp (-K * V)	[6]

where **K** is a constant.