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# Development and validation of a method for allantoin determination in liposomes and pharmaceutical formulations

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ABSTRACT

The aim of this work was to develop and validate an ultraviolet derivative spectrophotometric (UVDS) method for the quantitative determination of allantoin (ALL) in liposomes, gels and creams. Liposomes were prepared by methods of thin film hydration and mechanical agitation. Solutions of ALL in 0.1 mol/L NaOH with ethanol:water (70:30, v/v) were prepared in order to destroy liposome vesicles. Spectral interference from components of liposomes, cream, gel and ALL degradation products was eliminated using the second-order derivative of the zero-order spectrum. Characterization of ALL in 0.1 mol/L NaOH was carried out by direct infusion mass spectrometry. Absorbances of ALL solutions were measured at 266.6 nm of the second-derivative spectrum and linearity was observed in the ALL concentration range of 50–300 $\mu g\,mL^{-1}$ (correlation coefficient ($r$) = 0.9961). The mean recovery percentage was 100.68 ± 1.61, repeatability expressed as relative standard deviation (RSD) was 1.07 and 2.12%, and intermediate precision (RSD) was 2.16%. The proposed UVDS method was found to be linear, precise, accurate, robust and selective, providing rapid and specific determination of ALL in raw materials and in topical formulations.

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## 1. Introduction

Allantoin (ALL) is the final product of purine catabolism of some vertebrates by the oxidation of uric acid with the enzyme uricase. ALL is also a common constituent of plants, as in some Dioscorea species [1], *Symphytum officinale* L. [2] and *Zea mays* [3], and can be chemically synthesized by the alkaline oxidation of uric acid in a cold environment [4].

Known for its protective skin, anti-inflammatory and keratolytic properties due to its ability to promote cell proliferation, ALL has been widely used in the treatment of skin ulcers, acne, hemorrhoids and psoriasis [4–7]. Cosmetic products for skin-soothing, mouthwashes [8] and hand sanitizers [9], as well as formulations for the treatment of hypertrophic scars [10] and atopic dermatitis [11], can contain ALL.

Potentiometric titration can be used for the determination of ALL in raw materials [12]. Titration is a simple and low cost method but is usually less sensitive. Additionally, because of its poor selectivity, this technique cannot be applied to cosmetic and pharmaceutical formulations without the previous separation of ALL from other components [13,14].

Methods employing HPLC [15–17], hydrophilic interaction chromatography [18,19], capillary electrophoresis [20,21] or liquid chromatography with tandem mass spectrometry (LC–MS/MS) [22,23] have been developed to determine ALL in biological, cosmetic and pharmaceutical samples. Besides requiring expensive equipment, these methods are time-consuming since the previous separation of interfering substances is necessary.

Spectrophotometric methods are often used due to the availability of instrumentation and the simplicity and speed of the techniques. Derivative spectrophotometry in the UV–vis region (UVDS) is a useful process and has been widely employed for the quantitative analysis and quality control of pharmaceutical samples [24]. This procedure usually improves resolution and eliminates interference from other formulation components and degradation products [25–29].

Taking all of these aspects into consideration, the development of a simple alternative method that allows ALL determination, both as raw material and in pharmaceutical products, is of interest for use in the quality control routine of pharmaceutical compounding

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as well as in pharmaceutical industries. Among the advantages of the proposed UVDS method are the environmentally friendly and non-time consuming conditions; the proposed method does not require any previous separation of interfering substances and saves time and effort in ALL determinations.

In this work, a new derivative spectrophotometric method was developed and validated for ALL determination. This new method provides a rapid and specific determination of ALL in raw materials and in topical formulations, such as liposomes, cosmetic creams and gels.

## 2. Experimental

### 2.1. Chemicals

ALL (raw material-70804) was obtained from DEG (São Paulo, SP, BR), and its purity (98.6 ± 0.18%) was determined by potentiometric titration [12]. ALL standard (99.8% purity) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Solutions for the UVDS method were prepared with commercial reagents of analytical grade and ultrapure water obtained from a Milli-Q® system (Milipore®).

The raw materials used for liposome preparations were polyenylphosphatidylcholine (Phosal 75 SA®), purchased from PHOSPHOLIPID GmbH, soya phosphatidylcholine (Phospholipon 90 G®), kindly supplied by PHOSPHOLIPID GmbH, and cholesterol, obtained from Sigma–Aldrich (St. Louis, MO, USA). Urea, oxalic acid, nonionic self-emulsifying wax (Polawax®) and nonionic water-soluble polymer hydroxyethylcellulose (Natrosol®) were provided by Faculty of Pharmacy of Federal University of Rio de Janeiro (UFRJ) and used to assess the method selectivity.

### 2.2. Apparatus and experimental conditions

The absorbance of solutions containing 200 $\mu g\, mL^{-1}$ of ALL was measured by a UV–vis spectrophotometer (UV-12401 PC, Shimadzu®) within the range of 190–400 nm with a 2 nm bandwidth. The first and second derivative spectra were obtained by instrumental electronic differentiation (UV-PC software) in the 190–400 nm range. Absorbances of ALL solutions were measured at 266.6 nm of the second derivative spectrum using a 1.0 cm quartz cell.

Characterization of ALL in a 0.1 mol/L NaOH solution was accomplished using a triple quadrupole mass spectrometer (Quattro LC, Micromass, Altringham, Cheshire, UK) equipped with a syringe pump (KD Scientific, Model 100, Holliston, MA, USA). An electrospray ionization (ESI) source was used and operated in the negative-ion mode. Data from mass spectrometry was acquired using MassLynx v.3.2 (Micromass, Manchester, UK).

### 2.3. Formulations

Liposomes were prepared by the thin film hydration (TFH) and mechanical agitation (MA). In both methods, ALL was included in the aqueous phase of the liposome in a 42:2.7 × $10^{-2}$ molar ratio of soya phosphatidylcholine (PC) to ALL.

PC multilamellar vesicles were prepared using TFH [30]. Typically, 1.7 g of PC and 250 mg of cholesterol were dissolved in chloroform in a round flask. The solvent was evaporated using a rotary evaporator under reduced pressure at 45 °C, and the mixture was left to dry overnight in a vacuum container with activated silica. The dried mixture was hydrated for 30 h at 4 °C in 50 mL of TRIS buffer pH 6.8, with 400 mg of ALL.

Liposomes prepared by MA, as indicated by PHOSPHOLIPID GmbH, employed Phosal 75 SA®. Typically, 2.2 g of Phosal® was added to a 100 mL beaker and mixed for 15 min. To the mixture, 50 mL of TRIS buffer pH 6.8 with 400 mg of ALL was added and mixed for 2 h in a mechanical agitator.

Both liposome preparations were filtered twice through 0.4 μm and once through 0.2 μm polycarbonate membranes to standardize the size of the vesicles. During UVDS method development and validation, the determination of ALL content was performed immediately after liposome formulations were prepared.

However, during liposome formulation optimization, free ALL was removed by gel-filtration with Sephadex G-50. Fractions containing both the liposome and ALL were pooled and the ALL content was evaluated by the proposed UVDS method. Typically, 200 μL of liposomes were diluted in a 0.1 mol/L NaOH ethanol:water (70:30, v/v) solution to destroy the liposome vesicles, thereby allowing UV light transmission without precipitation of ALL. The encapsulation efficiency was calculated directly from the purified liposomes.

Particle size distribution of liposome vesicles was performed by dynamic light scattering (ZetaPlus Zeta Potential Analyzer – Brookhaven Instruments Corporation; previously calibrated according to the manufacturer).A Polawax® O/W emulsion was prepared with 10% Polawax® and 70% purified water. The aqueous phase was heated to 80 °C, added to the oil phase (75 °C), mixed and cooled.

Natrosol gel was prepared with 3% hydroxyethylcellulose (natrosol®) and 97% purified water. A cream containing 1% ALL was prepared using Polawax®. ALL was added to the aqueous phase of the emulsion and heated to 80 °C. The aqueous phase was then added to the oil phase (75 °C), mixed and cooled.

### 2.4. Standard and sample solutions

Approximately 50 mg of ALL (99.8% purity, Sigma–Aldrich) was transferred to a 50 mL volumetric flask and dissolved in NaOH (0.1 mol/L, pH 12.8). Working solutions with ALL concentrations of 50, 100, 150, 200, 250 and 300 $\mu g\, mL^{-1}$ were prepared in triplicate.

A cream containing approximately 50 mg of ALL was transferred to a 50 mL volumetric flask and dissolved in 0.1 mol/L NaOH. An aliquot of this solution was diluted in 0.1 mol/L NaOH to obtain a solution with a final ALL concentration of 200 $\mu g\, mL^{-1}$.

### 2.5. Method validation

The proposed method was validated for linearity, precision, accuracy, robustness and selectivity according to the guidelines of the International Conference on Harmonization (ICH) [31]. Student's *t*-test was used to compare the proposed and pharmacopeial methods [12].

#### 2.5.1. Linearity

Five different concentrations of ALL, within the targeted concentration range of 25–150%, were used to evaluate the linearity of the method. A calibration plot was obtained in the concentration range of 50–300 $\mu g\, mL^{-1}$ of ALL standard solutions. Linearity was assessed by the least squares regression method with triplicate determinations at each concentration.

#### 2.5.2. Precision

Precision was determined by the degree of repeatability from the analysis of six samples during the same day, covering the specified range from 80 to 120% of the target ALL concentration (200 $\mu g\, mL^{-1}$) (triplicates of three different concentrations). Creams containing approximately 4, 5 or 6 mg of ALL were transferred to 25 mL volumetric flasks and dissolved in a NaOH (0.1 mol/L) ethanol:water solution (70:30, v/v). The resulting solutions contained 160, 200 and 240 $\mu g\, mL^{-1}$ of ALL. Intermediate

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