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Physicochemical characterization of lapachol

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The aim of this study was to characterize three batches of lapachol (LAP), in order to establish specifications for the development of pharmaceutical forms. Physical methods including nuclear magnetic resonance (^{1}H and ^{13}C NMR), infrared spectroscopy (IR), scanning electron microscopy (SEM), x-ray diffraction, differential scanning calorimetry (DSC), determination of the melting range and drug assay were performed. All batches exhibited assays values that were statistically equal to the chemical reference substance (CRS). Furthermore, we found that lots evaluated had a mean purity value of 99.79%. The DSC curves for the three batches and CRS show an endothermic event in the range of 139.87 to 141.01°C. The IR and NMR spectra of all lots are overlapping. All diffraction profile results showed a series of intense and well-defined diffraction peaks, displaying the typical pattern of a compound with a crystal habit. SEM photographs showed that the crystals present stratified plates with visible separation between the layers. This study was fundamental for the gathering of information on the physical, physicochemical and thermal characteristics of lapachol. Thus, facilitating the monitoring of drug quality of LAP, given it is not yet described in official compendia.

Key words: Lapachol, physiochemical characterization, quality control.

INTRODUCTION

During the development process of dosage forms, it is essential to know the physiochemical properties of the drug; therefore, the characterization of the active pharmaceutical ingredient (API) should be the first stage in the development of new medicines. Changes in the API can reduce productivity, affect quality, and even spoil a production batch (Nery et al., 2008; Soares-Sobrinho et al., 2010; Cavalcanti et al., 2012).

When working with drugs that are derived from plant materials and have low solubility in an aqueous medium, the characterization stage is even more important (Cunha-Filho et al., 2007; Freitas-Neto et al., 2012). 2-hydroxy-3-(3-methylbut-2-enyl)naphthalene-1,4-dione (LAP), is an important naturally occurring naphthoquinone, which was isolated from the bark of *Tabebuia avellanedae* (Mart. Ex DC.) Standl. in the mid-1950s by the industrial chemist, Oswaldo Gonçalves de Lima (Hussain et al., 2007; Almeida, 2009; Ferreira et al., 2010). LAP is a yellow powder that is weakly acidic, highly lipophilic and has low solubility in water, though it is highly soluble in alkaline solutions (Lira et al., 2008). Its molecular weight is 242.26 g mol^{-1} and it possesses the

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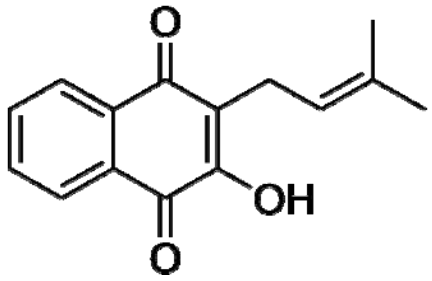

Figure 1. Lapachol chemical structure.

following chemical formula $C_{15}H_{14}O_3$ (Figure 1). Several pharmacological activities have been attributed to LAP and its semisynthetic derivatives, such as antimicrobial, antifungal, molluscicidal, leishmanicidal, trypanocidal, antimalarial, anti-inflammatory, anti-cancer, anti-ulcer and contraceptive (Almeida et al., 1990; Guerra et al., 2000; Andrade-Neto et al., 2004; Almeida, 2009).

Despite lapachol being well understood pharmacologically, but there is not any pharmacopeic method in official compendium and there is little information about its physical, physiochemical and thermal properties in the scientific literature.

Furthermore, a factor that should be considered in the quality control of plant products is their susceptibility to physical change, which can be extrinsic, including, time, temperature, light, oxygen, humidity, packaging and micro-organisms and intrinsic, which can be related to the nature of formulations, such changes create physical and/or chemical incompatibilities (Fonseca and Yoshida, 2009; Isaac et al., 2008). Consequently, their characterization is of great importance, so that high quality products may be developed (Soares-Sobrinho et al., 2010).

Therefore, an understanding of the different polymerphic forms, impurities and crystal sizes and morphologies obtained by techniques, such as, thermal analysis, particle size determination, spectrometric (UV and IR) and x-ray diffraction, may be allowed for the proper control of LAP (Nery et al., 2008; Storpirtis et al., 2009; Maximiano et al., 2010).

Thus, the objective of this study was to evaluate the physical and physiochemical characteristics of LAP, using several identification and characterization techniques, thereby establishing quality standards and technological improvements for this promising drug.

MATERIALS AND METHODS

The active pharmaceutical ingredient (API), lapachol (LAP), was provided by Departamento de Antibióticos da Universidade Federal de Pernambuco (UFPE), following the methodology developed by Lima et al. (1962). Three different batches were used, which for the purposes of this study were labelled: LAP 01, LAP 02 and LAP 03. LAP Sigma-Aldrich® (Brazil), batch 142905, 98% purity, was used as a Chemical Reference Substance (CRS) (Table 1). All solvents and reagents were of analytical grade, all glassware was calibrated and the tests described subsequently were performed in triplicate.

Assay using high performance liquid chromatography coupled with an ultraviolet detector (HPLC-UV)

To determine the assay of LAP, dilutions from a stock solution of 100 µg/ml of methanol were analysed using a calibration curve with concentrations ranging from 0.1 to 20 µg/ml.

Analyses were performed using a Shimadzu chromatograph with an SCL-10 controller, a SIL-10AD auto-injector, a LC-10AD pump, a SPD-10AV UV-VIS detector and a CTO-10 column oven. Chromatographic conditions were as follows: mobile phase of methanol and 5% acetic acid solution (80:20 v/v); isocratic pumped at a rate of 1.0 ml/min, with an 100 RP-18 column Lichrosoher (250 × 4 mm, 5 µm), an injection volume of 20 µl and ultraviolet detection at a wavelength of 278 nm and a 7-min evaluation period. This method was developed and validated by Núcleo de Desenvolvimento Farmacêutico e Cosmético - Universidade Federal de Pernambuco (NUDFAC – UFPE) (unpublished data).

Calorimetric profile and purity test using differential scanning calorimetry (DSC)

DSC curves were used for the thermoanalytical characterization and determination of the purity of the three batches of LAP. DSC curves were obtained with a Shimadzu® DSC-60 cell, using closed aluminium capsules with an ~2 mg sample under a N_2 dynamic atmosphere (50 ml/min) and a heating rate of 5°C min^{-1} from 30 to 300°C. The equipment was calibrated with zinc (m.p.: 419.6°C). All analyses were performed in triplicate and compared with the values of LAP CRS.

Purity was determined using the Van't Hoff equation (Equation 1), which correlates the depreciation of the melting point of the analyte in the presence of impurities (Oliveira et al., 2011; Gong et al., 2013).

$$T_s = T_0 - \frac{R T_0^2 X}{\Delta H} \frac{1}{F} \quad (1)$$

where T_S is the observed temperature of the sample; T_0 is the theoretical melting temperature of the pure analyte; χ is the molar fraction of impurities; and F is the melted fraction, that is determined by measuring the peak partial area of the experimental melting.

Melting range

The melting range was evaluated using a Melting Point Meter 430D in accordance with the general method of F. Bras. V (2010) and by DSC.

Infrared spectroscopy (IR)

Infrared spectroscopy with a KBr tablet was verified with a Fourier Transformed Infrared Spectrometer (model IFS66, Bruker, Madison, WI, USA). The API spectrum was set in the range of 4000 to 400 cm^{-1}.

Nuclear magnetic resonance spectroscopy (NMR)

A Varian® spectrometer (model Unity plus-400 MHz, Palo Alto, CA) as used to perform proton (^{1}H NMR, 399.74 MHz) and carbon-13

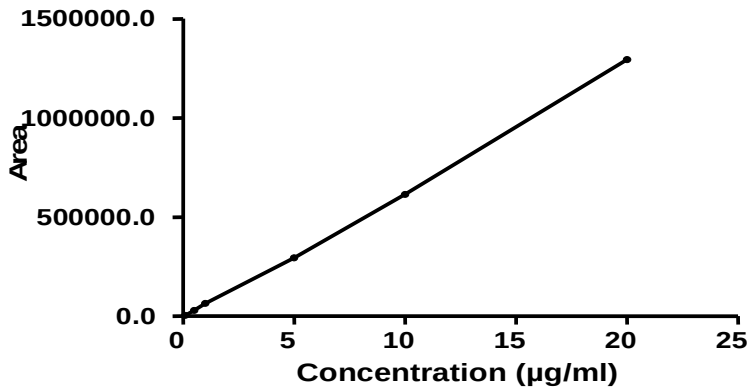

Figure 2. Linear regression line obtained from the mean of three authentic calibration curves.

(^{13}C NMR, 100.51 MHz) nuclear magnetic resonance. Samples were dissolved with deuterated chloroform.

Diffraction profile

A Siemens diffractometer (model D5000) operating under a current of 40 mA, a tension of 40 kV, with an analysis interval of 5 to 50° (2θ), 0.05° per step and a time step of 5 s, was used to obtain X-ray diffractograms (Carini et al., 2009).

Granulometric and crytal morphology analysis

Particle size distribution was determined by laser diffraction and photon correlation spectroscopy (PCS) in a DelsaTM Nano-S particle analyser (Beckman Coulter, Brea, CA) at 25°C with a detection angle of 90°. A dispersion of 12 mg of LAP in 20 ml of a 0.02% aqueous solution (v/v) of the surfactant Triton X-100® was kept in an ultrasonic bath (40 kHz) for 3 min. Due to the hydrophobicity of LAP, the surfactant was used to facilitate the dispersion of particles. The results are reported in terms of mean size ± standard deviation.

To enable morphological evaluation of the crystals, the samples were first coated in gold (25 nm) in a Quick Coater Metaliser (model SC701, Sanyu Electron) for 2.5 min. Photomicrographs were then obtained by scanning electron microscopy (SEM) using a Shimadzu SS-550 microscope coupled with an energy dispersive spectrometer (EDS). Images of backscattered and secondary electrons were obtained with an accelerating tension of 15 kV, a working distance (WD) of 16 mm and magnifications of 450, 1800 and 3500 times.

Statistical analysis

All data were analyzed using ANOVA (GraphPad Prism® v. 5.0). 95% confidence interval was use, that is, differences were considered statistically significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

Determination of assay using high performance liquid chromatography

Ensuring the quality of pharmaceutical inputs/ingredients is an essential prerequisite for the development of drugs, which contributes to their safety and efficacy. One of the most common tests to evaluate the quality of drugs is HPLC assay (Holfer et al., 2007).

The calibration curve obtained from the lapachol was linear (y = 64515x – 9830.6) with a correlation coefficient (r^2) of 0.9991. According to ANVISA (BRASIL, 2003) the correlation coefficient should be equal, or greater than, 0.99. Thus, the r^2-value obtained from the analysis of lapachol, using the developed HPLC-UV method, meets the established limits (Figure 2).

The amount of lapachol in the samples was determined from the standard lapachol chromatogram. Sample lapachol peaks were identified according to the standard lapachol retention time and its assay was calculated from the areas under the curves. The three batches of LAP were compared with the LAP CRS (Table 2). All batches exhibited assay values that were statistically equal to the LAP CRS.

Calorimetric profile and purity test using differential scanning calorimetry

Thermoanalytical methods have been widely applied to the study of drugs. While they do not constitute official techniques, there are many studies that use these methods for the characterization and quality control of pharmaceutical materials (Rodrigues et al., 2005). DSC is one of the most widely-used thermoanalytical techniques, employed in manifold studies and applied to a great variety of pharmaceutical materials. The application of thermoanalytical methods, particularly DSC, has played a fundamental role in the study of characterization, development and quality control of pharmaceutical products. The applications in this area have addressed the characterization of raw materials and finished products, the determination of purity and the testing of stability and decomposition kinetics (Rodrigues et al., 2005).

The DSC curves of the three batches of LAP CRS (Figure 3) show an endothermic event, in the temperature range T_{onset} 139.87 to T_{endset} 141.01°C, with an enthalpy of 114.02 J g^{-1}, which is characteristic of the melting of this substance. This result may suggest the drug is of a

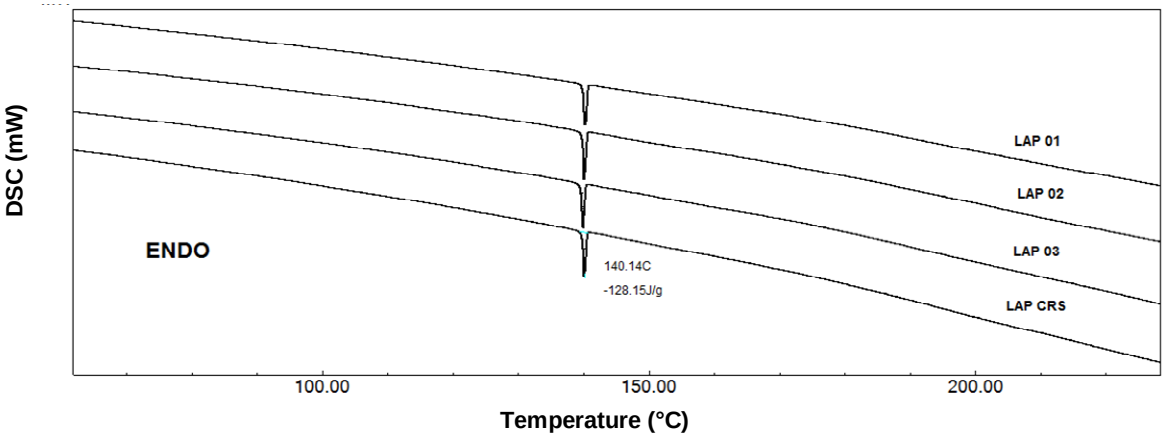

Figure 3. DSC curves for (a) LAP 01, (b) LAP 02, (c) LAP 03 and (d) LAP CRS.

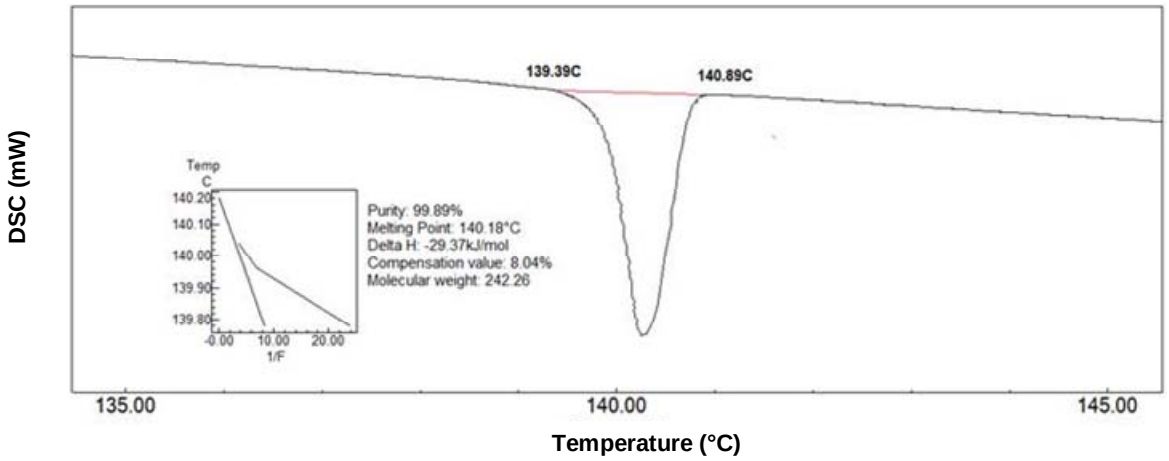

Figure 4. Analysis of CRS lapachol purity, using DSC.

crystalline nature, since such an event is characteristic of compounds with an organized structural arrangement (Freitas-Neto et al., 2012).

Another application of DSC is the evaluation of purity of chemical compounds. However, researchers should be aware that DSC results are only reliable for highly pure (≥98%) compounds with a sharp melting point, and that do not exhibit thermal events that interfere with their melting endotherms (Mathkar et al., 2009).

Figure 4 shows the DSC curve obtained for LAP 02, in which a well-defined, symmetrical endothermic peak characteristic of a melting event. This event indicates a constant transition temperature, indicating that the process occurs in thermodynamic equilibrium. Moreover, this confirms that the baseline was maintained, suggesting suggesting that the thermal capacity of the material does not change during the process. Furthermore, there was no other phase transition during the temperature scan. Thus, the sample exhibited ideal conditions for the performance of the purity calculation.

The purity of the three batches of LAP was calculated using Equation 1. Purity is determined by the deviation from linearity of the melting event, which occurs due to the presence of impurities. If the deviation from linearity is known, the linear correction factor can be inferred. The lapachol in the tested batches showed mean purity values of 99.79 ± 0.09%, with a mean correction factor of 8.83 ± 1.21%. The purity values obtained using DSC were compared with those obtained by chromatographic techniques (Table 2) and were found to be statistically equivalent. Thus, DSC may serve as an important alternative method for analysing the purity of pharmaceutical compounds, given it is faster and less costly than chromatography techniques.

Melting range

The observed melting range, using the general method of F. BRAS. V, was between 139 and 142°C (Table 3). This

Table 1. Lapachol melting enthalpies (J g^{-1}) and event temperatures (°C).

Batch	Onset (ºC)	Endset (ºC)	Energy consumption (J g^{-1})
LAP 01	139.80 ± 0.11	140.88 ± 0.33	-111.36 ± 2.84
LAP 02	139.54 ± 0.10	140.42 ± 0.17	-110.78 ± 1.64
LAP 03	140.44 ± 1.73	142.34 ± 1.50	-105.87 ± 4.36
LAP CRS	139.69 ± 0.32	140.41 ± 0.13	-114.32 ± 1.98

Table 2. A comparison of purity values obtained using DSC and those obtained by chromatographic techniques.

Batch	Assay (%)		Difference in purity (%)
	HPLC/UV	DSC	
LAP 01	98.79±1.23	99.75 ± 0.18	0.97
LAP 02	98.11±0.66	99.84 ± 0.04	1.76
LAP 03	99.04±1.34	99.76 ± 0.06	0.72
LAP CRS	98%*	99.81 ± 0.06	1.84

*Value declared by manufacturer. Analysis performed using thin layer chromatography (Sigma Aldrich®).

Table 3. Comparison of melting points/ranges obtained by the general method described in F. Bras. V and DSC.

Batch	F.Bras.V (°C)	DSC (°C)
LAP 01	139 – 140	139.80 – 140.88
LAP 02	140 – 142	139.54 – 140.42
LAP 03	140 – 142	140.44 – 142.34
LAP CRS	140 – 141	139.69 – 140.41

result was similar to that obtained by DSC (T_{onset} de 139.54°C, T_{endset} de 142.34°C) and are in agreement with the values reported by Macêdo and Nascimento (2001) and Parrilha et al. (2012).

Infrared spectroscopy

Infrared spectroscopy is reliant on chemical bonds having specific vibration frequencies. Today, this technique is a major analytical tool for the identification and structural elucidation of organic compounds (Burns and Ciurczak, 2001). The main pharmaceutical applications are in quality control of raw materials and excipients, drug characterization, identification of polymorphisms, among others (Freitas-Neto et al., 2012).

The infrared spectra of LAP 01, LAP 02 and LAP 03 overlap and have the following absorption bands: 3351.68 υ(OH), 166.81 and 1640.27 υ(C=O), 1369.03 to 159.60 υ(C=C).

Nuclear magnetic resonance spectroscopy

The nuclear magnetic resonance spectra of hydrogen (^{1}H) and carbon-13 (^{13}C) in LAP 01, LAP 02 and LAP 03 were similar, with the following chemical shifts: ^{1}H RMN (δ ppm) 1.66 (s; 3H; CH_3), 3.28 (d; 2H; CH_2), 5.19 (t; 1H; CH), 7.41 (s; 1H; OH), 7.68 (m; 2H; Ar) and 8.06 (m; 2H; Ar). NMR ^{13}C (75.45 MHz): (δ ppm) 19.9 (CH_3); 22.6 (CH_3); 25.7 (CH_2); 119.6 (H<u>C</u>=C); 123.4 (<u>C</u>=C – OH); 126.0 (CH Ar); 126.7 (CH Ar); 129.4 (C Ar); 132.8 (C Ar); 133.8 (<u>C</u>=CH); 134.8 (CH Ar); 152.7 (OH – <u>C</u>=C); 181.6 (C=O); 184.5 (C=O). The chemical structure of LAP was confirmed by the absorption peaks and the characteristic fragmentation of the molecule.

Diffraction profile

X-ray diffractometry (XRD) is one of the main techniques for microstructural characterization of crystalline materials. This analytical tool is capable of determining the physical characterization of crystalline materials. Diffraction angles and peak intensities are direct results of different crystalline structures (Freitas-Neto et al., 2012). The XDR technique is very important for the monitoring of crystalline forms of drugs during the various stages of their development, since any change in the

Table 4. Particle size distribution of the three lapachol batches.

Batch	Particle size (µm)			
	Mean ± D.P.	P10* ± D.P.	P50* ± D.P.	P90* ± D.P.
LAP 01	526.30 ± 15.46	179.65 ± 52.25	441.26 ± 44.33	910.25 ± 35.14
LAP 02	264.20 ± 62.50	61.94 ± 0.30	294.35 ± 54.95	467.40 ± 137.88
LAP 03	222.10 ± 83.58	46.28 ± 21.21	195.55 ± 78.55	411.30 ± 141.98

*Particle population (%) that has a diameter below the expressed diameter.

degree of crystallinity may alter the solubility of the drug and, consequently, its bioavailability (Karjalainen et al., 2005).

The diffraction profile results of the three batches of LAP showed a series of intense and well-defined diffraction peaks, displaying the typical pattern of a compound with a crystal habit. The main peak was found at 8.31° (2θ) with secondary peaks at 16.94, 25.26 and 26.41 (Figure 5).

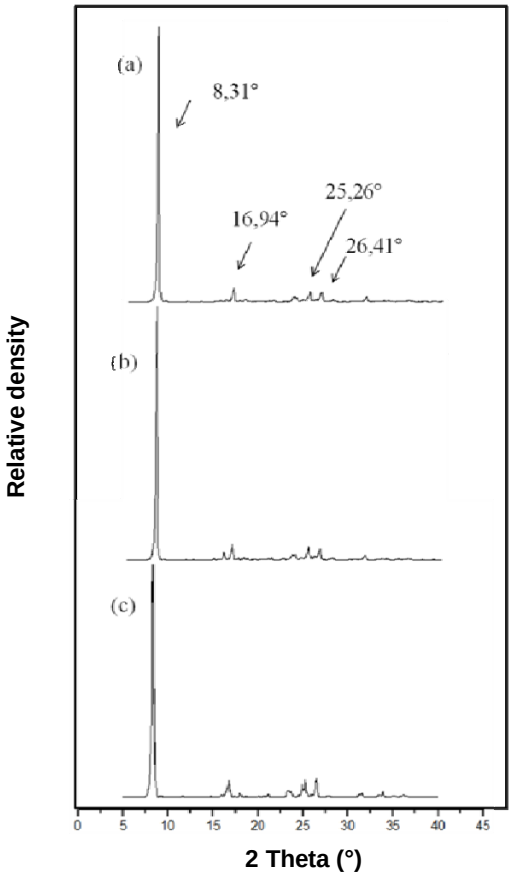

Figure 5. Diffractogram of LAP 01 (a), LAP 02 (b) and LAP 03 (c).

Granulometric and crytal morphology analysis

Laser diffraction is a commonly used method for the measurement of particle size. The particle size distribution can be represented by the mean value and percentile of the population that is below the expected diameter: 10 (P10), 50 (P50) and 90 (P90). Comparative data of mean diameters for different percentage populations, obtained from LAP 01, LAP 02 and LAP 03, are summarized as shown in Table 4.

Batches LAP 02 and LAP 03 exhibited statistically equivalent values. Batch LAP 01 was differentiated from the others, in that it had a higher mean particle size. Therefore, strict control of lapachol particle size is necessary, since this physical parameter can change the effectiveness of the dosage form and it has a direct relationship with its dissolution rate (Storpirtis et al., 2009).

SEM is an important technique for the characterization of drugs, because it is possible to observe both the crystal habit of a compound and its crystal size (Brittain et al., 1991). SEM images (Figure 6) clearly show that LAP forms stratified plates, with visible separation between the layers. A comparative analysis of the surface of the three batches of LAP, unlike the laser diffraction analysis, exhibited variation, albeit not statistically significant.

Qualitative analyses obtained by SEM/EDS for the three batches revealed carbon and oxygen, as would be expected for LAP. Chemical groups containing these elements were visualized using infrared spectroscopy diagram. No other elements were detected in the stoichiometric levels, as shown in Figure 7.

CONCLUSION

This study was fundamental for the gathering of information on the physical, physciochemical and thermal characteristics of LAP. Thus facilitating the monitoring of drug quality of LAP, given it is not yet described in official compendia.

Melting range, IR spectrophotometry, as well as NMR spectroscopy allowed the identification of LAP. Through high performance liquid chromatography and DSC, it was possible to evaluate the purity of the three batches of the drug. Particle size distribution and crystal habit tests are of great importance for the characterization of low-solubility drugs. The use of SEM and DR-X confirmed the

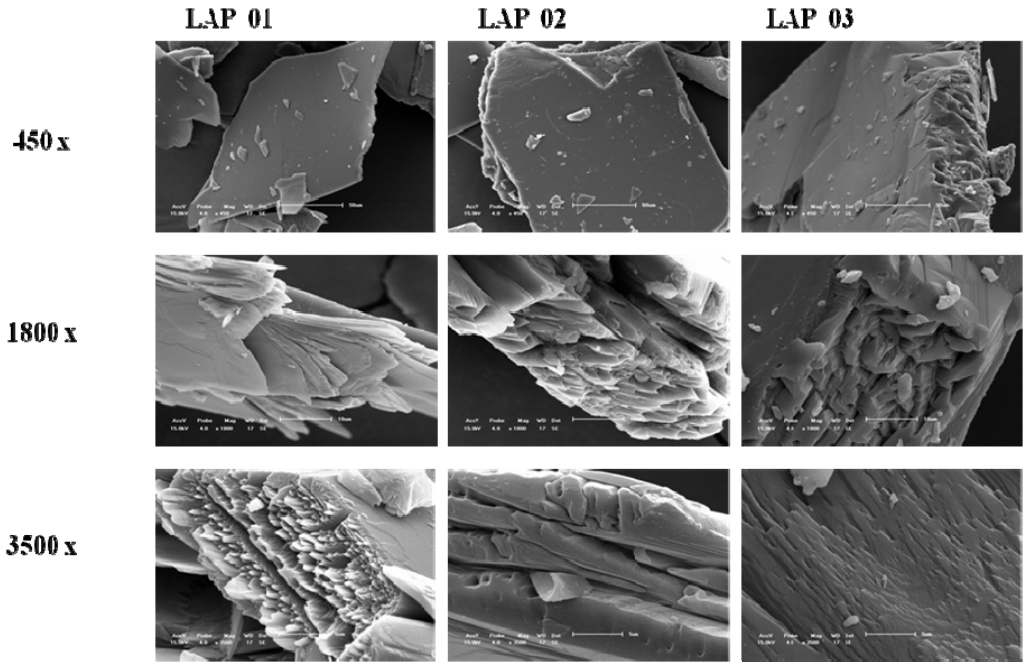

Figure 6. Crystal habit of LAP 01, LAP 02 and LAP 03.

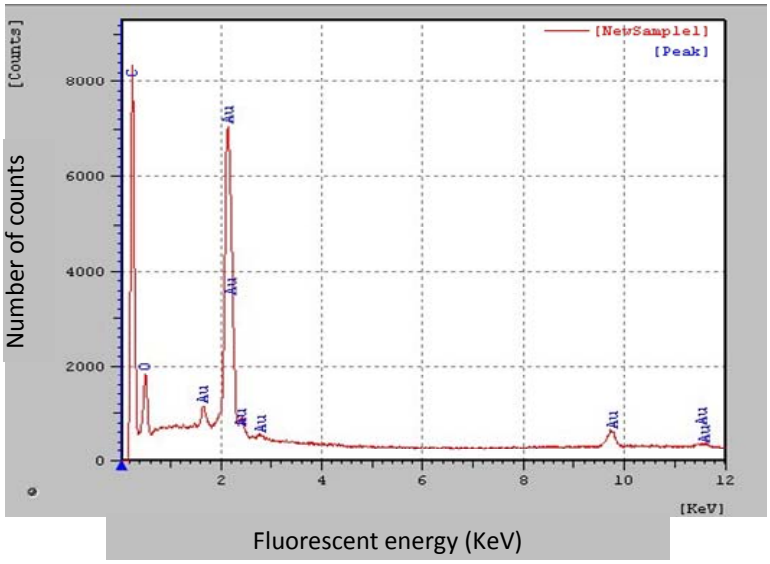

Figure 7. Qualitative elemental analysis (SEM/EDS) of lapachol (Au coating applied to the sample).

crystal habit of the drug.

Finally, the obtained results show that the use of a specific set of techniques is necessary to ensure the quality of lapachol.

Conflict of interest

Authors declare that there are no conflicts of interest.

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