Melatonin and Hyaluronic Acid Mixture as a Possible Therapeutic Agent in Dental Medicine

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The present work reports an efficient procedure for a simultaneous determination of melatonin (MEL) and hyaluronic acid (HA) mixture applying first derivative UV-Vis spectrophotometry. At selected wavelength (205 nm), the linearity range for each component of the mixture was established. The linear regression correlation coefficients for the direct UV-Vis spectra (R_{HA}^2 = 0.9627) and their first derivate (R_{HA}^2 = 0.9986) put in evidence a better fit for the derivative procedure. Through such simple analysis method, it was possible to emphasize that each component of MEL-HA mixture preserves its identity and characteristic properties. Consequently, the association of MEL and HA biomolecules could be used for obtaining a complex therapeutic agent with extensive applications in dental medicine.

Keywords: melatonin, hyaluronic acid, derivative UV-Vis spectrophotometry, oral pathology, therapeutic agent

Melatonin (MEL), is an endogenous hormone synthesized from tryptophan, mainly in the pineal gland, but also in other tissues such as retina, bone marrow, the gastro-intestinal track, the immune system and skin, in a circadian manner, showing the highest levels of secretion at night, in most species [1].

The role of MEL in the oral cavity (both in physiological and pathological processes) is basically related to its antioxidant and anti-inflammatory effects, as well as acting as a mediator in bone formation and resorption [2].

MEL was proven to directly neutralize reactive oxygen species [3] acting as an antioxidant in several conditions and tissues. Due to its antioxidant properties and its ability to detoxify free radicals, melatonin can also interfere in the bone resorption process, mediated by the osteoclasts, acting at the level of the osteoclast lacuna and blocking the reactive oxygen species produced by the superoxide dismutase [4].

With regard to bone formation, MEL promotes osteoblast differentiation [5] and stimulates the formation of a new mineralized matrix [6]. It was observed that, in human osteoblasts, *in vitro*, MEL has the ability to stimulate, at micromolar concentrations, the proliferation and synthesis of collagen type I, also upregulating other bone matrix proteins and bone markers (including alkaline phosphatase, osteopontin, and osteocalcin), and consequently reducing the osteoblast differentiation period from 21 days to 12 days [6].

Another role of MEL, important for bone regeneration, is that of mediator of angiogenesis [7] by increasing vascular endothelial growth factor (VEGF).

Hyaluronic acid (HA) is a glycosaminoglycan, produced primarily by mesenchymal cells but also by other cell types [8], widely found in the human body, mostly in the extracellular matrix, vitreous humor of the eye and synovial fluid of articular joints [9]. It is a highly attractive natural biomaterial due to its participation in cell behavior and cell signaling, being widely distributed throughout connective, epithelial, and neural tissues. HA's viscoelastic properties and high hydration ratio, plays an important role in the control of tissue hydration [10,11], stimulating bone wound healing.

Moreover, Sakay and co-workers reported the role of extracellular HA in mediating anti-Candida activity of epithelial-cells [12], this characteristic being of great interest for the oral environment.

Hyaluronic acid molecules of different weights may have different rheological and biological properties. For instance, according to Selvin and coworkers, low-molecular-weight HA (LMW-HA) enhances angiogenesis and increases collagen production by endothelial cells as compared to high-molecular-weight HA (HMW-HA) with inhibitor effect on angiogenesis [13]. Therefore, prior knowledge about the molecular weights correlated with the characteristics is essential for proper utilization of hyaluronic acids in the development of biomaterials and medications [14].

MEL and HA have both favorable effects on soft and hard tissues of the oral cavity.

Therefore, the aim of the present study was to investigate, using derivative UV-Vis spectrophotometry, if MEL and HA compounds could preserve their characteristics in a mixture (MEL-HA). This study could be consider the first step in evaluating the association of the two biomolecules for obtaining a complex therapeutic agent to be used in dental medicine and periodontology.

Experimental part

Equipment

The UV-Vis spectrophotometric studies were performed using a dual beam spectrophotometer Varian Cary® 50 (Victoria, Australia) UV-Vis with a spectral bandwidth 1.5 nm, resolution of 1 nm and at a scan rate of 300 nm/s, over wavelength range $\lambda \in$ (200 to 800 nm). Samples were put in quartz cells having 10 mm thickness. Working temperature was 25 ° C.

Materials

Hyaluronic acid (HA – reference standard, USP) solutions with concentrations between 1 - 4 mg/mL were used for the spectrophotometric calibration curves. The solutions

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have been obtained in deionized water (conductivity 128 μS/cm^2). The fresh prepared solutions of melatonin (MEL – Sigma-Aldrich, Missouri, USA), 3 mg/mL, were obtained in ethanol (Merck KGaA, Darmstadt, Germany). Taking into account the final target of our studies, namely to develop a topic treatment for oral lesions, specific quantities of MEL - 3 mg/L and HA-2 mg/mL were taken to prepare the MEL-HA composition. The mixture melatonin (MEL) -hyaluronic acid (HA) was prepared by initially dissolving melatonin and then adding hyaluronic acid. For homogenization, the mixture was maintained for one hour in a thermostatically controlled ultrasonic bath (25^{0}C). For preservation between experiments, the solutions were kept in a dark, cold place.

Investigation method - The first derivative of the UV-Vis spectrophotometric curve

An extensive literature review highlighted that, to date, no spectrophotometric methods have been applied to the simultaneous determination of MEL and HA in a mixture. Any determination of the two components in the presence of each other, applying conventional spectrophotometry [15,16] without prior separation procedures, is not possible due to the overlapping of the characteristic UV spectra and the possible interference due to the excipients (when formulations of the compounds are used). The application of derived spectrophotometric techniques is very useful when signal overlapping or interfering can occur [17,18], providing a versatile and effective working technique for both qualitative and quantitative analysis of various pharmaceutical mixtures [19-22]. Very often, in the spectrophotometric determinations performed on complex samples, the characteristic bands of the analyte are superimposed on a large, sometimes curved, background. In such cases, background could be reduced by differentiation [23].

Such spectrophotometric technique refers to the derivative spectra of basic, so-called zero-order, spectrum, with the results expressed through:

$$D^n_{a,\lambda} = \frac{d^n A}{d^n \lambda} = f \qquad (1)$$

for which: $D^n_{a,\lambda}$ is value of n-derivative order for an analyte (a) at a certain analytical wavelength (λ), A represents the absorbance, while *n* is the derivate order.

An important aspect of derivative spectrophotometry is the fact that it is characterized through the key features of spectrophotometry, namely: Lambert- Beer law, mathematically expressed by the following equation:

$$D^n_{a,\lambda} = \frac{d^n A}{d^n \lambda} = \frac{d^n \varepsilon}{d^n \lambda} \cdot c_a \cdot l \qquad (2)$$

where:ε is the molar absorption coefficient (cm^{-1}mol^{-1}l) , c_a-concentration of analyte (a) (mol/L), and l represents the thickness of solution layer (cm).

In addition, the law of absorbance additivity for mixtures applies. For a *n*-component mixture, the derivative spectrum is given by the sum of derivative spectra of each component according to:

$$D^n_{mixture,l} = D^n_{a1,\lambda} + D^n_{a2,\lambda} + \cdots + D^n_{am,\lambda} \qquad (3)$$

where a_m represents a certain analyte from the given mixture.

Derived spectrophotometry allows solving, identifying the components of complex mixtures having as main advantages the increased sensitivity and accuracy. However, the shape of the derived spectra is complicated compared with the zero-order one, as new peaks (maxima and minima) appear. Important is that the maxima from zero-order spectrum are situated at the same wavelength for the first order derivate extremes. Moreover, the presence of maxima and minima at certain wavelengths allows the identification of specific compounds in the presence of others even when interfering components are present.

In the present study, we propose the use of UV-Vis-derived spectrometry for quick, simple and accurate determination of mixed MEL and HA. In the meantime, we demonstrated that the two components do not interact, preserving their individuality and characteristic properties. Thus, such mixture could allow the preparation of an active therapeutic agent with applications in dental medicine and periodontology.

Results and discussions

The UV-Vis spectrophotometric analysis for hyaluronic acid gave the spectra shown in figure 1.

For each of these spectra, their first derivatives were calculated and traced (fig. 2). It could be easily noticed that the signal-to-background is increased in the case of derivate. The derivative curves allows spectral discrimination for mixture analysis.

Comparing the direct and the derived spectra, it is observed that the latter offer the possibility of accurately deciphering the absorption maxima, sensing better the spectral absorption characteristics [24]. We could consider this method as a qualitative fingerprinting analysis.

Applying direct UV-Vis spectrophotometry for hyaluronic acid, the absorbance for various concentrations within the range of 1-4 mg / mL was determined. The first derivative analysis exhibited the best linear response to HA concentration at 205 nm, where the maximum absorption for HA was registered, therefore this wavelength have been selected for measurements. Linear regression analysis was applied. Thus, the slope (0.183) and the correlation coefficient (R^2 = 0.9627) were obtained for the wavelength set at 205 nm.

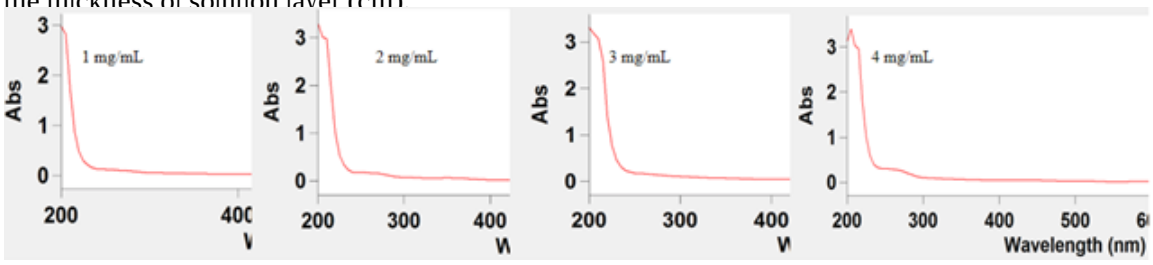

Fig. 1. UV-Vis spectra for HA at different concentrations

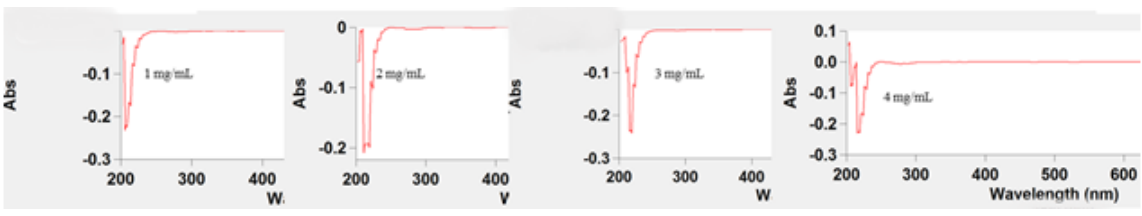

Fig. 2. UV-Vis first-derived spectra for HA at various concentrations

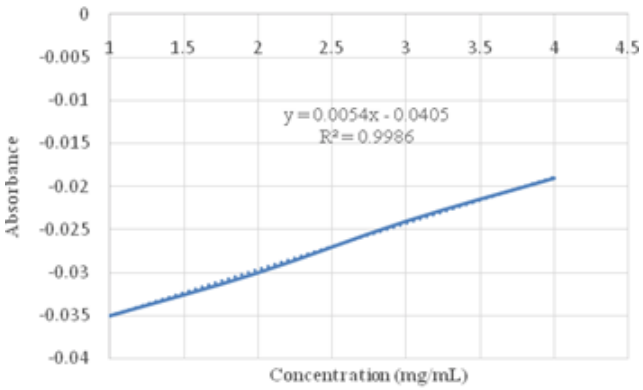

Fig. 3. The linearity of the absorbance against concentration for the first derivative spectra.

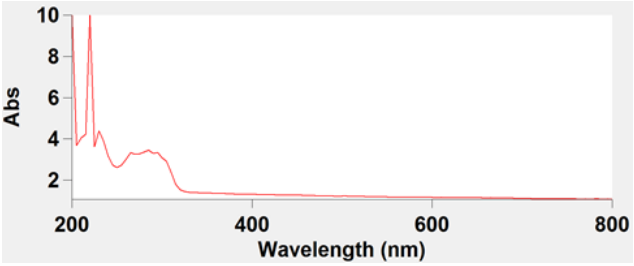

Fig. 4. UV-Vis spectrum for the MEL-HA mixture

The calibration curve of absorbance function of HA concentration resulted from the derived spectrophotometric analysis is presented in figure 3.

Applying the least squares method, a regression coefficient (R^2) of 0.9986 was calculated for the linearity of the derived spectrophotometric response, which complies with the quality standard requirements. The recorded results have shown a better linear regression coefficient obtained for the calibration curve based on the derived spectrophotometric spectra.

In figure 4 the zero-order UV-Vis spectrum for MEL-HA mixture in 200-800 nm region is presented. It can be noticed that at about 207, 218, 230 and 290 nm four bands appeared for the MEL-HA mixture. The bands from 218, 230 and 290 nm were not recorded on HA spectrum. In fact, MEL presents such bands in UV region at these wavelengths [25].

For the mixture, in the 200-400 nm region, a partial overlap of the components' spectra is noticed, and therefore determination of the two compounds is not possible directly from the absorbance measurements. This shortcoming could be solved by obtaining the first derivative of the UV-Vis spectrum shown in figure 5.c.

Comparing individual derived UV-Vis spectra (I) of hyaluronic acid and melatonin with that of their mixture, one could easily identify each specific components' maxima/minima (fig. 5).

A spectrum having a simple peak shape with a single maximum (spectrum for HA, fig. 1), presents sequential derivative, which exhibits a series of alternating maxima and minima. The number of these maxima and minima is one more than the derivative order [26]. We also noted that the y-axis values (where numerical magnitude of the derivative is recorded) is much less than the original signal, due to the procedure to calculate the derivatives.

The results of the present study clearly highlights that in the MEL and HA mixture, each individual component could be identified and no derivate component is observed. Taking into account the specific action of each compound, it is expected that a complex mixture containing both MEL and HA would result into an active formulation to be used in oral lesions' treatment.

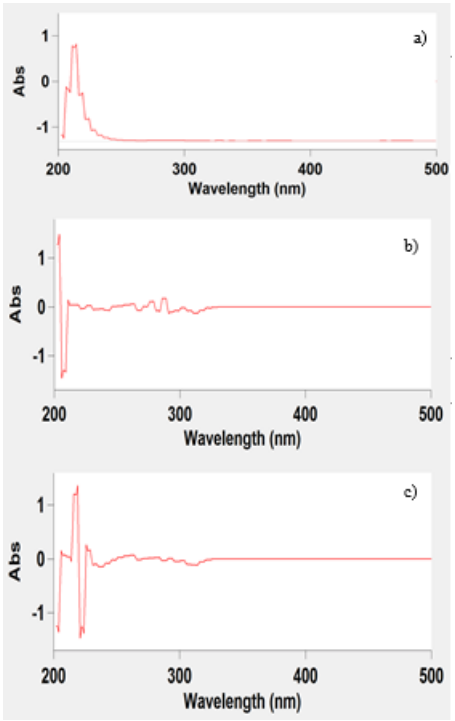

Fig. 5. Derived UV-Vis spectra for: a) MEL (3mg/mL); b) HA (1mg/mL); c) MEL-HA mixture

MEL actions at the cellular level are mediated by its G protein-coupled membrane bound melatonin receptors type 1 and type 2 (MT1 and MT2) as well as, indirectly by nuclear orphan receptors from the RORα/RZR family [27]. The presence of different levels of MEL in human saliva as well as the occurrence of both MT1 and MT2 receptors in salivary gland ducts and acini, oral epithelium, fibroblasts of the mucosal lamina and osteoblasts of maxilla alveolar bone, among other oral cells [28], could explain melatonin's effects in the oral cavity environment.

HA is bonded by various proteins (hyaladherins), widely distributed in the ectodermal matrix, the cell surface, the cytoplasm and the nucleus. HA receptors attached to the cell surface are the transmembrane glycoprotein cluster of differentiation 44 (CD44), found on virtually all cells, except red blood cells. Due to its intrinsic biocompatibility, lack of immunogenicity and viscoelastic physical properties, HA could be used as an important biomaterial for drug delivery [29]. HA can be lyophilized or esterified into a variety of different structural configurations such as sponges and membranes with various rate of biodegradation for obtaining resorbable grafting material in regenerative surgical procedures [30]. The bacteriostatic (mainly on *S. aureus* and *A. actinomycetemcomitans*) of both LMW-HA and HMW-HA [30] and fungistatic effects (on *C. albicans*) [12] of HMW-HA make it an attractive therapeutic agent to be used in various oral pathologies.

Conclusions

This experimental study is the first to investigate the application of the first derivative UV-Vis spectrometry for hyaluronic acid and melatonin simultaneously present in a complex mixture.

The first derived spectrophotometry is a selective, sensitive, rapid method, and can be easily used in routine analysis and quality control of hyaluronic acid in various formulations.

The proposed investigation technique allows the mixture of melatonin and hyaluronic acid to be quantified with good accuracy and improved sensitivity. Moreover, the derivation method offers the possibility of easy measurement of the separated peaks by recording larger analytical signals. The method allows the direct

discrimination of the mixtures without prior separation. In addition, it could be applied without solving equation systems or separation steps.

Taking into account the proven facts that MEL and HA have complementary effects on the soft and hard tissues of the oral and maxillofacial region, a therapeutic agent obtained by mixing both components could be beneficial.

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