Physico-Chemical Characterization and Analytical Development for Sodium Azumolene, a Potential Drug Designed to Fight Malignant Hyperthermia

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Abstract

Sodium azumolene is a drug designed to fight Malignant Hyperthermia (MH), which is characterized by genetic predisposition and triggered by the use of inhalational anesthetics. This drug is shown as a water-soluble analogue of dantrolene sodium, 30-folds more water soluble, which gives advantages for its emergency use. To our knowledge there is no analytical method for sodium azumolene raw material or dosage form published so far. The objective of the present investigation was to develop and validate analytical methods to achieve sodium azumolene chemical identification and quantification. The sodium azumolene was characterized regarding its thermal behavior, by differential thermal analysis and thermogravimetric analysis; Visible, UV and infrared absorption. To accurately assess the sodium Azumolene content three different analytical methods (visible and UV spectrophotometry and high performance liquid chromatography) were developed and validated. All methods showed to be linear, accurate, precise and reliable. Azumolene has shown to be equipotent to dantrolene in the treatment and prevention of an MH crisis and the great advantage compared to dantrolene is better water solubility. This study has characterized the sodium azumolene and presents new analytical methods which have not been reported so far.

Keywords: Malignant hyperthermia; Sodium azumolene; UV-VIS Spectrophotometry; Liquid chromatography; Quality control

Introduction

Malignant hyperthermia, which so far has no cure defined, is also known as malignant fever, malignant hyperpyrexia and anesthetic fever. It is hereditary (autosomal dominant) and latent, it is characterized by hypermetabolic response to volatile anesthetics (halothane, isoflurane, sevoflurane, isoflurane, enfurane and desflurane) and succinylochlorine, initially from a derangement of intracellular calcium homeostasis [1-4].

It can occur at any time of anesthesia procedure and even some hours after. Its incidence has been reported with variations from 1/20,000 to 1/100,000 users of the triggering anesthetic agents cited above. The incidence of malignant hyperthermia in aesthetician procedure is of 1/15,000 in children and 1/50,000 in adults. There are studies reporting that 50% of cases occur in people less than 15 years [5-7].

Whereas the evolution of the disease can be fatal and occurs quickly, early diagnosis and specific treatment are of great importance, reducing mortality from 80% to less than 10% of the cases [8,9]. Thus, encountering the signs described for the disease associated with the use of volatile anesthetics agents, it must be considered a case of malignant hyperthermia and specific treatment protocol must be initiated [8,9]. There should be immediate cessation of the exposure to possible agents triggering of malignant hyperthermia with replacement by safe anesthetic drugs, followed by administration of dantrolene sodium or succinylochlorine, since the latter shows poor water solubility and requires a dilution of 20 mg of the lyophilized drug in 60 ml water for injection. Sodium azumolene is many times more soluble in its administration vehicle than dantrolene and provides an easier and faster drug administration procedure which is necessary for emergency situations [9,16,17].

The chemical structure of sodium azumolene (1-(((5-(4-bromophenyl)-2-oxazolyl)methylene)amino)-2,4-imidazolidinedione sodium) is shown at Figure 1.

To our knowledge there is no analytical method for sodium azumolene raw material or dosage form published so far. There is no monograph for sodium azumolene in any pharmacopoeia. The aims of this study was to develop and validate analytical methods to achieve sodium azumolene chemical identification and quantification. The sodium azumolene was characterized regarding its thermal behavior, by differential thermal analysis and thermogravimetric analysis; Visible, UV and infrared absorption. To accurately assess the sodium Azumolene content three different analytical methods (visible and UV spectrophotometry and high performance liquid chromatography) were developed and validated. All methods showed to be linear, accurate, precise and reliable. Azumolene has shown to be equipotent to dantrolene in the treatment and prevention of an MH crisis and the great advantage compared to dantrolene is better water solubility. This study has characterized the sodium azumolene and presents new analytical methods which have not been reported so far.

Figure 1: Chemical structure of sodium azumolene.

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study were to provide reliable analytical methods as tools to analyze sodium azumolene for routine use.

**Material and Methods**

**Chemicals**

Sodium azumolene chemical reference (assigned purity of 100%) and the product as powder for injectable solution were kindly donated by Cristalria (Itapira, Brazil).

Methanol LC grade was purchased from Tedia (Fairfield, USA). Ultrapurified water was prepared in-house by using Direct-Q® water system (Millipore Corporation, Billerica, USA). Prior to use, mobile phase solvents were degassed in an ultrasonic bath for 30 min. Solvents were filtered through a 0.45 μm membrane filter.

**Apparatus**

Liquid Chromatography apparatus (Waters Corporation, Milford, MA, USA), equipped with a Waters 1525 binary pump, a Rheodyne Breeze 7725i manual injector, a Waters 2487 UV–VIS wavelength detector was used. In addition a Liquid Chromatography apparatus HPLC analysis was conducted by using a RP C18 column (Waters, Milford, MA, USA), with 5 μm particle size; 4.6×250 mm.

For thermal analysis the SDT 2960 (TA Instruments) was used to do the differential scanning calorimetry and thermal gravimetry analyses simultaneously. For UV–VIS analysis a spectrophotometer UV mini-1240 Shimadzu (Columbia USA) was used. Spectrometer of infrared Shimadzu FTIR 3000 (Columbia, USA) was used.

**Validation of Methods**

All LC, UV and VIS spectrophotometric methods were validated according to the International Conference on Harmonization (ICH) guidelines [18]. The following characteristics were considered for validation: Linearity, range, accuracy and precision.

Linearity was determined in the range 5.0 to 15.0 μg/mL (LC method); 7.0 to 12.0 μg/mL (UV spectrophotometric method) and 8.0 to 13.0 μg/mL (VIS spectrophotometric method). The least squared method was used to calculate the regression coefficient from peak area or absorption vs. concentration of standard solutions.

Accuracy was tested by calculating the percentage recoveries of sodium azumolene from sample solutions at different concentrations and by determining the relative standard deviation (RSD). Precision was assessed at different levels–repeatability (system repeatability by testing three curves constructed with five different standard solution concentrations high, intermediate and low, in a different day, with two days interval between repeatability and intermediate).

The limits of detection (LOD) and quantification (LOQ) were calculated as 3 and 10 σ/s, respectively, where S is the slope of the calibration curve and σ is the standard deviation of y-intercept of regression equation [18].

**LC conditions**

Chromatographic analysis was performed in isocratic mode. Mobile phase consists of methanol-water (75:25, v/v, pH 3.0 adjust with formic acid) at a flow rate of 1 mL/min. The injection volume was 20 μL and the detection wavelength was 340 nm. The retention time of sodium azumolene was 4.25 min (Figure 2). All experiments were performed at room temperature (25°C) and the total area of peak was used to quantify sodium azumolene.

**Preparation of standard and sample solutions to validate the LC method:** Six standard and sample aqueous solutions of sodium azumolene were prepared at the concentrations 5.0; 7.0; 9.0; 11.0; 13.0 and 15.0 μg/mL. All these solution were evaluated at 340 nm.

**UV Spectrophotometric conditions**

The UV spectrum of sodium azumolene was obtained using an aqueous solution of this drug at 10 μg/mL. The absorption peak could be observed at 339 nm. This wavelength was used for the drug determination.

**Preparation of standard and sample solutions to validate the UV Spectrophotometric method:** Solutions of sodium azumolene were prepared at the concentrations 7.0; 8.0; 9.0; 10.0; 11.0 and 12.0 μg/mL. Standard and sample solutions were evaluated at 339 nm.

**VIS Spectrophotometric conditions**

For the visible determination a purple color was obtained by addition of 0.1% chloranilic acid in a sodium azumolene solution prepared with acetonitrile at 10.0 μg/mL. This coloring reaction was described by Melby and coworkers [19]. After the staining process the visible spectrum of the solution was obtained and an absorption peak could be observed at 507 nm.

Charge transfer reactions can be ideal for visible analysis methodology. The chloranilic acid is an acceptor of electrons and it is widely used for photometric analysis in visible region of spectre to penicillins [20], cephalosporins [21], antimalarials [22], antifungals [23] and propranolol [24]. In a literature search of sodium azumolene it was not found the association of the drug with the chloranilic acid.

The mix of chloranilic acid and acetonitrile shows a VIS absorption peak near to 390 nm. However after addition of this mix to sodium azumolene the color immediately changes from yellow to purple and the absorption peak also changes to 507. This reaction may be explained by the occurrence of ion pairing [25,26].

**Preparation of standard and sample solutions to validate the VIS Spectrophotometric method:** Standard and sample solutions of sodium azumolene were prepared at 8.0; 9.0; 10.0; 11.0; 12.0 and 13.0 μg/mL using 0.1% of chloranilic acid in acetonitrile. All these solution were evaluated at 507 nm.

**Results and Discussion**

**Validation of LC Method**

The LC method validated is fast (retention time=4.25 min) and optimized method. Good symmetry was obtained by using endcapped
column (C18, 4.6×250 mm; 5 μm) and mix of methanol and water (75:25, v/v; pH 3.0 adjust with formic acid) as mobile phase.

Good linearity was obtained at the concentration range tested. It was evaluated by the analysis of variance (ANOVA) and results showed regression statistically significant (Fcalculated<Fcritical; P=0.05). The equation of the curve was y=44299X+137924 with a good coefficient of determination (r²=0.9990).

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day) and was expressed as R.S.D. (%) of a series of measurement. The repeatability test shows R.S.D. equal to 0.08 and the inter-day variability was equal to 1.23% (Table 1). The accuracy of the method was determined by the mean recovery, by adding standard to sample. The mean result was 98.14 % indicating an agreement between the true value and the value found.

LOD and LOQ were determined based on the standard deviation of the response and the slope, based on the calibration curve [18], and the values were 0.95 and 2.89 μg/mL, respectively (Table 1).

To evaluate the robustness of the proposed method low and deliberate changes were made on the flux rate of mobile phase. Sample and standard solutions were submitted at the following flux rate 0.9; 1.0 and 1.1 mL/min. No interference over assay test was found.

**Validation of UV Spectrophotometric Method**

The developed UV spectrophotometric method has an easy procedure; moreover it is fast and low cost. The UV method shows to be linear at the concentration range tested (7.0 to 12.0 mg/mL). The equation of the curve was y=0.0868X−0.0425 with a good coefficient of determination (r²=0.9995). The validity of the linearity test was evaluated by analysis of variance (ANOVA) which shows the statistically significance of the test.

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day) and was expressed as R.S.D. (%) of a series of measurement. The result obtained shows R.S.D. equal to 0.83%, indicating good intra-day precision. Inter-day variability was calculated from assays on 3 days and shows a mean R.S.D. of 2.22% (Table 1). The accuracy of the method was determined

| Parameters                     | LC method          | UV method         | VIS method       |
|-------------------------------|--------------------|-------------------|------------------|
| Usefull concentration, μg/mL  | 6                  | 6                 | 6                |
| Concentration, μg/mL          | 5.0-15.0           | 7.0-12.0          | 8.0-13.0         |
| Analytical curve              | 44299X+137924      | 0.0868X-0.0425    | 0.0255X-0.0257   |
| Determination coefficient (r²)| 0.9990             | 0.9995            | 0.9970           |
| R.S.D. of repeatability (%)   | 0.08               | 0.83              | 0.73             |
| R.S.D. intermediate precision (%) | 1.23              | 2.22              | 0.88             |
| Accuracy (%)                  | 98.14              | 98.1              | 98.63            |
| RSD of accuracy (%)           | 0.74               | 0.9               | 0.68             |
| LOQ, μg/mL                    | 2.89               | 0.73              | 3.82             |
| LOD, μg/mL                    | 0.95               | 0.24              | 1.26             |

Table 1: Validation parameters for chromatographic, visible and UV quantitation methods for azumolene.
by infrared spectroscopy and UV-VIS spectrophotometry. The infrared (IR) spectrum of sodium azumolene standard (A) and sample (B) are shown in Figure 3. The UV and VIS spectrum are shown in Figures 4 and 5. The extinction coefficient was determined by measurement of the spectrophotometric absorbance at 339 nm of sodium azumolene aqueous solution. The result was equal to 0.246 M⁻¹.

Moreover, sodium azumolene sample and standard were evaluated for its thermal behavior. A simultaneous differential scanning calorimetry (DSC) analysis and thermogravimetric (TG) analysis were made in atmosphere of oxygen and heating rate of 20°C/min. The results are shown in Figure 6.

Validation of VIS Spectrophotometric Method

At the same way of the UV method, the developed VIS method is fast and low cost. It shows to be linear at the concentration range tested (8.0 to 13.0 μg/mL). The equation of the curve was y=0.0255X–0.0257 with a good coefficient of determination (r²=0.9970). The validity of the linearity test was evaluated by analysis of variance (ANOVA) which shows the statistically significance of the test.

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day) and was expressed as R.S.D. (%) of a series of measurement. The result obtained shows R.S.D. of 0.73% (Table 1). Inter-day variability was calculated from assays on 3 days and shows a mean R.S.D. of 0.88% (Table 1). The mean recovery found was equal to 98.63% (Table 1). The LOD and LOQ values were 1.26 and 3.82 μg/mL respectively.

Sodium azumolene characterization and assay testing–A method comparison

Sodium azumolene sample and standard were well characterized by the mean recovery. The mean result was 98.10% (Table 1) indicating an agreement between the true value and the value found. The LOD and LOQ were calculated at the same way used for LC method. Their results were 0.24 and 0.73 μg/mL respectively.
Our study leads to conclude that the three validated method can be used to determine sodium azumolene and applied as quality control tools. The choice of the method to use should be made considering cost, simplicity, equipment, solvents, speed, and application to large or small workloads.

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