DEVELOPMENT AND VALIDATION OF A RP- HPLC METHOD FOR THE QUANTITATION STUDIES OF BROMADIOLONE IN RATITOX F

DEZVOLTAREA ŞI VALIDAREA METODEI RP- HPLC DE DETERMINARE CANTITATIVĂ A BROMADIOLONE DIN PRODUSUL RATITOX F

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Summary

An isocratic high-performance liquid chromatography (HPLC) procedure was developed for the quantitative determination of bromadiolone (hydroxycoumarins) in Ratitox F product – rodenticide. HPLC separation was carried out by reversed phase chromatography ODS 2 Hypersil C_{18} (250 mm x 4.6 mm i.e.; 5 µm particle size), held in thermostat at 25°C. The mobile phase consisted of methanol/ 0.1% aqueous solution phosphoric acid (90/ 10 v/ v), with a flow rate of 1 ml/ min and with UV detection at 265 nm. In order to validate the method, the following parameters have been investigated- linearity (r^2=0.9999), range, precision, accuracy, specificity, limit of detection and limit of quantification. The described method can be successfully applied for the analysis of Ratitox F – rodenticide.

Key words: *bromadiolone, Ratitox F, reversed phase high performance liquid chromatography RP-HPLC UV - VIS, validation*

Rezumat

A fost dezvoltată şi validată o metodă isocratică de lichid cromatografie de performanţă înaltă pentru determinarea cantitativă a bromadiolone din produsul Ratitox F - raticid. Separarea HPLC a fost realizată prin cromatografie în fază inversă pe o coloana ODS 2 Hypersil C18 (mărimea particulelor 5 µm; 250 x 4.6 mm diametrul intern), termostatată la 25°C. Faza mobilă a fost metanol/ acid fosforic 0.1% solutie apoasa (90/ 10 v/ v), cu debit de 1 ml/ min. şi detecţie UV la 265 nm. Pentru validarea metodei au fost urmăriţi următorii parametri - linearitatea (r^2=0,9999), intervalul, precizia, acurateţea, specificitatea, cantitatea minimă decelabila LOD, cantitatea minimă masurabilă LOQ. Metoda descrisă poate fi utilizată cu succes pentru analiza compusului activ din produsul Ratitox F

Cuvinte cheie: *bromadiolone, Ratitox F, UV-VIS cromatografie de lichide de performanţă ridicată de fază inversă, validare*

This paper aimed to develop and validate an HPLC sensitive applicable method to determine the quantity of bromadiolone in Ratitox F, contributing to the quality and safety control of these types of pharmaceutical preparations.

Materials and methods

Reagents

The standard reference bromadiolone has been provided by SIGMA (Germany). Methanol, dimethylformamide and phosphoric acid have been provided by MERCK (Germany).

Ratitox F has been provided by Romvac Company and used during shelf-life.

All the chemical substances used had pharmaceutical or analytical degree. Double distilled water, filtered on 0.45 µm membrane was used.

System and chromatographic conditions

HPLC method was carried out on a LC SURVEYOR (Thermo Electron Corporation, USA) provided with quaternary pump, auto sampler, 25 µl loop and UV-VIS detector – diode array (Thermo Electron Corporation, USA). The integration of chromatographic peaks has been carried out with the ChromQuest soft (Thermo Electron).

The analyses have been performed by using an ODS 2 Hypersil C18 (5 µm particle size; 250 x 4.6 mm inner diameter).

The samples have been isocratically eluted in methanol and phosphoric acid aqueous solution 0.1% (90/ 10 v/ v), with flow of 1 ml/min. Each sample has been filtered before injection with PVDF 0.45 µm filter (Thermo Electron).

The injection volume of the sample was 5 µl, and detection was carried out at 265 nm, at 25°C.

Preparing the standard reference solutions

Bromadiolone standard working solution had a final concentration of 0.025 mg/ ml, prepared in methanol.

The standard solutions for linearity fell within the area of 0.005 – 0.05 mg/ ml starting from a stock solution of bromadiolone of 1 mg/ ml prepared in dimethylformamide. All samples have been triplicated. The stock solution of bromadiolone is kept at +4°C for one week.

Preparing the test solutions

Weigh 30 g of product and add over 5 ml dimethylformamide and keep at ultrasound for 15 minutes; add 10 ml methanol and keep at ultrasound for 15 minutes. Filter through filter paper. Before injection filter the solutions through a 0.45 µm PVDF filter.

Chromatographic method validation

After establishing the chromatographic conditions, the method has been validated by observing the following parameters: linearity, working range, precision, accuracy, limit of detection, limit of quantification, specificity and system compliance, using ICH guide.

Linearity and working range

The analytical curve has been obtained with 5 different concentrations of bromadiolone placed between 0.005–0.05 mg/ml, prepared in triplicate.

The linearity was evaluated by linear regression analysis. The system has been balanced for minimum 30 minutes. 3 replicates have been injected from each concentration of standard bromadiolone at a volume of 5 µl, in order to verify the reproducibility of the detector response at each level of concentration.

Precision

The method precision has been determined through repeatability (same day) and intermediate precision (different days). The repeatability has been determined through 12 repeated analyses of the same test sample of Ratitox F, on the same day, in the same experimental conditions. The intermediate precision of the method has been determined through the analysis during 2 days (same day), and by other analyst within the same laboratory (different analysts).

Accuracy

In order to certify the accuracy of the recommended method, 9 samples have been analyzed using 3 levels of concentration which cover the working range.

System compliance

In order to ensure the validity of the analytical method, the test of system compliance has been carried out. 6 samples with 0.025 mg/ ml bromadiolone have been injected on this purpose at a volume of 5 µl.

The evaluation of the system compliance has been carried out with the ChromQuest soft, by analyzing the parameters – area, retention time and asymmetry.

The analysis of bromadiolone in the product

The analysis of the content in bromadiolone in Ratitox F has been carried out under the developed method recommended for validation using the reference standard.

Results and discussions

In order to determine the quantity of bromadiolone in RATITOX F a HPLC method of reversed phase has been suggested, choosing the optimum conditions of chromatographic separation.

The analysis of the chromatograms reveals that there are no interferences between the compound of interest and the rest of the matrix constituents, the retention time being 4.292 min. The asymmetry of the peak was good, equal to 1.0.

The calibration curves for bromadiolone have been formed by representing the peak area towards concentration.

The linearity has been observed in the selected reference field.

The concentration range was 20 – 200% towards the working concentration.

By applying the linear regression for the calibration curve, a coefficient of determination r^2 = 0.99929 has been established.

The method precision represents the degree of compliance between the results of

the individual tests, through repeated application of the method on multiple samples of a homologue batch.

Repeatability has been studied by calculating the relative standard deviation (RSD) of 12 samples with a concentration of 0.1 mg/ ml bromadiolone, carried out on the same day and experimental conditions.

The intermediate precision involves the estimation of the variability of analysis when the method is used in different laboratories, on different days, by different analysts or with different equipment.

The results are detailed in Table 1.

Table1

Concentration, precision and intermediate precision in HPLC method for bromadiolone

Parameter	Value
Concentration	0.025 mg/ ml
RSD% (same day)	0.489%
RSD% (different day)	0.458%

The accuracy of method is the degree of similarity between the results practically obtained with the method, compared to the theoretical value.

The accuracy has been determined by analyzing 9 samples with bromadiolone in concentration of 80, 100, 120% towards the suggested working concentration (0.02, 0.025, 0.03 mg/ ml).

Table 2

Recovery of bromadiolone from samples analyzed through RP-HPLC

Theoretical amount mg/ ml	% Recovery	% Accuracy
0.02	106.666%	
0.025	126.673%	121.594%
0.03	116.516%	

[a] mean of three replicates

The analysis of data presented in Table 2 reveals that the method is accurate within the recommended range, the average recovery rate being 121.594% for the compound of interest - bromadiolone.

In order to evaluate the resolution and reproducibility of the recommended system of analysis, compliance tests have been carried out.

The results presented in Table 3 prove that the parameters are within the limits of compliance.

Table 3

Results of the system compliance test for bromadiolone

Parameter	Minimum	Maximum	RSD (%)	Status
Asymmetry	0.91104	0.91993	0.384	complies
Retention time	4.282	4.292	0.105	complies
Area	2013881	2032493	0.377	complies

The limits of detection and quantification have been calculated, reaching the following values:

Table 4

Limit of detection and limit of quantification for bromadiolone

Component	LOD	LOQ
Bromadiolone	0.000384 mg/ml	0.001280 mg/ml

Conclusions

1. The results presented for the validation of RP–HPLC method prove its accuracy, linearity and precision and show the limits of detection and quantification.
2. The method can be successfully used for the quantification of bromadiolone as active substance.
3. The recommended method provides the advantage of using a comfortable analytical method, which requires a simple preparation of samples. Therefore, the method can be used for the routine analysis.

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