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Short communication

Quantitative determination and sampling of azathioprine residues for cleaning validation in production area

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Abstract

Cleaning validation is an integral part of current good manufacturing practices in any pharmaceutical industry. Nowadays, azathioprine and several other pharmacologically potent pharmaceuticals are manufactured in same production area. Carefully designed cleaning validation and its evaluation can ensure that residues of azathioprine will not carry over and cross contaminate the subsequent product. The aim of this study was to validate simple analytical method for verification of residual azathioprine in equipments used in the production area and to confirm efficiency of cleaning procedure. The HPLC method was validated on a LC system using Nova-Pak C18 (3.9 mm × 150 mm, 4 μm) and methanol–water–acetic acid (20:80:1, v/v/v) as mobile phase at a flow rate of 1.0 mL min^{-1}. UV detection was made at 280 nm. The calibration curve was linear over a concentration range from 2.0 to 22.0 μg mL^{-1} with a correlation coefficient of 0.9998. The detection limit (DL) and quantitation limit (QL) were 0.09 and 0.29 μg mL^{-1}, respectively. The intra-day and inter-day precision expressed as relative standard deviation (R.S.D.) were below 2.0%. The mean recovery of method was 99.19%. The mean extraction-recovery from manufacturing equipments was 83.5%. The developed UV spectrophotometric method could only be used as limit method to qualify or reject cleaning procedure in production area. Nevertheless, the simplicity of spectrophotometric method makes it useful for routine analysis of azathioprine residues on cleaned surface and as an alternative to proposed HPLC method.

Keywords: Cleaning validation; Qantitative determination; Liquid chromatography; Azathioprine residues; Production area

1. Introduction

The azathioprine is an immunosuppressive agent widely used in renal transplant patients. It inhibits the T and B cell proliferation that are involved in kidney rejection mechanism. However, its use is limited due to associated high toxicity [1,2].

Azathioprine is chemically 6-((1-methyl-4-nitroimidazol-5-yl)thio)purine. Its molecular weight is 277.26, slightly soluble in water (0.272 mg mL^{-1}) and insoluble in alcohol and in chloroform. The chemical structure azathioprine is illustrated in Fig. 1.

For the production of azathioprine, production areas of larger complexity is necessary due to the numerous risks associated to occupational exposure and related to cross contamination. The production and cleaning operations involved in the production area should follow strict good manufacturing practices. Among these are cleaning validations, which is critical for patients' safety and person involved in the production. Moreover, the cleaning validation is integral part of quality assurance that embodies all the necessary steps to guarantee the quality of medications be inside the adopted standards, be safe and effective for therapeutic application [3].

The objective of cleaning validation is to prove, through validated analytical method, that the cleaning procedure is efficient in removing product residues and excipients, degradation products, cleaning substance and other possible contaminants. This way cross contamination risk in production area can be reduced substantially [4].

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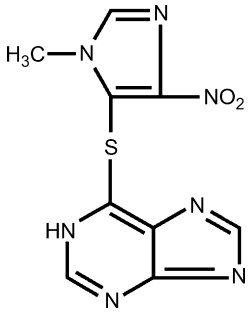

Fig. 1. Molecular structure of azathioprine.

During the cleaning validation following factors should be taken into consideration: equipment construction material, sealing part and parts that offers greater risk of contamination. It is important to standardize cleaning procedures and cleaning material, verification of residues chemical products and post-cleaning microbial load. Other factors such as time that the equipment can be considered clean, sampling procedure and analysis of contaminating residues in the equipment should also be considered. The analysis method and selected sampling procedure should be validated and presents adequate extraction-recovery to favor the analysis of possible contaminating residues [6].

The acceptable limit for residue in the equipments is not established in the current regulations. However, Food and Drug Administration (FDA) mention that the limit should be based on logical criteria, involving the risk associated to residues of a determining product [5]. The calculation of acceptable residual limit for active products in production equipments should be based on therapeutical doses, pharmacological activity and toxicological index. Several mathematical formulas were proposed that can be used to establish acceptable residual limit [6].

The aim of this study was to validate simple analytical method and its application in the determination of residual azathioprine in production area equipments and to confirm efficiency of cleaning procedure.

2. Materials and methods

2.1. Reagents

The azathioprine raw material was received from local pharmaceutical industry and was used as standard (99.7%). Methanol (HPLC grade) and acetic acid (analytical grade) were obtained from Merck (Darmstadt, Germany). High purity water was prepared by using Waters Milli-Q® plus purification system. The extraction-recovery sampling was done with "Alpha® Swab" (6.8 mm × 16.8 mm) model TX761 TEXWIPE® and stainless steel standard plates AISI 316 (25 cm^2) were used to simulate equipment surface.

2.2. Equipment

High performance liquid chromatographic system, model 5000 (Varian Associates, CA, USA); rotatory injection valve, Model 7125, Rheodyne® with a loop of 20 μL; variable ultraviolet detector, model Varian 4000; integrator model Varian 4400 (Varian Associates).

2.3. Chromatographic condition

Analytical conditions were optimized through the LC system using Nova-Pak C18 (3.9 mm × 150 mm, 4 μm) WATERS®. The mobile phase was constituted of methanol–water–acetic acid (20:80:1, v/v/v), at a flow rate of 1.0 mL min^{-1}. UV detection was made at 280 nm. The volume of injection was fixed at 20 μL. All analyses were done at room temperature 25 ± 2 °C, approximately. The mobile phase was prepared fresh each day, vacuum-filtered through a 0.45 μm Millex® (HV) hydrophilic membrane and degassed by ultrasonication for 20 min.

2.4. Standard solution preparation

Amount of azathioprine standard, equivalent to 25.0 mg was accurately weighed and transferred to a 100 mL volumetric flask. Approximately, 40 mL of methanol was added and content of the flask was ultrasonicated for 10 min. The solution in the flask was diluted to volume with the same solvent. The final concentration of solution was 250.0 μg mL^{-1} of azathioprine. All solutions were stored refrigerated until injection (2–8 °C). Appropriate dilutions were made with mobile phase to obtain solution containing 50.0, 20.0 and 10.0 μg mL^{-1} of azathioprine.

2.5. Recovery of sample from stainless plates

Stainless steel standard plates (25 cm^2) were contaminated with 1 mL of solution containing 10.0 μg mL^{-1} of azathioprine and plates were dried in oven at 60 ± 3 °C. Sampling was done with polyester swab previously humidified with purified water.

In brief, swab was passed on the board in zig-zag manner from right to left, returning from left to right, from top to bottom and returning upwards. For recovery of residues removed from plate, the sampling swab was immersed in 2.0 mL of mobile phase and the solution with swab immersed was put in ultrasonication bath for 10 min. The resulting solutions were filtered through Millex® filtration units and injected into the chromatographic system.

2.6. Sample solution for determination of inter and intra-day repeatability

The sample solutions were prepared as described in procedure for recovery of samples from stainless steel plates. The precision, intermediate precision and reproducibility were determined by inter and intra-day repeatability of responses and are expressed as standard deviation (S.D.) and relative standard deviation (R.S.D.).

2.7. Sample preparation for recovery test

A standard solution was prepared separately as described above to obtain solution containing 20.0 μg mL^{-1} of azathioprine.

The sample solution containing 10.0 μg mL^{-1} was obtained from stock solution containing 50.0 μg mL^{-1} of azathioprine. For that, an aliquot of 2.0 mL was transferred to a 10 mL volumetric flask and volume was completed with mobile phase. A polyester swab was immersed into the flask and ultrasonicated during 10 min in order to simulate samples to be analyzed. Three separate aliquots of sample solution (2.0 mL) were spiked with 1.0, 2.0 and 3.0 mL of standard solutions in three separate 10 mL volumetric flasks. The volumes were completed with mobile phase to obtain solutions containing 4.0, 6.0 and 8.0 μg mL^{-1} of azathioprine, approximately.

Method accuracy was assessed by determining the agreement between the measured standard concentration and known concentration of standard actually used to spike the sample solutions (Table 3). All determinations were made in triplicate at three concentration levels. All standard and sample solutions were filtered through 0.45 μm Millipore® (Millex HV) hydrophilic membrane, before injection into the system.

3. Results and discussion

3.1. Acceptance limit calculation (LA)

The acceptance limit was calculated based on total production line equipments area for azathioprine, the least unit therapeutical dose that promotes effect, maximum daily doses and size of produced batch in question [5,6].

For azathioprine, least unit therapeutical dose (DT) is 4 mg while minimum production batch size is 25,590,000 mg, taking into consideration production of reduced batches, which can be 50% of the original batch size. The maximum daily dose (LD) is 600 mg with a safety factor of 1/1000. The safety factor depends on route of administration and the toxicity of the product. For an oral formulation, the safety factor is generally set at 1000 or a higher value [6]. The calculated maximum acceptable residue (MA) value was found to be 170.6 mg.

The simulated standard sampling area (A) was a stainless steel plate surface of 25 cm^2 and simulated total production line area (AT) was 76,652.32 cm^2. As the sampling method recovery factor is yet unknown, a value of 30% was used [6]. An acceptance limit value of 16.7 μg of residue in each swab analyzed was obtained. Therefore, the initial value for the recovery tests with swab will be 10.0 μg, so that interferences below standardized concentration (16.7 μg) could be verified. The calculation was made using Eqs. (1) and (2). The lot size, daily dose are expressed in milligram units, total surface area is expressed in cm^2 units. While the acceptance limit for residue (LA) is expressed in μg/cm^2.

$$\mathrm{MA} = \frac{\mathrm{DT}Bf}{\mathrm{LD}} \quad (1)$$

$$\mathrm{LA} = \frac{\mathrm{MA}A\mathrm{fr}}{\mathrm{AT}} \quad (2)$$

where MA is the maximum acceptable residue, DT the least unit therapeutical dose, B the minimum production batch size, f the safety factor, LD the maximum daily doses of the subsequent product, A the sampling area, fr the recovery factor of sampling method, AT is the total production line area and LA is acceptance limit.

Table 1
Linear regression data in the analysis of azathioprine

Statistical parameters	Obtained values
Concentration range (μg mL^{-1})	2.0–22.0
Regression equation	$y = 1235.4x - 214.94$
Correlation coefficient (r)	0.9998
DL (μg mL^{-1})	0.09
QL (μg mL^{-1})	0.29

3.2. Validation of proposed method

3.2.1. Linearity

Linearity of method was studied by analyzing standard solutions at seven different concentration levels ranging from 2.0 to 22.0 μg mL^{-1}, with triplicate determination at each level. The calibration curve was constructed by plotting mean response area against corresponding concentration injected, using the least square method. The calibration curve values of slope, intercept and correlation coefficient for azathioprine are presented in Table 1 and indicate good linearity.

3.2.2. Detection limit (DL) and quantitation limit (QL)

The detection limit (DL) and quantitation limit (QL) were determined based on the standard deviation amongst response and slope of the curve at low concentration levels [7]. The DL and QL were 0.09 and 0.29 μg mL^{-1}, respectively (Table 1). The obtained theoretical values for DL and QL were actually prepared and were cross checked by actual analysis using proposed methods. At QL, standard deviation and relative standard deviation amongst responses was 30.2 and 1.81%, respectively.

3.2.3. Precision

The precision of the method was evaluated by inter and intra-day repeatability. Intermediate precision was determined by two analysts. The intra-day repeatability was determined by analyzing 10 replicates of extraction-recovery samples and is expressed in terms of R.S.D. The results are presented in Table 2.

The inter-day repeatability (reproducibility) was determined by analyzing same sample solutions on 2 consecutive days, at the same concentration level. The inter-day repeatability is expressed in terms of R.S.D. values and indicates a good reproducibility of method (Table 2).

Two different analysts used the same method and same equipment to analyze same sample and R.S.D. amongst responses on same day was calculated. The method presented good intermediate precision amongst analysts (Table 2).

3.2.4. Specificity

Specificity is the ability of the method to accurately measure the analyte response in the presence of all potential sample components (excipients). In this case, sample solutions containing 10.0 μg mL^{-1} of azathioprine were prepared using 50 mg

Table 2
Precision results and statistical data obtained in the determination of azathioprine extracted samples

	Intra-day	Intra-day	Intra-day	Inter-day	Between analysts
Number of samples	03	01	10	1	10 (5 each)
Number of injections	06	10	6	10	1
R.S.D.	0.83	0.75	5.83	1.07	6.01
Extraction-recovery (%)	83.5		79.2		79.3

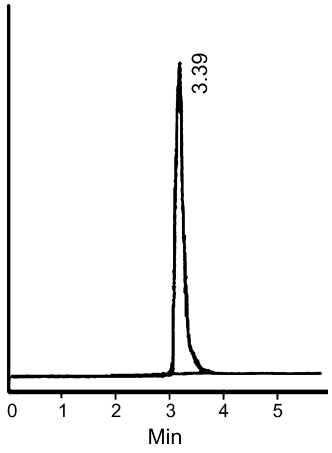

Fig. 2. Chromatogram of 10.0 μg mL^{-1} azathioprine tablet sample.

tablet and were analyzed using proposed method (Fig. 2). The results were compared with those obtained in the analysis of placebo and standard solution (10.0 μg mL^{-1}). No interference from excipients was observed within 5 min chromatographic run.

3.2.5. Accuracy

The accuracy of the present method was determined by spiking sample solutions with known standard. The accuracy of the method was checked at three concentration levels, i.e. at 4.0, 6.0 and 8.0 μg mL^{-1}. Triplicate analyses were done with HPLC method and accuracy is expressed as percentage of standard recovered from sample matrix with corresponding R.S.D. [8]. The recovery data is presented in Table 3.

3.3. UV spectrophotometric method

A UV spectrophotometric method was also developed and validated (results not shown). The proposed UV spectrophotometric for azathioprine presented promising results for cleaning validation as an alternative method to present HPLC method.

Table 3
Results obtained in the recovery of azathioprine standard added to sample solution and analyzed by the proposed HPLC method

Amount added (μg mL^{-1})	Amount found (μg mL^{-1})	Recovery (%)	Mean recovery
2.00	1.98	99.00	
4.00	3.95	98.75	99.19 ± 0.57
6.00	5.99	99.83	

However, the UV spectrophotometric method was unable to quantify azathioprine below 10.0 μg with precision (R.S.D. near 3%). Nevertheless, the method could be used as a limit method, where concentration values above established limit should be considered rejected.

4. Conclusions

The proposed method for quantitative determination of azathioprine residue on production area equipments is efficient and sensitive. The results showed that the proposed method is suitable for quantitative determination of azathioprine in production area well below the calculated limit of contamination.

The cleaning validation sample impurities as well as excipients of the commercial sample did not interfere in the analysis, which proved the specificity of the method. The ease of sample preparation permits fast and efficient application of the proposed method in quantitation of azathioprine residue with precision and accuracy. The method can be used in routine cleaning validation processes and for quantitative determination of azathioprine in commercial samples.

The simplicity of UV spectrophotometric method makes it useful for routine analysis of azathioprine residues on cleaned surface and can be used as limit method to qualify or reject cleaning procedure in production area.

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