Determinación de azathioprina en forma de producto farmacéutico por HPTLC

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ABSTRACT

Introducción: Se describió un método HPTLC para la determinación de azathioprina en forma de producto farmacéutico. Los valores de respuesta fueron proporcionados como una función lineal de la concentración de azathioprina en el rango de 200-1200 ng/banda. El límite de detección y cuantificación para azathioprina fueron de 18.58 y 59.14 ng/banda, respectivamente. La recuperación promedio fue de 100.1%, lo que muestra que el método fue libre de interferencia de excipientes presentes en la formulación. Conclusión: El método establecido permitió un análisis preciso y rápido de azathioprina en forma de producto farmacéutico.

Palabras clave: Azathioprin, HPTLC, validación

Cómo citar este artículo: Jain PS, Thakre P, Chaudhari AJ, Chavhan ML, Surana SJ. Determination of azathioprine in bulk and pharmaceutical dosage form by HPTLC. J Pharm Bioall Sci 2012;4:318-21.
temperature (25°C ± 2). The length of the chromatogram run was approximately 8 cm. Subsequent to development, the thin layer chromatography plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed on a Camag TLC scanner 3 and was operated by winCATS software.

**Stock and working standard solution**

Azathioprine (25 mg) was accurately weighed into a 25 mL volumetric flask and dissolved in a minimum volume of methanol, and diluted to the required volume with methanol to furnish a solution of concentration 1000 ng/µL. This was used as stock solution. Calibration standards were prepared over the concentration range 200-1200 ng/band for Azathioprine by appropriate dilutions of the above-mentioned standard stock solution in a 10 mL volumetric flask with methanol.

**Calibration curve**

Separate stock standard solutions of Azathioprine were used for the preparation of calibration standard solutions. All calibration standards were prepared freshly every day and were found to be stable during the analysis time. The plate was developed, dried and scanned as described above. After densitometric scanning, the peak area was recorded for each concentration and a calibration plot was obtained by plotting average peak area against concentration of Azathioprine (ng/spot). The slope and correlation coefficient were also determined.

**Method Validation**

Validation of the optimized HPTLC method was carried out with respect to the following parameters:

**Precision**

Repeatability of sample application and measurement of peak area were carried out using six replicates of the same band (800 ng/band of Azathioprine). The intra-and inter-day variation for the determination of Azathioprine was carried out at three different concentration levels of 600, 800 and 1000 ng/band.

**Limit of detection and limit of quantification**

In order to determine the limit of detection (LOD) and limit of quantification (LOQ), Azathioprine concentrations in the lower part of the linear range of the calibration curve were used. Azathioprine solutions of 200, 240, 280, 320 360 and 400 ng/band were prepared and applied on the plate. The LOD and LOQ were calculated using the equation LOD = 3.3 × N/B and LOQ = 10 × N/B, where, N is the standard deviation of the peak areas of the drugs (n = 3), taken as a measure of noise, and B is the slope of the corresponding calibration curve.

**Specificity**

The specificity of the method was ascertained by analyzing standard drug and sample. The spot for Azathioprine in the sample was confirmed by comparing the Rf values and spectra of the spot with that of the standard. The peak purity of Azathioprine was assessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M) and peak end (E) positions of the spot.

**Ruggedness**

Ruggedness of the method was performed by spotting 800 ng/band of Azathioprine by two different analysts keeping the same experimental and environmental conditions.

**Accuracy**

The analyzed samples were spotted with extra 80, 100 and 120% of standard Azathioprine, and the mixture was analyzed by the proposed method. This was done to check the recovery of the drug at different levels in the formulations.

**Robustness**

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions of ethyl acetate: methanol:triethylamine (5:2.5:0.5 v/v) were tried and chromatograms were run. The amount of mobile phase, temperature and relative humidity was varied in the range of ± 5%. The plates were pre-washed by methanol and activated at 80 ± 10°C for 2, 5 and 7 min prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied from 10, 15 and 20 min.

**Application of the proposed method to the tablet formulation**

To determine the concentration of Azathioprine in tablets (labelled claim: 100 mg per tablet), the contents of 20 tablets were weighed, their mean weight was determined and they were finely powdered. The powder, equivalent to 100 mg of Azathioprine, was weighed. The drug from the powder was extracted with methanol. To ensure complete extraction of the drug, it was sonicated for 30 min and the volume was made up to 10 mL. The resulting solution was filtered using a 0.45 μm filter (Mill filter, Milford, MA, USA). The above solution (800 ng/band) was applied on a TLC plate, followed by development and scanning, as described above.

**Results and Discussion**

**Development of optimum mobile phase**

The TLC procedure was optimized with a view to developing a stability-indicating assay method. Initially, ethyl acetate: methanol (4:1v/v) gave good resolution with Rf value of 0.48 for Azathioprine, but typical peak nature was missing and tailing appeared. Finally, the mobile phase consisting of ethyl acetate: methanol: triethylamine (4:1:0.5 v/v) gave a sharp and well-defined peak at Rf value of 0.49 [Figure 1]. Well-defined spots were obtained when the chamber was saturated with the mobile phase for 15 min at room temperature.
Calibration curve

The linear regression data for the calibration curves showed a good linear relationship over the concentration range 200–1200 ng/band. Linear regression equation was found to be $Y = 7.154X + 1293.9$ and $R^2 = 0.998$.

Validation of the method

Precision

The precision of the developed HPTLC method was expressed in terms of %relative standard deviation (%RSD). The results depicted revealed high precision of the method, which is presented in Table 1.

LOD and LOQ

Detection limit and quantification limit was calculated by the method described above. The LOQ and LOD were found to be 18.58 and 59.14 ng/spot, respectively. This indicated adequate sensitivity of the method.

Specificity

The peak purity of Azathioprine was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot, i.e., $r^2(S, M) = 0.999$ and $r^2(M, E) = 0.999$. Good correlation ($r^2 \approx 0.999$) was also obtained between standard and sample spectra of Azathioprine [Figure 2].

Ruggedness

When the method was performed by two different analysts under the same experimental and environmental conditions, it was found to be rugged [Table 2].

Recovery study

The proposed method when used for extraction and subsequent estimation of Azathioprine from the pharmaceutical dosage form after overspotting with 80, 100 and 120% of additional drug, affording good recovery of Azathioprine. The amounts of drug added and determined and the % recovery are listed in Table 3.

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Table 1: Precision

| Conc. of drug (ng/band) | Intraday* | Interday* |
|-------------------------|-----------|-----------|
|                         | Mean of area | %RSD   | Mean of area | % RSD   |
| 600                     | 5577       | 0.82     | 5596         | 0.30    |
| 800                     | 7058.33    | 0.53     | 7092.33      | 0.32    |
| 1000                    | 8453.67    | 0.46     | 8406.2       | 0.89    |

*Mean of three estimations at each level

Table 2: Summary of validation parameter

| Parameters                             | % RSD |
|----------------------------------------|-------|
| Linearity range (ng per spot)          | 200–1200 |
| Correlation coefficient                | 0.998 |
| Limit of detection (ng per spot)       | 18.58 |
| Limit of quantification (ng per spot)  | 59.14 |
| Recovery $(n = 6)$                     | 100.1 |
| Ruggedness                             | 0.56  |
| Robustness                             | Robust |
| Specificity                            | Specific |

Table 3: Recovery study*

| Drug/label claim Initial amount of drug (ng/band) | Amount of standard drug added (%) | Drug recovered | % RSD |
|--------------------------------------------------|----------------------------------|----------------|-------|
| Azathioprine (10 mg/tab)                         | 400                              | 80             | 99.89 | 1.80  |
|                                                  | 100                              | 10.4           | 100.2 | 1.33  |

*Mean of three estimations at each level

Table 4: Robustness of the method*

| Parameters                           | % RSD |
|--------------------------------------|-------|
| Mobile phase volume (±2 mL)          | 0.94  |
| Development distance (±5 cm)         | 0.79  |
| Duration of saturation (±2)          | 0.81  |
| Time from spotting to chromatography (±10 min) | 0.43  |
| Time from chromatography to scanning (±10 min) | 0.82  |

*Mean of three estimations
Robustness of the method

The standard deviation of peak areas was calculated for each parameter, and the %RSD was found to be less than 2%. The low values of %RSD [Table 4] indicated that the method was robust.

Analysis of the marketed formulation

A single spot at Rf 0.49 was observed in the chromatogram of the drug samples extracted from the tablets. There was no interference from the excipients commonly present in the tablet. The %drug content and %RSD were calculated. The low %RSD value indicated the suitability of this method for the routine analysis of Azathioprine in pharmaceutical dosage forms.

Conclusion

This HPTLC method is simple, precise, specific and accurate. Statistical analysis proved that the method is reproducible and selective for the analysis of Azathioprine as the bulk drug and in tablet formulations.

Acknowledgment

The authors are thankful to the Principal, R.C. Patel Institute of Pharmaceutical Education and Research, Shirpur (Maharashtra), India, for providing the required facilities and valuable guidance to carry out this research work.

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Source of Support: Nil, Conflict of Interest: None declared.
