Drug Development and Industrial Pharmacy, 26(3), 267–273 (2000)

RESEARCH PAPER

Determination of Azathioprine and Its Related Substances by Capillary Zone Electrophoresis and Its Application to Pharmaceutical Dosage Forms Assay

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ABSTRACT

*The development of a stability-indicating capillary zone electrophoresis (CZE) method for the determination of the drug azathioprine (AZA) and its related substances in bulk and dosage forms is described. Theophylline was used as an internal standard to improve quantitative results. The method was fully validated in terms of repeatability (*n = *10, RSD for migration time and peak area ratio were 0.15% and 0.60%, respectively), reproducibility (*n = *5, RSD of peak area ratio was 0.84%), linearity at two ranges of the azathioprine concentration, limits of detection (LOD) and quantitation (LOQ), and robustness. The method was applied for determination of the drug in bulk and a commercial tablet dosage form (recovery 98.3–101.3%) and in powder for injection (recovery 98.7–100.6%). The method was fast and reliable for the analysis of AZA and its related substances in bulk and dosage forms.*

INTRODUCTION

Capillary electrophoresis (CE) (1) has been applied to the separation of a wide variety of compound types, including peptides, proteins, carbohydrates, nucleic acids and nucleotides, vitamins, organic acids, amino acids, inorganic ions, synthetic polymers, clinical samples, and pharmaceuticals (2). The possibility of employing CE for the analysis of pharmaceuticals was demonstrated by Fujiwara and Honda in 1987 (3). The popularity for the use

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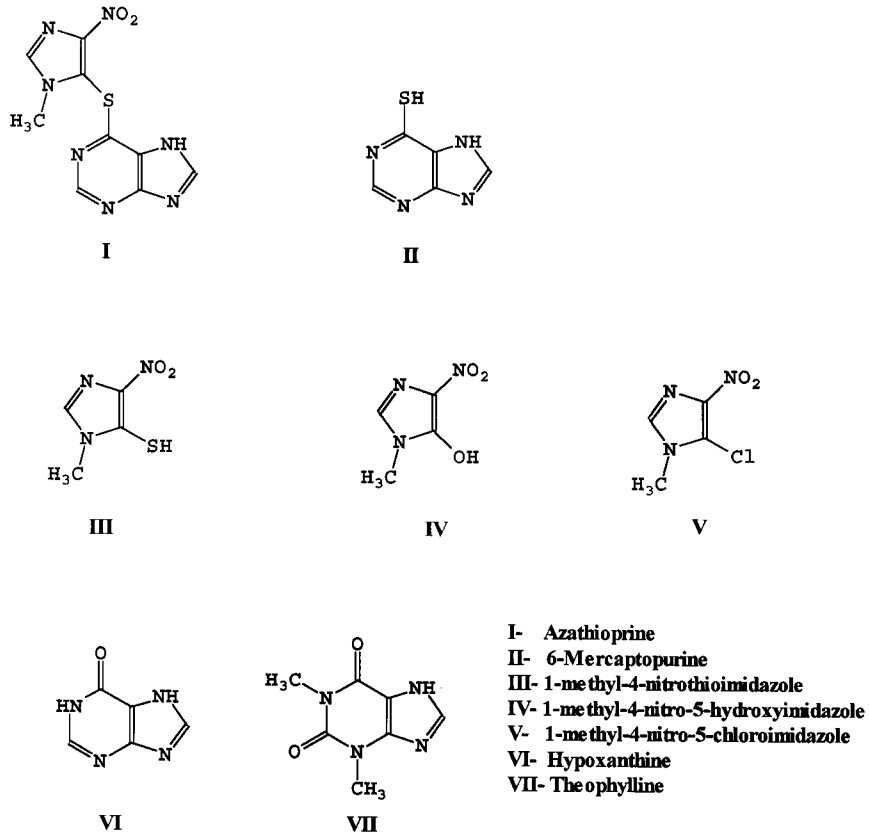

Figure 1. Chemical structure of azathioprine (I), 6-mercaptopurine (II), their related substances (III–VI), and theophylline (VII).

of CE in the pharmaceutical field has been accelerated by its simplicity, high efficiency and selectivity, large separatory capacity, and relatively lower operational cost. Several excellent reviews have addressed the wide application of CE analysis to pharmaceutical products and biologically active proteins and peptides (4–9).

Azathioprine (AZA), 6-(1-methyl-4-nitroimidazol-5-ylthio)purine (structure I in Fig. 1), is a potentially useful antileukemic and immunosuppressive drug (10). It is commercially available as the freeze-dried sodium salt for injection and in tablets as the neutral product. Intact AZA is reported to be split readily by sulphydryl compounds when in solution (11). In vivo, AZA is transformed to the active moiety 6-mercaptopurine (6MP) (structure II in Fig. 1), which is itself commercially available in tablet form and may be administered as an antineoplastic agent (10).

The analysis of AZA in pharmaceutical dosage forms presents a major challenge due to the complexity of impurities and degradation products potentially present (Fig. 1). Few of the published methods for AZA consider the problem of potential interference by contaminants (structures II–VI in Fig. 1) in pharmaceutical dosage forms. Fell et al. (12) presented a reversed-phase high-performance liquid chromatography (HPLC) method for the separation and quantitation of AZA, 6MP, and their related substances and degradants present in dosage forms. A previously reported spectrophotometric method for the determination of AZA in the presence of 6MP was evaluated for its specificity in the presence of other major degradation products (13).

The principal in vitro degradation product of AZA is 6MP, which is limited by the official British and American compendia to less than 1% (w/w) by thin-layer chromatography (TLC) (14,15). 1-Methyl-4-nitro-5-chloroimidazole (V) was also limited by the British Pharmacopoeia to less than 1% in bulk drug and tablet dosage form. Although potentially present as trace impurities, the associated degradants 1-methyl-4-nitro-5-thioimidazole (III) and the 5-hydroxyimidazole (IV) and the

synthetic precursor and hypoxanthine (VI) are not officially controlled in AZA or its pharmaceutical formulations.

This paper presents the development and validation of a rapid and selective capillary zone electrophoresis (CZE) method for the determination of AZA and its related substances. To obtain better quantitative results, theophylline (THP) was used as an internal standard. The method was also applied to the assay of AZA in a tablet dosage form and powder for injection.

EXPERIMENTAL

Capillary Zone Electrophoresis Conditions

For the CZE, a model P/ACE 2210 Beckman capillary electrophoresis instrument was connected to Beckman System Gold Chromatography Software on a PS/2 IBM PC, and an uncoated fused silica capillary (Composite Metal Services, Worchester, UK) of 570 mm total length (500 mm to the detector) and 0.05 mm i.d. was used. The capillary was kept at a constant temperature using a thermostated liquid coolant. Samples were introduced into the capillary by hydrodynamic injection (applying high pressure at the inlet), and the injection time was 5 sec. Sodium phosphate buffer (50 mM) was used for the pH range 6–8, and sodium tetraborate buffer (20 mM) was used for the pH range 8–10. The buffers were prepared freshly on a daily basis and filtered through a 0.45-μm membrane filter. Other conditions were as follows: detection wavelength 214 nm, voltage 25 kV, and temperature 25°C. Capillary conditioning procedures were discussed in our previous work (16).

Materials and Dosage Forms

Azathioprine and its related substances were supplied by Wellcome Foundation Limited (London, UK). Imuran freezed-dried powder containing equivalent to 50 mg azathioprine sodium and Imuran tablets (25 mg) were also supplied by Wellcome Foundation. Theophylline was purchased from Sigma Chemical Company (St. Louis, MO). All reagents used in this work were analytical grade (BDH Ltd., Dorset, UK).

Standard and Sample Solutions

Note that all standard and sample solutions were stored in light-resistant containers in the refrigerator and discarded after 2 days.

Standard Solutions

A standard solution of AZA was prepared by dissolving 10 mg AZA in 10 ml NaOH 0.02 M. A standard solution of the internal standard, THP (VII in Fig. 1), in 0.02 M NaOH at a concentration of 1 mg/ml was prepared. Then, 1 ml of AZA solution and 1 ml of THP were transferred to a 20-ml volumetric flask, and buffer was added to the volume (both compounds at 0.05 mg/ml). Standard solutions used in the linearity assessment were prepared similarly.

For developing and optimizing the separation system, standard solutions of AZA and related substances in 0.02 M NaOH (all at 1 mg/ml) were prepared. Then, a standard test mixture of AZA (0.2 mg/ml), its impurities, and THP (all at 0.05 mg/ml) in buffer was prepared from concentrated standard solutions of each compound.

Sample Solution

Imuran tablets (20), each containing 25 mg AZA, were accurately weighed and ground to fine powder, and the amount equivalent to 1 tablet was weighed and transferred to a flask. Then, 10 ml NaOH solution (0.02 M) was added, and the mixture was shaken in the sonic bath for 10 min. The mixture was filtered into a 25-ml volumetric flask, and NaOH solution was added to volume. A standard solution of THP in 0.02 M NaOH (1 mg/ml) was prepared. Tablet extract solution (1 ml) and 1 ml of THP solution were transferred to a 20-ml volumetric flask, and the solution was made up with the buffer (both AZA and THP at a concentration of 0.05 mg/ml).

The content of one Imuran powder for injection vial (50 mg) was dissolved in 10 ml NaOH 0.02 M, and the solution was made up to 50 ml with NaOH solution. Then, 5 ml of the solution was transferred to a 100-ml volumetric flask. THP solution (5 ml at 1 mg/ml) was also added, and the solution was made up with the running buffer (final concentration 0.05 mg/ml AZA and 0.05 mg/ml THP). A standard mixed solution of AZA and THP was prepared in the same way to assess the recovery of AZA from the dosage form.

RESULTS AND DISCUSSION

Capillary Zone Electrophoresis Method Development

Preliminary experiments using complete factorial design to study the effects of the pH, ionic strength of the buffer, and voltage revealed that pH has a dramatic effect

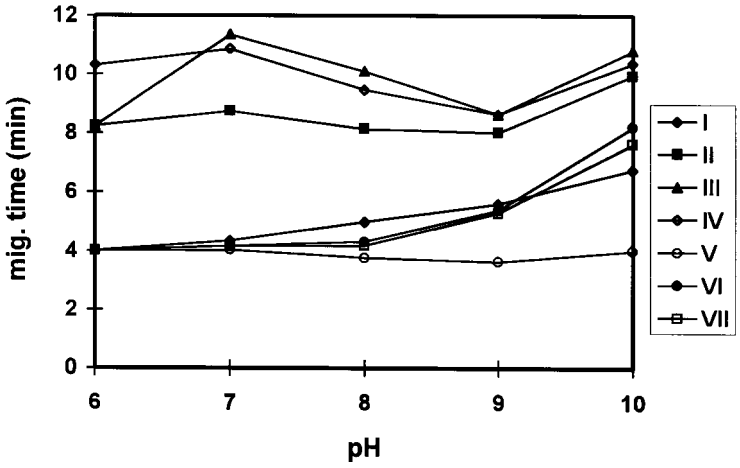

Figure 2. Effect of pH on the migration times of azathioprine (I), its related substances (II–VI), and theophylline (VII) in the CZE method. The running buffer was sodium phosphate (20 mM) and sodium tetraborate (20 mM). Other conditions were as follows: voltage 25 kV, detection wavelength 214 nm, temperature 25°C. Definition of the compounds as in Fig. 1.

on the resolution of the AZA from the other six components of the sample solution (including the internal standard). The resolution of the sample components was studied over a range of pH between 6 and 10 (Fig. 2). Partial resolution at pH 8 and complete resolution of all compounds at pH 10 was achieved (Fig. 3). Further experiments using borate buffer over a range of pH between 8 and 9 led to resolution of the analytes at pH 8.2, but at this pH, the peak relevant to 6MP was very broad and split to several peaks (Fig. 4). The efficiency of the separation with regard to AZA was 1.85×10^5.

Stability of Azathioprine Solution at pH 10 at 25°C

A standard mixture solution of AZA and THP in borate buffer pH 10 was prepared and stored in an incubator at 25°C for 2 days. The solution was subjected to CZE immediately after preparation and every 12 hr. No addi-

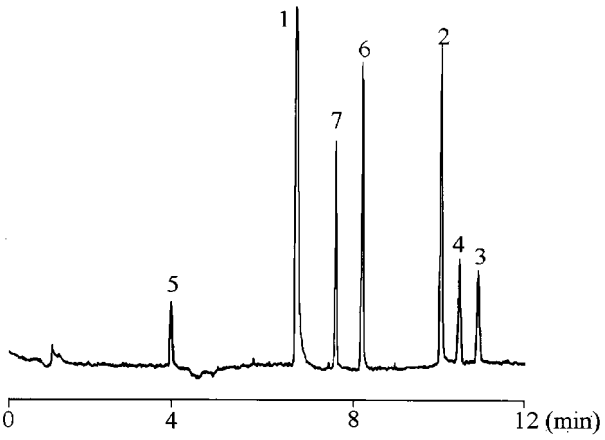

Figure 3. Resolution of azathioprine and its related substances using the CZE method at pH 10. Other conditions and peak definitions as described in Figs. 1 and 2.

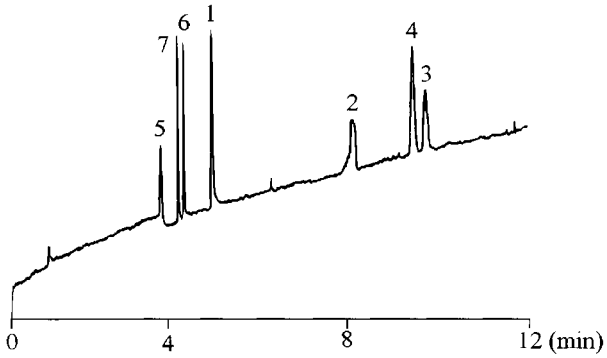

Figure 4. Resolution of azathioprine and its related substances using the CZE method at pH 8.2. Note that the peak relevant to 6-mercaptopurine (peak number II) is broad and split. Other conditions and peak definitions as described in Figs. 1 and 2.

Table 1

Repeatability Assessment of the Capillary Zone Electrophoresis Method Developed for the Determination of Azathioprine and Related Substances (n = 10)

	AZA				THP				AZA/THP	
	Time (min)	Area	Area[a]	Height	Time	Area	Area[a]	Height	Area[a]	Height
Mean	6.89	0.586	0.085	0.0122	7.79	0.571	0.073	0.0108	1.159	1.125
SD	0.01	0.010	0.001	0.0001	0.01	0.010	0.001	0.0001	0.007	0.002
RSD%	0.15	1.71	1.18	0.82	0.13	1.75	1.37	0.93	0.60	0.18

[a] Normalized area.

tional peak was observed. The RSD value for normalized peak area ratio of AZA to THP was found to be 1.03%.

Validation Assessments

To demonstrate the benefits of using an internal standard in quantitative CZE, THP was used in all validation assessments and in dosage form assays. Normalized peak area ratios and peak height ratios of the compound of interest to THP were used for quantitative measurements. The CZE method for assay of AZA was validated in terms of repeatability, linearity (at two ranges of AZA concentration), limit of detection (LOD) and limit of quantitation (LOQ), reproducibility, and robustness. The experiments were carried out as described in our previous work (16).

Repeatability

Complete results of the repeatability assessment, including 10 consecutive injections of a standard solution of AZA and THP, are given in Table 1.

Linearity

Linearity of the peak area/area ratio of AZA/THP over two ranges of concentration of AZA (0.01–0.07 and 0.05–0.50 mg/ml, THP at 0.05 mg/ml), and peak area/area ratios of 6MP/THP and compound V/THP, each over a range of 0.005–0.050 mg/ml (THP at 0.02 mg/ml), were assessed. The complete results are given in Table 2.

Reproducibility

To demonstrate the reproducibility of the method, five Imuran tablet extracts were injected into the capillary in duplicate. The RSDs for normalized peak area ratios and peak height ratios were 0.84% and 1.27%, respectively.

Limit of Detection and Quantitation

The values of LOD and LOQ for AZA and its related substances are given in Table 3.

Robustness

Preliminary experiments showed that the buffer pH is the factor that most affects repeatability of the method.

Table 2

Results for Linearity Experiments for Capillary Zone Electrophoresis Method Developed for the Analysis of Azathioprine

	AZA (0.01–0.07 mg per ml)	AZA (0.05–0.50 mg per ml)	6MP	Compound V
n	7	6	7	7
r	.9998	.9985	.9963	.9978
r^2	.9996	.9970	.9926	.9956
Y intercept	0.60 ± 0.03	0.14	0.85	0.82
Slope	0.19 ± 0.04	0.27	0.90	0.64

Table 3

Limit of Detection (LOD) and Quantitation (LOQ) for Azathioprine and Its Related Substances

	AZA	6MP	III	IV	V	VI
LOD ($\times 10^{-6}$ M)	2.71	6.58	6.29	6.99	6.19	7.35
LOQ ($\times 10^{-5}$ M)	0.91	2.19	2.09	2.33	2.06	2.45

The method was employed at different times with at least 2-month intervals and by different operators. In these experiments, five known standard mixed solutions of AZA and THP (each at 0.05 mg/ml) were assessed, and the results showed no significant differences.

Recovery Assessment

Three tablet extracts and a standard solution of AZA (prepared in exactly the same way as the tablet extracts were prepared) were analyzed with the CZE method. Then, the concentrations were determined from the calibration data. The recovery of AZA from tablets was 98.3% to 101.3% (w/w).

A similar experiment was carried out using the sample solution prepared from Imuran powder for injection (only one vial), and the recovery of AZA was found to be 98.7–100.6%.

Determination of Impurities in the Analytical Samples

Three solutions of the tablet extract were prepared at a concentration of 0.5 mg/ml, and each was injected into the capillary in replicate at 5-sec injection times. No trace of impurities was observed in any of the samples.

Quantitation Procedure

To demonstrate the impact of using an internal standard on the quantitative results in CZE, among the few suitable compounds to be applicable, THP was selected for this purpose. THP is chemically similar to the main compound and its major related substance, 6MP, and migrates in the CZE buffer similarly (i.e., it possesses negative charge at pH 10).

In this study, the quantitation of AZA and its official impurities, including 6MP and compound V (14), were based on the use of calibration curves. All quantitative results in this work are reported as the ratio of peak area or peak height of the compound of interest (AZA or its related compounds) to that of the THP. However, as THP migrates with different mobility in the capillary, it is necessary to normalize the peak area of the test compound and the internal standard before calculating the peak area ratios. The complete results of the repeatability assessment, which are given in Table 1, clearly show that using an internal standard in this CZE method can greatly improve the quantitative results.

Application of the Method for Dosage Form Assays

As illustrated in Fig. 3, AZA and 6MP were well resolved from all their known or potential impurities, thus enabling the method to be used for quantitative assay of AZA in bulk and dosage forms. The results of validation experiments showed that the developed CZE method for the determination of AZA and its related substances was robust and reliable. Therefore, the method was applied to assay bulk drug and two AZA dosage forms, the Imuran tablets (25 mg) and Imuran powder for injection (50 mg). The results are given above (validation assessments). It is shown that the method was fast, easy, and reliable for the assay of AZA bulk and dosage forms.

CONCLUSION

The potential contribution of CZE to the rapid and precise separation and quantitation of the drug azathioprine and its related substances is demonstrated by the method described in this paper. The developed and fully validated method was applied to determine azathioprine in bulk and two commercial pharmaceutical dosage forms.

Also, the usefulness of employing an internal standard for improving the quantitative results in CZE was demonstrated.

ACKNOWLEDGMENT

We thank the Ministry of Health and Medical Education of I.R. of Iran for the financial support of this work.

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