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Rapid determination of nitazoxanide in tablets using reversed-phase ultra-performance liquid chromatography (UPLC) and high-performance liquid chromatography

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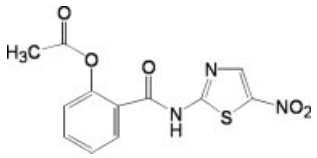
A simple and rapid determination method for nitazoxanide (NTZ), an antiprotozoal agent, was developed using reverse-phase HPLC and Ultra Performance Liquid Chromatography™ (UPLC™). Only six minutes gradient condition for NTZ analysis using UPLC was achieved. The mobile phase consisted of a mixture of phosphate buffer (pH 6.0) and acetonitrile. The repeatability (relative standard deviation (RSD), $n = 6$) and the correlation coefficient from linearity (the range from 80% to 120% of amount) were 0.25% and 0.9963 for UPLC and 0.15% and 0.9988 for HPLC, respectively. The quantitative values of NTZ in tablets were 103.2% for HPLC and 98.7% for UPLC. The RSDs of quantitative values of sample solution were calculated to be 4.06% to 4.64% for HPLC and 0.15% to 0.36% for UPLC.

1. Introduction

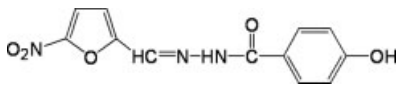
Nitazoxanide (2-acetyloxy-N-(5-nitro-2-thiazolyl) benzamide, NTZ), a nitrothiazole derivative, was first synthesized by Rossignol in 1974 (Rossignol et al. 1984), and is an effective compound for treating a wide variety of parasitic infections in animals and humans (Dubreuil et al. 1996). NTZ in oral suspension (Alinia® for oral suspension, Romark Laboratories L.C., Tampa Florida, USA) was approved by US-FDA as a treatment for diarrhea caused by *Cryptosporidium parvum* infection and *Giardia lamblia* infection in children (1–11 years old) in 2002. Moreover, it has also been approved for *Cryptosporidium parvum* infectious disease for infants and adults (12 years old or older) since 2005. Oral suspensions and tablets (Alinia® tablets) of 500 mg and 100 mg have been available for treatment of Cryptosporidiosis in cases of immunodeficiency.

Cryptosporidium parvum infects a wide variety of mammalian hosts (Fayer et al. 1997), and Cryptosporidiosis is a ubiquitous diarrheal disease of animals and humans (Fayer et al. 1997; Casemore et al. 1997). Cryptosporidiosis generally results in acute but self-limiting gastroenteritis in humans who are in an immuno-competent condition. However, the lives of infected patients who are immunocompromised or have non-functional immuno-systems can be threatened from the insufficiency of multiple organ systems (Current et al. 1983).

Since the 1990s, cryptosporidiosis has been recognized increasingly as a cause of severe, life-threatening diarrhea in patients with AIDS (Brandonisio et al. 1993; Sorvillo et al. 1994; Pedersen et al. 1996; Anand et al. 1996; Ananthasubramanian et al. 1997; Agarwal et al. 1998; Ballal et al. 1999). Hence, NTZ products will become more important for the treatment of Cryptosporidiosis diarrhea in AIDS patients, whose numbers are rapidly increasing worldwide. These important medicines for the treatment of infectious diseases are highly in demand in developing countries. However the supply-demand imbalance in these countries often leads to problems with counterfeit drugs. In the case of NTZ tablets, almost no simple analytical procedures for measuring levels of NTZ in pharmaceutical products have been reported. Therefore, it would be desirable to develop a simple analytical method that could check the quality of NTZ tablets on the market.



Nitazoxanide



Nifuroxazide (IS)

Liquid chromatography (LC) such as HPLC is commonly applied to the quality analysis of pharmaceuticals, because it can achieve highly specific qualitative and quantitative analysis of an active drug. Development of an analytical method using HPLC provides the advantage that it can be used in various sites. Detection of NTZ metabolites in human plasma, urine etc, using HPLC or LC/MS has been reported (Stockis et al. 1996; Lunn et al. 2005). Recently,

an assay for NTZ to validate stability using reversed phase LC has been developed (Jadhav et al. 2007). However, a rapid quantification of NTZ in pharmaceutical formulations has not been reported. In this study, the quantification of NTZ in tablets using HPLC was developed using the modified analytical condition for NTZ metabolites in human plasma (Stockis et al. 1996). The mobile phase was composed of phosphate buffer aqueous solution and acetonitrile. The gradient condition achieved not only rapid detection but also good separation between NTZ and decomposed compounds. The typical ODS column and UV detection was selected for this method.

In this study, the authors also applied Ultra Performance Liquid Chromatography™ (UPLC™) for development of rapid NTZ analysis. UPLC has been applied to rapid analysis and/or a simultaneous detection of similar compounds, because it can achieve analysis within a few minutes (Sherma et al. 2005). The UPLC column, which contains sub-2.0 μm (e.g. 1.7 μm) silica gel particles, will contribute to the development of a very rapid analytical condition with a high theoretical plate number. Several papers dealing with applications of UPLC including mass spectrometry have been reported in the analysis of food, environmental substances, chemicals, crimes and pharmaceuticals (Leandro et al. 2006; Ryan et al. 2005; Benvenuti et al. 2005; Lee et al. 2005; Apollonio et al. 2006; Antwerp et al. 2005; Wren et al. 2006). Especially, UPLC/MS has contributed to rapid and high-sensitivity analysis of metabolites in biological specimens (Rainville et al. 2005; Yin et al. 2006; Al-Dirbashi et al. 2006; Wang et al. 2006). Thus, UPLC can provide time savings for chromatography in all fields.

This paper describes two LC gradient methods. One is the development of a quantitative analytical procedure for NTZ using HPLC, and the other is the application of UPLC, which is a relatively new technology of LC separation for rapid analysis of NTZ.

2. Investigations and results

Approximately ten times or more volume of acetonitrile against volume of water is needed to prepare the NTZ solution. However, such a high proportion of acetonitrile in solution (acetonitrile/water, 9 : 1) affects the retention of NTZ to the column, and a suitable peak shape was not obtained. Finally, the water/acetonitrile ratio of 3 : 2 was selected because of appropriate peak retention.

A time course of the amount of the decomposed compound for 45 min analytical intervals is shown in Fig. 1. The ratio of the decomposed compound to NTZ increased for every measurement, and 19.6% of decomposed compound was detected from the solution at 6 h after the preparation. It was suggested that the decomposition of NTZ was caused by water, because the color of the acetonitrile solution of NTZ was immediately changed to deep yellow by the addition of water. However, the ratio of acetonitrile and water was finally set at 3 : 2, in order to keep appropriate retention of NTZ in the column. Therefore, the latter part of the sample preparation was performed under cool conditions, and the prepared solutions were kept under 4 °C.

In order to check the column performance for HPLC and UPLC, the tailing factor was calculated using the following equations.

$$\text{Tailing factor} = W_{0.05}/2f$$

The tailing factor of the NTZ peak was 2.0 for HPLC and 1.2 for UPLC, respectively.

Typical HPLC and UPLC chromatograms of the NTZ standard solution are shown in Fig. 2 (A, B). The retention times of IS (nifuroxazide) and NTZ were 22.1 and 24.8 min for HPLC, and 3.2 and 3.5 min for UPLC, respectively. The relative standard deviation (RSD) of system repeatability ($n = 6$) and the intermediate precision ($n = 30$) were 0.15% and 1.30% for HPLC, 0.25% and 1.56% for UPLC, respectively.

The linearity was calculated from 15 successive measurements of the reference standard solutions (5 concentrations

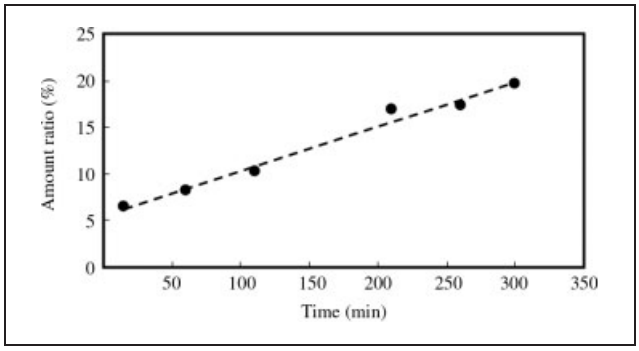


Fig. 1: Time course of the amount ratio of decomposed compound in room temperature. The ratio of decomposed compound reached approximately 20% 6 h after preparation of sample solution

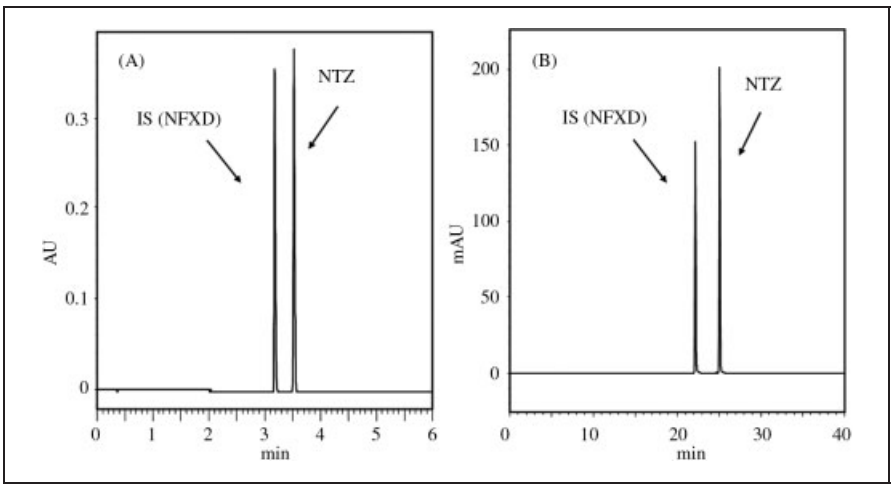


Fig. 2:
Typical chromatograms of NTZ and IS (NFXD) obtained from standard solution. (A) UPLC chromatogram; (B) HPLC chromatogram. It took 40 min to achieve NTZ detection by HPLC; it took only 6 min to detect NTZ by UPLC analysis

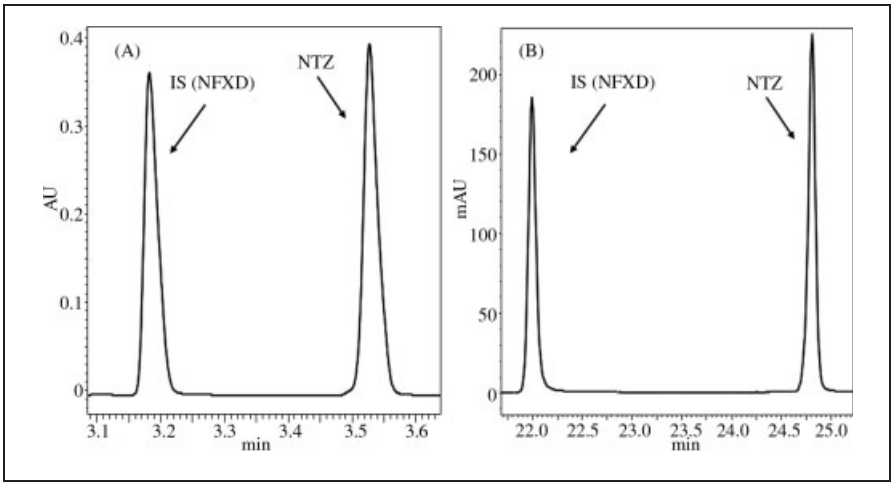


Fig. 3:
Chromatograms of NTZ obtained from extract of Alinia® Tablets. (A) UPLC chromatogram (B) HPLC chromatogram. No interference peaks were observed for these gradient conditions

at 80% to 120% of the NTZ concentration in the sample solution) by HPLC or UPLC. The relative standard deviations (RSDs) of ratios of peak areas were 0.32% to 1.32% for HPLC, and 0.23% to 0.57% for UPLC, respectively. Relatively good linearity was obtained from both LC conditions (the correlation coefficients were 0.9988 (HPLC) and 0.9963 (UPLC). Each calibration curve was used for the quantitation of NTZ.

Enlarged HPLC and UPLC chromatograms of the extract of Alinia® Tablets are shown in Fig. 3 (A, B). No interference peaks were observed from the sample solution in the measurement range. The label for the provided Alinia® Tablets was 500 mg, and the content of NTZ in each tablet was approximately 70% (the average weight of each tablet ($n = 15$) was 724.6 mg). The quantitative values of NTZ in Alinia® Tablets ($n = 3$ and 5 tablets) ranged from 102.7% to 105.4% by HPLC, and from 98.4% to 99.9% by UPLC, respectively (Table). The quantitative values of standard solution prepared together with the sample solution showed 103.2% for the HPLC and 98.7% for the UPLC, respectively. The RSDs of quantitative values of sample solution were calculated from 4.06% to 4.64% (HPLC) and from 0.15% to 0.36% (UPLC), respectively. In the case of the standard solution, the indicated RSDs were 3.31% for HPLC, and 0.27% for UPLC.

In another HPLC measurement, a range of NTZ quantitative values from 96.7% to 100.1% was obtained, and the standard solution gave an average result of 98.8% from 4 injections.

The unknown small peak that was presumed as the decomposed compound was found at the lead of the NTZ peak in the HPLC (Fig. 4) and UPLC (Fig. 5) chromatograms of time-elapsed sample solutions. This small undesirable peak would affect the precise quantitation of NTZ.

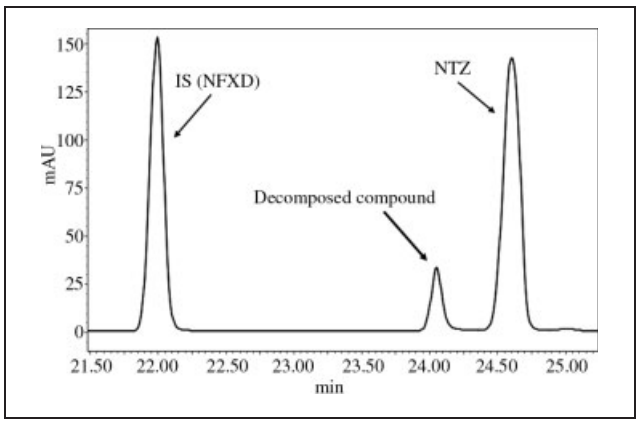


Fig. 4: HPLC chromatogram of the time-elapsed sample solution. The decomposed compound was detected at 24.1 min

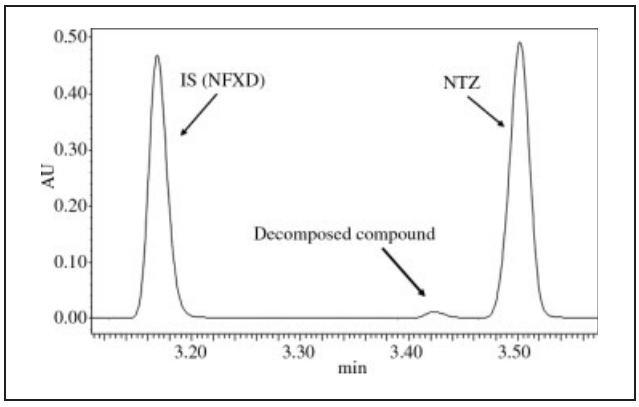


Fig. 5: UPLC chromatogram of the time-elapsed sample solution. This small undesirable peak was detected at 3.4 min

Table: Gradient conditions of UPLC (A) and HPLC (B)

A: Gradient condition for UPLC			B: Gradient condition for HPLC		
PB/AcCN			PB/AcCN		
0.00 min	97	3	0 min	97	3
0.72 min	97	3	3 min	97	3
2.00 min	95	5	10 min	95	5
3.50 min	48	52	20 min	60	40
4.00 min	40	60	23 min	60	40
4.02 min	97	3	24 min	97	3
6.00 min	Stop		40 min	Stop	

3. Discussion

Sample preparation under light-protection and maintenance of the sample solution under cool conditions (4 °C) was needed to prevent decomposition of NTZ. Moreover, delicate adjustment of the portion of acetonitrile in water is required to achieve appropriate retention of NTZ in the ODS column. An internal standard method should be adopted in order to carry out precise NTZ quantitative analysis because the volume of acetonitrile solution is changeable in ambient temperature. However, the low solubility of NTZ in the prepared solution causes low quantitative precision of NTZ due to decomposition during re-

peated HPLC measurements. A comparatively long analytical time was unavoidable for achieving the appropriate condition to separate out the unexpected decomposed compound.

Although a rapid method cannot be achieved given the troublesome characteristics of NTZ, the developed gradient condition for popular HPLC should contribute not only to a simple quality check procedure but also to screening of counterfeit NTZ tablets on the market.

Using UPLC, the analytical time was successfully shortened to about one-seventh that of HPLC. Moreover, precise quantitative results of NTZ were obtained under ultra high column pressure condition. The HPLC and UPLC spent 40 ml and 2.4 ml of mobile phase at each analytical cycle; UPLC reduced the consumption of mobile phase to approximately one-seventeenth the volume for HPLC. Thus, UPLC technology will greatly reduce the consumption of organic solvent, which will also contribute to protecting the environment from chemical pollution.

These advantages suggest that the UPLC analytical technology would be useful for achieving high-precision, rapid quantitative analysis with good separation.

4. Experimental

4.1. NTZ reference standard, NTZ tablets and reagents

NTZ of 99.54% purity guaranteed by the manufacturer was purchased from Shinyang Co. Ltd. (Hangzhou, China) and used as a reference standard in this study. Alinia® Tablets 500mg (Romark Pharmaceutical, Tampa, FL, USA) were provided from the Human Science Research Group on Chemotherapy of Tropical Diseases. Nifuroxazide (5-Nitro-2-furaldehyde p-hydroxybenzoylhydrazone), used as an internal standard (Stockis et al. 1996), was purchased from Wako Chemical Inc. (Tokyo, Japan). General reagents and solvents, potassium dihydrogenphosphate (analytical grade), dipotassium hydrogenphosphate (analytical grade), and acetonitrile (HPLC grade) were purchased from Wako Chemical Inc.

4.2. Apparatus and chromatographic condition

4.2.1. HPLC system

The Shimadzu Class-VP HPLC system consist of a gradient pump (LC-10ADvp pump unit), an auto-injector unit equipped with a sample cooler (SIL-10ADve), and a photodiode array detector (SPD-M10Avp). Separation was achieved with a Waters symmetry C18 Column (150 mm × 4.6 mm I.D., 5 μm particle size, Waters Co., Milford, MA, USA), and the temperatures of column oven and sample cooler were set using the same conditions as for UPLC (see below).

4.2.2. UPLC system

A Waters ACQUITY Ultra Performance LC™ system equipped with photodiode array (PDA) was used. The UPLC column, Acquity UPLC BEH C18 (50 mm × 2.1 mm I.D., 1.7 μm particle size, Waters Co., MA, USA) was used, and the column oven was kept at 40 °C. The sample cooler was set at 4 °C. Detection was carried out at 360 nm of wavelength.

4.2.3. Mobile phase

A 1.76 g portion of potassium dihydrogen phosphate was dissolved in 900 ml of water. A 1.74 g portion of dipotassium hydrogen phosphate was dissolved in 1000 ml of water. The latter solution was added to the former solution until the pH was adjusted to 6.0. Then, water was added to 1000 ml, and this solution was used as the 10 mM phosphate buffer (pH 6.0). A mixture of acetonitrile and 10 mM phosphate buffer solution was filtered through a 0.2 μm pore-size membrane filter for use as the mobile phase for HPLC and UPLC.

The mobile phase consisted of the mixture of 10 mM phosphate buffer (pH 6.0) and acetonitrile, and the measurement was performed by gradient condition. In the gradient condition for HPLC, a 97 : 3 mixture of 10 mM phosphate buffer (pH 6.0)/acetonitrile was maintained for 3 min; then, over the next 7 min, the ratio of acetonitrile was increased up to 5%. Over the following 10 min, the volume ratio of acetonitrile was linearly increased up to 40% and was kept at that level for 3 min (phosphate buffer/acetonitrile, 3 : 2) (Table).

For the UPLC gradient condition, the initial condition was kept for 0.72 min, and the ratio of acetonitrile was gradually increased up to 5% over 2 min. In the next 1.5 min, the ratio of acetonitrile was linearly increased up to 52%, and the ratio was further increased up to 60% in the next 0.5 min (phosphate buffer/acetonitrile, 2 : 3) (Table). The required time of each measurement was 40 min for HPLC and 6 min for UPLC. The flow rates of the mobile phase for HPLC and UPLC were set at 1.0 ml/min and 0.4 ml/min, respectively.

4.3. Standard solutions for calibration curve and internal standard solution (IS solution)

Quantities of 80, 90, 100, 110 and 120 mg of the NTZ reference standard were put into 100 ml light-resistant volumetric flasks, and acetonitrile was added to volume and well mixed. Then, 5 ml of each solution, 10 ml of IS solution and 5 ml of acetonitrile were added into 50 ml light-resistant volumetric flasks. Water was added into each light-resistant volumetric flask to volume, and these solutions were used as the standard solutions for a calibration curve.

For the IS solution, 12.5 mg of nifuroxazide was put into a 100 ml light-resistant volumetric flask, and acetonitrile was added to volume and well mixed. The end concentration of nifuroxazide was 0.125 mg/ml.

4.4. Sample preparation

Five NTZ tablets were precision-weighed, and were powdered finely by hand using a mortar. Then an amount equal to 100 mg of NTZ was put into a 100 ml light-resistant volumetric flask, and acetonitrile was added to volume. Each mixture solution was kept under ultrasonication for 10 min, and approximately 100 ml of each mixture solution were centrifuged at 4,000 rpm for 5 min. Exactly 5 ml of each supernatant liquid was transferred into 50 ml light-resistant volumetric flasks. Then the IS solution, acetonitrile and water were added, and the solution described above was prepared. A suitable volume of this solution was filtered through a synthetic membrane filter with a pore size of 0.20 μm. The filtrate was used for the HPLC or UPLC measurement as the sample solution. Volumes of 10 μl or 2 μl of sample solution were automatically injected into the HPLC or UPLC for the quantitative analysis.

4.5. Solubility of NTZ into water

NTZ is described as "practically insoluble" in water in the document attached to pharmaceutical products. In order to decide the final ratio of water in the sample solution, the solubility of NTZ in a water/acetonitrile mixture was examined. A 0.5 ml volume of water was added to 50 mg of NTZ, and 0.5 ml of acetonitrile was added to this mixture, and it was shaken well for 5 min by hand. An additional 0.5 ml of acetonitrile was added to this mixture, and it was shaken well for 5 min. This operation was continued until NTZ dissolved completely.

4.6. Stability of NTZ in sample solution

About 10 mg of NTZ was dissolved in 100 ml of a 60 : 40 mixture of water/acetonitrile. Ten μl of this solution were injected into HPLC after 15, 60, 120, 210, 260 and 300 min of preparation.

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