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Short communication

Determination of diphenytriazol (DL111-IT) and its related impurities by RP-HPLC with DAD

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Abstract

An analytical method was developed for determining diphenytriazol and its related impurities in oil injection by using RP-HPLC with DAD and diazepam as internal standard. The C_{18} column was used as analytical column. Mobile phase consisted of methanol–potassium dihydrogen phosphate solutions (10 mmol l^{-1}, pH 7.5) (7:3, v/v). The standard curve was linear in the concentration range from 2 to 100 μg ml^{-1} for diphenytriazol. The analytical method afforded average recoveries of $100.3 \pm 1.9\%$ ($n = 9$) and the relative standard deviation (RSD) was less than 2% for within-day and between-day precision. The limit of detection and of quantitation for the assay were 15 and 40 ng, respectively. The method was simple, accurate and allowed to be used as analytical method for the routine quality control of diphenytriazol injection. Diphenytriazol injection showed a high stability to the heat (60 °C) and the light (4000 lx).

Keywords: Diphenytriazol; Related impurities; DL111-IT; Reversed phase high-performance liquid chromatography; Photodiode array detection

1. Introduction

Diphenytriazol, 3-(2-ethyl phenyl)-5-(3-methoxy phenyl)-1*H*-1,2,4-triazole, was reported with the name DL111-IT as non-hormonal contragestional and immunosuppressive agent [1]. Diphenytriazol combining with mifepristone or RU486 has the synergistic action in termination of early pregnancy [2,3]. It performed the action through inhibiting progesterone synthesis by suppressing the conversion of pregnenolone to progesterone [4]. It shows low embryotoxic and teratogenic effects on fetuses of gestation [5,6]. But the mechanism of early pregnancy-terminating has not been clarified [5]. The structure of diphenytriazol is shown in Fig. 1. There were no reports about the methods of determining diphenytriazol injection and its impurities so far. The ultraviolet spectrophotometry (UV), reversed-phase high-performance liquid chromatography (RP-HPLC) and gas chromatography (GC) were tested and compared for the determination of diphenytriazol based on its chemical structure in our lab. The oil solvent shows strong ultraviolet absorption and

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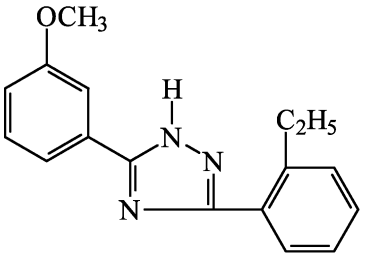

Fig. 1. Structure of DL111-IT.

interferes for determining of diphenytriazol by UV. The accuracy and sensitivity of GC was not better than RP-HPLC. The purpose of this study was to develop a rapid, accurate, sensitive RP-HPLC method for determining diphenytriazol and its related impurities in injections simultaneously.

2. Experimental

2.1. Materials and reagents

Diphenytriazol reference standard (99.96%, batch 000803) and its injections were donated by Zhejiang Xianju Pharmaceutical Factory (Zhejiang, China). Diazepam, the internal standard (I.S.) was obtained from the National Institute for Drug Control of China (Beijing, China). All other chemicals and solvents were analytical reagent or chromatographic grade and obtained from common commercial sources.

2.2. The RP-HPLC system

The HPLC system was composed of a HP-1100 pump, a Rheodyne injector with final volume loop of 20 μl and a HP-1100 photodiode-array detector (DAD) with HP Chemstation (Agilent, USA). The analytical column was packed with C_{18} standard phase (ODS 250 × 4.6 mm I.D. 5 μm particle size, Agilent, USA). The mobile phase consists of methanol–potassium dihydrogen phosphate solutions (10 mmol l^{-1}, pH 7.5) (7:3 v/v). The wavelength of DAD was operated at 235 nm. The flow rate of mobile phase was set at 1.0 ml per minute. Chromatographic assay was carried out at room temperature.

2.3. Assay for injection

A stock solution of the internal standard (0.1 mg ml^{-1}) was prepared by dissolving 10 mg of the diazepam in 100 ml methanol. The diphenytriazol injection (5 mg ml^{-1}) was accurately weighed about 0.45 g to a 10 ml screw-capped tube. Five millilitres of methanol was added to the injection sample. The sample was vortex-mixed for 10 min and centrifuged (3500 × *g*) for 10 min. After the sample was frozen at −20 °C for 30 min, 0.2 ml of the supernatant was transferred into another test tube. The methanol was evaporated to dryness at 85 °C water bath under air flow then 0.5 ml of internal stock solution and 4.5 ml of mobile phase were added to the residues. The solution was vortex-mixed for 10 min and centrifuged (3500 × *g*) for 10 min. An aliquot of 20 μl supernatant with the diphenytriazol concentration about 20 μg ml^{-1} and I.S. concentration 10 μg ml^{-1} was injected into the HPLC system. The content of diphenytriazol in injection was calculated by comparing of the ratio of diphenytriazol peak area to IS peak area of the test sample with that of the standard.

2.4. Determination of related impurities

The procedure was carried out as the 'Assay for injection' but the I.S. was not added. The mobile phase was added to the residues and the final concentration of diphenytriazol was made 0.25 mg ml^{-1}. The control solution was obtained by diluting 0.1 ml of the test solution to 10.0 ml. The content of related impurities of diphenytriazol in injection was calculated by comparing the sum peak-area of related impurities in test solution with the main peak area of diphenytriazol in control solution.

2.5. Stability testing for injection

The diphenytriazol injection was exposured to the 60 °C for 10 days and the light (4000 lx) for 10 days, respectively, in order to investigate the stability of diphenytriazol injection to light and heat. Diphenytriazol and related impurities in injections stressed or decomposed were determined

according to the methods as described in Sections 2.3 and 2.4 and the results were compared with those injection stored at room temperature.

3. Result and discussion

3.1. Optimization of chromatographic conditions

3.1.1. Detection wavelength

The detection wavelength for diphenytriazol and its impurities was investigated from the ultraviolet absorption spectrum of diphenytriazol and its related impurities measured by photodiode array detection (Fig. 2). There was little absorbance difference for diphenytriazol at 248 and 235 nm. I1, I2 and I3 were intermediates formed in synthesis of diphenytriazol and may exist in final product. Those three compounds showed that the absorption at 235 nm was higher than that at 248 nm in methanol. So, the 235 nm was used as analytical wavelength.

3.1.2. Mobile phase

An aliquot of 20 μl mixture solution of diphenytriazol and diazepam in methanol was injected into a C_{18} column. The chromatographic parameters were compared for diphenytriazol and diazepam under three different systems of mobile phase (Table 1). The three systems of mobile phase included: (1) a mixture solution of methanol and water at the ratio of 70:30 (v/v); (2) a solution consisting of methanol and 0.1% acetic acid solution at the ratio of 70:30 (v/v); and (3) a system consisted of methanol–potassium dihydrogen phosphate solutions (10 mmol l^{-1}, pH 7.5) (7:3, v/v). From the data of the theoretical plates, tailing factor and resolution between diphenytria-

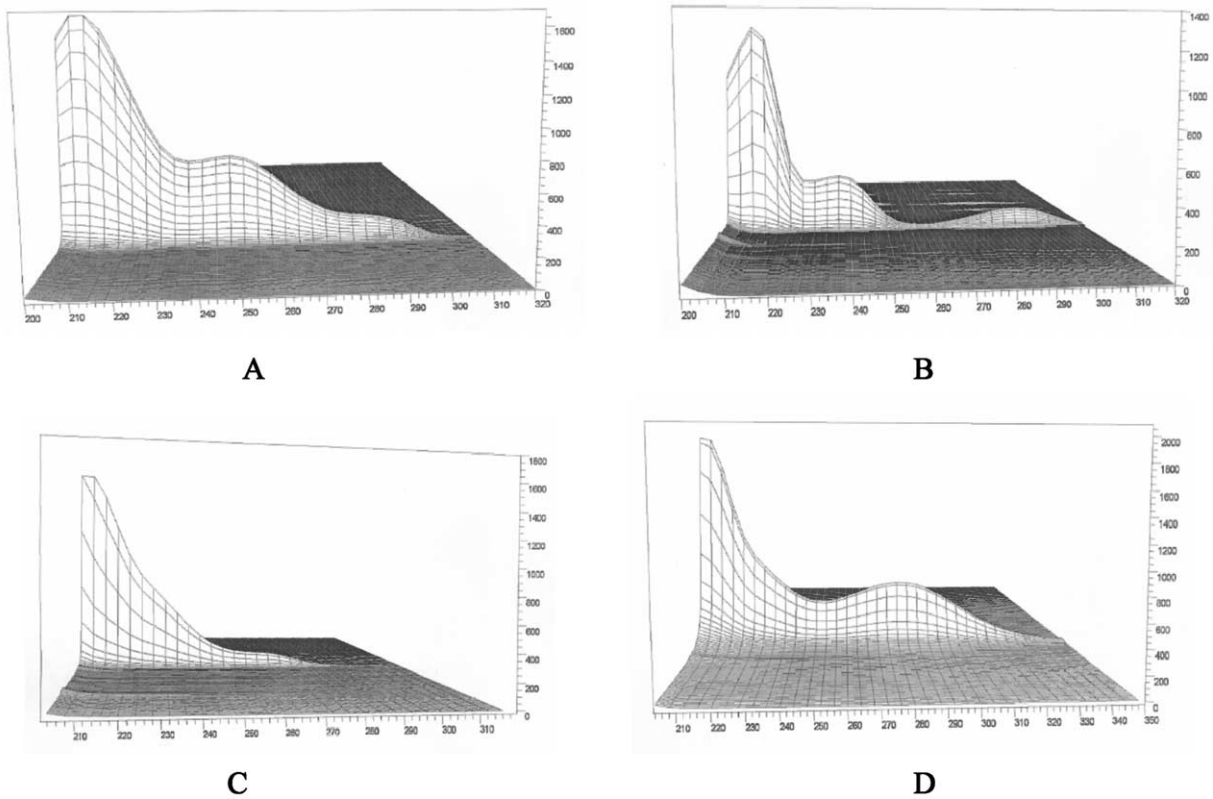

Fig. 2. The UV spectra of DL111-IT and its related impurities measured by DAD: (A) the UV absorption spectrum of DL111-IT by DAD; (B, C and D) the UV absorption spectrum of related impurities by DAD.

Table 1
The chromatographic parameters of diphenytriazol for different mobile phase system

Mobile phase (70:30)	Theoretical plates (n)	Tailing factor (T)	Resolution (R) between diphenytriazol and IS
CH_3OH:H_2O	3966	0.873	9.24
CH_3OH:0.1%HAc	4845	0.958	8.69
CH_3OH:KH_2PO_4 (10 mmol/l, pH 7.5)	5575	1.087	10.01

zol and internal standard, it was found that the system (3) was the best one among three systems. Diphenytriazol and its related impurities or internal standard were separated on base line by using the system (3) as the mobile phase (Fig. 3). Therefore, the mixture solution of methanol–potassium dihydrogen phosphate solution (10 mmol l^{-1}, pH 7.5) (7:3, v/v) was chosen as appropriate mobile phase for subsequent investigations.

3.1.3. Selection of internal standard substance

The chromatographic parameters of diphenytriazol and I.S. including diazepam, dexametha-

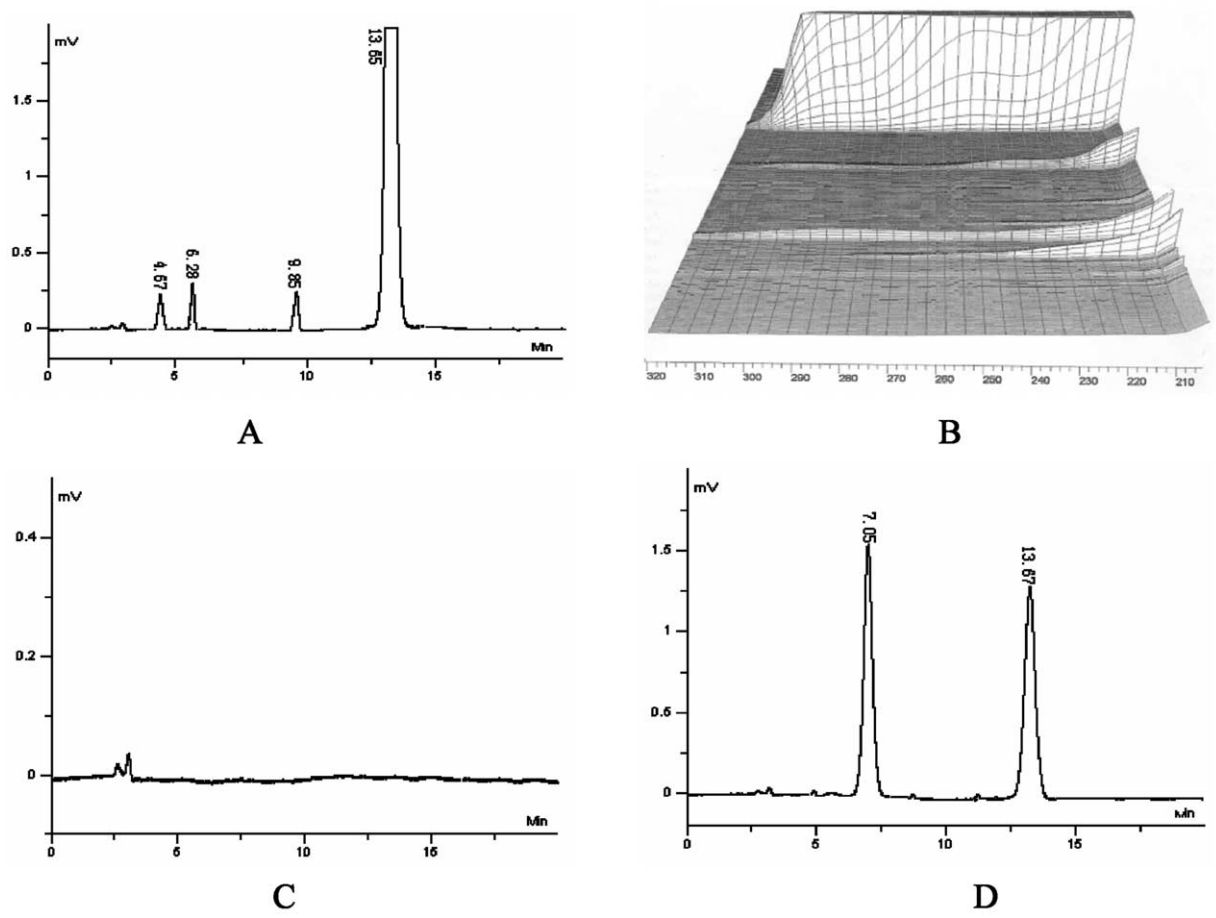

Fig. 3. The chromatogram of DL111-IT and its related impurity measured by DAD: (A, B) bulk drug spiked with 0.1% of I1, I2 and I3; (C) blank injections; (D) injection samples and the internal standard (diazepam).

sone, testosterone and 18-methyl norethisterone were measured. The data listed in Table 2 indicated diazepam and dexamethasone could be separated with diphenytriazol. Diazepam had more suitable α and k value than dexamethasone.

3.2. Validation of the method

3.2.1. Standard curve and sensitivity of the method

A reference stock solution of diphenytriazol (1.0 mg ml^{-1}) was prepared by dissolving 50 mg diphenytriazol in 50 ml methanol. The stock solution was diluted with mobile phase to 2, 5, 10, 20, 50, and 100 μg ml^{-1} with 10 μg ml^{-1} of internal standard for construction of the standard curve. The standard curve for diphenytriazol were constructed by plotting peak area ratio (y) of diphenytriazol over internal standard vs. Diphenytriazol concentration (x). The standard curve of assay for diphenytriazol showed an acceptable linearity in the concentration range from 2 to 100 μg ml^{-1} with a correlation coefficient over 0.9989. The regression equation was $y = 19.521x + 1.118$. Diphenytriazol concentration in injection samples were calculated from the equation.

The limit of quantitation for the assay of diphenytriazol base on 10/1 of the signal to noise ratio, was 40 ng. The detection limit, base on 3/1 of the signal to noise ratio, was 15 ng.

With the same assay, we obtained the LOD of the three intermediates: 25, 40 and 20 ng, respectively. The LOQ of the three intermediates were 80, 140 and 60 ng, respectively.

3.2.2. Recovery and repeatability

The recovery of the method was determined as described in 'assay for injection' by analyzing a series of blank oil injection spiked with various concentrations of standard diphenytriazol at the 80, 100 and 120% of the prescription amount. The recovery of diphenytriazol was calculated by comparing the peak area ratio of diphenytriazol vs. I.S. with that resulting from the corresponding diphenytriazol standard solution in mobile phase. The results were shown in Table 3.

The within-day variability was determined by analyzing the blank oil injection spiked with various concentrations of standard diphenytriazol in five replicates and between-day variability by analyzing the blank oil injection spiked with various concentrations of standard diphenytriazol in triplicate on separated days. The results were listed in Table 3.

The data in Table 3 showed that the method of assay for diphenytriazol was accurate and repeatable. The average recovery of the assay for diphenytriazol was $100.3 \pm 1.9\%$ and the relative standard deviation (RSD) for assay precision was less than 2%.

3.2.3. Stability of injection

The results indicated that there were few impurities unidentified in diphenytriazol injection and the total impurities were less than 0.16% comparing with the injection stored at room temperature. The impurities are not intermediates I1, I2 and I3. The diphenytriazol injection was stable for stress testing under light (4000 lx) and heat (60 °C) condition (Table 4).

Table 2
The HPLC chromatographic parameters of diphenytriazol and other drugs

Compound	Retention time (t_R, min)	Separation factor (α)	Capacity factor (k)
Diphenytriazol	13.67	1.00	4.26
Diazepam	7.05	2.49	1.71
Dexamethasone sodium phosphate	5.40	3.94	1.08
Testosterone	13.06	1.06	4.02
18-Methyl norethisterone	13.86	0.98	4.33

Table 3
Recovery and reproducibility of the assay for diphenytriazol injections ($n \geq 3$)

Target concentration	Concentration (mg ml^{-1})	Recovery (%)	Within-day RSD (%)	Between-day RSD (%)
3.90	3.99 ± 0.088	102.1 ± 1.86	0.78	1.82
4.95	4.97 ± 0.021	100.4 ± 0.42	0.57	1.80
6.07	5.95 ± 0.036	98.3 ± 0.82	0.40	0.83

Table 4
Content of diphenytriazol and related impurities in injections exposure to stress conditions

Stress testing	Batches	Diphenytriazol (%)	Total impurities (%)
Room temperature	000601	99.86	0.24
	000602	99.92	0.22
	000603	99.98	0.22
60 °C for 10 days	000601	99.41	0.37
	000602	99.40	0.30
	000603	99.39	0.38
Light for 10 days	000601	99.61	0.30
	000602	99.28	0.36
	000603	99.46	0.32

4. Conclusions

A simple and accurate RP-HPLC analytical method was developed for determining diphenytriazol and its related impurities in oil injections simultaneously. The method was successfully applied to the routine quality control of diphenytriazol injection. Diphenytriazol injection showed a high stability to the high temperature (60 °C) and the light (4000 lx).

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