Asian Journal of Chemistry Vol. 19, No. 5 (2007), 3375-3381

High Performance Thin Layer Chromatographic Determination of the Related Substances in Alprazolam Drug

Y. ANJANEYULU†, R. MARAYYA, D. LINGA RAO* and P. KRISHNA RAO
Quality Control and Assurnace, Natco Pharma Ltd., Chemical Division
Road No. 2, Banjara Hills, Hyderabad-500 033, India
Tel: (91)(40)23058729; E-mail: mekaguda@natcopharma.co.in

The thin layer chromatographic method described in United State Pharmacopoeia (USP-28) do not quantify in the separation of starting material with thionordiazepam and the substance 2-(2-aceto hydrazinyl)-7-chloro-5-phenyl-3H-1,4 benzodiazapine with alprazolam drug substance. An alternative high performance thin layer chromatographic method is developed for the separation and estimation of the starting material, synthesis related intermediates in alprazolam drug substance. The HPTLC method is capable of detecting the impurities at a level of 0.05 % (with respect to test concentration).

Key Words: 2-Chloro acetamide-5-chloro benzophenone, Nordiazepam, Thionordiazepam, 2-(2-aceto hydrazinyl)-7-chloro-5-phenyl-3H-1, 4-benzodiazapine, 8-chloro-1-methyl-6-phenyl-4H-s-triazolo[4,3-α][1,4]benzodiazepine (alprazolam).

INTRODUCTION

Alprazolam drug substance is used to treat anxiety disorders and panic attacks. Alprazolam is categorized under benzodiazepines class of compounds. It works by decreasing abnormal excitement in the brain. Alprazolam is also used to treat depression, fear of open spaces (agoraphobia) and premenstrual syndrome[1].

The alprazolam drug substance monograph is described[2] in USP-28. The monograph has not referred any specific impurities. The possible related substances (refer 2.0) as described in the synthetic route (Fig. 1) were subsequently evaluated by using the thin layer chromatographic analytical method described in USP-28.

The TLC method described in USP[3,4] uses chloroform, acetone, ethyl acetate and methanol (50:50:50:5) as the mobile phase; 40 mg/mL as test concentration and the evaluation against 0.1, 0.3 and 0.5 % standard solutions (alprazolam) under short-wavelength UV light (254 nm).

†Institute of Science & Technology, Jawaharlal Nehru Technological University, Kukatpally, Hyderabad-500 072, India

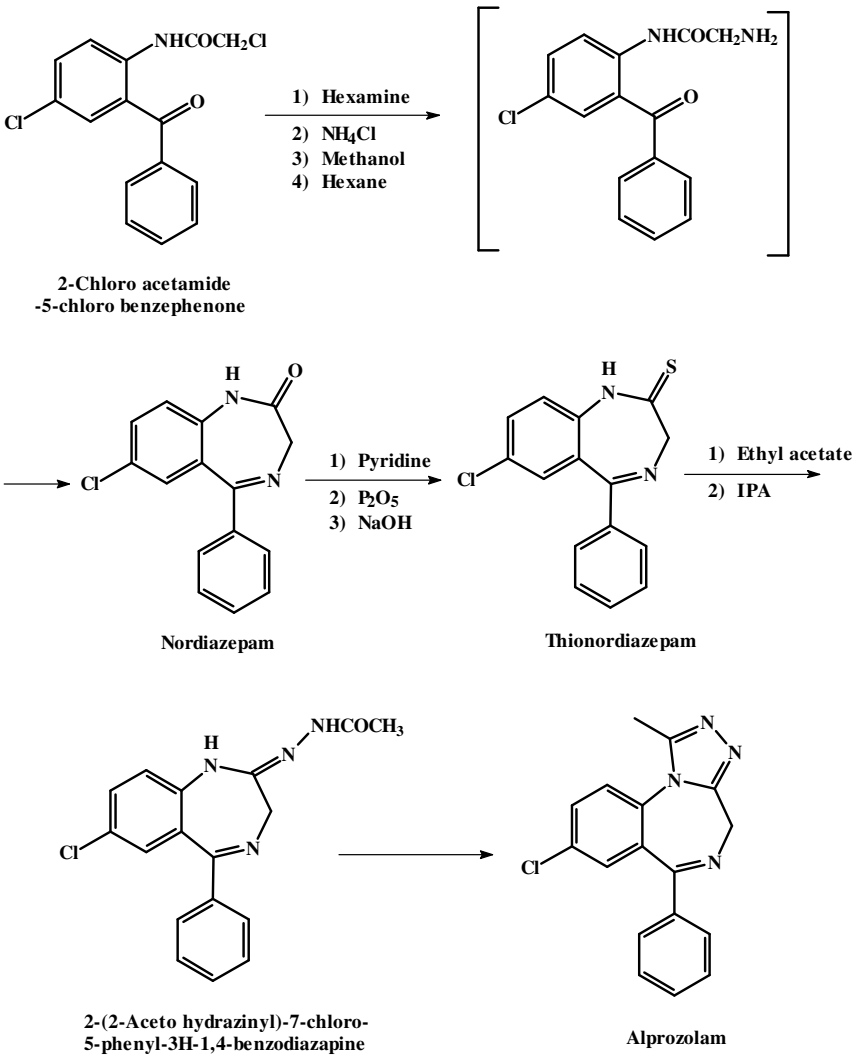

Fig. 1. Synthetic route

The starting material 2-(2-aceto hydrazinyl)-7-chloro-5-phenyl-3H-1,4-benzodiazapine is found merging with the alprazolam main spot. Two other substances *i.e.*, 2-chloro acetamide-5-chloro benzophenone and thionordiaze-pam were also found merging with each other and hence no separation (Table-1) of these compounds as per TLC method described in USP-28.

As sufficient separations were not achieved as per USP method, attempts were to develop an alternative method for the detection, separation and estimation of these impurities by HPTLC.

TABLE-1
R_f-Values Of Impurities Present In Alprazolam

Component	Identification	R_f value
2-Chloro acetamide-5-chloro benzophenone	Impurity-1 (starting material)	0.08
Nordiazepam	Impurity-2	0.72
Thionordiazepam	Impurity-3	0.86
2-(2-Aceto hydrazinyl)-7-chloro-5-phenyl-3H-1,4-benzodiazapine	Impurity-4	0.19
Alprazolam	Drug substance	0.23

A more specific and an alternative high performance thin layer chromatographic method is developed for the separation of all compounds listed in the route of synthesis (Fig. 1).

Sufficient levels of detection, separation and estimation were obtained at the below-described, optimized chromatographic conditions.

From the above synthetic reaction scheme (Fig. 1) the related substances considered for evaluation in the alprazolam drug substance are as described below:
a) 2-chloro acetamide-5-chloro benzophenone (starting material or impurity-1) *b*) Nordiazepam (impurity-2) *c*) Thionordiazepam (impurity-3) *d*) 2-(2-aceto hydrazinyl)-7-chloro-5-phenyl-3H-1, 4-benzodiazapine (impurity-4). All the above listed substances can be identified and estimated with current novel HPTLC analytical method (Table-1).

EXPERIMENTAL

The drug substance alprazolam and all the above listed related substances were manufactured by M/S Natco pharma limited (Hyderabad, India).

Analytical grade methylene chloride, methanol and ethyl acetate was obtained from Merck. Pre-coated Silica Gel $60F_{254}$ TLC plates from Merck were used.

This developmental study is conducted using high performance thin layer chromatograph equipment (CAMAG, Germany).

Diluent: A mixture of 4: 1 chloroform and methanol.

System suitability solution: A mixture having 0.08 mg/mL each of impurity-1, impurity-2, impurity-3, impurity-4 and alprazolam (0.2 % with respect to test concentration) in diluent.

Reference solution (a): A mixture having 0.04 mg/mL each of impurity-1, impurity-2, impurity-3, impurity-4 and alprazolam (0.1 % with respect to test concentration) in diluent.

Reference solution (b): A mixture having 0.08 mg/mL each of impurity-1, impurity-2, impurity-3, impurity-4 and alprazolam (0.2 % with respect to test concentration) in diluent.

Reference solution (c): A mixture having 0.12 mg/mL each of impurity-1, impurity-2, impurity-3, impurity-4 and alprazolam (0.3 % with respect to test concentration) in diluent.

Test solution: 400 mg of alprazolam in 10 mL of methanol (40 mg/mL).

Mobile phase: Mix 8 mL of methylene chloride, 0.5 mL of methanol and 0.25 mL of ethyl acetate.

Application volume: 5 µL.

Elution: The TLC plate shall be developed in closed chamber lined the walls with filter paper for pre-saturation with the mobile phase vapours. Upon elution the TLC plate shall be air-dried and is visualized under UV light at 254 nm.

The TLC plate shall be subjected for densitometric scanning using Camag Linomat-IV scanner.

Scan parameters: The typical densitometric scan parameters are as below.

Distance between tracks	: 14.0 mm
Bandwidth	: 20 nm
Lamp	: Mecury
Wavelength	: 254 nm
Slit dimention	: 8.0 × 0.9 mm
Data step resolution	: 50 µm
Measurement mode	: Absorption/Reflection

The HPTLC system was deemed suitable for use if the system suitability solution has shown five completely separated spots corresponding to impurity-1, impurity-2, impurity-3, impurity-4 and alprazolam.

The components under study will be identified with system suitability solution.

Reference solution (a), Reference solution (b), Reference solution (c) shall be used for estimating the respective impurity level in test application.

RESULTS AND DISCUSSION

System suitability

The HPTLC chromatographic system is deemed suitable when five fully separated spots are obtained with the application of system suitability solution having alprazolam and all the related impurities 1, 2, 3 and 4 at about 0.2 % (0.08 mg/mL) with respect to test concentration of 40 mg/mL.

The system suitability solution resulted 5 distinctively separated spots and thus acceptance criteria specified has been met. Thus the system suitability of the HPTLC method is established.

Power of the method-specificity or resolution

The power of the developed HPTLC analytical method in separating the components is established by calculating the R_f value of each component. Each of the components under study is individually applied at a concentration of 0.2 % (0.08 mg/mL) with respect to test concentration of 40mg/mL. Identified each of the component by its retardation factor (R_f).

Alprazolam drug substance (40 mg/mL) spiked with each of the impurity at 0.2 % (0.08 mg/mL) has been analyzed as per the documented method. All the components under study were got well resolved which can be evidenced by the respective R_f values (Table-2).

TABLE-2

Component	R_f value	Spot. No.
2-Chloro acetamide-5-chloro benzophenone (Impurity-1)	0.83	5
Nordiazepam (Impurity-2)	0.45	3
Thionordiazepam (Impurity-3)	0.77	4
2-(2-Aceto hydrazinyl)-7-chloro-5-phenyl-3H-1,4-benzodiazapine (Impurity-4)	0.16	1
Alprazolam	0.25	2

From the R_f values obtained and from the chromatogram it can be observed that the method is capable of separating all the components with sufficient resolution. Thus this HPTLC method is novel and specific for the application.

The precision is determined by 6 replicate applications of alprazolam sample spiked with the related impurities each at 0.2 % (0.08 mg/mL) with respect to test concentration. The developed TLC plate is scanned on HPTLC. The responses are recorded and calculated the RSD (%) (Table-3). Based on RSD (%) results for all five compounds, listed in Table-2, it is evident that the method is precise.

The linearity of the method is studied over a range of 0.05 % (0.02 mg/mL) to 0.50 % (0.2 mg/mL) with respect to test concentration (40 mg/mL). Applications were made with a mixture of alprazolam sample spiked with impurities at 0.05 % (0.02 mg/mL), at 0.1 % (0.04 mg/mL), at 0.15 % (0.06 mg/mL), 0.2 % (0.08 mg/mL), 0.30 %, (0.12 mg/mL) and at 0.50 % (0.2 mg/mL) concentration levels with respect to test concentration of 40 mg/mL.

TABLE-3

S. No.	Impurity-1	Impurity-2	Impurity-3	Impurity-4	Alprazolam
1	7896.1	5296.8	6477.7	8489.1	82389.6
2	8042.9	5017.6	6562.7	8495.0	86177.0
3	7906.2	5066.6	6668.8	8347.2	79591.4
4	7872.6	5068.7	6713.0	8422.2	76848.5
5	8058.1	5255.7	6747.3	8576.1	80984.8
6	8104.0	5213.5	6934.5	8300.4	85199.0
Average	7980.0	5153.2	6684.0	8438.3	81865.1
RSD (%)	1.25	2.26	2.37	1.21	4.27

The TLC plate eluted is air-dried and is densitometrically scanned on Camag TLC Scanner. The correlation coefficient for the concentration taken and area response obtained on HPTLC (Table-4).

TABLE-4

Impurity-1		Impurity-2		Impurity-3		Impurity-4	
Conc. (%)	Area response	Conc. (%)	Area response	Conc. (%)	Area response	Conc. (%)	Area response
0.05	3797.1	0.05	2363.4	0.05	1022.6	0.05	4335.9
0.10	5060.0	0.10	3928.4	0.10	2992.1	0.10	6371.7
0.15	7312.9	0.15	5591.7	0.15	4975.4	0.15	7328.0
0.20	8434.5	0.20	6224.6	0.20	6041.5	0.20	8067.4
0.30	10707.7	0.30	8376.8	0.30	8620.2	0.30	9537.8
0.50	13804.9	0.50	13259.6	0.50	12159.1	0.50	13526.9
CC	0.9820	CC	0.9970	CC	0.9854	CC	0.9909

Linear regression equation : $Y = 22143.09X + 3388.514$ for Impurity-1
$Y = 23446.63X + 1543.981$ for Impurity-2
$Y = 22143.09X + 3388.514$ for Impurity-3
$Y = 19038.25X + 4069.663$ for Impurity-4

CC = Correlation coefficient.

The recoveries of impurities were assessed by spiking to alprazolam sample at 3 concentration such levels as 0.1 % (0.04 mg/mL), 0.2 % (0.08 mg/mL) and 0.3 % (0.12 mg/mL). The % recovery ranges from 89.1 to 101.5 % for all the impurities (Table-5).

The detection level (L.O.D.) for each component is studied by application of each component at a level of 0.025 % (0.01 mg/mL) with respect to test concentration (40 mg/mL). All the components were detected. The detection of impurities is also verified in alprazolam sample matrix at test concentration of 40 mg/mL.

TABLE-5

Description	0.1 % Level	0.2 % Level	0.3 Level
	Impurity-1		
Conc. obtained	0.102	0.205	0.270
Conc. taken	0.101	0.202	0.303
Recovery (%)	101.000	101.500	89.100
	Impurity-2		
Conc. obtained	0.098	0.184	0.281
Conc. taken	0.100	0.200	0.300
Recovery (%)	98.000	92.000	93.700
	Impurity-3		
Conc. obtained	0.090	0.194	0.286
Conc. taken	0.101	0.202	0.303
Recovery (%)	89.100	93.000	94.400
	Impurity-4		
Conc. obtained	0.099	0.200	0.278
Conc. taken	0.100	0.200	0.300
Recovery (%)	99.000	100.000	92.700

Conclusions

From the above it is established that the HPTLC method developed is sufficiently specific for the detection, separation and estimation of the possible impurities in alprazolam drug substance.

ACKNOWLEDGEMENTS

The authors wish to thank the management of M/s Natco Pharma Limited for providing facilities and support for carrying out this work and permission for publishing this information.

REFERENCES

1. Physician Desk Reference (PDR).
2. United States Pharmacopoeia, USP-28 (2005).
3. US Department of Health and Human Resources, Food and Drug Administration, Guidance to Industry: Q2A Validation of Analytical Procedures, Center for Drug Evaluation and Research, Rockville, MD (1994). www.fda.gov/cder/guidance/index.htm
4. US Department of Health and Human Resources, Food and Drug Administration, Guidance to Industry: Q2B Validation of Analytical Procedures-methodology, Center for Drug Evaluation and Research, Rockville, MD (1996). www.fda.gov/cder/guidance/index.htm

(*Received*: 5 January 2006; *Accepted*: 15 February 2007) AJC-5414