

Development and Validation of a Stability-Indicating RP-HPLC Assay Method and Stress Degradation Studies on Dapiprazole

Thippani Ramesh and Pothuraju Nageswara Rao*

Department of Chemistry, National Institute of Technology, Warangal 506004, Andhra Pradesh and India

*Author to whom correspondence should be addressed. Email: pnr.nitw@gmail.com; tippaniramesh.1@gmail.com

Received 11 April 2012; revised 26 August 2012

Dapiprazole (DPZ) was subjected to different stress conditions prescribed by the International Conference on Harmonization. A stability-indicating high-performance liquid chromatography method was developed for the analysis of the drug in the presence of its degradation products. The degradation was found to occur in hydrolytic, and to some extent, photolytic conditions, however, the drug was stable to oxidative and thermal stress. The drug was particularly labile under neutral and alkaline hydrolytic conditions. The assay was involved an isocratic elution of DPZ in a Kromasil 100C18 column using a mobile phase composition of water (pH 6.5, 0.05%, w/v, 1-heptane sulfonic acid) and acetonitrile (40:60, v/v). The flow rate was 0.8 mL/min and the detection was conducted at 246 nm. The assay method was found to be linear from 5 to 30 µg/mL. The method was validated for linearity, range, precision, accuracy, specificity, selectivity, limit of detection and limit of quantitation.

Introduction

Dapiprazole (DPZ), 3-{2-[4-(2-methylphenyl)piperazin-1-yl]ethyl}-5,6,7,8-tetrahydro-[1,2,4]triazolo[4,5-a]pyridine (Figure 1), is a potent α -adrenergic blocking drug, the general pharmacological properties of which have been described in detail (1, 2). It is a white, crystalline powder. It is freely soluble in ethanol, methanol and water, but partially insoluble in acetonitrile. It melts at approximately 162 °C and is reported to be stable at ambient temperature (3). It is used in topical eye therapy for the treatment of chronic simple glaucoma, for the induction of pre-operative miosis and for the reversion of pharmacologically induced mydriasis (4). DPZ causes the pupil of the eye to constrict. It reverses pupil dilation caused by other drugs that are given during eye examinations. The drug is also endowed with a unique psychopharmacological profile in mice and rats. It inhibits amphetamine toxicity and alcohol and morphine withdrawal syndromes, produces sedation, blocks conditioned avoidance reflexes and reduces the response to noxious stimuli (2). Human tests have proved the efficacy of DPZ in psychotic conditions, such as the withdrawal syndrome from opioids (5); the orally administered daily dose varied from 30 to 90 mg.

The parent drug stability test guideline Q1A (R2) was issued by International Conference on Harmonization (ICH) (6). It suggests that stress studies should be conducted on a drug to establish its inherent stability characteristics, leading to the identification of degradation products, and hence, supporting the suitability of the proposed analytical procedures. It also

requires that analytical test procedures for the stability of samples should be stability-indicating and fully validated.

Accordingly, the aims of the present study are to establish the inherent stability of DPZ through stress studies under a variety of ICH recommended test conditions (6), and to develop a stability-indicating assay (7). In literature, analytical methods have reported limited stress testing in which a single product was indicated to be formed under alkali conditions. Instead, more intensive stress studies during the current study showed that the drug was decomposed to almost six products under different stress conditions. Accordingly, a stability-indicating method was developed that could separate various degradation products (8).

Experimental

Materials

DPZ was a gift sample from MSN Laboratories (Hyderabad, India). Acetonitrile [high-performance liquid chromatography (HPLC) grade] was obtained from Qualigens Fine Chemicals (Mumbai, India). 1-Heptane sulfonic acid was purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals and solvents of analytical grade were used for further studies. Ultra-pure water, obtained from an ELGA water purification unit (Bucks, UK), was used in making solutions throughout the analysis.

Instrumentation

The HPLC system consisted of a Shimadzu LC-20T Prominence liquid chromatograph (Shimadzu, Columbia, MD) equipped with a Prominence DGU-20A5 degasser and a SPD-20A Prominence ultraviolet (UV)-visible detector. Data acquisition was performed by the Millennium 32-bit software operated on a Pentium IV microprocessor. Analysis was conducted at 246 nm with a Kromasil 100C18 reversed-phase column with dimensions of 250 × 4 mm i.d., 5 µm (VDS Optilab, Chromatographie technik GmbH, Germany) at ambient temperature. Precision water baths equipped with MV controllers (Julabo, Seelbach, Germany) were used for hydrolytic studies. Photostability studies were conducted under sunlight and stability studies were conducted in a humidity chamber (KBF 760; WTB, Binder, Tuttlingen, Germany). Thermal stability studies were performed in a dry air oven (Smart Lab Tech., Hyderabad, India).

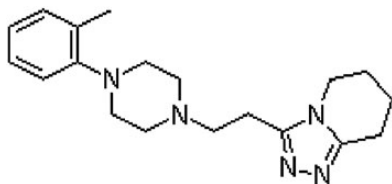


Figure 1. Chemical structure of DPZ.

Degradation studies

All stress decomposition studies were performed at an initial drug concentration of 0.5 mg/mL in water containing 25% acetonitrile. The study in alkaline condition was conducted in 0.2M NaOH at 70°C for 9 h. Acid hydrolysis was performed in 0.1 and 1M HCl at 70°C for 9 h. These experiments were repeated at a lower temperature of 40°C, keeping all other conditions constant. To study the neutral condition, the drug was dissolved in water and heated at 70°C for 9 h. Photodegradation studies were performed in water and in 1M HCl. The solutions were exposed to sunlight during the day for three days. Oxidative studies were conducted at room temperature in 5 and 20% H₂O₂ for 24 h. Additionally, the drug powder was exposed to dry heat at 60°C for 40 days and at 50°C for eight days. One milliliter of each of these solutions was transferred into a 10 mL volumetric flask and the volume completed with mobile phase. The final solution was injected for analysis.

Analytical method validation

The method was validated for precision, linearity, specificity, selectivity, accuracy, limit of detection (LOD) and limit of quantitation (LOQ) according to the United States Pharmacopeia (10) and ICH guidelines (ICH Q2 R1) (6).

Linearity and range

A stock solution of DPZ was prepared at strength of 0.5 mg/mL. It was diluted to prepare solutions containing 5–30 µg/mL of the drug. The solutions were injected in triplicate into the HPLC column, keeping the injection volume constant (20 µL).

Precision

Six injections of three different concentrations were made on the same day and the percentage values of relative standard deviation (RSD) were calculated to determine the intra-day precision. These studies were also repeated on different days to determine the inter-day precision. The intermediate precision was established through a study on different chromatographic systems using different columns.

Accuracy

The accuracy of an analytical procedure is the closeness of agreement between the values that are accepted either as conventionally true values or accepted reference values. The accuracy was evaluated by fortifying a mixture of degraded solutions with six known concentrations of the drug. The recovery of the added drug was determined.

Specificity and selectivity

The specificity of the method was established through a study of the resolution factors of the drug peak from the nearest resolving peak and among all other peaks.

LOD and LOQ

The LOD is defined as the lowest concentration of an analyte that an analytical process can reliably differentiate from background levels. The LOQ is defined as the lowest concentration of the standard curve that can be measured with acceptable accuracy, precision and variability (9). The LOD and LOQ were calculated as $LOD = 3.3 \sigma/S$ and $LOQ = 10 \sigma/S$, where σ is the standard deviation (SD) of the lowest standard concentration and S is the slope of the standard curve.

Results and Discussion

Degradation behavior

HPLC studies on DPZ under different stress conditions suggested the following degradation behavior. Acidic, basic and neutral studies were conducted in an RB flask with a condenser on a temperature-programmable electromagnetic stirrer, and a UV-light chamber was used for the photolytic studies.

Acidic condition

The drug gradually decreased with time on heating at 70°C in 1M HCl. The degradation products were observed at retention times (RTs) of 1.82 and 7.06 min. The rate of hydrolysis in acid was slower than that of alkali or water.

Degradation in alkali

The drug was found to be highly labile to alkaline hydrolysis. The reaction in 0.2M NaOH at 70°C was so fast that the whole drug was degraded in 5 min. Subsequently, studies were performed in 0.02M NaOH at 40°C. Drug degradation was associated with the rise of a major degradation product at RT of 8.79 min. Complete degradation of the drug was observed in 5 h. Minor degradation products at RTs of 1.82 and 3.15 min emerged after 5 h.

Neutral or water condition

Upon heating the drug solution in water at 70°C for 1 h, a steep fall in the drug peak area was observed. At the end of 1 h, almost complete degradation of the drug was observed with a corresponding rise in the major degradation peak at 14.21 min.

Oxidative conditions

The drug was stable to hydrogen peroxide (5 and 20%) at room temperature.

Photolytic conditions

No major degradation product was observed after exposure of the drug solution in 1M HCl to sunlight for three days, and only minor degradation products were observed at RTs of 3.15, 7.06 and 21.06 min. The nature of degradation in light and dark was found to be similar, indicating that light had no effect on the degradation of the drug in acid. On the other hand, the samples in water degraded under sunlight for three days to a

major product at RT of 14.21 min, along with minor degradation products. The corresponding rate of degradation in the dark was much slower.

Solid-state study

The solid-state studies showed that DPZ was stable to the effect of temperature. When the drug powder was exposed to dry heat at 60°C for 40 days and at 50°C for eight days, no decomposition of the drug was observed.

Stability-indicating method development and optimization

DPZ is hydrophobic and almost insoluble in aqueous solutions, whereas it is freely soluble in organic solvents like methanol and acetonitrile. During the development phase, the use of acetonitrile and water as the mobile phases resulted in asymmetric peaks with a greater tailing factor (>2). Ion-pair chromatography is a good approach to minimize this effect, and the successful use of 1-heptane sulfonic acid as an ion-pair reagent for the separation of bio-chemicals and pharmaceuticals has previously been described (8). The addition of 1-heptane sulfonic acid to the mobile phase resulted in a drastic reduction in peak tailing. At the reported concentration (0.05%, w/v), the tailing factor was within the acceptable limit, resulting in good peak symmetry and resolution. Where the flow rate was increased from 0.5 to 0.8 mL/min, a flow rate of 0.5 mL/min resulted in a drug retention time beyond 50 min, which was more time consuming. Hence, the mobile phase was optimized at 0.8 mL/min with the retention time of the drug at approximately 30 min. Also, the low flow rate and shorter run time consumed comparatively fewer mobile phase solvents, which will prove to be cost-effective during the routine analysis of

drug samples. The peak shape and symmetry were found to be good when a mobile phase composition of 40:60 (v/v) water (pH 6.5, 0.05%, w/v 1-heptane sulfonic acid) and acetonitrile was used, which also resulted in better resolution of the drug. The resulting chromatogram is shown in Figure 2. This indicates that the isocratic method successfully separated drug and all degradation products. The proposed chromatogram and the method were sufficiently specific to the drug. The resolution factor for the drug peak was >3 from the nearest resolving peak. Intermediate precision was performed to confirm that the separation was satisfactory under external conditions. Good separations were always achieved, indicating that the method remained selective for all components under the tested conditions.

Validation of stability-indicating method

In the current study, an HPLC method was used to determine the percentage of the drug release. HPLC was used to separate, identify and determine the concentration of a specific component in a mixture; moreover this method was very fast, reproducible and easy to operate (11). The developed method was validated to meet the requirements for a global regulatory filing. The validation parameters such as precision, linearity, specificity, accuracy, LOD and LOQ were conducted in accordance with ICH and U.S. Pharmacopoeia guidelines.

Linearity

The linearity of DPZ was evaluated from the range of 5.0 to 30.0 $\mu\text{g/mL}$ and showed a good correlation coefficient (r^2) = 0.9997. The standard curve of DPZ was constructed by plotting concentration ($\mu\text{g/mL}$) versus area response (AU) which is shown in Figure 3. The linearity regression and slope were calculated, and these results are shown in Tables I–III.

Precision

The precision of an analytical procedure express the closeness of the agreement between a series of measurements obtained from multiple samples of the same homogeneous sample under the prescribed conditions. Repeatability is a measure of the precision under the same operating conditions over a short interval of time; it is also known as intra-assay precision. A minimum of six determinations at 100% of the standard

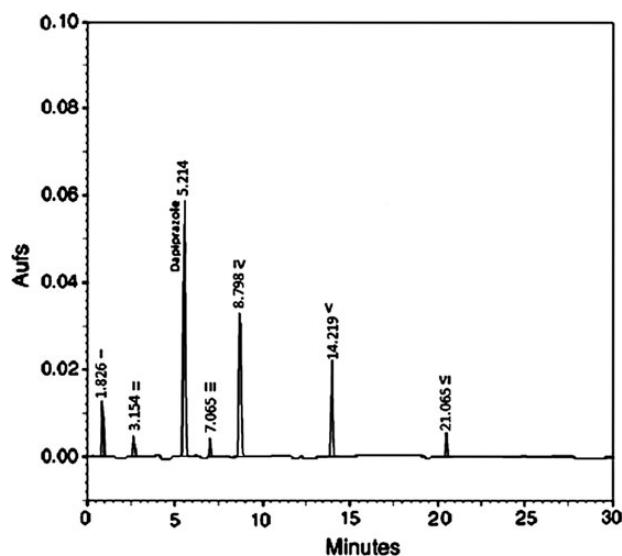


Figure 2. Chromatograms showing the separation of different degradation products of DPZ contained in a mixture of reaction solutions. Peaks: I, photolytic product in acid; II, hydrolytic degradation product in alkali and photolytic product in acid; III, photolytic product in water; IV, product formed in all types of hydrolytic and photolytic conditions; V, product formed in all types of hydrolytic and photolytic conditions; VI, product formed in water under dark and photolytic conditions.

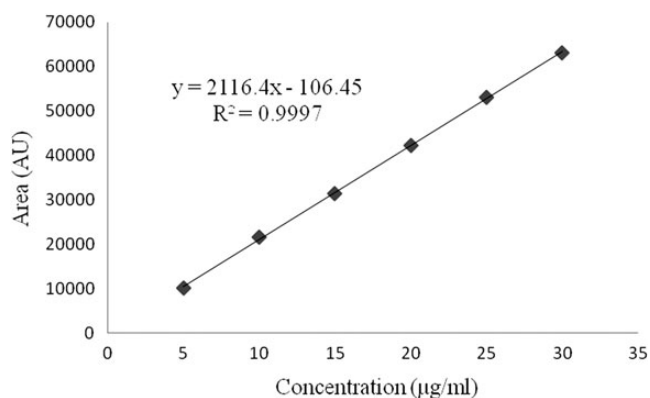


Figure 3. Linearity graph of DPZ.

Table I

Linear Regression of DPZ

Concentration ($\mu\text{g/mL}$)	Area* (in AU)	SD	RSD
5	10,145.25	60.89	0.16
10	21,568.76	66.32	0.18
15	31,447.88	60.55	0.16
20	42,154.66	57.53	0.15
25	53,124.53	53.45	0.14
30	63,145.54	61.27	0.17

*Average of five determinations.

Table II

Calculation of Regression Line

X (concentration in $\mu\text{g/mL}$)	Y* (area in AU)	XY	X ²	Y ²
5	10,145.25	50,726.25	25	102,926,097.6
10	21,568.76	215,687.6	100	465,211,407.9
15	31,447.88	471,718.2	225	988,969,156.5
20	42,154.66	843,093.2	400	1,777,015,360
25	53,124.53	1,328,113.3	625	2,822,215,688
30	63,145.54	1,894,366.2	900	3,987,359,222

*Average of five determinations.

Table III

Slope Calculation

X-axis	Y-axis*	Y-intercept	Slope
5	10,145.25	106.45	2,081.2
10	21,568.76	106.45	2,102.0
15	31,447.88	106.45	2,093.4
20	42,154.66	106.45	2,102.6
25	53,124.53	106.45	2,109.8
30	63,145.54	106.45	2,110.2

*Average of five determinations.

concentration were tested to determine the average, SD and RSD, and all calculated parameters were within the prescribed limit. Intra-day precision and intermediate precision were studied to ensure the robustness of the method. The RSDs of both tests were within the desirable limit of 2.0%, which clearly indicates that the developed method is robust. Intra-day and intermediate precision results are shown in Table IV.

Accuracy

Accuracy is usually reported as the percent recovery of an assay by using the proposed analytical procedure of adding a known amount of the analyte to the sample. The ICH also recommends assessing a minimum of three determinations over a minimum of three concentration levels covering the specified range. The common method to determine accuracy is to apply the analytical procedure to the drug substance and to quantitate it against the reference standard of known purity. The range for the accuracy limit should be within the linear range. Typical accuracy of the recovery of the drug substance in the mixture is expected to be approximately 99–102%. Values of accuracy of the recovery data beyond this range were to be investigated. The precision concentration was 7 $\mu\text{g/mL}$; hence, the linearity range was selected from 5 to 10 $\mu\text{g/mL}$.

Table IV

Intermediate and Intra-Day Precision of DPZ*

Replicates	Chemist 1 (area)	Chemist 2 (area)	Column 1 (area)	Column 2 (area)
Area 1	11,478.2	11,256.2	11,478.2	11,256.3
Area 2	11,451.1	11,458.5	11,451.1	11,245.7
Area 3	11,485.1	11,458.6	11,485.1	11,247.9
Area 4	11,456.2	11,458.3	11,456.2	11,458.8
Area 5	11,458.7	11,258.4	11,458.7	11,478.5
Average	11,465.86	11,378.0	11,465.86	11,337.44
SD	14.87	110.18	14.87	120.04
RSD	0.13	0.97	0.12	1.05

*Area: average of five determinations.

Table V

Accuracy of DPZ

Solution	Quantity added (known, %)	Area*	Average area	SD	RSD	Recovery	Accuracy
1	100 (5 $\mu\text{g/mL}$)	7,582.1 7,481.1 7,458.2 9,584.1	7,507.13	65.92	0.87	—	—
2	120 (6 $\mu\text{g/mL}$)	9,568.2 9,475.1 11,855.5	9,542.46	58.88	0.61	119.72	99.76
3	140 (7 $\mu\text{g/mL}$)	11,635.4 11,895.6 13,254.5	11,795.53	140.09	1.18	141.96	101.40
4	160 (8 $\mu\text{g/mL}$)	13,251.1 13,125.6 15,238.9	13,210.42	73.45	0.55	158.89	99.30
5	180 (9 $\mu\text{g/mL}$)	15,485.6 15,458.6 17,985.6	15,394.36	135.31	0.87	179.55	99.75
6	200 (10 $\mu\text{g/mL}$)	17,452.3 17,512.4	17,650.10	292.10	1.65	201.67	100.83

*Average of three determinations.

The known concentrations of 20, 40, 60, 80 and 100% were added to the standard preparation (5 $\mu\text{g/mL}$). The obtained percentage recoveries were considered to be under the range, as per ICH guidelines (Table V).

LOD and LOQ

The LOD and LOQ for DPZ were determined based on the SD of the response (y -intercept) and the slope of the calibration curve at low concentration levels, according to ICH guidelines. The LOD and LOQ for DPZ were found to be 0.81 and 2.43 $\mu\text{g/mL}$ (RSD: 0.7%), respectively. The tailing factor and number of theoretical plates (N) were calculated for 5.0 $\mu\text{g/mL}$ of DPZ, i.e., 0.72 and 15,572, respectively.

Recovery Studies

The percentage recovery was calculated from the differences between the peak areas obtained for fortified and unfortified solutions. As shown by the data in Table V, excellent recoveries were made at each added concentration.

System Suitability

System suitability is an important parameter to ensure whether the method is valid. The limit of theoretical plates and tailing factor were fixed as not less than 6,000 and not more than 2, respectively. In all chromatograms, the number of theoretical

plates was above 6,000 and the tailing factor was <2 . These results indicated that the developed method is valid and can be used for routine lab analysis.

Conclusions

The study shows that DPZ is a labile molecule in water and alkali and shows lability in water under light conditions. It is stable to oxidation and dry heat. A stability-indicating method was developed that separates all of the degradation products formed under a variety of conditions. The method has been proven to be simple, accurate, precise, specific and selective. Hence, it is recommended for the industry for the analysis of the drug and its degradation products in stability samples.

References

1. Lisciani, R., Baldini, A., Silvestrini, B.; General pharmacological properties of dapiprazole, a potential psychotropic agent; *Arzneimittel-Forschung/Drug Research*, (1982); 32: 668–673.
2. Monti, D., Saettone, M.F., Giannacini, B., Galli-Angeli, D.; Enhancement of transdermal penetration of dapiprazole through hairless mouse skin; *Journal of Controlled Release*, (1995); 33: 71–77.
3. Mahadik, M.V., Dhaneshwar, S.R.; Application of a stability-indicating HPTLC method for the quantitative determination of ezetimibe in pharmaceutical dosage forms; *Asian Journal of Pharmaceutical Sciences*, (2007); 2(5): 182–190.
4. Saettone, M.F., Alderigi, C., Giannacini, B., Galli-Angeli, D.; Preparation and evaluation of a sustained-release ophthalmic vehicle for dapiprazole; *Drug Development and Industrial Pharmacy*, (1989); 15: 2621–2637.
5. Fogaro, R., Corradi, L., Ferri, F., Pasotti, C., Tettamanti, F.; Dapiprazole nella terapia delle crisi di astinenza da eroina; *Farmaco*, (1987); 43: 279–289.
6. Guideline on validation of analytical procedures: text and methodology. In: Proceedings of the International Conference on Harmonization, Geneva, ICH (Q2R1), (2007).
7. Bakshi, M., Singh, S.; Development of validated stability-indicating assay methods critical review; *Journal of Pharmaceutical and Biomedical Analysis*, (2002); 28: 1011–1040.
8. Sistla, R., Tata, V.S.S.K., Kashyap, Y.V., Chandrasekar, D., Diwan, P.V.; Stress degradation studies on ezetimibe and development of a validated stability-indicating HPLC assay; *Journal of Pharmaceutical and Biomedical Analysis*, (2005); 39: 517–522.
9. Argekar, A.P., Sawant, J.G.; Determination of cisapride in pharmaceutical dosage forms by reversed-phase liquid chromatography; *Journal of Pharmaceutical and Biomedical Analysis*, (1999); 21: 221–226.
10. United States Pharmacopoeial Convention Inc., Rockville, USA, United States Pharmacopoeia 31st ed, (2007).
11. Pharmacopoeial forum, Pharmacopoeial previews (<http://www.usp.org/usp-nf/pharmacopeial-forum>) Vol.1, (2004).