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Development and Validation of Stability Indicating Method for Darunavir with Forced Degradation Studies Using LC-ESI-MS/MS

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Darunavir is a non-peptidic, second generation protease inhibitor used to treat HIV infection. Stability testing is main component in the pharmaceutical development program for a new drug as well as new formulation. Herewith forced degradation studies were carried out in accordance with the ICH guideline Q1A (R2). Darunavir was found to degrade in basic and oxidative stress conditions. Resolution of the drug and degradation products was achieved on a Hi-Q Sil C-18 column (4.6 × 250 mm, 5 µm) utilizing acetonitrile, water (90:10 % v/v) of pH 5 at a flow rate of 1 mL/min and at the detection wavelength 266 nm.

Keywords: Oxidative degradation, Darunavir, Stability indicating method, LC-ESI MS/MS.

INTRODUCTION

Stability testing is main component in the pharmaceutical development program for a new drug as well as new formulation. Drugs undergo physicochemical degradation upon storage. Forced-degradation studies (stress testing) are carried out by pharmaceutical companies during preformulation to help in selection of compounds and excipients for further development, to facilitate in salt selection or formulation optimization and to produce samples for developing stability-indicating analytical methods. Hence, stability testing of a drug under various temperature and humidity conditions is crucial during the drug development process. A stability-indicating method is "a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substance and drug product". Stability-indicating method measures accurately the active ingredients, without interference from degradation products, process impurities, excipients, or other potential impurities. Guidelines issued by International Conference on Harmonization (ICH) and other international agencies [1-5] for stability testing require the reporting, identification and characterization of degradation products (DPs). Tandem mass spectrometry (MS^n) and LC coupled with mass spectrometry (LC-MS, LC-MS/MS) are becoming the most versatile techniques for characterization of pharmaceutical degradation products and impurity profiling [6].

Darunavir ethanolate (Fig. 1) is an antiviral drug and inhibitor of the human immunodeficiency virus (HIV).

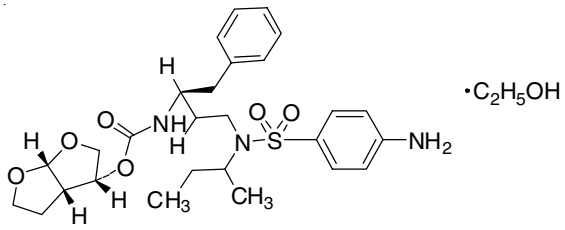

Fig. 1. Structure of darunavir ethanolate

Chemically it is [(1*S*,2*R*)-3-[[(4-aminophenyl)sulfonyl](2-methylpropyl)amino]-2-hydroxy-1-(phenyl methyl)propyl]-carbamic acid (3*R*,3a*S*,6a*R*)hexahydrofuro[2,3-b]furan-3-yl ester monoethanolate. Darunavir is a second generation protease inhibitor and is used with ritonavir and other medications to treat HIV. Darunavir selectively inhibits the cleavage of HIV-1 encoded Gag-Pol polyproteins in infected cells, thereby preventing the formation of mature virus particles.

Darunavir can be estimated by analytical methods, which have been reported for the quantification and determination of the drug individually. Literature survey reveals that, till date, there are many RP-HPLC [7-12] methods and few stability indicating methods for determination of darunavir [13,14]. However the identification of the degradation compounds of darunavir found during forced degradation studies needs to be carried out. The present manuscript describes the (i) degradation behaviour of darunavir under hydrolysis (acid, base and neutral), oxidation, photolysis and thermal stress conditions, (ii) optimization of LC conditions to separate the drug and its degradation

product on a reversed-phase C18 column, (iii) method validation, (iv) characterization of degradation product and (v) proposed fragmentation pathway of degradation product using LC-ESI-MS/MS.

EXPERIMENTAL

Darunavir was gifted by Ranbaxy pharmaceuticals, India. Acetonitrile (HPLC grade) was procured from Thomas Baker Ltd; India and used without purification. Analytical reagent grade (AR) hydrochloric acid, sodium hydroxide pellets, hydrogen peroxide solution (30 %) were purchased from Omkar Traders (Mumbai, India). Ultrapure water was obtained from water purification unit Elga option Q/OQ007XXm1 (Elga Ltd., Bucks, England). All other chemicals were of AR grade. The marketed pharmaceutical tablet dosage form of darunavir *i.e.* daruvir, by Cipla Ltd., Mumbai, India was purchased from local market.

The HPLC-MS analyses were carried out on an Agilent 1200SL Series liquid chromatographic system interfaced to an Agilent 6410B Triple Quad LC-ESI-MS/MS system (Mass Hunter Data Acquisition, Qualitation and Quantitation software, USA) equipped with an Agilent XDB-C column (1.8 µm, 4.6 × 50 mm) at a column temperature of 40 °C was used for the confirmation of atomic mass number of unknown compound/s formed during forced degradation studies in oxidative stress conditions in the mobile phase consisting of acetonitrile: methanol with pH 5.0 (90:10), v/v with 1.0 mL/min as flow rate was optimized.

An HPLC system used for analysis of stressed samples consisted of quaternary pump (PU-2089), solvent mixing module (MX-2080-31), multi-wavelength PDA detector (MD-2018), interface box (LC-NET II/ADC), rheodyne manual injector (7725i, USA) with 20 µL capacity, chromNAV data system software 1.8.1.6 version (all from Jasco, Tokyo, Japan) were used.

In all studies, separations were achieved on Hi-Qsil C-18 (4.6 × 250 mm, 5 µm) column (Thermo Scientific, Japan). Carousel six stage reaction station (Radleys Tech, UK) was used for generating hydrolytic degradation products. The thermal degradation study was performed using a high precision hot air oven (Pathak Electrical Works, Mumbai, India) capable of controlling temperature within ± 2 °C. Photo degradation study was carried out in a photostability chamber (Labin, Mumbai, India).

A pH meter (Equip-tronics, Mumbai, India) was used to check and adjust the pH. Also sonicator (UCB40, Spectralab, Mumbai, India) and precision analytical balance (ME 204, Mettler Toledo Group, India) were used in the present studies.

MS (500-MS IT) system consisted of direct infusion mass with positive as well as negative APCI ionization (+APCI and -APCI) modes, mass ranging from 50-2000 *m/z*. The system was controlled by 500-MS Workstation software. In LC-MS studies the separation was carried out on Hypersil Gold C-18 (4.6 × 250 mm, 5 µm).

Stress decomposition studies: Forced degradation studies of bulk drug and drug formulation included appropriate solid state and solution state stress conditions in accordance with regulatory guidelines. The stressors, choice of their concentration and preparation of samples were based on published guidelines. As the drug was insoluble in water, it was dissolved in mobile phase; acetonitrile, methanol (90:10 % v/v) of pH 5 to a final concentration of 2 mg/mL. The stock was diluted 50:50 (v/v) with the stressor (*e.g.* HCl, NaOH, H_2O_2 and water). All hydrolytic studies were conducted at 60 °C. The oxidative study was carried out in 30 % (v/v) H_2O_2 at room temperature. For thermal stress testing, the drug was sealed in glass vial and placed in a thermostatic block at 50 °C for 21 days. Photolytic studies on the drug in the solid and solution state were carried out by exposure to a UV lamp in a chamber set at accelerated conditions of temperature and humidity (40 °C/75 % RH). Parallel blank set was kept in dark for comparison [15]. After subjecting to stress, samples were withdrawn at appropriate time interval. The optimized stressed conditions are enlisted in Table-1.

Sample preparation for HPLC and LC-MS analysis: The stressed samples of acid and base hydrolysis were neutralized with NaOH and HCl, respectively to obtain 1000 µg/mL solutions. Neutral hydrolysis, thermal and photolytic samples were diluted with mobile phase to obtain concentration at 1000 µg/mL solutions. The oxidative stress sample was diluted with mobile phase to obtain 100 µg/mL solution. All the prepared samples were passed through 0.45 µm membrane filter before HPLC and LC-MS analyses.

Characterization of degradation products: The stressed solutions, in which sufficient amounts of products formed, were subjected initially to LC-PDA and further to LC-MS analyses for characterization of degradation product.

RESULTS AND DISCUSSION

LC-MS conditions: The main aim of present research work was to separate darunavir and its degradation products. An Hi-Q-sil C-18 column (4.6 × 250 mm, 5 µm) was found to be suitable for this analysis after having tried with different columns. During the optimization process on above-mentioned column, several conditions with various mobile phases like methanol/water and acetonitrile/water in different proportionalities were tried in an isocratic mode. To get acceptable separation between the drug and its degradation products, studies were carried out using varied proportions of acetonitrile (A) and methanol (B) The pH, flow rate and composition of the mobile phase were systematically varied to optimize the method. To detect drug and degradation products with suffi-

TABLE-1
OPTIMIZED STRESS CONDITIONS FOR THE DRUG

Stressors	Hydrolytic at 60 °C			Oxidative at room temperature	Photolytic	Thermal at 50 °C
	Acid	Neutral	Base		Solid	
Concentration of stressor	0.5 N HCl	H_2O	0.1 N NaOH	30 % H_2O_2	-	-
Duration	2 h	12 h	6 h	3 d	13 d	21 d

cient peak intensity, the wavelength at 266 nm was chosen. Finally, a mobile phase consisting of A and B (pH 5.0) (90:10 % v/v) at a flow rate of 1 mL/min and PDA detection at 266 nm, in an isocratic mode gave good separation of drug and its degradation product. The advantage of the method was simple and rapid (Fig. 2). Validation of the optimized LC method was done with respect to various parameters outlined in ICH guideline Q1A (R2) and was extended to LC–MS studies.

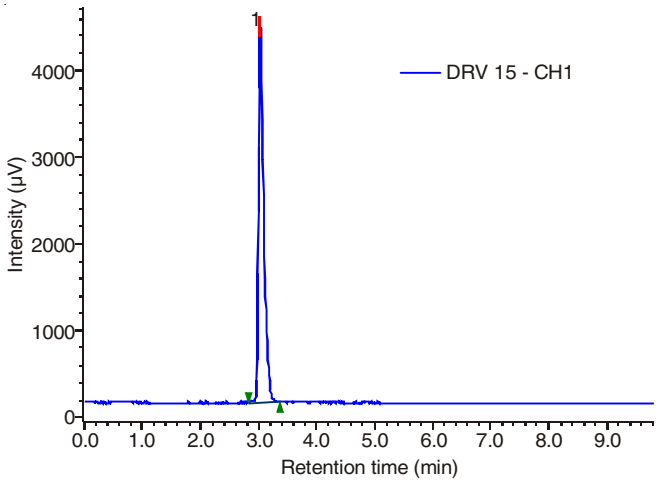

Fig. 2. Chromatogram of darunavir

LC-MS studies: LC-MS/MS studies were carried out in +APCI ionization mode in the mass range of 50-2000 amu. High purity helium was used as carrier gas and nitrogen was used as nebulizer. Mass parameters were optimized to the following values: R_f loading: 80 %; capillary voltage: 80 volts; syringe volume: 250 µL; spray chamber temperature: 50 °C; nebulizer pressure: 35 psi; drying gas temperature: 300 °C; drying gas pressure: 10 psi; vapourizer gas temperature: 350 °C; vapourizer gas pressure: 20 psi; spray shield voltage: ± 600 volts.

Evaluation of Method validation parameters

Specificity: Specificity is the ability of the analytical method to measure the analyte concentration accurately in presence of all potential degradation product. The specificity was determined by subjecting darunavir to stress degradation under various conditions. The degradation product was well separated, peak purity assessment was carried out on the stressed samples of darunavir by using diode-array-detector and the specificity was also established by subjecting the degradation sample to LC–MS analysis using same method.

The mass detector also showed an excellent mass purity for darunavir and its degradation product which unambiguously proves the specificity of the method.

Linearity: Linearity test solutions were prepared from stock solution at six concentration levels of analyte (15, 30, 45, 60, 75 and 90 µg/mL). The peak area *versus* concentration data was performed by least squares linear regression analysis. The calibration curve was drawn by plotting darunavir average area for triplicate injections and the concentration expressed as a percentage. Linearity was checked over the same concentration range for three consecutive days. Good linearity was observed in the concentration range from 15 to 90 µg/mL of darunavir. The data is subjected to statistical analysis using a linear regression model; the linear regression equation and correlation coefficient (r^2) were y = 1172x + 8813 and 0.999 respectively. These results indicate estimable linearity. The LOD and LOQ for darunavir were estimated at a signal-to-noise ratio of 3:1 and10:1, respectively. The LOD and LOQ were 5.18 µg/mL and 13.70 µg/mL respectively.

Precision: Precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were carried out by analyses of three different concentrations of the drug in hexaplicate on the same day. Intermediate precision of the method was checked by repeating the studies on three different days. Additionally, the developed HPLC method was checked through separation studies on the mixture of reaction solutions on a different chromatographic system on a different day. The results of repeatability and intermediate precision experiments are shown in Table-2. The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were < 1.5. Separation of the drug and its degradation product in a mixture of stressed samples was found to be similar when analyses were performed on a different chromatographic system on different days.

TABLE-2
PRECISION STUDIES

Concentration taken (µg/mL)	Measured concentration (µg/mL) ± S.D., RSD (%)	
	Repeatability (n = 6)	Intermediate precision (n = 3)
30	30.15 ± 0.63, 1.25	50.40 ± 0.69, 1.37
45	45.75 ± 0.59, 0.98	39.78 ± 0.60, 0.75
60	60.55 ± 0.70, 1.05	60.79 ± 0.45, 1.18

Accuracy: Accuracy of the method was assessed employing the standard addition method at three different levels (80, 100, 120 %). The mixtures were analyzed in triplicate and the percentage of added drug obtained from difference between peak areas of unfortified and fortified samples of darunavir. The HPLC area responses for accuracy determination are depicted in Table-3. Good recoveries (99.65 ± 0.38) of the spiked drugs were obtained at each added concentration, indicating that the method was accurate.

TABLE-3
RECOVERY STUDIES

Spiked concentration (µg/mL)	Measured concentration (µg/mL) ± S.D., RSD (%)	Recovery (%)
24	23.78 ± 0.12, 0.043	99.10
30	30.05 ± 0.122, 0.367	100.17
35	34.90 ± 0.165, 0.382	99.73

Robustness: To determine the robustness of the method, experimental conditions were purposely altered. Three parameters selected were flow rate, composition of mobile phase and solvent from different lots. The mobile phase flow rate was 1 mL/min which was changed to 0.9 and 1.1 mL/min and the effect was studied. The effect of mobile phase composition was analyzed by use of acetonitrile and methanol and in ratio of 85:15 (v/v) and 95:5 (v/v). Also acetonitrile and methanol of different lots from same manufacturer was used. For all

changes in conditions, the sample was analyzed in triplicate. When the effect of altering one set of conditions was tested, the other conditions were held constant at the optimum values. In all the calculated varied chromatographic conditions, no significant change in retention time and tailing factor of darunavir was observed (Table-4).

TABLE-4
ROBUSTNESS STUDIES

Factors	Level	Darunavir (RT, min)
A: Flow rate (mL/min)		
0.9	-0.1	3.183
1.0	0	3.03
1.1	+0.1	2.86
Mean ± SD		3.023 ± 0.191
B: Mobile phase v/v (acetonitrile:methanol)		
8.5:1.5	-0.5	2.96
9:1	0	3.03
9.5:0.5	+0.5	3.13
Mean ± SD		3.046 ± 0.051
C: Solvents of different lots		
First lot	–	3.03
Second lot	–	3.03
Third lot	–	3.03
Mean ± S.D. (n = 3)	–	3.03 ± 0.148

System suitability test: The system suitability parameters with respect to theoretical plates, capacity factor, resolution factor, tailing factor were calculated and all the peaks were well resolved.

Degradation behaviour: The drug degraded into degradation product's under basic and oxidative stress. There was insignificant degradation in acidic, neutral and photo degradation (< 0.45 %). The extent of degradation for oxidative medium was 26.69 %. The chromatograms of the degraded samples in stressed is shown in Fig. 3.

Study of the stability of commercial tablets: The assay content of darunavir, commercially available marketed formulation was analyzed by the proposed method after exposure to accelerated storage condition (*i.e.* 40 °C/75 % RH). The peak at retention time 3 min for the drug was observed in the chromatogram of the drug samples extracted from tablets and no additional peak was found Fig. 4. Experimental results of the amount of darunavir in tablets, expressed as percentage of label claim were in good agreement with the label claims as reported in Table-5 thereby suggesting that there is no interference from any excipients, which are normally present in tablets and packaging material is of good quality which is reported in.

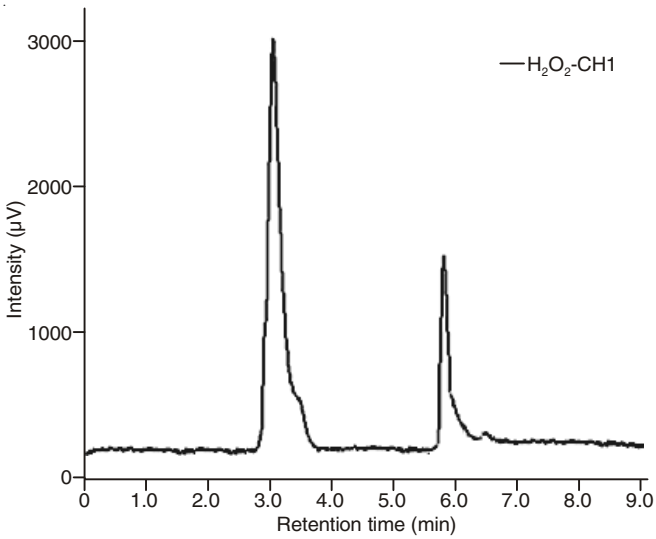

Fig. 3. Chromatogram of oxidative stress

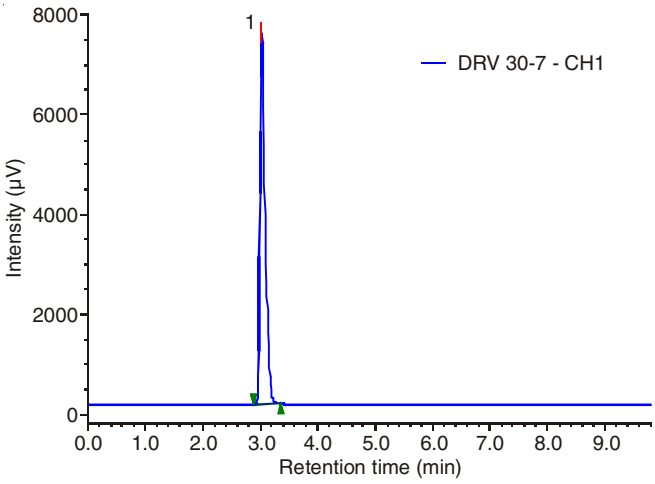

Fig. 4. Peak of marketed formulation

Mass fragmentation pathway of the drug: Darunavir on oxidation yielded one degradation product (DP). The fragment ions of degradation product were at *m/z* 485.52 amu, 456.52 amu, 414.44 amu, 268.29 amu and 116.12 amu (Fig. 5). The peak at *m/z* 456 was due to the loss of N-oxide (*m/z* 30) from the fragment ion *m/z* 485. Loss of the ion C_3H_7 from *m/z* 456 resulted in the formation of peak at *m/z* 414. Further, loss of hexahydrofuro[2,3-b]furan moiety with elimination of one molecule of water yielded *m/z* of 268.29 amu. The fragmentation pathway is proposed in Fig. 6.

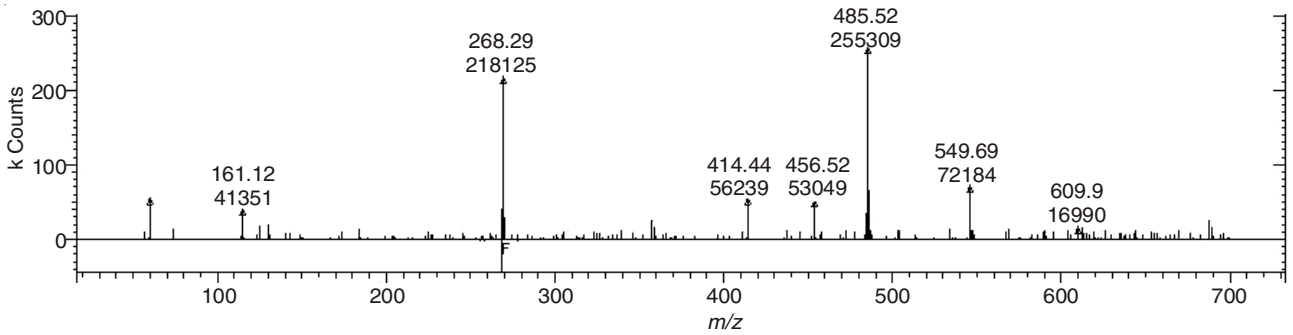

Fig. 5. Mass spectra for degradation product

TABLE-5
STUDY OF STABILITY OF COMMERCIAL TABLETS (n = 3)

Marketed formulation	Duration	Taken (µg/mL)	Found (µg/mL)	% Label claim (± SD)	RSD (%)
Daruvir by Cipla Ltd., Mumbai, India	1 month	30	29.97	99.90 ± 0.5536	1.051
	2 months	30	29.91	99.70 ± 0.6732	0.981
	3 months	30	29.87	99.56 ± 0.7872	1.029

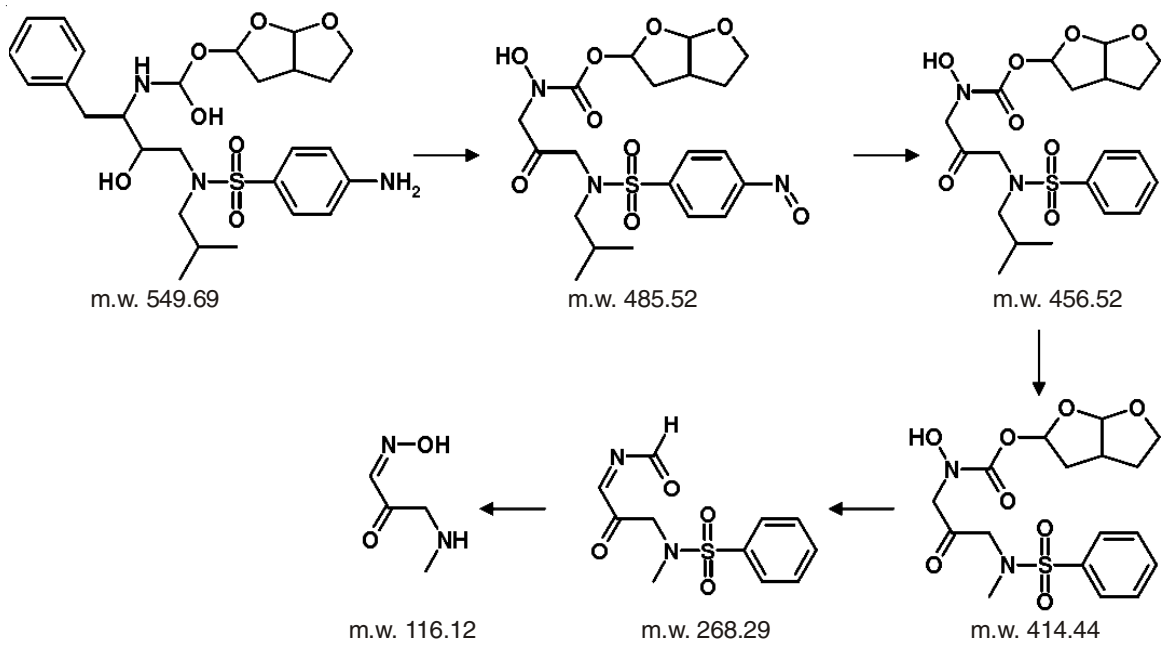

Fig. 6. Proposed mass fragmentation pathway of the drug

Conclusion

It was possible, in this study, to develop a stability-indicating RP-HPLC assay method for darunavir by subjecting the drug to ICH recommended stress conditions. Degradation product was formed in oxidative stress condition and was separated in a single run, by an isocratic LC method. The method proved to be simple, accurate, precise, specific and robust. It was successfully employed for the analysis of marketed formulation stored for three months under accelerated conditions of temperature and humidity. The developed method was extended to LC-MS/MS for characterization of degradation product. The complete degradation pathway of the drug and mechanism of the formation of degradation product is proposed. The proposed method can thus be used for routine analysis, quality control and for studies of pharmaceutical tablets containing this drug.

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