Analytical Research[1], Custom Pharmaceutical Services, Dr. Reddy's Laboratories Ltd., Andhra University[2], Visakhapatnam, India

Development and validation of a high performance liquid chromatographic method for the separation of exo and endo isomers of granatamine (9-methyl-9-azabicyclo[3.3.1]nonan-3-amine); a key intermediate of granisetron

S. Radha Krishna[1], P. Suresh babu[1], B.M. Rao[2], N. Someswara Rao[2]

Received May 2, 2009, accepted May 25, 2009

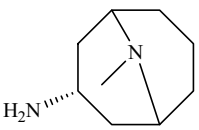
Dr. B. M. Rao, Research Supervisor, Department of Analytical Chemistry, School of Chemistry, Andhra University, Visakhapatnam 530 003, India
drbmrao@hotmail.com

Pharmazie 64: 800–803 (2009) *doi: 10.1691/ph.2009.9142*

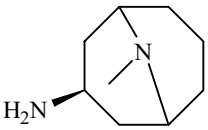
A simple and accurate high-performance liquid chromatographic method was developed for the determination of exo-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine in endo-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine, commercially known as grantamine and used as a key intermediate in the preparation of granisetron bulk drug. Chromatographic separation of the exo and endo isomers of 9-methyl-9-azabicyclo[3.3.1]nonan-3-amine was achieved on an Inertsil C8 column using a mobile phase containing 0.3% trifluoroacetic acid. The resolution between the two isomers was found to be more than 4. The limit of detection (LOD) and limit of quantification (LOQ) of exo isomer were 0.8 and 2.5 μg. mL^{-1} respectively, for a 10 μL injection volume. The percentage recovery of exo-isomer ranged from 99 to 102% *w/w* in the endo-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine sample. The test solution and mobile phase were observed to be stable up to 48 h after preparation. The validated method yielded good results for precision, linearity, accuracy, robustness and ruggedness. The proposed method was found to be suitable and accurate for the quantitative determination of exo-isomer in bulk samples of endo- 9-methyl-9-azabicyclo[3.3.1]nonan-3-amine.

1. Introduction

Granisetron, chemically known as endo-*N*-(9-methyl-9-azabicyclo [3.3.1] non-3-yl)-1-methyl-1*H*-indazole-3-carboxamide is a 5-HT3-receptor antagonist having significan antiemetic activity against chemotherapy-induced nausea and vomiting (Yarker and McTavish 1994). Granisetron is prepared by condensation of endo-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine (granatamine) and 1-methyl-1H-indazole-3-carboxylic acid. One of the key requirements is to control the level of exo granatamine present in endo granatamine in order to control the overall impurity content in granisetron drug substance. The regulatory requirement for exo-isomer in granisetron active pharmaceutical ingredient is not more than 0.10% (European Pharmacopeia 2008). Hence the control of the exo isomer content of granatamine is critical and important to meet the regulatory specificatio for residual exo isomer. The HPLC method in the European Pharmacopeia is not able to detect the exo isomer of granatamine. A 1H NMR method is available in the literature to determine the ratio of endo and exo isomers (Kim et al. 2006; Gil et al. 2007). However it is very difficul to quantify the impurity accurately at the 0.1% level using an NMR technique. Separation of endo and exo-isomers of iohexol in canine serum and rat urine samples by using C30 HPLC has been described (Klenner et al. 2007). Gas chromatographic separation using a 60 M length column has been reported for a similar type of compound, 3-amino-8-methyl-8-azabicylco [3.2.1] octane (Allegretti et al. 2001).
To our present knowledge no high-performance liquid chromatography (HPLC) methods heve been reported in the literature for the determination of exoisomer in endo-9-methyl-9-azabicyclo [3.3.1] nonan-3-amine. The present research work focused on the development of a simple and rapid HPLC method for separation of the exo and endo isomers of granatamine using an RP HPLC column.



Exo-9-methyl-9-azabicyclo [3.3.1]nonan-3-amine



Endo-9-methyl-9-azabicyclo [3.3.1]nonan-3-amine

2. Investigations, results and discussion

The objective of this work is to quantify accurately the residual level of exo isomer present in endo-9-methyl-9-azabicyclo [3.3.1] nonan-3-amine. Exo and endo isomer samples were prepared individually for use in the development of the method. As granatamine is non-chromophoric in nature, initial trials were made with gas chromatography using various polar, mid-polar and non-polar stationary phases. In all the trials both exo and endo isomers of granatamine were eluting together and no separation was observed. The details are summarized in

Table 1: Results of different Gas Chromatograph trials

Trail No.	GC conditions	Remarks
1	Column: DB-624, (30 m × 0.53 mm, 3.0 μm), oven: 100 °C-10 °C/min-250 °C (15 min), injector: 200 °C, Det: FID, 240 °C, carrier: He, fl w: 2.0 mL. min^{-1}, split: 20:1, injection volume: 1.0 μL, sample conc.: 100 mg. mL^{-1} in methanol.	No separation between exo and endo isomers
2	Column: Beta-Dex (30 m × 0.25 mm, 0.25 μm), oven: 100 °C-10 °C/min-150 °C (15 min), injector: 200 °C, Det: FID, 240 °C, carrier:He, fl w:2.0 mL. min^{-1} split: 20:1, injection volume: 1 μL, sample conc.: 100 mg. mL^{-1} in methanol	No separation between exo and endo isomers
3	Column: Carbowax TM Amine, (30 m × 0.53 mm), Oven: 100 °C (5 min)-10 °C/min-200 °C (20 min), injector: 200 °C, Det: FID, 240 °C, carrier: He, fl w: 1.0 mL. min^{-1}, split: 20:1, injection volume: 1.0 μL, sample conc.: 100 mg. mL^{-1} in methanol.	No separation between exo and endo isomers
4	Column: DB-17, (30 m × 0.25 mm, 5.0 μm), oven: 100 °C (20 min)-10 °C/min-240 °C (17 min), Injector: 240 °C, Det: FID, 260 °C,carrier: N_2, fl w:1.0 mL. min^{-1}, split: 10:1, injection volume: 1.0 μL, sample conc.: 100 mg. mL^{-1} in methanol.	No separation between exo and endo isomers
5	Column: DB-17, (30 m × 0.25 mm, 5.0 μm), oven: 70 °C (10 min)-5 °C/min-240 °C (10 min), injector: 240 °C, Det: FID, 260 °C,carrier: N_2, fl w:1.0 mL. min^{-1} split: 10:1, Injection volume: 1.0 μL, sample conc.: 100 mg. mL^{-1} in methanol.	No separation between exo and endo isomers
6	Column: DB-1, (30 m × 0.25 mm, 3.0 μm), oven: 50 °C (5 min)-20 °C/min-250 °C (15 min), injector: 220 °C, Det: FID, 260 °C, carrier: He, fl w:1.0 mL. min^{-1} split: 10:1, Injection volume: 1.0 μL, sample conc.: 100 mg. mL^{-1} in methanol.	No separation between exo and endo isomers

Table 1. Various attempts were made to separate the exo and endo isomers of granatamine using different reverse phase and normal phase HPLC columns. No peak elution was observed even if the mobile phase contained a very low level of organic modifie . Separation between the exo and endo isomers was observed only when a mobile phase containing trifluoroaceti acid was employed. Superior resolution between the two isomers was observed by increasing the concentration of TFA from 0.05% to 0.3%. The details are summarized in Table 2.

Chromatographic separations were achieved only on an Inertsil C8, 250 × 4.6 mm; 5 μm column using a mobile phase containing 0.3% v/v trifluoroaceti acid in water. The fl w rate of the mobile phase was 0.5 mL. min^{-1}. The test sample concentration was 5.0 mg. mL^{-1} in the mobile phase. The column temperature was maintained at 25 °C and detection was by a refractive index detector. The injection volume was 10 μL. The total analysis time for each run was about 20 min. Good separation within a short runtime was observed on an Inertsil C8, 250 × 4.6 mm, 5 μm column (Resolution > 4.0). Typical retention times of the exo and endo isomers of granatamine were 11.5 and 14.6 min, respectively (Fig.). The system suitability (USP 2008) results are presented in Table 3.

Table 3: System suitability test results

Name	Retention time (t_R) in min	Resolution (R_s) by Tangent method (*USP*)	*USP* Tailing factor (*T*)
Exo-Isomer	11.5	–	1.5
Endo-Isomer	14.6	4.5	2.2

3. Experimental

3.1. Chemicals and reagents

Samples of endo-9-methyl-9-azabicyclo [3.3.1] nonan-3-amine and exo-9-methyl-9-azabicyclo [3.3.1] nonan-3-amine samples were obtained from the Process Research Department of Custom (Fig.) Pharmaceutical Services, a business unit of Dr. Reddy's Laboratories Ltd., Hyderabad, India.

The HPLC grade acetonitrile methanol was purchased from Qualigens Fine Chemicals, Mumbai, India, trifluoroaceti acid (TFA) extra pure was purchased from Across Organics, New Jersey, USA and HPLC grade water was produced by a Milli-Q water purificatio system.

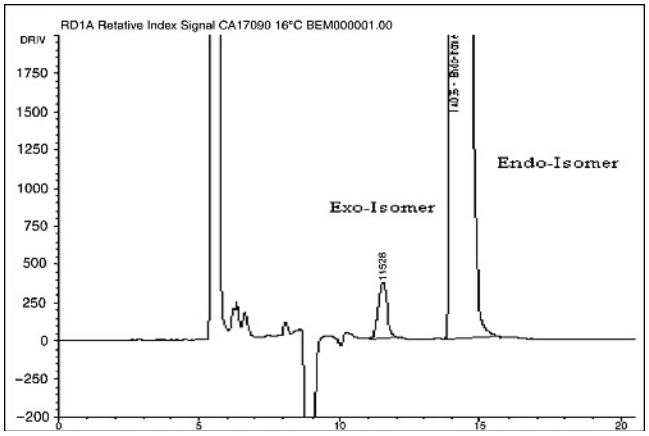


Fig.: HPLC chromatogram representing fina optimized conditions

3.2. Instrumentation

The LC system used for method development and validation was an Agilent 1100 series (Agilent Technologies Inc., Palo Alto, CA, USA). The output signal was monitored and processed using Chemstation software on a Pentium computer (IBM) (Laboratory A).

The LC system used in the ruggedness study was an Agilent 1100 series (Agilent Technologies Inc., Palo Alto, CA, USA). The output signal was monitored and processed using Chemstation software on a Pentium computer (HP) (Laboratory B).

3.3. Sample preparation

Stock solutions of the exo and endo isomers of granatamine were prepared separately by dissolving the appropriate amounts of the substances in diluent (mobile phase). The target analyte concentration was fi ed as 5.0 mg. mL^{-1}.

3.4. Method validation

3.4.1. Precision

The precision of an analytical procedure expresses the closeness of agreement among a series of measurements obtained from multiple samplings of the same homogenous sample under prescribed conditions

Table 2: Results of different HPLC trials

Trail No.	HPLC conditions	Remarks
1	Column: Zorbax SB C8, 150 mm × 4.6 mm, 3.5 μm mobile phase: water:aceonitrile (80:20, *v/v*), fl w rate: 1 mL. min^{-1}, detector: RID, injection volume: 10 μL	No peak eluted
2	Column: Zorbax SB C8, 150 mm × 4.6 mm, 3.5 μm mobile phase: water:aceonitrile (20:80, *v/v*), fl w rate:1 mL. min^{-1}, detector: RID, injection volume: 10 μL	No peak eluted
3	Column: Zorbax SB C8,150 mm × 4.6 mm, 3.5 μm, mobile phase: aceonitrile, fl w rate:1 mL. min^{-1}, detector: RID, injection volume: 10 μL	No peak eluted
4	Column: Zorbax SB phenyl, 150 mm × 4.6 mm, 3.5 μm mobile phase: water:aceonitrile (80:20, *v/v*), fl w rate: 1 mL. min^{-1}, detector: RID, injection volume: 10 μL	No peak eluted
5	Column: Zorbax Silica, 250 mm × 4.6 mm, 5 μm mobile phase: acetonitrile, fl w rate: 1 mL. min^{-1}, detector: RID, injection volume: 10 μL	No peak eluted
6	Zorbax SB C18 150 mm × 4.6 mm, 3.5 μm, mobile phase: 10 mm n-decane sulphonate: ACN;(80:20) (*v/v*), fl w rate:1 mL. min^{-1}, detector: RID, injection volume: 10 μL	No peak eluted
7	Column: Zorbax SB C8, 150 mm × 4.6 mm, 3.5 μm mobile phase: 0.05% TFA in water, f ow rate: 1 mL. min^{-1}, detector: RID, injection volume: 10 μL	Peaks eluted at early retention times ie. 2.2 & 2.8 min., negative peak observed close to exo-isomer peak and poor resolution (R: about 0.5)
8	Column: Zorbax SB C8, 150 mm × 4.6 mm, 3.5 μm mobile phase: 0.05% TFA in water, f ow rate: 0.5 mL. min^{-1}, detector: RID, Injection volume: 10 μL	To increase the retention fl w decrease to 0.5 mL. min^{-}, retention increased to 4.6& 5.2, negative peak interference still persists and one more close eluting peak observed with exo-isomer.
9	Column: Zorbax SB C8, 250 mm × 4.6 mm, 5 μm Mobile phase: 0.05% TFA in water, f ow rate: 0.5 mL. min^{-1}, detector: RID, injection volume: 10 μL	Increased column length is resolving close eluting peak with exo-isomer. Negative peak interference still persists.
10	Column: YMC pro C18, 150 mm × 4.6 mm, 5 μm mobile phase: 0.05% TFA in water, f ow Rate: 0.5 mL. min^{-1}, detector: RID, injection volume: 10 μL	Close eluting peak with exo-isomer not resolved. Negative peak interference still persists.
11	Column: Ace C8, 250 mm × 4.6 mm, 5 μm mobile phase: 0.05% TFA in water, f ow rate: 0.5 mL. min^{-1}, detector: RID, injection volume: 10 μL	Close eluting peak with exo-isomer not resolved. Negative peak interference still persists.
12	Column: Hypersil BDSC18, 250 mm × 4.6 mm, 5 μm mobile phase: 0.1% TFA in water, f ow rate: 0.5 mL. min^{-1}, Detector: RID, injection volume: 10 μL	Close eluting peak with exo-isomer not resolved. Negative peak interference still persists and high tailing of peaks observed.
13	Column: Inertsil C8-3, 250 mm × 4.6 mm, 5 μm mobile phase: 0.05% TFA in water, f ow rate: 0.5 mL. min^{-1}, detector: RID, injection volume: 10 μL	Close eluting peak with exo-isomer not resolved, but more separation observed when compared to previous trials. Negative peak interferance was still persists.
14	Column: Inertsil C8-3, 250 mm × 4.6 mm, 5 μm mobile phase: 0.2% TFA in water, f ow rate: 0.5 mL. min^{-1}, detector: RID, injection volume: 10 μL	Increased concentration of TFA resolved the close eluting peak and exo-isomer with resolution less than 1.0, also observed increase in the retention of peaks due to which the negative peak seperated from Exo-isomer.
15	Column: Inertsil C8-3, 250 mm × 4.6 mm, 5 μm mobile phase: 0.3% TFA in water, f ow rate: 0.5 mL. min^{-1}, detector: RID, injection volume: 10 μL	Further increase in concentration of TFA resolved the close eluting peak and exo-isomer with resolution more than 1.5.

(ICH Q2R1 2005). The system and method precision for exo-isomer was checked at the 0.5% level with respect to analyte concentration, which is 5.0 mg. mL^{-1}. The percentage RSD of method repeatability and system repeatability for exo-isomer were found to be 2.9 and 0.94, respectively, confirmin the good precision of the method.

3.4.2. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of the analyte in the sample (ICH Q2R1 2005). The linearity of the method for exo-isomer was checked at six concentration levels, i.e., from LOQ (0.05%) to 1.0%, which is with respect to analyte endo-isomer concentration 5.0 mg. mL^{-1}. The coefficien of regression of the calibration curve was found to be 0.999, thus confirmin the excellent correlation between the peak area and concentration of the exo-isomer.

3.4.3. Limit of detection and limit of quantification

The limit of detection (LOD) represents the concentration of analyte that would yield a signal to noise ratio of 3 (ICH Q2R1 2005). The limit of detection for exo-isomer was found to be 0.8 μg. mL^{-1} for a 10 μL injection volume. The limit of quantificatio (LOQ) represents the concentration of analyte that would yield a signal to noise ratio of 10. The limit of quantifi cation for exo-isomer was found to be 2.5 μg. mL^{-1} for a 10 μL of injection volume.

3.4.4. Quantification of exo-isomer in bulk sample

Standard addition and recovery experiments were conducted to determine the accuracy of the present method for the quantificatio of exo-isomer in samples of endo-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine. The study was carried out at 0.25, 0.50 and 0.75 % with respect to the analyte concentration. The percentage recoveries of exo-isomer ranged from 99.3 to 102.4 in samples of endo-9-methyl-9-azabicyclo[3.3.1] nonan-3-amine.

3.4.5. Ruggedness and robustness

The ruggedness of a method is define as the degree of reproducibility of results obtained by analysis of the same sample under a variety of normal test conditions such as different laboratories, different analysts, different instruments, different days and different lots of reagents. Precision studies were carried out for exo-isomer in endo-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine bulk samples at the same concentration levels tested in Laboratory A and repeated at laboratory B using a different instrument by a different analyst. The data obtained from Laboratory B were well in agreement with the results obtained in Laboratory A, thus proving the method's ruggedness. The robustness of an analytical procedure is a measure of its capability to remain unaffected by small, but deliberate, variations in method parameters and provides an indication of its reliability during normal usage. Under varied chromatographic conditions i.e. fl w rate and mobile phase composition, resolution between the peaks of endo and endo-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine was found to be > 4.0, illustrating the robustness of the method.

3.4.6. Solution stability and mobile phase stability

Solution stability was studied by keeping the test solution in a tightly capped volumetric flas at room temperature ($25 \pm 2\,^{\circ}C$) on a laboratory bench for 48 h. Content of exo-isomer was checked every 12 h and compared with freshly prepared solution. No variation was observed in the content of exo-isomer over the study period, indicating that endo-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine sample solutions prepared in diluent were stable for up to 48 h at room temperature ($25 \pm 2\,^{\circ}C$). The mobile phase stability experiment data, showing no variation in the content of exo-isomer in endo-9-methyl-9-azabicyclo [3.3.1] nonan-3-amine over the study period, confir that the prepared mobile phase was stable for up to 48 h at room temperature ($25 \pm 2\,^{\circ}C$).

Acknowledgments: The authors wish to thank the management of the Dr. Reddy's Group for supporting this research work. The authors wish to acknowledge the Process Research group for providing the samples for our research.

References

Allegretti M, Curti R, Cesta MC, Nicolini L (2001) A stereoselective process for the preparation of endo-3-aminozabiycyloalkanes. PCT patent WO/2001/02536.(to Dompe' SpA).

Gil AM, Buñuel E, Cativiela C (2007) ARKIVOC (iv) 157-169.

European Pharmacopeia 6.0, 2008, monograph, 1695.

International Conference on Harmonization tripartite guideline Q2R1, "Validation of Analytical Procedures: Text and Methodology", November 2005.

Klenner S, Bergmann C, Strube K, Ternes W, Spillmann T (2007) SPE for endo- and exo-iohexol analysis with HPLC in canine serum and rat urine Chromatographia, 65: 733–736.

Kim S-G, Park T-H Kim B J (2006) Efficien total synthesis of (+)-exo, (−)-endo-brevicomin and their derivatives via asymmetric organocatalysis and olefi cross-metathesis Tetrahedron Letters 47: 6369–6372.

United States Pharmacopeia (2008) pp. 621, 1225.

Yarker YE, McTavish D (1994) Granisetron: an update of its therapeutic use in nausea and vomiting induced by antineoplastic therapy Drugs 48(5): 761–93.