Asian Journal of Chemistry; Vol. 30, No. 6 (2018), 1349-1353
ASIAN JOURNAL OF CHEMISTRY
https://doi.org/10.14233/ajchem.2018.21247

Liquid Chromatographic Estimation of Leuprolide Acetate in Bulk and Microsphere Formulation

SANDHYA PITTALA[1], VEDULA GIRIJA SASTRY[1], RAPALLI VAMSHI KRISHNA[2], SUNIL KUMAR DUBEY[2] and GAUTAM SINGHVI[2,*]

[1]Department of Pharmaceutical Sciences, College of Pharmacy, Andhra University, Visakhapatnam-530003, India
[2]Department of Pharmacy, Birla Institute of Technology & Science, Pilani-333 031, India

*Corresponding author: E-mail: singhvigautam@gmail.com

Received: 21 January 2018; *Accepted*: 15 February 2018; *Published online*: 30 April 2018; AJC-18892

Development of a sensitive, robust and validated analytical method for determination of an active drug in sample matrix is the key component to ensure identity, purity and quality of drug and formulations. The aim of the proposed work was to develop and validate a simple, rapid, sensitive, robust and cost effective high performance liquid chromatographic method for the estimation of leuprolide acetate in bulk and formulations for routine analysis. The method was developed using Supelco-C18 (150 × 4.6 mm, 5 µm) analytical column and mobile phase consisting of a mixture of 10 mM sodium acetate buffer and acetonitrile in 70:30 ratios. Developed method was validated as per ICH guidelines. The method has demonstrated linearity over the range of 1 to 50 µg/mL with regression equation, peak area = 7364.6 × concentration (µg/mL) - 10051 (R^2 = 0.998). The method demonstrated selectivity with no interference of formulation excipients and mobile phase. The developed method showed good and consistent recovery (98.85 to 101.05 %). Method indicated acceptable repeatability with % RSD not more than 1.58. The method was found to be sensitive with detection and quantification limit 0.26 and 0.79 µg/mL, respectively. Also, developed method was successfully applied for compatibility study and determination of entrapment efficiency of prepared microsphere formulation. The method was found to be accurate, precise, sensitive and selective for the determination leuprolide acetate in bulk and formulations.

Keywords: Leuprolide acetate, High performance liquid chromatography, Analytical method, Validation.

INTRODUCTION

Leuprolide acetate is the potent synthetic luteinizing hormone analogue used in the treatment of prostate cancer and is approved by FDA, Germany, European regulatory authorities. It is also used to treat hormonal related mammary cancer, endometriosis and precocious puberty. It is analogue of luteinizing hormone so it increases gonadotropin hormone secretion by pituitary and steroidogenesis, but at higher doses, contradictly produces antagonist effect on pituitary and inhibit gonadotropin secretion which results in temporary or reversible down regulation of receptors [1]. Leuprolide acetate is chemically N-ethyl-1-[(5-oxopyrrolidone-2-carbonyl)histidyl tryptophyl seryl tyrosyl leucyl leucyl arginglyl]pyrrolidone-2-carboxamide and the structure of molecule is shown in Fig. 1.

Some liquid chromatographic methods for determination of leuprolide acetate in analytical aqueous matrix and human plasma have already been published. Liquid chromatographic techniques published for estimation of leuprolide acetate in formulations and plasma concentrations (Pharmaco kinetic study) along with USP official monograph are utilizing gradient flow with long retention time. Zhan *et al.* [2] have reported tandem mass spectroscopy method utilizing electrospray ionization. The reported method is for estimation of low leuprolide acetate content in blood samples. Park *et al.* [3] have reported fluorescence spectroscopy method for *in vitro* drug release analysis utilizing intrinsic fluorescent amino acid tryptophan. Some reverse phase HPLC methods are reported for quantification of leuprolide utilizing dibasic ammonium phosphate buffer and acetonitrile at flow rate of 2 mL/min with separation time of more than 20 min [4,5]. Hayao Ueno and Shigeki Matsuo reported HPLC method followed by radioimmunoassay for determination of leuprolide and its metabolite for determination of leuprolide in blood plasma samples [6]. Above reported analytical methods are having certain limitations such as long run time, need of sophisticated instruments, derivatization of sample, complex extraction protocols, gradient elution and cost of analysis. Extensive literature survey did not reveal any suitable simple liquid chromatographic method for routine estimation of leuprolide acetate in aqueous in-process matrix and finished formulation samples. Therefore, the purpose of this study was to develop and validate a rapid simple and sensitive liquid chromatographic method for the determination of leuprolide acetate in bulk as well as in formu-

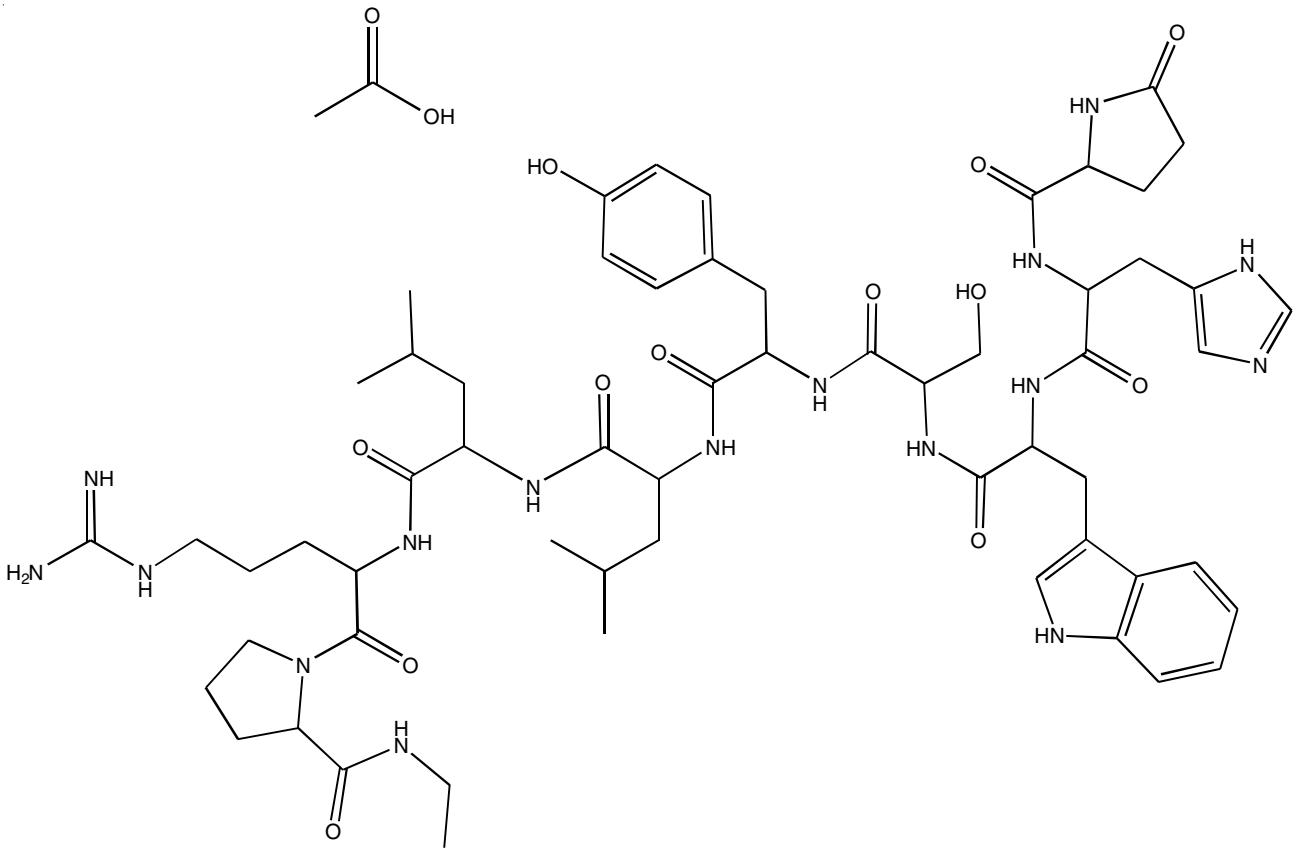

Fig. 1. Leuprolide acetate

lations as per the standard guidelines [7] and published reports [8-10]. It is also desirable that such method should be cost effective and robust enough for easy acceptability at industry and academic research environment.

The present paper describes the development and the validation of a rapid reverse phase isocratic HPLC method with UV detection, which is certainly less complicated and requires less expensive instrumentation than other available methods while being equally reliable, precise and accurate.

EXPERIMENTAL

Leuprolide acetate was purchased from PolyPeptide Pvt Ltd, Mumbai, India. All chemicals used were of analytical grade and solvents were of HPLC grade. Methanol, acetonitrile, triethylamine and orthophosphoric acid were procured from Merck (India). All other chemicals were used of analytical grades. Ultrapure water (Milli-Q Plus, Millipore®, India) was used throughout the analysis.

Mobile phase preparation: 10 mM Sodium acetate buffer and acetonitrile were used in 70:30 ratios as mobile phase for chromatographic separation. Sodium acetate buffer was prepared by dissolving appropriate quantity of sodium acetate in milli-Q water and acetic acid is added to adjust pH of buffer to 5.00. Buffer is filtered through 0.22 micron filter using filter assembly, the filtered buffer and acetonitrile are degassed using bath sonicator.

Standard solution preparation: Primary stock solution of leuprolide acetate 1 mg per mL solution was prepared, secondary stock solution 250 µg/mL was prepared from primary stock solution in water. Various standards solutions 1, 3, 5, 10, 15, 20, 35 and 50 µg/mL were prepared from secondary stock solution. Three quality control samples 7, 18, 45 µg/mL were prepared as lower quality control (LQC), middle quality control (MQC) and higher quality control (HQC) concentration respectively.

Chromatographic conditions: The HPLC system (Shimadzu®, Japan) consisted of LC-10ATVP liquid chromatographic pump, SPD-10AVP UV-Vis detector, CTO-10ASVP column oven and SIL-HT auto sampler was used for method development. Separations were carried out on Supelco-C18 (150 × 4.6 mm, 5 µm) analytical column. Chromatographic peaks were integrated using LC-Solutions® work station loaded on a computer system.

Analytical method development: Various chromatographic columns and different composition of mobile phase were evaluated to get the best chromatographic condition for method development. The C_{18} column showed good separation performance and was selected as the stationary phase for the reversed phase (RP) LC method. A mixture of sodium acetate buffer (pH 5) and acetonitrile in 70:30 ratios as mobile phase provided the best chromatographic performance. Aliquots of 30 µL were injected into the system with mobile phase at a constant flow rate of 1.0 mL/min and temperature maintained at 40 °C. The run time of the proposed assay was 10 min under isocratic elution. A retention time of 3.5 min was observed with injections of standard solutions of leuprolide acetate.

Method validation: The developed method was validated in terms of linearity, system suitability, limit of detection, limit of quantification, specificity, precision, accuracy, stock solution stability and robustness as per USP and ICH guidelines.

System suitability: The suitability of the system for the intended application was evaluated with injecting five replicates of the standard preparation (18 µg/mL) into the liquid chromatographic system. Various parameters including retention time, tailing factor, capacity factor and number of theoretical plates were determined for system suitability.

Linearity: The linearity was determined with linear regression analysis. Calibration curve of leuprolide acetate was obtained with seven concentrations of 1, 3, 10, 15, 20, 35 and 50 µg/mL and peak area of respective concentration were analyzed for linearity.

Specificity: Specificity is of an analytical method indicated the ability to measure the analyte in presence of excipients or degradation products. The possible interferences were analyzed by the peak purity of leuprolide acetate in sample matrix.

Sensitivity: For the sensitivity of proposed HPLC method, the limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the intercept (σ) and the slope (s) by using three calibration curves. Limit of detection and limit of quantification were calculated as 3.3 σ/s and 10 σ/s respectively.

Accuracy: The accuracy of the method was determined by placebo spiking method. In placebo spiking method, known amount of drug was added to placebo blank to obtain the sample solutions with concentration of 7, 18 and 45 µg/mL. The percentage of drug recovered was calculated from the mean concentrations obtained, which was a measure of the accuracy of the developed method.

Precision: Precision of the developed method was determined by replicate analysis of the sample, expressed as repeatability (intra-batch) and intermediate precision (inter-batch). For precision study, three quality control sample *viz*. lower (LQC = 7 µg/mL), middle (MQC = 18 µg/mL) and higher (HQC = 45 µg/mL) were prepared and analyzed in triplicates under same experimental conditions three times on the same day and on three different days. From the results obtained, the precision was expressed as percentage relative standard deviations (% RSD) from mean intra and inter-day assays.

Robustness: The robustness of developed HPLC method was determined under different analytical conditions and the chromatographic parameters of the main peak were evaluated. Changes in flow rate between 0.8-1.2 (1 ± 0.2) mL/min and pH between 4.8-5.2 (5 ± 0.2) were evaluated for robustness of proposed method.

Stock solution stability: The stability of leuprolide acetate in mobile phase was determined by injecting standard (18 µg/mL) at 0, 12, 24, 48, 72 and 96 h in triplicates into the system and analyzing under the optimized conditions. The obtained results were compared with the results of fresh stock solution and % RSD was calculated.

Method application

Compatibility study: Drug-excipient compatibility study was conducted prior to selection of formulation composition. In this study, binary mixtures of the leuprolide acetate with each selected excipient (1:1 w/w) were prepared by geometric mixing. All the compatibility admixtures were passed through #40 sieve and mixed well to ensure the uniform mixing. Excipients selected include poly(lactic acid) (PLA), poly(lactic glycolic acid) (PLGA), poly(vinyl alcohol) (PVA), poloxamer, lactose, sucrose and gelatine. All the samples were stored at 2-8 °C, 25 ± 2 °C/60 ± 5 % RH and 40 ± 2 °C/75 ± 5 % RH in both open and closed containers for 30 days. Samples were withdrawn at 0, 7, 15 and 30 days for analysis using thermal and non-thermal analysis methods. Developed HPLC method was used assay and degradation determination.

Drug entrapment evaluation: Developed and validated method was applied for leuprolide acetate determination in formulation. leuprolide acetate loaded microspheres were prepared with double emulsion technique. Drug entrapment was evaluated with developed method.

RESULTS AND DISCUSSION

Method development and validation

Linearity and range: The retention time of leuprolide acetate was found to be 3.5 min in the selected mobile phase. Peak was having good resolution with asymmetric factor of 1.05 ± 0.20. Calibration curve was plotted with peak area and respective standards solutions 1, 3, 10, 15, 20, 35 and 50 µg/mL of leuprolide acetate. The average equation for calibration curves was, Peak area = 7364.6 × concentration (µg/mL) – 10051. The data confirm the linearity of the standard curves over the range studied (1-50 µg/mL) with regression coefficient of 0.998. The representative overlaid chromatograph of quality control concentrations are shown in Fig. 2. The results of regression analysis are as shown in Table-1.

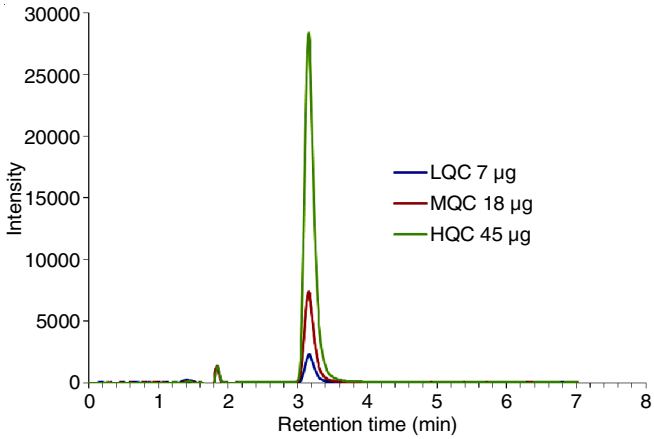

Fig. 2. Overlay of chromatograms of quality control standards of leuprolide acetate

TABLE-1
CALIBRATION CURVE DATA OF LEUPROLIDE ACETATE FOR HPLC METHOD DEVELOPMENT (EACH VALUE IS RESULT OF NINE SEPARATE DETERMINATION)

Standard concentration (µg/mL)	Mean peak area	± SD	% RSD	Predicted concentration
1	2949	28.16	0.95	1.77
3	16216	58.31	0.36	3.57
5	27203	233.05	0.86	5.06
10	59604	394.32	0.67	9.46
15	97920	525.96	0.54	14.66
20	132494	72.02	0.05	19.36
35	241292	320.37	0.13	34.13
50	365376	730.98	0.20	50.98

System suitability: The developed HPLC method indicated excellent system suitability with acceptable chromatographic peak parameters such as capacity factor (k > 2.0), number of theoretical plates (N > 9000) and tailing factor (Tf ≤ 1.5). In repetition of standard samples, a low variability in peak area and retention time were observed which further confirmed the suitability of developed method for leuprolide acetate determination.

Specificity: In specificity study, the chromatogram showed absence of any peaks at drug RT due to inactive ingredients when standard solution of leuprolide acetate and placebo were injected (Fig. 3). A single peak was obtained for leuprolide acetate, which indicated that there was no interference from the excipients used and also from the mobile phase.

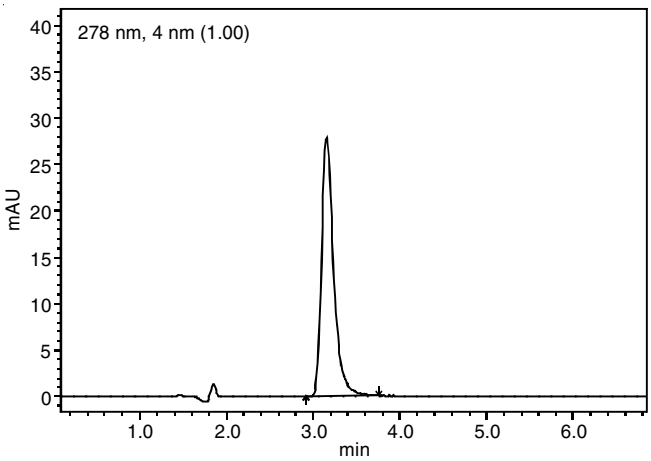

Fig. 3. Representative HPLC chromatogram of leuprolide acetate (45 µg/mL) for specificity study

Sensitivity: The sensitivity parameters, LOD and LOQ of the method were found to be 0.26 and 0.79 µg/mL, respectively. The results indicated that method is sensitive enough to detect and determine the leuprolide acetate in bulk and formulation.

Accuracy: The recovery results at all quality control levels of accuracy determination was found to be in range of 98.85 to 101.05 % and % RSD was found to be less than 1.85. High recovery and low % RSD showed the accuracy of developed method (Table-2).

TABLE-2
RESULTS OF ACCURACY STUDY
USING PLACEBO SPIKING METHOD

Concentration of drug (µg/mL)	Mean absolute recovery (%)	% RSD	% Bias
LQC	98.85 ± 0.57	0.57	0.15
MQC	100.24 ± 1.32	1.32	0.24
HQC	101.05 ± 0.45	0.45	1.05

Precision: The results of precision studies indicated high reproducibility with the % RSD values not more than 1.58. At all the quality control levels, variation observed was not significant indicating the repeatability of the developed method. The data obtained from precision studies are shown in Table-3.

Robustness: The results obtained in the new conditions of robustness study were in accordance with the initial method developed conditions as compiled in Table-4. The variation in peak RT was found to be less than 0.02 min and there was no significant change found in peak area. The method was found to be robust with respect to change in mobile phase pH and flow rate.

TABLE-3
RESULTS OF PRECISION STUDY

Actual conc. (µg/mL)	Measured conc. (µg/mL) ± S.D; RSD (%)	
	Repeatability	Intermediate precision
LQC	7.18 ± 0.25; 1.28	7.05 ± 0.14; 1.58
MQC	18.11 ± 1.05; 1.23	18.36 ± 0.94; 0.59
HQC	45.68 ± 2.44; 0.87	45.17 ± 2.12; 0.30

TABLE-4
RESULTS OF ROBUSTNESS STUDY (n = 3)

Parameter	Recovery (%)	S.D	% RSD
Change in mobile phase flow rate (mL/min)			
0.8	98.78	0.85	0.35
1.0	99.15	1.02	0.27
1.2	99.75	0.73	0.71
Change in mobile phase pH			
5.0	100.12	1.12	0.65
4.8	100.73	0.93	0.71
5.2	99.89	0.87	0.38

Stock solution stability: The stock solution of drug in optimized mobile phase was found to be stable for 72 h at room temperature. The results demonstrated the stability of drug in mobile phase with variation less than ± 1.15 %.

Method application

Compatibility study: Drug-excipient interaction was determined using developed HPLC method. Solid admixtures of all the storage conditions were analyzed with assay and degradation determination. Table-5 summarizes the stability results for binary mixtures stored at 40 ± 2 °C/75 ± 5 % RH in open containers. After 30 days storage, the assay was found to be 98.75 to 100.12 % which indicated that the selected excipients are compatible with leuprolide acetate and can be used for formulation development. There was no degradation found at all the storage conditions with selected excipients. Further, DSC and FTIR analysis also supported the compatibility results of HPLC analysis. These observations clearly suggested the absence of incompatibility between selected excipients and leuprolide acetate.

TABLE-5
DRUG-EXCIPIENT COMPATIBILITY STUDIES OF
SAMPLES STORED AT 40 ± 2 °C/75 ± 5 % RH

Sample	Leuprolide acetate	
	Assay ± SD[a] (% w/w)	Degradant (% w/w)
Pure drug	100.87 ± 0.15	Not observed
Drug + PLA	99.35 ± 1.22	Not observed
Drug + PLGA	100.12 ± 0.33	Not observed
Drug + PVA	98.75 ± 0.37	Not observed
Drug + Lactose	99.88 ± 0.15	Not observed
Drug + Sucrose	98.75 ± 0.45	Not observed
Drug + Gelatin	99.20 ± 1.35	Not observed

[a]Each value is average of three separate determination

Drug entrapment evaluation: Leuprolide acetate loaded lyophilized microspheres were dispersed into methanol and centrifuged at 10,000 rpm for 15 min. The dissolved samples were filtered, diluted appropriately and analyzed under developed HPLC conditions. The entrapment efficiency (EE) was next determined using the given equation:

$$EE\ (\%) = \frac{w \times 100}{W}$$

where, 'w' represents the actual amount of drug entrapped in the microspheres and 'W' as the total amount of drug added during preparation.

The method determined 55 ± 1.25 % entrapment efficiency which was further confirmed with mass balance. In addition, there was no interference of excipient matrix in the estimation of leuprolide acetate by developed HPLC method.

Conclusion

Reverse phase HPLC method for estimation of leuprolide acetate was developed and validated as per standard guidelines. The developed HPLC method was found to be specific, accurate, precise, simple and highly sensitive for determination of leuprolide in bulk and microsphere formulation. The developed and validated method can be conveniently used for the routine quality control analysis of leuprolide.

ACKNOWLEDGEMENTS

The authors are thankful to Crenza Pharma, Hyderabad, India for providing financial support for this project.

REFERENCES

1. A.C. Wilson, S. Vadakkadath Meethal, R.L. Bowen and C.S. Atwood, *Expert Opin. Investig. Drugs*, **16**, 1851 (2007); https://doi.org/10.1517/13543784.16.11.1851.
2. Y. Zhan, X. Chen, X. Zhao and D. Zhong, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **877**, 3194 (2009); https://doi.org/10.1016/j.jchromb.2009.08.023.
3. Y.M. Báez-Santos, A. Otte and K. Park, *Anal. Chem.*, **88**, 4613 (2016); https://doi.org/10.1021/acs.analchem.6b00190.
4. J. Singh, S.K. Rastogi, S.N. Singh and J.S. Bhatia, *J. Liq. Chrom. Rel. Technol.*, **23**, 3023 (2000); https://doi.org/10.1081/JLC-100101840.
5. J.W. Sutherland and G.N. Menon, *J. Liq. Chromatogr.*, **10**, 2281 (1987); https://doi.org/10.1080/01483918708068911.
6. H. Ueno and S. Matsuo, *J. Chromatogr. A*, **566**, 57 (1991); https://doi.org/10.1016/0378-4347(91)80110-X.
7. ICH Q2 (R1) Guideline-Validation of Analytical Procedures: Text and Methodology (2017).
8. G. Singhvi, G. Gampa and R.N. Saha, *Curr. Pharm. Anal.*, **9**, 191 (2013); https://doi.org/10.2174/1573412911309020010.
9. G. Singhvi, V. Kumar, R. Ukawala and R.N. Saha, *Curr. Pharm. Anal.*, **6**, 574 (2017); https://doi.org/10.2174/1573412913666161129145436.
10. M. Singh, S.G. Kaskhedikar, G. Singhvi and L. Soni, *Asian J. Chem.*, **23**, 3909 (2011).