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High-performance Liquid Chromatographic Method with Diode Array Detection for Quantification of Haloperidol Levels in Schizophrenic Patients During Routine Clinical Practice

Tarun Jain[1]*, Anil Bhandari[1], Veerma Ram[1], Sanjay Sharma[1], Ratendra K Chaudhary[1], Manish Parakh[2]

[1]Faculty of Pharmaceutical Sciences, Jodhpur National University, Jhawar Road, Boronada, Jodhpur-342001, Rajasthan, India
[2]Pediatric Department, Ummed Hospital, Saivanchi Gate, Jodhpur-342001, Rajasthan, India

Abstract

Rationale: Till date, very few HPLC methods are available with short run time for monitoring of haloperidol, facilitating large number of sample analysis within short time frame.

Objective: A selective and sensitive reverse phase high-performance liquid chromatographic assay has been developed for monitoring of Haloperidol levels.

Methodology: Chromatogram separation of Haloperidol and Loratidine (Internal Standard) was achieved using C18 column as stationary phase. Mobile phase consists of Acetonitrile and Water (50:50), pH-2.5 with 0.1% Acetic Acid and 0.05 M KHPO$_4$ at a flow rate of 1.6 mL min^{-1}. Detection was carried out at 240 nm using UV-PDA detector. Retention time for Haloperidol and Internal Standard was found to be 2:13 and 3:16 minutes respectively. The method has been validated for linearity, specificity, robustness, stability, accuracy and precision.

Results: Linearity for Haloperidol was in the range of 03-200 ng mL^{-1}. The total run time of analysis was 5 minutes and the lower limits of detection and quantification were 1.0 and 3.0 ng mL^{-1} respectively. In present study, most of the enrolled patients were clinically stable on Haloperidol (as evident from various rating scales applied during the study period) at therapeutic range of 5-19 ng mL^{-1} [13.04 ± 4.03]

Discussion: This validated high-performance liquid chromatographic method using a simple mobile phase has been successfully applied for clinical monitoring of Haloperidol in psychiatric patients. The method is economic and its time sparing ability using simple RP-HPLC speaks its utility in clinical and toxicological management over other analytical methods.

Introduction

The wide fluctuation in biological levels of narrow therapeutic index drugs have led to the idea of therapeutic range. This enables there to be a subsequent rationale for making a dosage adjustment. This is predictably achievable if pharmacokinetic principles are applied. Too rigid an interpretation is inappropriate: some individuals respond beyond the range and some need to be above the range [1].

Antipsychotic drugs are used for the treatment of schizophrenia and other psychoses. In general, anti-psychotics are administered in oral doses of only a few milligrams per day and they are widely metabolized in the body. Therefore, the concentration of these drugs in plasma is very low (pg-ng mL^{-1} levels). In addition, Therapeutic Drug Monitoring (TDM) of anti-psychotic drugs has proven to be of notable value for determining poor compliance of patients and addressing the challenges associated with considerable genetic variability in their metabolism. Thus, in order to conduct the pharmacology, toxicology studies and clinical TDM of anti-psychotics, along with the challenges associated with poly pharmacy - A highly sensitive, selective and accurate bioanalytical methods are essential [2].

Haloperidol [HPL] is butyrophenone series of major tranquilizers. The chemical designation is 4-[4-(p-chlorophenyl)-4-hydroxypiperidino]-4'-fluorobutyrophenone (Figure 1). HPL possesses a strong activity against delusions and hallucinations, most likely due to an effective dopaminergic receptor blockage in the mesocortex and the limbic system of the brain. It blocks the dopaminergic action in the nigrostriatal pathways, which is the probable reason for the high frequency of extrapyramidal-motoric side-effects like dystonias, akathisia and pseudoparkinsonism. It has minor antihistaminic and anticholinergic properties, therefore cardiovascular and anticholinergic side-effects such as hypotension, dry mouth and constipation are seen quite infrequently [3].

Various analytical procedures and studies explore different plasma levels for therapeutic action. The determination of plasma levels can be
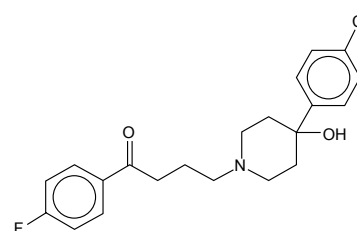

Figure 1: Haloperidol.

Corresponding author: Tarun Jain, Faculty of Pharmaceutical Sciences, Jodhpur National University, Jhawar Road, Boronada, Jodhpur-342001, Rajasthan, India, Tel: +91 9784155675; E-mail: jain0291@gmail.com

used to calculate dose adjustments and to check compliance, particularly in long-term patients. Plasma levels in excess of the therapeutic range may lead to a higher incidence of side-effects or even pose the risk of HPL intoxication [4].

A lot of analytical procedures have been described for determination of antipsychotics using HPLC, but these methods have some or other disadvantages in terms of run time, expensive solvent and complex sample extraction [5-7].

Various methods have been employed for routine monitoring of HPL in biological samples have some or other disadvantages like they either have longer run time [8,9], employing solid phase extraction method - making the process time consuming and expensive [8], have narrow linearity range: eventually not suitable for toxicological studies [8-10], high biological matrix volume [11], and higher LLQ makes the method unfit to cover the entire therapeutic range effectively. [9-12]

The aim of this study was to establish a simple, rapid, economic and accurate HPLC method for the identification and measurement of HPL in plasma correlating the plasma concentration with dose and clinical picture of patients; beneficial to certain group of population. The assay requires a small sample volume, involves a single step liquid-liquid extraction with a specific internal standard and a short chromatographic run.

Experimental

Study site and approval

The study was conducted at three sites, Mathuradas Mathur Hospital (Site 1), Jodhpur Hospital and Multi-specialty Centre (Site 2) and Faculty of Pharmaceutical Sciences (Site 3), Jodhpur. Before initiating the study, ethical approval was obtained from site 1st and 2nd, sample analysis at 3rd Site. The patients (n=105) were enrolled in study based on inclusion criteria like patient willing to participate in study, at least come for one follow up, between 18-70 year of age, no hepatic or renal failure and exclusion criteria like patients which did not fell under above age range, patient suffering from other chronic disease/ infections, on drug regimen known to alter the metabolism of studied drugs.

Chemicals and standard solutions

HPL (purity 99.9%) was gifted by Zydus-Cadila Pharmaceutical Pvt. Ltd, Ahemdabad (Gujarat) and Loratidine [I.S. to calculate a relative response factor for HPL, selected on the basis of similar solubility profile] from Alembic Pharmaceuticals, Baddi, Himachal Pradesh. All reagents used were of HPLC grade except acetic acid which was of analytical grade. Water was glass triple-distilled and further purified with a 0.44μ filtration membrane using vacuum pump. Stock solutions of HPL and IS (10μg mL^{-1}) were prepared in methanol. Working standards of HPL (3–100ng mL^{-1}) and IS (100ng mL^{-1}) were prepared by serial dilution of the stock solution in methanol and stored at 4°C.

Equipments

The HPLC system used consisted of Cecil ADEPT CE 4700 solvent delivery system, a system controller (CE 4900), a UV–PDA detector (CE 4200) operated at wavelength of 240 nm, a degasser and a data processor all from Cecil, England, United Kingdom.

Chromatographic conditions

The method was developed and validated using C_{18}-ODS column (Thermo), 250mm×4.6mm I.D., 5μm particle size which was protected by a guard column (1 cm×4.0mm I.D., 5 μm particle size). A mixture of Acetonitrile and water containing 0.1% Acetic Acid (pH adjusted to 2.5 using 0.05 M KHPO$_4$) [50:50 v/v] was used as the mobile phase (isocratic separation). The detection wavelength was 240 nm. The mobile phase was filtered, degassed and pumped at a flow rate of 1.6 mL/min.

Preparation of sample solution

1.0 mg of pure HPL was weighed accurately and transferred into round bottom flask. 100 ml of methanol was added and sonicated for 10 minutes for complete dissolution of drug. Now, the solution was filtered through a 0.45μm pore size Nylon 66 membrane filter using vacuum pump and further diluted using methanol to give stock solutions 10μg mL^{-1}. The stock solution of IS was prepared in similar manner.

Extraction of HPL from human plasma

Extraction of HPL from human plasma sample was carried out using liquid- liquid extraction technique. 300μL of human plasma sample was mixed with 50μL of IS working standard (100ng mL^{-1}) and is vortexed for 60s.Then 300μL of Isopropyl Alcohol was added and centrifuged at 10,000rpm for 5 min. The clear organic layer was separated and evaporated in turbo vap LV Evaporator (Zymark, Hopkinton, MA, USA) at 50°C under stream of nitrogen. The dried residue was then reconstituted with 300μL of mobile phase and 100μL was injected for HPLC analysis.

Method validation

The proposed method was validated as per FDA guidelines for Bioanalytical method validation. [13]

Specificity and selectivity

Specificity of the method was determined by analyzing six replicates of blank human plasma obtained from six different sources. Each blank sample was tested for interference, and selectivity was ensured at the lower limit of quantification [LLQ]. The other possible interfering substances like co administered drugs, blood components like hemoglobin e.t.c, metabolites and excipients were also tested and it was found that no endogenous/external substances interfere with the assay.

Accuracy and precision

The accuracy and precision of the method can be determined by analyzing the spiked control samples with analyte concentrations around the LLQ, 2-5 times the LLQ, 0.5 times the ULQ, ULQ and above ULQ. For acceptance criteria, accuracy should be within 85–115% of nominal concentration and for precision, the coefficient of variation (CV%) values should be <15% over the calibration range, except at the LLOQ, where accuracy should be between 80 - 120% and %CV should not be more than 20%. Choosing three concentrations from above range, each spiked sample was analyzed in a minimum of 6 replicates for within run and between run accuracy and precision.

Recovery

Recovery experiments were performed by comparing the analytical results for extracted samples at low, medium, and high concentration with un-extracted standards injected directly that represent 100%

recovery. Each observation was determined in triplicate. Recovery of IS was evaluated by comparing the mean peak areas of extracted samples to mean peak areas of reference solutions (un-extracted) of the same concentration.

Linearity, range of the calibration curve

Calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. Calibration curve was prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte including a blank sample (matrix sample without internal standard), a zero sample (matrix sample with internal standard), and six to eight non-zero samples covering the expected range, including LLQ.

LOD and LLQ

The LOD is the lowest concentration of analyte in the sample that can be detected but not quantified under the stated experimental conditions. Blank samples were measured together with samples with concentrations of the analyte at the expected LOD. The signals of the blank and the analyte samples were compared and expressed as a signal-to-noise ratio. The LLQ is the lowest concentration of the analyte in the sample that can be measured with acceptable accuracy and precision under the stated experimental conditions. The signals of the blank samples were compared with the signals from samples which contain known low concentrations of the analyte. Next, a signal to noise-ratio at which the analyte can be reliable quantified (3:1 for LOD and 10:1 for LLQ) was determined. The procedure for LOD and LLQ was replicated 6 times.

Stability

In order to determine the stability of HPL in plasma samples, they were studied at three different stability conditions such as short term, freeze-thaw, and long term stability which were examined by replicate analysis of the low, medium and high concentration samples spiked in plasma matrices. Short term stability was carried out by keeping replicates samples for approximately 6 h. Freeze–thaw stability of the samples was obtained over three freeze-thaw cycles, by thawing at room temperature for 2–3 h and refrozen for 12 h for each cycle. Long term stability of HPL in human plasma was tested after storage at −80°C for 30 days. For each concentration and each storage condition, six replicates were analyzed in one set of batch.

Ruggedness and robustness

To evaluate the ruggedness and robustness in chromatographic methods, the effects of variations of certain variables (varied ± 2%) were investigated like instrument, analyst, material, flow-rate, column temperature, mobile phase composition, detection wavelength and extraction solutions.

Sample pretreatment; for evaluation of therapeutic levels

For quantitative estimation, a reliable pretreatment of biological samples (n=105) was done using liquid –liquid extraction. 300μL of human plasma sample was mixed with 50μL of IS working standard (100ng mL^{-1}) and is vortexed for 60s. Then 300μL of Isopropyl Alcohol was added and centrifuged at 10,000rpm for 5 min. The clear organic layer was separated and evaporated in turbo vap LV Evaporator at 50°C under stream of nitrogen. The dried residue was then reconstituted with 300μL of mobile phase and 100μL was injected for HPLC analysis (Figure 2 & 3).

Results and Discussion

Selectivity and specificity

HPL and IS were well separated from the co-eluted components and there were no interferences from the endogenous material which was observed at retention time of both HPL and IS. The peaks were of good shape, completely resolved from plasma components. It indicates that the developed method was highly selective for the matrices like plasma (Figure 2). The developed method was very specific as inferred from the co-administered anti-psychotic drugs like aripiprazole, olanzapine, lamotrigine, clozapine and sodium valproate e.t.c.
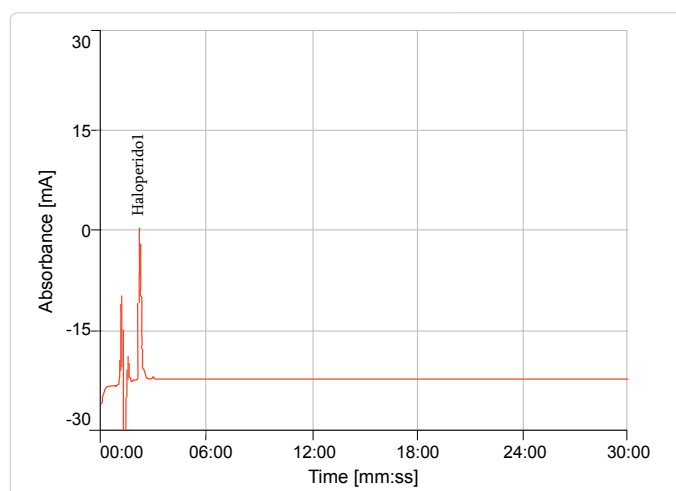

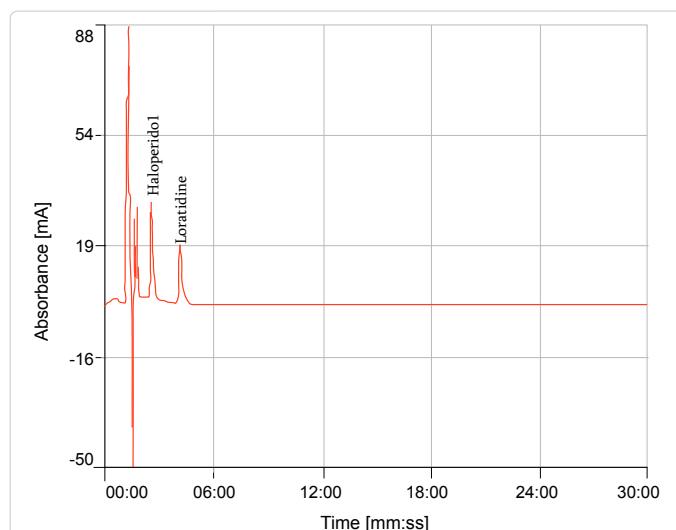**Figure 2:** Typical Chromatogram of Haloperidol (5.71 ng mL^{-1}) in Patient Plasma.

Figure 3: Chromatogram of plasma sample from patient on Haloperidol (8.38 ng mL^{-1}) spiked with I.S. Loratidine (50 μL).

PARAMETERS	WITHIN RUN			BETWEEN RUN		
Added Conc. (ng mL^{-1})	Nominal Conc. (ng mL^{-1}) [Mean +- SD]	Accuracy (%)	Precision (%RSD)	Nominal Conc. (ng mL^{-1}) [Mean +- SD]	Accuracy (%)	CV %
3	2.86±0.06131	95.33%	2.144	2.71±0.07574	90.27%	2.7965
10	10.61±0.48926	106.11%	4.611	10.30±0.6098	103.00%	5.9188
30	28.62±1.47356	95.41%	5.1481	27.95±1.65107	93.19%	5.9054

Table 1: Accuracy and Precision Studies (n=6) of HPL.

Accuracy and precision

The accuracy and precision values for within and between run studies at low, medium and high quality control concentrations of HPL in plasma were within acceptable limits (Table 1). The results also indicated that the assay method was reproducible, accurate and precise for replicate analysis of HPL within the same batch and on different batches.

Extraction recovery

Over the concentrations studied the minimum extraction recovery of HPL and IS found to be 93.43 ± 4.731 % and 88.32 ± 4.521% respectively. The details for other concentration studied are given in Table 2.

Linearity, range of calibration curve

The developed and validated method was linear over the range of 3-200 ng/mL for plasma with coefficient greater than 0.999 for the selected matrices.

LOD and LLQ

The signals of the blank samples were compared with the samples containing known low concentrations of the analyte. The LOD and LLQ were found to be 1.1 ± 0.1154701 and 3.0 ± 0.1154701 [mean ± RSD] respectively on the basis of signal to noise ratio.

Stability

The stability experiments were aimed at testing all possible conditions that the samples might experience after collecting and prior the analysis. The stability of the drug spiked at three QC levels evaluated for: short term (6 h), freeze thaw (3 cycles), and long term (30 days). The results (Table 3) showed that the HPL was stable in human plasma for about one month when stored in the frozen state (-80°C).

Name of the Analyte	Added Conc. (ng mL-1)	Plasma Nominal Conc. (ng mL-1) [Mean ± SD]	% Recovery	CV %
HPL	3	2.85±0.08216	95%	2.8828
	10	10.68±0.5982	106.80%	5.6011
	30	28.03±1.42	93.43	5.0660
Internal Std.	10	8.832±.4521	88.32%	5.1189

Table 2: Recovery studies of HPL (n=3) in human plasma.

Sample Conc. (ng mL-1)	Plasma Conc.	% Accuracy	CV %
Short Term Stability (6h)			
3	3.21±0.1105	107%	3.44%
10	10.13±0.7821	101.30%	7.72%
30	26.87±1.2251	89.56%	4.55%
Long Term Stability (- 80 °C, 1 Month)			
3	2.67±0.2123	89%	7.95%
10	9.13±0.9128	91.30%	9.99%
30	26.53±1.4121	88.40%	5.32%
Freeze and Thaw Cycles			
3	2.91±0.1319	97%	4.53%
10	9.77±0.6718	97.70%	6.87%
30	27.90±1.4413	93%	5.16%

Table 3: Stability Studies of HPL (n=6) in Human Plasma.

Ruggedness and robustness

The ruggedness of the method at various parameters was found to be ± 1.94%. For robustness, evident from ANOVA statistical test: the calculated F value (0.768637) found to be less than tabulated F value (4.06618) indicating that method was robust enough (± 1.98%) for the analysis of HPL within the specified range of deviation in the experimental conditions.

Clinical application of the method

The method was applied for routine estimation of haloperidol in psychiatric patients. After ethical approval, 205 patients were enrolled in study and further 116 included as per inclusion-exclusion criteria. Out of 116, 105 patients [95 male, 10 female] were on haloperidol regimen (20-30 mg daily).

In present study, therapeutic range in which most of patients (n=95) were stable was 5-19 ng mL^{-1}. Amongst the 10 outliers, 3 patients were below range (<5 ng mL^{-1}) and 7 patients above range (>19 ng mL^{-1}). The outliers were either therapeutically compromised or showed adverse effect associated with high concentration of drug like tradive dyskinesia, rigidity, sedation and QT prolongation without clinical benefits. The regimen of these patients was changed later as per their clinical condition.

Discussion

Various bio-analytical methods were reported for the estimation of HPL in human plasma using HPLC, but the working range of some methods was narrow i.e. from 1-30 ng mL^{-1} unlike this method with a range 3-200 ng mL^{-1}. This working range is justified since the reported range in literature as well as our study confirmed the need for a wider working range [8-10]. The current method used a simple liquid-liquid extraction procedure unlike many methods which reported use of solid cartridges which will increase the cost of analysis [8]. The present method has a lower limit of quantification of 3 ng mL^{-1}. Many reported methods have much higher lower limit of quantification [9,11,12] which may not be suitable for studies where lower concentrations are expected.

Many reported method used higher volume (1-2 mL) for sample processing [14-16]. The present method used a sample processing volume of 300μL which will help in optimal use of human blood samples in clinical studies where some amount of samples have to be archived after analysis for future reference. Thus the developed method has relative advantages when compared to previously reported methods. The therapeutic range of clinical significance found to be 5-19 ng mL^{-1} within which most of the enrolled patients showed maximum clinical benefit and further HPL plasma levels that substantially exceed 19ng mL^{-1} may be counter-therapeutic as evident from adverse effects observed in outliers (7) in current study. In particular, increase dose beyond this level are not efficacious for patients who have not responded to lower doses.

Conclusion

A highly sensitive, specific, accurate, and precise RP-HPLC assay method with UV-PDA detection was developed and validated to quantify HPL in human plasma. The developed method involves simple liquid-liquid extraction procedure and its shorter run time (<5 Min) makes it feasible and economic for routine clinical and toxicological monitoring of HPL. The method had adequate sensitivity (3 ng mL^{-1}) to detect trough concentrations in patients. In present study, most of the enrolled patients were clinically stable on HPL (as evident from various

rating scales applied during the study period) at therapeutic range of 5-19 ng mL^{-1} [Mean ± R.S.D. (13.04 ± 4.03)] which is slight different from range reported by other authors like 5-15 ng mL^{-1} [17], 8-18 ng mL^{-1} [18], 5-20 ng mL^{-1} [19], 4-20 ng mL^{-1} [8], 5-12 ng mL^{-1} [19], 5-17 ng mL^{-1} [20]. The individual population genomics and other factors could be responsible for wide variation of drug response based on therapeutic level but in a nut-shell, from above reported studies - a range of 5-20 ng mL^{-1} seems to be most promising for clinical monitoring of HPL.

In a nutshell, the method is simple (liquid-liquid extraction of sample), rapid (retention time of HPL 3 min and total run time 5 min.) and can easily applied in an analytical laboratory like hospital lab which is involved in routine analysis of patients sample, requires simple chromatographic equipment (liquid chromatography with diode array) and cheaper chemicals which can be available at a low cost in different countries.

Acknowledgements

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