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Research Article

In-silico Toxicity Assessment and Trace Level Quantification of Veratryl Chloride a Potential Genotoxic Impurity in Ivabradine Hydrochloride using LC-MS/MS

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ABSTRACT

A sensitive and selective liquid chromatography-mass spectrometry (LC-MS/MS) method using multiple reaction monitoring (MRM) mode was developed and validated for trace analysis of 4-(chloromethyl)-1, 2-dimethoxybenzene (Veratryl chloride) a potential genotoxic impurity in Ivabradine hydrochloride (IVB). Chromatographic separation was performed on a Poroshell 120EC C18 (50 × 3.0 mm, 2.7 µm) column using a mixture of 10 mM ammonium formate and acetonitrile in isocratic elution mode at a 0.25 mL/min flow rate. A simple pre-column derivatization with di-ethylamine was employed for the derivatization of the veratryl chloride. The developed LC-MS/MS method was linear and accurate in the 1.5–10.0 ppm concentration range with $r^2 > 0.999$ and percent recoveries greater than 90%. The developed method was precise with RSD (%) of not more than 4.5%. *In-silico* genotoxicity and carcinogenicity potential of veratryl chloride was assessed using ICH M7 principles found to be positive. The developed method can identify and quantify veratryl chloride in IVB, hence can be applied by quality control labs of pharmaceutical industries for trace quantification.

Introduction

Pharmaceutical manufacturers and global regulatory agencies are focusing on controlling the genotoxic impurities by identification and assessment, in both drug substances and products.[1] Drug substances may contain trace amounts of impurities that yield no therapeutic benefit to the patient/sufferer but may potentially threaten the patient. Some of them may create lethal changes in the genetic cell material. Therefore, the exact levels of such potential genotoxic impurities (PGIs) present in the drug substances/products are to be assessed and controlled for patient safety. The International Council on Harmonization (ICH) covers the safety and quality frameworks for establishing acceptable limits that will assure negligible patient hazard.[2] The maximum daily dose (MDD) of the drug substance and the period of treatment are used to define the limit for any genotoxic impurity.

IVB is a pacemaker current inhibitor used for the symptomatic management of heart-related chest pain and heart failure in cases where beta blockers do not manage the heartbeat.[3,4] IVB reduces the heart rate by allowing negative chronotropic in the sinoatrial structure by inhibiting the pacemaker current (I_f) in a dose-dependent manner, which is different from the mechanisms of beta blockers and calcium channel blockers, two commonly prescribed cardiac pharmaceuticals with antianginal

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effects. I_f is the combination Na^+/K^+ inward current which is activated by hyperpolarization and modulated by the automatic nervous system, and it is critical ionic currents for regulating the pacemaker activity of the sinoatrial node. IVB selectively inhibits this channel by reducing cardiac pacemaker activity and slowing the heart rate.[5,6] As mentioned in Fig. 1, veratryl chloride is used as a starting material for the synthesis of the advanced intermediate of IVB. As per the structural alerts classification,[7,8] veratrine chloride belongs to the alkylating agent class of genotoxic impurities and the unreacted residue traces can be present in IVB as an impurity.

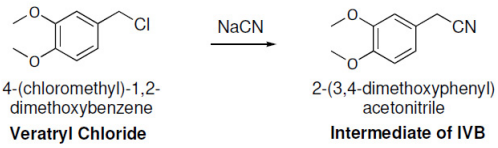

Fig. 1: Synthetic scheme of intermediate of IVB from veratryl chloride

There is some reported literature for the *in-silico* toxicity assessment for the genotoxic impurities,[9,10] but no published reports available on the genetic toxicity information of veratryl chloride in the literature. Accordingly, *in-silico* toxicity assessment was carried out according to the ICH M7 principles using expert knowledge-based (DEREK) and statistical-based (SARAH) approaches.

Veratryl chloride contains active chloride group and found to be unstable in multiple solvents and thus making it difficult for direct determination. Hence, a pre-column derivatization method by using diethyl amine was optimized for detecting in low level. In the present study, LC-MS/MS with multiple reaction monitoring (MRM) mode was selected for the analysis of veratryl chloride. There are some reported methods for the purity determination of IVB[11-13] and related substances [14,15] in the literature. However, no reported methods exist for estimating the trace levels of veratryl chloride. This study aims to perform the assessment of veratryl chloride followed by development and validation of LC-MS/MS method for trace level quantification in IVB using simple sample pre-column derivatization with diethyl amine.

Material and Methods

Materials

Veratryl chloride (Purity >99%), ammonium formate (HPLC grade), ammonia solution (AR grade), acetonitrile (HPLC grade), and diethyl amine (DEA) (HPLC grade) were procured from Merck (Mumbai, India). Ivabradine hydrochloride (Purity > 99%) samples were obtained from a local pharmaceutical company as gift samples. Polyvinylidene difluoride (PVDF) syringe filter was procured from Merck Millipore (Millipore® MA, USA).

Equipments

This study used an agilent chromatographic separation unit (Agilent Inc., USA) attached with 6460 triple quadrupole mass analyzer (Agilent Inc., USA). Data was acquired and integrated using automated software (Mass hunter., Agilent Inc., USA).

Chromatographic Conditions

Separation was performed on Agilent Poroshell 120EC C18 (50 × 3.0 mm, 2.7 µm) column in reverse phase mode using isocratic elution with 0.25 mL/min eluent flow. The mobile phase solution containing a mixture of an organic phase and aqueous phase. The aqueous phase comprised 10 mM ammonium formate in water (75%), while the organic phase contained ACN (25%). The sample injection volume was 5 µL. Auto sampler and column oven temperatures were set at 25°C (± 2°C) and 35°C (± 2°C), respectively. Method conditions are detailed in Table 1.

Mass Spectrometer Conditions

Electron-spray ionization (ESI) is used as source in positive mode and employed multiple reaction monitoring (MRM) mode for the ionization of veratryl chloride. Source and gas temperatures were set at 150 and 320°C, respectively. Ultra-high pure nitrogen gas (99.95% purity) was used for nebulizing and drying at a flow rate of 40 psi and 11 L/min, respectively. MRM transition of m/z 224.2 > 150.9 and 224.2 > 77.0 was used for the quantification. Method conditions are detailed in Table 1.

Preparation of Standard and Sample Solutions

A primary concentrated stock solution of 0.5 mg/mL of veratryl chloride was prepared using 1% DEA in ACN as the diluent. The stock solution was diluted to produce a secondary stock solution containing 5 µg/mL of veratryl chloride. The primary stock solution and secondary stock solutions were further diluted using 1% DEA in ACN to yield system suitability, calibration curve standard and accuracy spiking solutions.

Test sample solutions of IVB were prepared at 1-mg/mL in 1% DEA in ACN solution. All the solutions were kept at 60°C for 2 hours to complete the derivatization process. Cooled sample solutions were injected.

Method Validation

The developed method was validated according to the regulatory guidelines.[16,17] Validation parameters such as sensitivity, selectivity, accuracy, precision, linearity, robustness, and solution stability were studied. Six different concentrations were prepared and studied ranging from 1.5 to 10.0 ppm to establish linearity of the method for veratryl chloride. Statistical parameters have been applied for calibrating the linearity of the calibration curves obtained from the least-square regression analysis of the calibration data. The LoD and LoQ were determined by injecting the solutions containing veratryl chloride in

Table 1: Summary of method conditions

Parameter	*Conditions*
Eluent	10 mM ammonium formate in water/Acetonitrile – 25/75 (%v/v)
Flow rate	0.25 mL/min
Auto-sampler temperature	25°C
Injection volume	5 µL
Column temperature	35°C
Elution	Isocratic
Run time	7 minutes
Source	ESI
Ionization mode	Positive
Capillary voltage	4000 V
Nebulization gas	Nitrogen
Gas temperature	350°C
Gas flow	11 L/min
Nebulizer pressure	40 psi
Drying gas flow	15 mL/min
CID gas	Argon
Collision energy	40 V
Acquisition mode	MRM

Acquisition parameters for veratryl chloride amine derivative

Precursor Ion	*Product ion*	*Fragmentor voltage*	*Collision energy*	*Cell Accelerator voltage*
224.2	150.9	80	8	4
224.2	77.0	80	72	4

the 0.2–2.0 ppm range to obtain a signal-to-noise (S/N) ratio greater than or equal to 3:1 and 10:1, respectively. The accuracy and precision of the method were evaluated by comparing the percentage recovery values and RSD (%) values for veratryl chloride. For this study, the responses obtained in triplicate determination at four levels ranging from LoQ – 200% of the specification limit were compared for both un-spiked and spiked samples. Percentage recovery values should be within 100 ± 20%, while the RSD (%) values should be less than 10% to establish that the method is accurate. The solution stability of the spiked sample was proven by storing the solutions at room temperature at different intervals of time

Results and Discussion

In-silico prediction of Mutagenicity and Carcinogenicity of Veratryl Chloride

For veratryl chloride, no genetic toxicological data reported in the literature. Toxicity approximation was carried out by performing *in-silico* toxicity studies using knowledge-based (DEREK Nexus) and statistical-based (SARAH Nexus) tools using ICH M7 principles. The DEREK Nexus prediction for veratryl chloride is "Plausible". Alkyl halide structural alert for veratryl chloride, like example alert 027, a corresponding alkylating agent from DEREK data base. From DEREK knowledge base 2022 2.0, an *In-vitro* mutagenicity in bacterium and *in-vitro* mammalian chromosome damage is plausible. Sarah model 2.0 was used for predicting the statistical based (SARAH), a prediction with 42% positive was obtained for veratryl chloride with confidence for the mutagenicity *in-vitro* (i.e., Ames test positive). The training set has similar examples with supporting hypothesis. In summary of results for veratryl chloride using both DEREK and SARAH Nexus are presented in Table 2 and Fig. 2 veratryl chloride was classified as ICH M7 class-3 impurity based on the prophecies from the DEREK and SARAH. The boundary for veratryl chloride was considered as per ICH M7 principles using the threshold of toxicological concern (TTC) and the MDD of IVB. Using TTC of 1.5 µg/day and MDD of 15 mg/day, the veratryl chloride should meticulously have a boundary at 100 ppm. Nevertheless, in the current study a strict limit of 5 ppm for the quantification of veratryl chloride in IVB drug substance was carefully chosen to have improved control approach.

Optimization of Derivatization Conditions

Alkyl halides readily reacts with primary and secondary amines via nucleophilic addition mechanism to yield a tertiary amine.[18] Active chloride group in the veratryl chloride makes difficult for the direct determination due to instability in the solvent used for the analysis. In this study, diethyl amine was selected for the derivatization of veratryl chloride and considering the solubility acetonitrile used as a solvent media for the derivatization. Derivatization reaction completion was evaluated by conducting the reaction at different temperatures and the time intervals. Diethyl amine was maintained at a higher concentration in the reaction conditions, significant difference in the conversion rate was observed at different temperatures. Results from the derivatization optimization study showed that temperature is important to complete the reaction in a short time. Based on the evaluation, derivatization temperature of 60°C with 2 hours of reaction time was

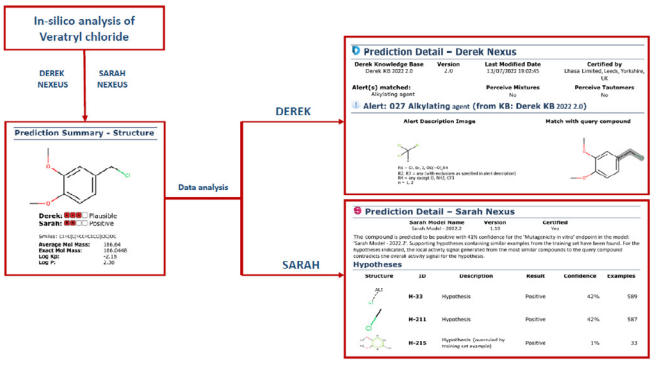

Figure 2: *In-silico* toxicity results for veratryl chloride

Table 2: *In-silico* toxicity prediction results for Veratryl chloride using Derek and Sarah Nexus software

Impurity	*DEREK Prediction*	*SARAH Prediction*
Veratryl chloride	• Chromosome damage *in vitro* in mammal is "PLAUSIBLE" [a]. • Alert matched to 27 Alkylating agent. • Mutagenicity *in vitro* in mammal is "PLAUSIBLE".	• The compound is predicted to be "POSITIVE" [b] with 42% confidence for the "Mutagenicity *in vitro*". • Hypotheses analysis was found to be "POSITIVE" with structure ID# H-33 and H-211

[a] As per DEREK prediction outcome definition, "PLAUSIBLE" indicate that the weight of evidence supports the proposition.

[b] As per SARAH prediction outcome definition, "POSITIVE" indicate that the query structure is predicted to be positive in a bacterial reverse mutation assay (Ames test).

selected for the derivatization. An optimized derivation reaction for veratryl chloride using diethyl amine is presented in Fig. 3.

Optimization of Liquid Chromatographic and Mass Spectrometric Method Conditions

Liquid chromatography united with either a UV detector or mass analyzer is the most appropriate analytical technique for the quantification of non-volatile impurities present in drug substances.[19] In optimizing an LC-MS/MS method for the identification/quantification of veratryl chloride in IVB, the LC conditions were first adjusted followed by the mass spectrometric conditions. In the optimization of LC conditions, first different LC-MS suited aqueous mobile phase (mobile phase A), two different solvents, namely, 10 mM ammonium acetate in water and 0.1% v/v formic acid were tried. Methanol and ACN were tried to identify the suitable non-aqueous mobile phase (mobile phase B). Optimization of LC conditions was done based on elution time, peak area using the above-mentioned mobile phase compositions. Based on the evaluation experiments, Poroshell 120 EC C18 column (50 mm × 3.0 mm × 3.0 µm) used premixed eluent containing 10 mM ammonium formate in water and ACN in the 75:25 (%v/v) ratio.

In the development of MS conditions, ionization of the sample was achieved using electrospray ionization technique and a m/z range of 50–500 was selected in scan mode for analysis. The parent ion (Q1) has good MS signal with excellent intensity. For fragmenting the parent ion (Q1) a collision energy was provided with the help of nebulization gas available in the second quadrupole (MS2) to yield daughter ion (Q3) with decent intensity. The collision energy parameters were fine tuned to get stable daughter ion (Q3). Further, instrument-dependent parameters like nebulization gas, capillary voltage, source temperature, nebulization gas temperature and drying gas flows were adjusted for the quantification of veratryl chloride. Two different transitions at m/z values of 224.2(Q1)/150.9(Q3) and 224.2(Q1)/77.0(Q3) were monitored during the optimization studies. The response obtained for the transition 224.2(Q1)/77.0 (Q3) was not intense and hence it was not considered for additional experiments. In the concluding optimized method, 224.2(Q1)/150.9(Q3) was carefully chosen for sample analysis.

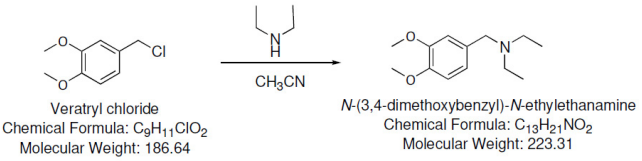

Fig. 3: Derivatization scheme for veratryl chloride

Method Validation

The regulatory guidelines[16,17] were referred for developed method validation. As shown in Fig. 4a and 4b, the specificity study demonstrates that veratryl chloride eluted at 1.6 minutes. The LoD and LoQ values for veratryl chloride were calculated from the S/N data. The LoD and LoQ values were 0.4 and 1.5 ppm,, respectively. The RSD (%) values of LoQ precision were found to be less than 7.4%. The LoQ results are summarized in Table 3.

Linear regression analysis of veratryl chloride demonstrated high r^2 (> 0.999) and small % Y-intercept (< 10.0%). The random scatter of residual plots of the calibration curve data of the analyte indicate that the method is linear over the corresponding calibration ranges. The RSD (%) values were found to be less than 5% for veratryl chloride for both method precision and intermediate precision studies. The experimental results from the precision study were summarized in Table S3. The recoveries of veratryl chloride from the accuracy experiments were found to be in the range of 82.5–104.6%. RSD (%) for the peak area response from the triplicate preparations at LoQ, 40, 60, 80, 100, and 200% levels were found to be less than 2.1%. The percentage recovery values, and the RSD (%) values were within the acceptance criteria of 80–120% and less than 10.0%, respectively. Deliberate changes for both flow rate and initial oven temperatures (robustness) for the method conditions have shown that there is no substantial impact on the resolution and/or the peak shape of veratryl chloride. The retention times of veratryl chloride altered with the deliberate change in flow rate and the collision energy. In the solution stability studies, the maximum percentage deviation was found to be less than 10.0% in the standard and spiked sample solutions; demonstrating that the solutions are stable for 48 hours when stored at ambient laboratory conditions (25 ± 5°C) and refrigerated conditions. Experimental results

from accuracy and robustness study are summarized in Table 3. Chromatograms of sample and LoQ level spiked sample solution are presented in Figs 5a and b, respectively.

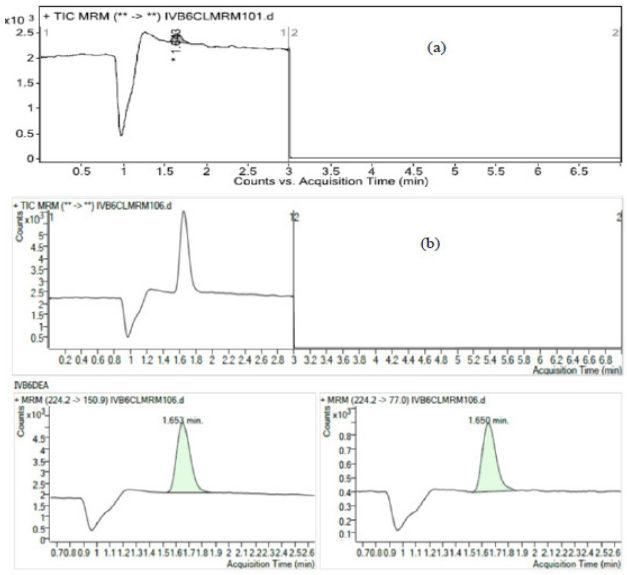

Figure 4: Chromatograms of (a) Blank, and (b) Standard solution

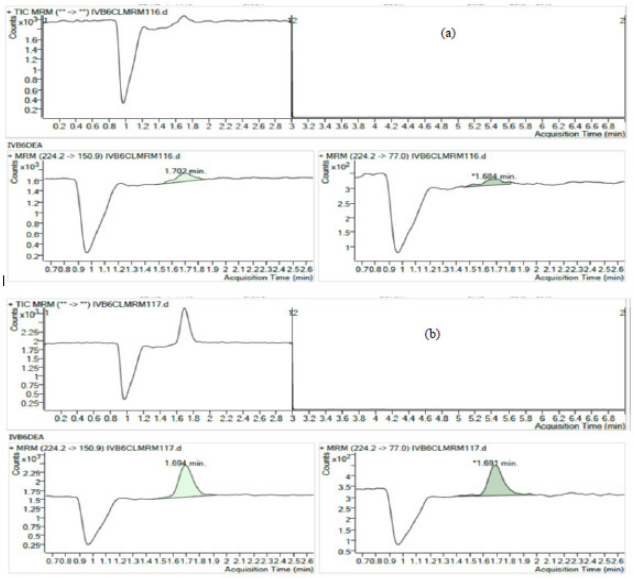

Fig. 5: Chromatograms of (a) Sample, and (b) Spiked sample solution

Table 3: Method validation data summary

Test Parameter	*Typical acceptance criteria*	*Veratryl chloride*
System suitability	RSD (%) for peak area response (n=6)	0.51%
Cumulative RSD (%) for peak area response (all injections) 0.47%		
System suitability (for Intermediate precision)	RSD (%) for peak area response (n=6)	2.97%
	Cumulative RSD (%) for peak area response (all injections)	2.89%
Specificity	Blank interference	No significant interference observed
Sensitivity	Concentration	LoD – 0.4 ppm LoQ – 1.5 ppm
	S/N for LoD solution should be > 3:1	4:1
	S/N for LoQ solution should be > 10:1	11:1
	RSD (%) for six replicate injections of LoQ solution should be ≤ 15.0%	7.4%
Linearity	Range	1.5 – 10.0 ppm
	Calibration Equation	y = 4803.46 x – 2138.77
	r^2	0.9996
	Residual plots	Random scatter
Accuracy	Average recovery (n=3) from the spiked samples performed at 5 levels should be between 80 – 100%; RSD (%) should be ≤ 10.0%	LoQ – 82.5%; 1.8% 40% – 88.7%; 2.1% 60% – 91.3%; 1.6% 80% – 93.8%; 1.0% 100% – 93.9%; 0.9% 200% – 104.6; 2.0%
Precision	RSD (%) for six preparations at 100% spike level should be ≤ 10.0%	4.9%
Intermediate Precision	RSD (%) for six preparations at 100% spike level should be ≤ 10.0%	4.4%
Robustness 0.20 mL/min Flow	RSD (%) for peak area response (n=6) %Recovery (n=3) for 100% spiked solution	0.93% 96.6%

Robustness 0.30 mL/min Flow	RSD (%) for peak area response (n=6) %Recovery (n=3) for 100% spiked solution	1.24% 93.1%
Robustness 35 V collision energy	RSD (%) for peak area response (n=6) %Recovery (n=3) for 100% spiked solution	0.34% 97.4%
Robustness 45 V collision energy	RSD (%) for peak area response (n=6) %Recovery (n=3) for 100% spiked solution	0.78% 98.5%

Conclusion

A simple and sensitive LC-MS/MS method was developed and validated for the trace level quantification of veratryl chloride in IVB using a simple pre-column derivatization with N, N-diethyl amine to overcome the stability and sensitivity issues. Toxicology studies revealed that veratryl chloride is genotoxic and classified under class 3 impurity as per ICH. The developed method was sensitive, precise, and specific for the quantification of the veratryl chloride in IVB. The developed method is suitable and qualified enough for being implemented in the quality control lab for respective analysis and can be applied for estimating and controlling of veratryl chloride in other drug substances with nominal sample preparation tweaking.

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