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Analytical Method Validation of Vilazodone with Spectrofluorimetric Detection in Rabbit Plasma

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Abstract: Vilazodone (VLZ) is an antidepressant agent approved in 2011 by Food and Drug Administration (FDA) for the treatment of major depressive syndromes. Its chemical structure derives from trazodone which is used as an off-label drug for dogs and cats in order to induce sedation and reduce anxiety, behavioural and pre-and post-operative stress. VLZ could be a potentially useful compound in reducing severe anxiety responses in pet animals. The aim of the present research was to develop and validate a method to quantify VLZ in rabbit plasma. A 500 µL aliquot of rabbit control plasma was added to 50 µL of IS (0.1 ug/mL) and 100 µL of NaOH 0.1M. Then 1 mL of Et_2O:EtOAc (70:30 v/v) was added and the sample, shaken and centrifuged. The organic layer was transferred into a clean polypropylene vial. This procedure was repeated twice. The combined supernatants were evaporated under nitrogen at 40°C and reconstituted with 100 µL of mobile phase. 50 µL of this latter solution was injected onto HPLC-FL. The mobile phase consisted of Na_2HPO_4 (0.02M, pH 5):ACN (67:33 v/v) at a flow rate of 1 mL/min. The analytical column (C18) was maintained at 40°C. Excitation and emission wavelengths were set at 353 and 486 nm, respectively. Times of retention of VLZ and IS were 3.26±0.09 and 4.70±0.08 minutes, respectively. The recovery of VLZ was about 83%. Limits of quantification and detection were 0.005 µg/mL and 0.001 µg/mL, respectively. This method was verified by determining VLZ concentration in rabbit plasma after a 1 mg/kg single IV administration. The analysis of samples allowed the calculation of the main pharmacokinetic parameters. In conclusion, the present research was able to quantify VLZ in rabbit plasma after VLZ administration. This method might have application for pharmacokinetic or toxicological studies.

Keywords: VLZ, Antidepressant, Fluorescence, Rabbits, Pharmacokinetics

Introduction

In veterinary medicine, SSRIs such as fluoxetine and fluvoxamine are frequently used to control aggression, obsessive-compulsive disorder and separation anxiety in pets (Sargisson, 2014; Overall, 1997). Phenylpiperazine compounds such as trazodone are used as off-label drugs in pet animals, in order to reduce anxiety, behavioural stress, sedation and pre- and post-operation stress (Chea and Giorgi, 2017). In the past few years Vilazodone (VLZ), a drug belonging to the same class as trazodone, has been issued on the human drug market. It has a similar chemical structure to trazodone (Fig. 1), shares the same mechanism of action, but has been reported to be more active and to possess a better safety profile. It is available as 10 mg, 20 mg or 40 mg tablets. Thanks to the favourable PK/PD features shown in humans, this compound appears to be a likely option in pets for anxiety disorders such as travel, separation, noise phobia, veterinary visits and hospitalization.

Science Publications

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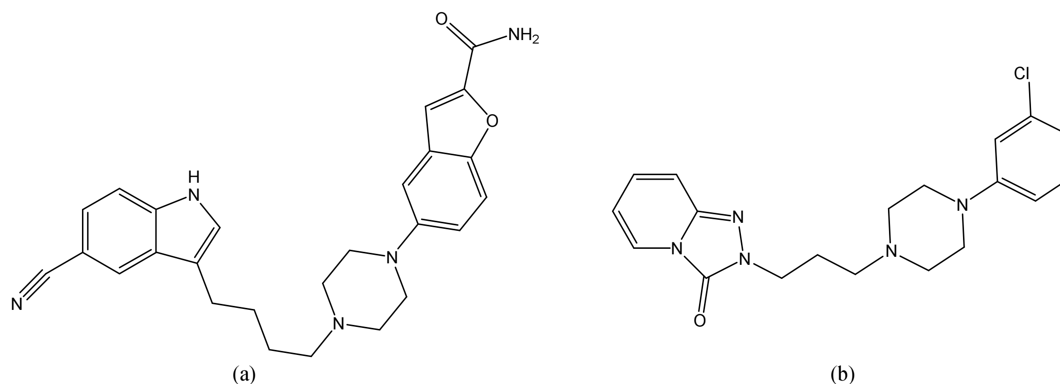

Fig. 1: Chemical structures of trazodone (a) (2-[3-[4-(3-chlorophenyl)-1-piperazinyl] propyl]-1,2,4-triazolo [4,3-a] pyridine-3 (2H)-one) and VLZ (b) (5-(4-[4-(5-cyano-1H-indol-3-yl) butyl] piperazin-1-yl) benzofuran-2carboxamide)

A number of analytical methods to detect VLZ in plasma have been reported in the literature (El-Bagary *et al.*, 2016) (Iqbal *et al.*, 2015) mainly based on mass spectrometry detection. Only a single study concerning its detection using HPLC with Fluorescence (FL) in human plasma is present (Studies *et al.*, 2012). The aim of the present research was to develop and validate a new simple and reproducible method to quantify VLZ in rabbit plasma using HPLC with spectrofluorimetric detection which could have application for further pharmacokinetic or toxicological studies in pets.

Materials and Methods

Chemical and Reagents

VLZ hydrochloride 95% was supplied by Abcr GmbH (Karlsruhe, Germany) and the IS, trazodone hydrochloride (≥ 99% purity), was supplied by Sigma-Aldrich (ST. Louis, MO, USA). Acetonitrile (ACN), Methanol (MeOH), diethyl ether (Et_2O), ethyl acetate (AcOEt) and orthophosphoric acid 85% (H_3PO_4) were purchase from VWR International (Radnor, Pennsylvania, US) and sodium monohydrogen phosphate (Na_2HPO_4) from Baker Analyzed® ACS, J.T. Baker® (Deventer, Holland).

HPLC-FL Conditions

The HPLC system was a LC Jasco (Como, Italy) consisting of ternary gradient system (PU 980), in line degasser (DG-2080-53), an autosampler (AS-2055) and an in line multilamda fluorescent detector (FP-1520). The chromatographic separation assay was performed with a Luna C18 analytical column (150×4.6 mm, inner diameter 3 µ particle size, Phenomenex, Bologna, Italy) preceded by a security guard column with a C18 cartridge, 4×3 µm (AJ0-4287). The mobile phase consisted of Na_2HPO_4 (0.02M, pH 5): ACN (67: 33 v/v) at a flow rate of 1 mL/min. The injection volume was 50 µL and the system was maintained at 40°C. A range of buffer pH (3.9, 4.2, 4.6, 5.0 and 6.0) was assayed to optimize the chromatographic separation. Excitation and emission wavelengths were set at 353 and 486 nm, respectively.

Standard Solutions

A stock solution of VLZ was prepared at a concentration of 1000 µg/mL, by adding 2.3 mg of compound to 2.3 mL of MeOH. This solution was subsequently diluted in MeOH to reach the final concentrations: 0.005, 0.05, 0.1, 0.5, 1, 2.5, 5, 10, 100 µg/mL. The solutions were stored at -20°C. Stock solution (1000 µg/mL) of internal standard (IS), trazodone, was prepared by adding 1 mg of IS to 1 mL of MeOH. The solution used as IS (0.1 µg/mL) was obtain by appropriately diluting the stock solution with MeOH and it was stored at+4°C.

Sample Extraction

The procedure was performed in a 2 mL polypropylene vial. A 500 µL aliquot of rabbit control plasma was added to 50 µL of IS (0.1 ug/mL) and 100 µL of NaOH 0.1M and vortexed for 10 seconds. Then 1 mL of Et_2O: EtOAc (70:30 v/v) was added and the sample was vortexed for 30 seconds, shaken for 10 minutes (60 osc/min) and centrifuged for 10 minutes at 5,000 *g*. The organic layer was transferred into a clean polypropylene vial. This procedure was repeated twice. The supernatants were combined, evaporated under a gentle stream of nitrogen at 40°C and reconstituted with 100 µL of mobile phase. The solution was sonicated for 3 minutes and centrifuged at 5,000 *g* for 3 minutes. Then 50 µL of this latter solution was injected onto HPLC-FL.

Bioanalytical Method of Validation

The described method was validated in terms of linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), recovery, specificity, stability, precision and accuracy according to the international guidelines on the bioanalytical method validation (Anonymus, 2011). Calibration curves were obtained by spiking the blank

matrix with a known concentration of each analyte and IS to provide concentration of 0.001, 0.005, 0.01, 0.05, 0.1, 0.25, 0.5, 1 and 2.5 µg/mL. The calibration curve of peak area versus concentration (µg/mL) of VLZ was plotted. Within- and between-run accuracy and precision were assessed on quality control sample (QC samples) and determined by replicate analyses using 3 determinations of different concentration levels (0.05, 0.1, 0.5, 1, 2.5 µg/mL). Stability of VLZ in rabbit plasma was demonstrated for at least 30 days at -20°C, as well as during 3 consecutive thawing-freezing cycles.

Animal Treatment and Sampling

In order to avoid variabilities linked to the unknown oral bioavailability of the drug in the rabbit, VLZ was administered intravenously via the marginal ear vein as an ethanol solution. Three healthy female adult New Zealand rabbits were administered with a single IV administration (1 mg/kg). Blood (1 mL) was collected via catheter, previously inserted in the central ear artery at assigned times: 0.5, 1, 2, 4, 6, 8, 10, 24 hours. The blood was then placed into collection tubes containing lithium heparin. The samples were centrifuged (1,500 *g*) for 10 minutes. The harvested plasma was immediately frozen and stored at-20°C. Prior to the analysis the samples were thawed at room temperature. Pharmacokinetic calculations were performed by the ThothPro^{TM} software (www.ThothPro.com), by a non-compartment analysis.

Results

Method Development

The method was developed by combining data from early reports with some modifications (Studies *et al.*, 2012). The mobile phase constituted by Na_2HPO_4 (0.02M) and ACN (67: 33 v/v) with a 1 mL/min flow rate was found to optimally provide separation between VLZ, IS and matrix interference peaks. A range of buffer pH (3.9, 4.2, 4.6, 5.0, 6.0) was tested in order to optimize the chromatographic separation (Fig. 2). The pH was modified by orthophosphoric acid 85%. At pH 6 the VLZ peak overlapped other matrix peaks. At pH 3.9 VLZ and IS peaks merged. At pH 4.2 the IS peak moved to a retention time of 3.26 min. The two peaks of interest were well resolved but if the concentration of VLZ was high, they sometimes merged. Finally, pH 5 was chosen as optimal with retention time for VLZ and IS of 3.17±0.09 and 4.59±0.08 minutes (Fig. 3).

Chromatographic C 18 columns with different lengths (250 and 150 mm) and particle sizes (5, 4 and 3 µm) were tested. The column Luna Phenomenex 3 µm C18 110A, 150x4.6 mm provided the best separation. Furthermore, different temperature conditions were tested (30°C, 40°C and 50°C). A temperature of 40°C showed the best peak resolution and separation.

Different compounds were evaluated as ISs: sulpiride, metoclopramide, trazodone, tapentadol and tramadol. Trazodone was found to be the best candidate with an excellent resolution, repeatability and a suitable retention time, providing the shortest chromatographic course with peaks well separated from both VLZ and matrix interferences. Trazodone has similar molecular structure compared to VLZ and consequently possesses the same maximum excitation and emission wavelengths. Trazodone was stable in solution over time and displayed also an excellent recovery (92±4%) if extracted with the current extraction solvent method.

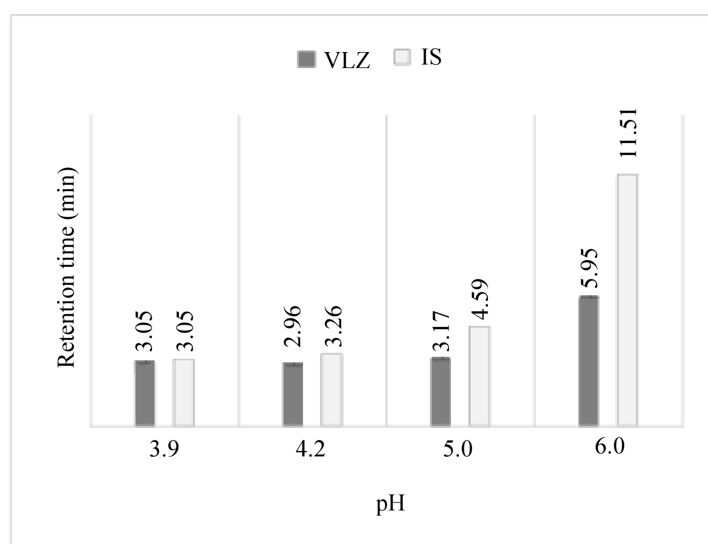

Fig. 2: Effect of the pH on the retention time of VLZ (grey bars) and IS (white bars)

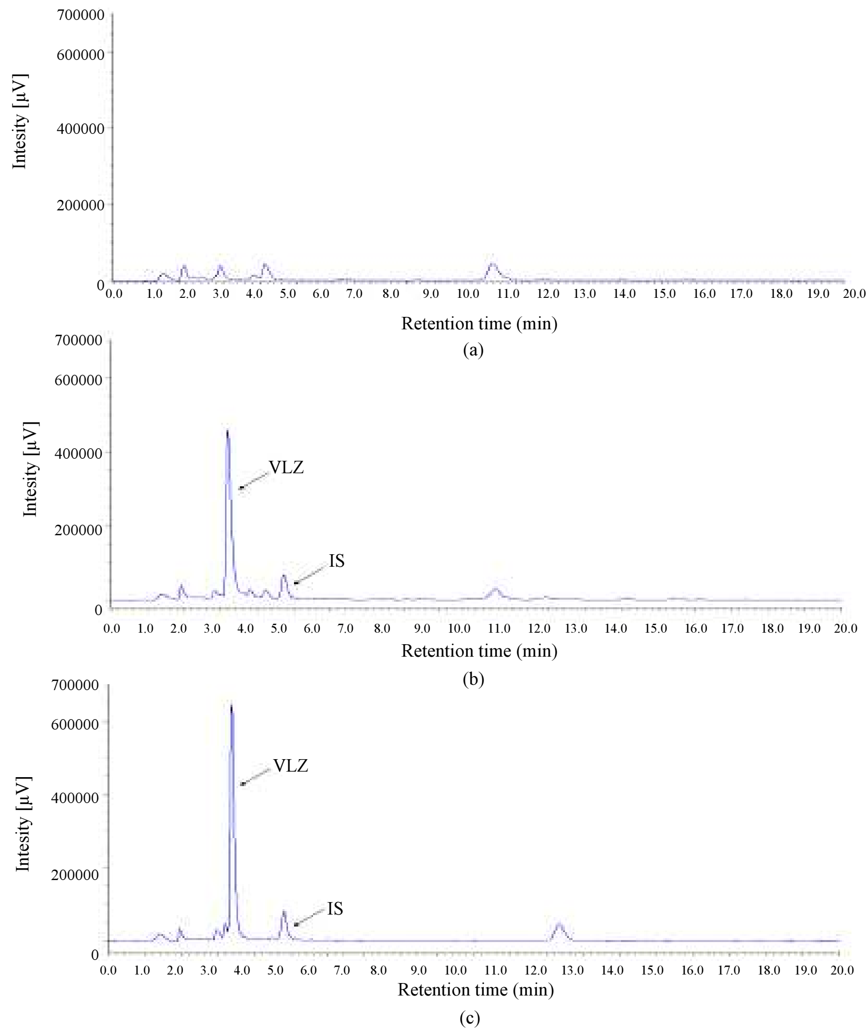

Fig. 3: HPLC-FL chromatogram from (a) rabbit control plasma, (b) from spiked sample (VLZ 0.25 µg/mL; IS 0.1 µg/mL) and (c) plasma sample collected in a rabbit after a single IV administration at 4 mg/kg (4 h)

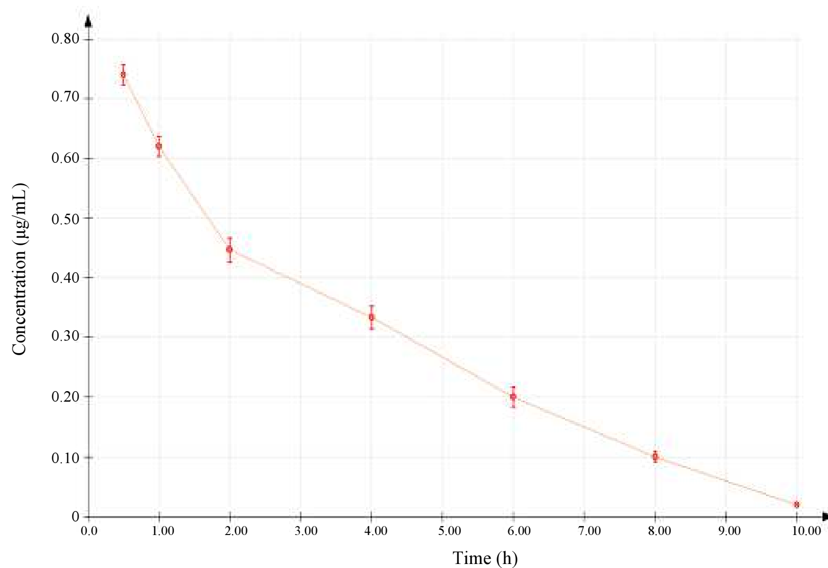

Fig. 4: Average of plasma VLZ profile after 1 mg/mL bolus injection in rabbits (n = 3)

Optimization of the Extraction Method

Solvents such as ACN, tetrahydrofuran, n-Hexane, CH_2Cl_2, Et_2O, EtOAc were examined singly and in combination. Et_2O and EtOAc were selected as the most suitable organic solvents in term of analyte extraction and minimization of matrix components. The other solvents were characterized by low recoveries and/or a low clean-up of matrix impurities, reducing the resolution of the analytes. Variations in the proportion of the selected extraction solvent (Et_2O/EtOAc 3:7, 7:3 v/v) were also assessed in terms of recovery and selectivity. Et_2O/EtOAc 7:3 v/v showed the best recovery for VLZ (83.20±3.2%) and the IS.

Method Validation

The calibration curve was constructed by plotting the ratio of the peak area versus concentrations in the working range of 0.005-0.25 µg/mL for VLZ. The calibration equation was y = 27.761x-0.1905 and the correlation coefficient was r^2 = 0.996. A good linearity was achieved in the investigated range of 0.01-2.5 µg/mL. According to EMA guidelines, LOD and LOQ were calculated based on a signal-to noise approach. The typical signal-to noise ratios were 10:1 and 3:1 for LOQ and LOD, respectively. LOQ and LOD was 0.005 and 0.001 µg/mL, respectively. The specificity was investigated in regard to the other co-eluting components by comparing the chromatograms of different batches of blank matrices to those from spiked solutions. Under optimized chromatography conditions, peaks due to the matrix did not interfere with VLZ and IS (Fig. 3). Typical retention time for VLZ and IS was 3.17±0.13 and 4.59±0.18 minutes, respectively. Intraday value consistency (repeatability) was evaluated for three replicates of the QC samples during the same day. It resulted in values lower than 7.4%. Inter-day value consistency (intermediate precision) was evaluated by quantization of the QC sample on three different day and resulted in values lower than 12.6%. Table 1 reports a summary of validation data for VLZ

Application of the Method

The applicability of this method has been verified by determining VLZ in rabbit plasma after a single bolus administration of VLZ (1 mg/kg). HPLC analysis of the plasma samples confirmed the presence of the molecule in time-related amounts. The average amount of VLZ in plasma ranged between 0.02 µg/mL (10h) and 0.74 µg/mL (0.5h). At 24h the VLZ concentration was lower than the LOD of the present method. The main pharmacokinetic parameters are reported in Table 2.

Table 1: Summary of validation data for VLZ

Property	Units	VLZ
Linear range	µg/mL	0.005-2.5 µg/mL
Calibration equation		y = 27.761x-0.1905
Correlation coefficient	r^2	0.9955
LOQ	µg/mL	0.005
LOD	µg/mL	0.001
Intra-day precision	%	2.1-7.4
Inter-day precision	%	4.2-12.6
Specificity		Specific

Table 2: Main pharmacokinetic parameters calculated in rabbits (n = 3) after 1 mg/kg IV administration of VLZ

Parameter	Unit	Average	SD
R^2		0.99	0.02
AUC(0-t)	µg*h/mL	2.79	0.11
Kel	1/h	0.30	0.03
$t_{1/2}$kel	h	2.32	0.25

AUC(0-t), area under the plasma concentration- time curve; $t_{1/2}$kel, half-life of the elimination phase; Kel, elimination constant; R^2, correlation between observed/predicted points

Discussion

VLZ has been approved for treatment of major depressive syndromes in humans. VLZ derives from trazodone and it might also be of value as an antidepressant agent in veterinary medicine. Recently analytical methods based on mass spectrometry detection have been employed for the detection of VLZ (Sui *et al*., 2014; Zeng *et al*., 2014; Iqbal *et al*., 2015; Kalariya *et al*., 2015; El-Bagary *et al*., 2016; Chavan *et al*., 2017). All these methods are carried out with chromatographic devices (HPLC, UPLC, UHPLC) coupled with expensive mass spectrometry detectors such as electrospray ionization (ESI) or quadrupole time-of-flight (QTOF). Although these method report LOQs lower than that reported in the present study, the high cost of the apparatus makes these methods unaffordable for low-budged laboratories.

A few studies have been presented based on other cheaper types of detection. For instance, a HPLC-DAD method was validated for the quantification of venlafaxine, VLZ and their main metabolites in human serum samples (Petruczynik *et al*., 2017). The LOQ was 14.33 ng/mL and the selectivity good. Thangabalan *et al*. (2015) reported a HPLC-UV method for the estimation of VLZ in tablet dosage form. LOQ was 0.33 µg/mL, although several details of the procedure are missing in this report. To the best of the author's knowledge there is only a single study concerning the estimation of VLZ in bulk and pharmaceutical formulations, namely El-Bagary *et al*. (2014). Higher LOD and LOQ (0.063 µg/mL and 0.191 µg/mL, respectively) than in the present study were reported. However, the fact that the method from El-Bagary *et al*. (2014) is not employed for the quantification of the drug in biological matrices prevents other comparisons with the present work.

Albeit VLZ can be considered a potential agent in veterinary medicine, there are not still enough PK/PD

studies regarding its use in pet animals. Indeed, it is not possible to discuss on its clinical practice use.

Conclusion

The analytical method described in this work provides a sensible, selective and accurate analysis of VLZ without the need for expensive clean up steps, solvent consuming flows or expensive devices. The described method could be suitable for pharmacokinetic investigations. In summary, this method is simple and efficacious for the determination of VLZ in rabbit plasma.

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Author's Contributions

I. Sartini: Performed the analytical study, participated in the *in vivo* study, in the data-analysis and contributed to the writing of the manuscript.

M. Salvadori: Coordinated/performed *in vivo* study.

B. Łebkowska-Wieruszewska: Supervised the project and contributed to the final manuscript.

A. Poapolathep: Involved in planning, contributed in pharmacokinetic analysis and supervised the study.

M. Giorgi: Contributed to the HPLC study, coordinated the whole study, contributed to the writing of the manuscript.

Conflict of Interest

None of the authors of this paper does have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Abbreviations

IV	Intravenous
VLZ	Vilazodone
HPLC-FL	High-performance liquid chromatography with spectrofluorimetric detection
UPLC	Ultra performance liquid chromatography
UHPLC	Ultra high-performance liquid chromatography
HPLC UV	High-performance liquid chromatography with ultraviolet detection
HPLC-DAD	High-performance liquid chromatography with diode-array detection
IS	Internal standard
AUC (0-t)	Area under the plasma concentration – time curve
$t_{1/2}kel$	Half-life of the elimination phase
Kel	Elimination constant
R^2	Correlation between observed/predicted points
LOQ	Limit of quantification
LOD	Limit of detection
PK	Pharmacokinetics
PD	Pharmacodynamic
ACN	Acetonitrile
MeOH	Methanol
Et_2O	Diethyl ether
AcOEt	Ethyl acetate
H_3PO_4	Orthophosphoric acid 85%
Na_2HPO_4	Sodium monohydrogen phosphate
ESI	Electrospray ionization
QTOF	Quadrupole time-of-flight