Journal of Pharmaceutical Analysis 2014;4(2):107–116

Contents lists available at ScienceDirect

Journal of Pharmaceutical Analysis

www.elsevier.com/locate/jpa
www.sciencedirect.com

ORIGINAL ARTICLE

Selective separation, detection of zotepine and mass spectral characterization of degradants by LC–MS/MS/QTOF

M.V.N. Kumar Talluri[a,*], Naveen Reddy Kandimalla[a], Raju Bandu[b], Divya Chundi[a], Ramesh Marupaka[b], Ragampeta Srinivas[a,b,]**

[a]*Department of Pharmaceutical Analysis, National Institute of Pharmaceutical Education & Research, Balanagar, Hyderabad 500037, India*
[b]*NCMS, Indian Institute of Chemical Technology, Tarnaka, Hyderabad 500007, India*

Received 15 July 2012; accepted 7 April 2013
Available online 28 May 2013

KEYWORDS

Zotepine;
Stability-indicating RP–HPLC method;
Characterization;
ESI-Q-TOF-MS;
Bulk drugs and formulations

Abstract A simple, precise, accurate stability-indicating gradient reversed-phase high-performance liquid chromatographic (RP–HPLC) method was developed for the quantitative determination of zotepine (ZTP) in bulk and pharmaceutical dosage forms in the presence of its degradation products (DPs). The method was developed using Phenomenex C_{18} column (250 mm × 4.6 mm i.d., 5 μm) with a mobile phase containing a gradient mixture of solvents, A (0.05% trifluoroacetic acid (TFA), pH = 3.0) and B (acetonitrile). The eluted compounds were monitored at 254 nm; the run time was within 20.0 min, in which ZTP and its DPs were well separated, with a resolution of >1.5. The stress testing of ZTP was carried out under acidic, alkaline, neutral hydrolysis, oxidative, photolytic and thermal stress conditions. ZTP was found to degrade significantly in acidic, photolytic, thermal and oxidative stress conditions and remain stable in basic and neutral conditions. The developed method was validated with respect to specificity, linearity, limit of detection, limit of quantification, accuracy, precision and robustness as per ICH guidelines. This method was also suitable for the assay determination of ZTP in pharmaceutical dosage forms. The DPs were characterized by LC–MS/MS and their fragmentation pathways were proposed.

1. Introduction

The parent drug stability test guidelines Q1A (R2) issued by International Conference on Harmonization (ICH) [1–4] requires the stress stability studies to be done on a drug to establish its inherent stability characteristics. This helps to identify the likely degradation pathways and degradation products (DPs) of the drug. It is a prerequisite that analytical test procedures should be stability indicating and fully validated. Accordingly, the aim of the present study was to establish inherent stability of zotepine (ZTP) and to develop a stability-indicating assay method through stress studies under a variety of ICH recommended test conditions [1,5–8]. The chemical name of

---

[*] Corresponding author. Tel.: +91 40 23423749x2012; fax: +91 40 23073751.

[**] Corresponding author at: Department of Pharmaceutical Analysis, National Institute of Pharmaceutical Education and Research, Balanagar, Hyderabad 500037, India. Tel.: +91 40 27193482; fax: +91 40 27193156.

E-mail addresses: narendra.talluri@gmail.com, narendra@niperhyd.ac.in (M.V.N. Kumar Talluri), srini@iict.res.in (R. Srinivas)

Peer review under responsibility of Xi'an Jiaotong University.

ELSEVIER Production and hosting by Elsevier

2095-1779
http://dx.doi.org/10.1016/j.jpha.2013.04.002

ZTP is 2-((8-chlorodibenzo[b,f]thiepin-10-yl)oxy)-N,N-dimethylethanamine (Fig. 1). It is an atypical antipsychotic drug which is highly effective in acute exacerbation of schizophrenia. It has fewer adverse effects than conventional antipsychotics [9]. Green et al. [10] reviewed research studies on ZTP and its adverse reactions related to metabolic effects and movement disorders. A thorough literature search revealed that few LC and LC–MS methods are available for determination of ZTP in plasma, serum and other biological matrices [11–13]. A liquid chromatography quadrupole time-of-flight mass spectrometry (LC–QTOF–MS) method was reported for the analysis of ZTP in 77 blood samples [14]. Nozaki et al. [15] investigated the electrochemical oxidation behavior of ZTP and its fragmentation using electrospray ionization–mass spectrometry (ESI–MS) coupled with a microflow electrolytic cell. Capillary electrophoresis method was reported for determination of ZTP and its metabolite in human plasma [16]. GC and GC–MS methods were also reported for quantification of ZTP in biofluids [9,17,18]. As there are no reports available on the degradation behavior, identification and characterization of DPs of ZTP formed under various stress conditions, the present work has been undertaken on development of an HPLC-UV stability indicating assay method for separation and determination of ZTP in the presence of DPs and characterization of degradants by using LC–QTOF–MS.

2. Experimental

2.1. Materials and stability equipments

ZTP was obtained as a gift sample by Symed Laboratory Limited, Hyderabad, India. Acetonitrile (HPLC grade) was purchased from Merck (Lichrospher, Darmstadt, Germany). Water was purified by using a Milli-Q Gradient ultrapure water system (Billerica, MA 01821, USA). Analytical reagent grade trifluoroacetic acid (TFA), hydrochloric acid (HCl), sodium hydroxide (NaOH), and hydrogen peroxide (H_2O_2) used in the present study were purchased from S.D Fine chemicals (Mumbai, India). Photo stability studies were carried out in a OSWORLD (model JRIC-11C) photo stability chamber with humidity and temperature control. The chamber was equipped with an illumination bank made of light sources, viz., a cool white fluorescent lamp designed for emitting significant radiation same as that specified in ISO 10977 (1993) 320 nm and a near UV fluorescent lamp with a maximum energy emission between 350 nm and 370 nm [19] for providing an overall illumination of not less than 1.2 million lux hours and irradiation density of not less than 200 W/m^2. Thermal stability studies were performed in a dry air oven (Osworld Scientific Equipments Pvt. Ltd., Mumbai, India).

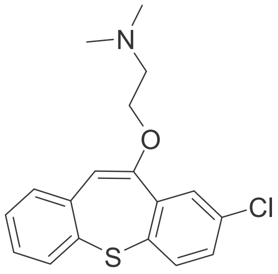


Fig. 1 Chemical structure of zotepine.

2.2. Instrumentation

The analysis was carried out using an Agilent 1200 series HPLC instrument (Agilent Technologies, USA) coupled to a quadrupole time-of-flight (Q-TOF) mass spectrometer (Q-TOF LC/MS 6510 series classic G6510A, Agilent Technologies, USA) equipped with an ESI source. The data acquisition was under the control of Mass Hunter workstation software. The chromatographic data were recorded using a computer system with chemstation data acquiring software. The HPLC-UV data were used for quantitative determination of ZTP and the same chromatographic conditions were used for mass spectral identification and characterization of its degradants.

2.3. LC–MS conditions

The chromatographic separations were carried out on a reversed-phase Phenomenex Luna C18 (250 mm × 4.6 mm i.d.) column with particle size of 5 μm (Phenomenex, Hyderabad, India) and the column was maintained at an ambient temperature (30 °C). HPLC separation was achieved with gradient elution (Table 1) using 0.05% TFA buffer (pH adjusted to 3.0), and acetonitrile as mobile phase. The mobile phase was filtered through 0.45 μm nylon membrane and degassed by using an ultra sonicator before use. The injection volume was 20 μL and the mobile phase flow rate was at 1 mL/min. A splitter was placed before the ESI source, allowing entry of only 35% of the eluent. The typical operating source conditions for MS scan of ZTP in positive ESI mode were optimized as follows: the fragmentor voltage was set at 80 V; the capillary at 3000 V; the skimmer at 60 V; nitrogen was used as the drying (300 °C; 9 L/min) and nebulizing (45 psi) gas. Ultra high pure nitrogen was used as collision gas. All the spectra were recorded under identical experimental conditions, and are an average of 25 scans.

2.4. Stress degradation studies

All stress decomposition studies were performed with an initial drug concentration of 1 mg/mL in methanol and water (8:2). Acid hydrolysis was performed in 1 M HCl at room temperature for 18 h. The study in alkaline condition was carried out in 5 M NaOH at 70 °C for 5 days under reflux. For neutral degradation study, the drug was dissolved in a mixture of methanol and water (8:2) and was heated at 60 °C for 7 days under reflux. Oxidative studies

Table 1 The optimized gradient elution program for ZTP and its DPs.

Time (min)	Mobile phase	
	A (%)	B (%)
0.01	75	25
2.50	50	50
10.0	40	60
12.5	0	100
20.0	0	100
22.5	75	25
30.0	75	25

A: 0.05%TFA (trifluoroacetic acid, pH 3); B: acetonitrile.

Table 2 System suitability data of zotepine and its DPs.

Drug/DPs	Retention time (Rt)	Resolution (Rs)	Tailing factor (T_f)	Capacity factor (k^1)	$[M+H]^+$ ions (m/z)
ZTP	9.5	–	1.20	–	332.0
Z1	9.9	1.95	1.32	0.04	348.0
Z2	14.0	18.0	1.10	0.56	318.0
Z3	18.8	22.0	1.02	1.09	261.0

Table 3 Recovery data of ZTP by RP–HPLC.

Amount added (μg/mL)	Mean of amount found (μg/mL, $n=3$)	Recovery (amount found/added × 100)	SD	RSD (%)
25	25.33	101.33	0.21	0.81
100	99.83	99.83	0.21	0.21
150	150.03	100.02	0.17	0.11
200	199.63	99.82	0.42	0.21
250	250.00	100.00	0.80	0.32

Table 4 HPLC analysis results of zotepine in SIRILEPT® Tablets.

S. no.	Zotepine labeled amount (mg)	Measured[a] amount ±SD (mg)	Mean recovery (%) (RSD%)
1	Batch 1: 50	49.97±0.17	99.93 (0.34)
2	Batch 2: 50	49.23±0.12	98.47 (0.25)
3	Batch 3: 50	50.27±0.17	100.53 (0.34)

[a] Average of three determinations.

were carried out at room temperature in 1% H_2O_2 for 3 h. Degradation was also carried out in solid state by exposing pure drugs to dry heat at 70 °C for 2 weeks, and in photo stability chambers for 6 h. Samples were withdrawn periodically and subjected to immediate analysis after suitable dilution.

2.5. *Sample preparation*

Acid and base hydrolyzed samples were neutralized with NaOH and HCl, respectively. The samples were further diluted to 10 times. Other products of degradation viz., thermal, photolysis, oxidation and neutral hydrolysis were also diluted to 10 times from their initial concentration. All the samples were filtered through 0.22 μm membrane filter before HPLC and LC–MS analysis.

3. Results and discussion

3.1. *HPLC method development and optimization*

For optimization of chromatographic conditions using C_{18} column, various buffers such as KH_2PO_4 (10 mM, pH 3–5) were tried. Since ZTP is a polar compound, it was eluted early in most of the conditions. We have avoided further usage of phosphate buffer as it is non-volatile and unamenable with MS detector. Ammonium acetate buffer (10 mM, pH adjusted to 3.5 with CH_3COOH) with organic modifiers, viz. methanol and acetonitrile (ACN) was tried. It was observed that ACN was found to be better in terms of resolution and peak shapes as compared to methanol. ACN has higher elution strength and the early retention times were achieved with less percentage than methanol. TFA, an ion-pairing reagent as mobile phase additive was used for the separation of ZTP from its degradants on C_{18} column because TFA provides better separation of basic analytes from other components in the sample [20]. TFA can be used as a mobile phase pH stabilizer and as an effective ion-pair reagent to control retention and selective separation of small ionizable compounds by RP–HPLC. Of the various percentages of TFA (0.05%, 1% and 2%), 0.05% was found to be highly suitable for better separation of the chromatographic peaks of all the analytes from each other with symmetrical peak shapes. Further studies were carried out on the pH effect of the buffer (0.05% TFA, pH 3, 4 and 5) on retention times, resolution and peak tailing. The symmetrical peaks were observed at pH 3 with improved resolutions. In addition, the gradient analysis, in which the strength of the mobile phase increased with time during sample elution, was suited for complex samples containing analytes of wide polarities and better separation [21]. At an initial content of 5% of ACN in mobile phase, ZTP was not eluted even after 15 min. When it was increased to 25%, ZTP eluted at 9.5 min. It was further tried to decrease the retention time by increasing ACN percentage but zotepine S-oxide (Z1) was co-eluted with ZTP due to similar polarity. Therefore the initial ACN was kept constant at 25% and the required resolution 1.95 was achieved for Z1. The mobile phase was used at a flow rate of 1 mL/min and the injection volume was 20 μL. The TFA (0.05%, pH 3) and ACN mobile phases were optimized with gradient elution mode since the degradants possessed varying polarities. Thus the peak shapes ($T_f<1.3$) and resolutions (>1.95) were further improved. The method was amenable for high-throughput screening of ZTP and for characterization of its degradants.

Table 5 Intra-day and inter-day data of ZTP.

Assay	Amount added (μg/mL)				
	25.00	100.00	150.00	200.00	250.00
Intra-day assay					
Day 1					
Mean conc. (mg/mL, *n*=3)	25.27	99.87	150.07	199.87	250.27
SD	0.17	0.29	0.12	0.17	0.12
RSD (%)	0.67	0.29	0.08	0.09	0.05
Day 2					
Mean conc. (mg/mL, *n*=3)	25.43	99.87	149.97	199.90	249.60
SD	0.12	0.21	0.26	0.24	0.57
RSD (%)	0.49	0.21	0.18	0.12	0.23
Day 3					
Mean conc. (mg/mL, *n*=3)	25.20	100.20	150.30	200.40	249.60
SD	0.08	0.08	0.16	0.29	0.57
RSD (%)	0.32	0.08	0.11	0.15	0.23
Inter-day assay					
Mean conc. (mg/mL, *n*=3)	25.50	100.50	150.63	200.50	250.63
SD	0.16	0.16	0.25	0.16	0.25
RSD(%)	0.64	0.16	0.17	0.08	0.10

3.2. *Validation*

3.2.1. *Linearity, LOD and LOQ*

Linearity test solutions were prepared from stock solution at eight concentration levels of analyte (0.1, 1, 10, 25, 50, 100, 150, 250 μg/mL). The peak area versus concentration data is performed by least squares linear regression analysis. The calibration curve was drawn by plotting average areas for triplicate injections of ZTP versus the concentrations in μg/mL. Linearity was checked over the same concentration range for three consecutive days. Good linearity was observed in the concentration range from 0.1 to 250 μg/mL of ZTP. The data were subjected to statistical analysis using a linear regression model; the linear regression equation and correlation coefficient (r^2) were $y=56{,}227.6\ (\pm 69.59)x+86{,}403\ (\pm 737.40,\ t_{\text{calculated}}: 0.50<t_{\text{theoretical}}: 3.18)$ and >0.9997, respectively. These results indicate a good linearity. The limits of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield a signal-to-noise ratio of 3 for LOD and 10 for LOQ[22,23]. The LOD and LOQ values were found to be 0.03 and 0.10 μg/mL, respectively.

3.2.2. *Specificity*

Specificity is the ability of the analytical method to measure the analyte concentration accurately in the presence of all potential DPs. The specificity was determined by subjecting ZTP to stress degradation under various stress conditions. All the DPs were well separated with good resolution (Table 2).

3.2.3. *Accuracy (recovery) and precision*

The recovery of ZTP was determined by spiking to the placebo sample at five different concentration levels i.e., 25, 100, 150, 200, 250 μg/mL each in triplicate. The recovery was determined by the following equation: recovery = amount found/amount taken × 100. The percentage recovery range and RSD values were found to be 99.0–101 and <1% respectively (Table 3). This method was applied to ZTP tablets and observed results were comparable with label claim of the formulations with an accuracy range of 98.4–100.5% and RSD values are found to be less than 1.0% (Table 4). The precision in determination of the assay was studied by repeatability, intermediate precision and reproducibility (ruggedness). Repeatability is the intra-day variation in assay obtained at different concentration levels of ZTP expressed in terms of RSD calculated for each day. Values were found to be below 1.0% indicating a good repeatability (Table 5). The intermediate precision is the inter-day variation at the same concentration level determined on successive days. The inter-day variations calculated for five concentrations levels from above data of 3 days are expressed in terms of %RSD values. At each concentration level, the RSD values were below 1.0%, indicating a good intermediate precision. The ruggedness of the method is defined as the degree of reproducibility obtained by analysis of the same sample under a variety of conditions at different labs, with different analysts, instruments and lots of reagents. The same samples of three concentrations were analyzed in triplicate on 2 days by another instrument (LC-20A Module HPLC, Shimadzu system containing two pumps and a UV detector) by a different analyst with different lots of reagents and columns. The data obtained were within 1% RSD.

3.3. *Degradation of ZTP*

The degradation behavior of ZTP under various stress conditions was investigated using HPLC. Typical chromatograms are shown in Fig. 2. Accurate knowledge regarding the relative response factors (RRF) of the degradants is vital for peak area mass-balancing during stability studies. A recent study on the degradation of β-artemether [24] reported a new approach for pharmaceutical stress conditions by using Matlab software mathematical model. This approach provided a good model fit with good correlation between the calculated and experimental residual mass of β-artemether. This approach may be ideal to calculate the RRF of degradants for mass balancing during stability studies of pharmaceuticals like β-artemether. However, the present study is not focused on the RRF calculations for mass balance of the ZTP stability study.

3.3.1. *Hydrolysis*

Initially ZTP was refluxed in 0.01 and 0.1 M HCl for 1 week, but no degradation was observed. When the strength of the acid was increased to 1 M HCl, 30% degradation was observed in 24 h. One degradation product was formed on acid hydrolysis, which was coded

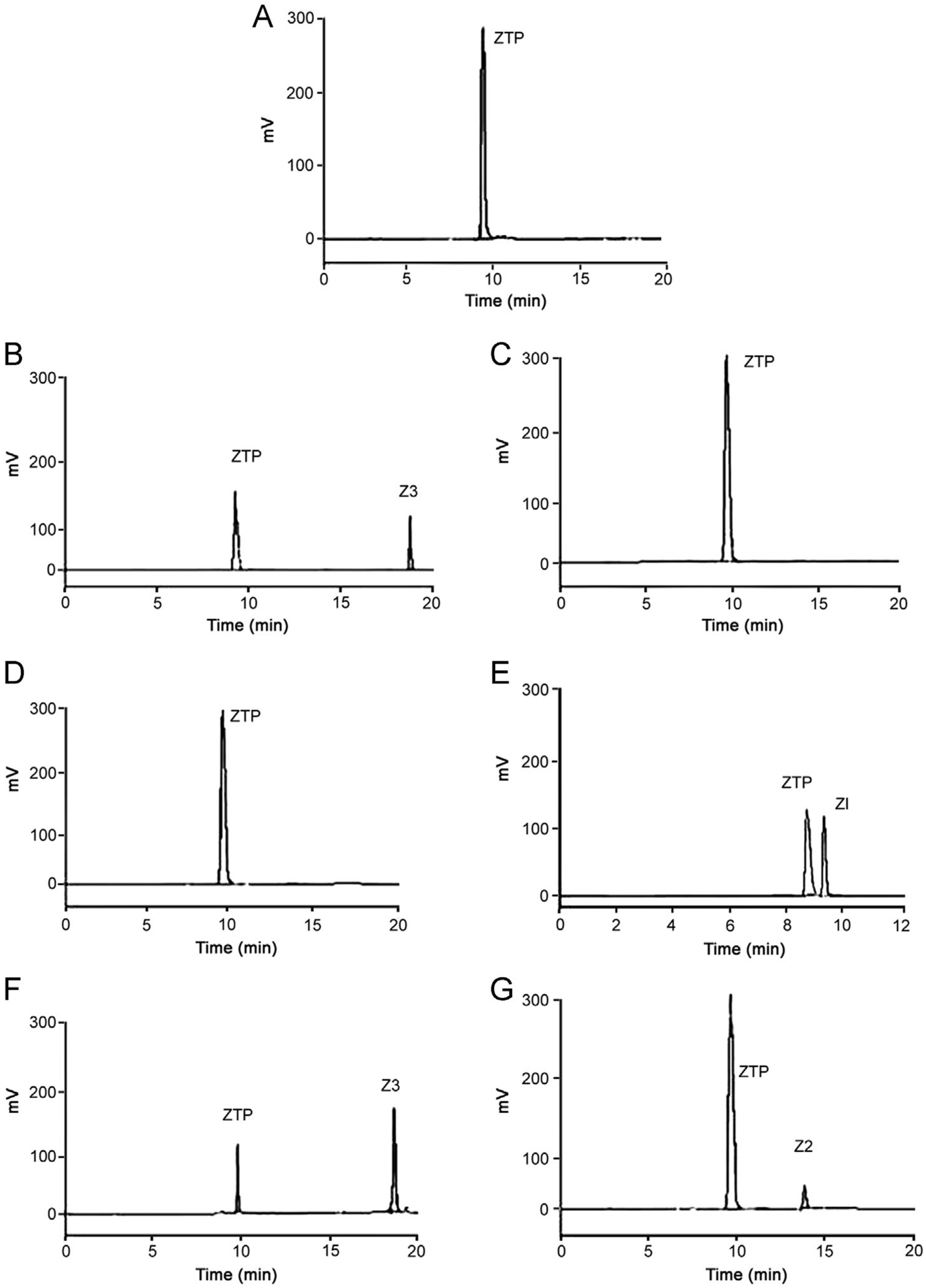


Fig. 2 Typical HPLC chromatograms of (A) ZTP std, (B) acid hydrolysis, (C) base hydrolysis, (D) neutral hydrolysis, (E) oxidation, (F) photolytic and (G) thermal conditions.

as Z3 and eluted with Rt of 18.8 min. No degradation occurred for 7 days in neutral condition upon heating the sample solution in methanol and water (80:20) at 60 °C. ZTP was refluxed in (1–5 M) NaOH at 60 °C for 7 days. But no degradation was observed and the drug was found to be highly stable in both neutral and alkaline hydrolysis.

3.3.2. Oxidative conditions

The drug was found to be highly labile in oxidative degradation conditions. The reaction in 3% hydrogen peroxide at room temperature was so fast that within 45 min the drug degraded to the extent of 23.2% and 100% in 4 h. Subsequently, studies were performed in low concentration. With 1% hydrogen peroxide solution at room temperature, degradation was observed to the extent of 5.8% and 10% in 1 h and 3 h, respectively and the degradation product (DP) was coded as Z1 and eluted at 9.9 min.

3.3.3. Photolytic conditions

The solid form of the drug was stable on exposure to a cool white fluorescent lamp emitting significant radiation below 320 nm and 0.1% degradation was observed after 72 h exposure. However, 25% degradation was observed in solution form within 3 h, and 2.3% degradation was observed in 8 h when it was exposed to direct sunlight and the DP (Z3) was eluted at 18.8 min.

3.3.4. Thermal conditions

The drug was found to be stable under thermal degradation conditions. Degradation was carried out in solid form by exposing

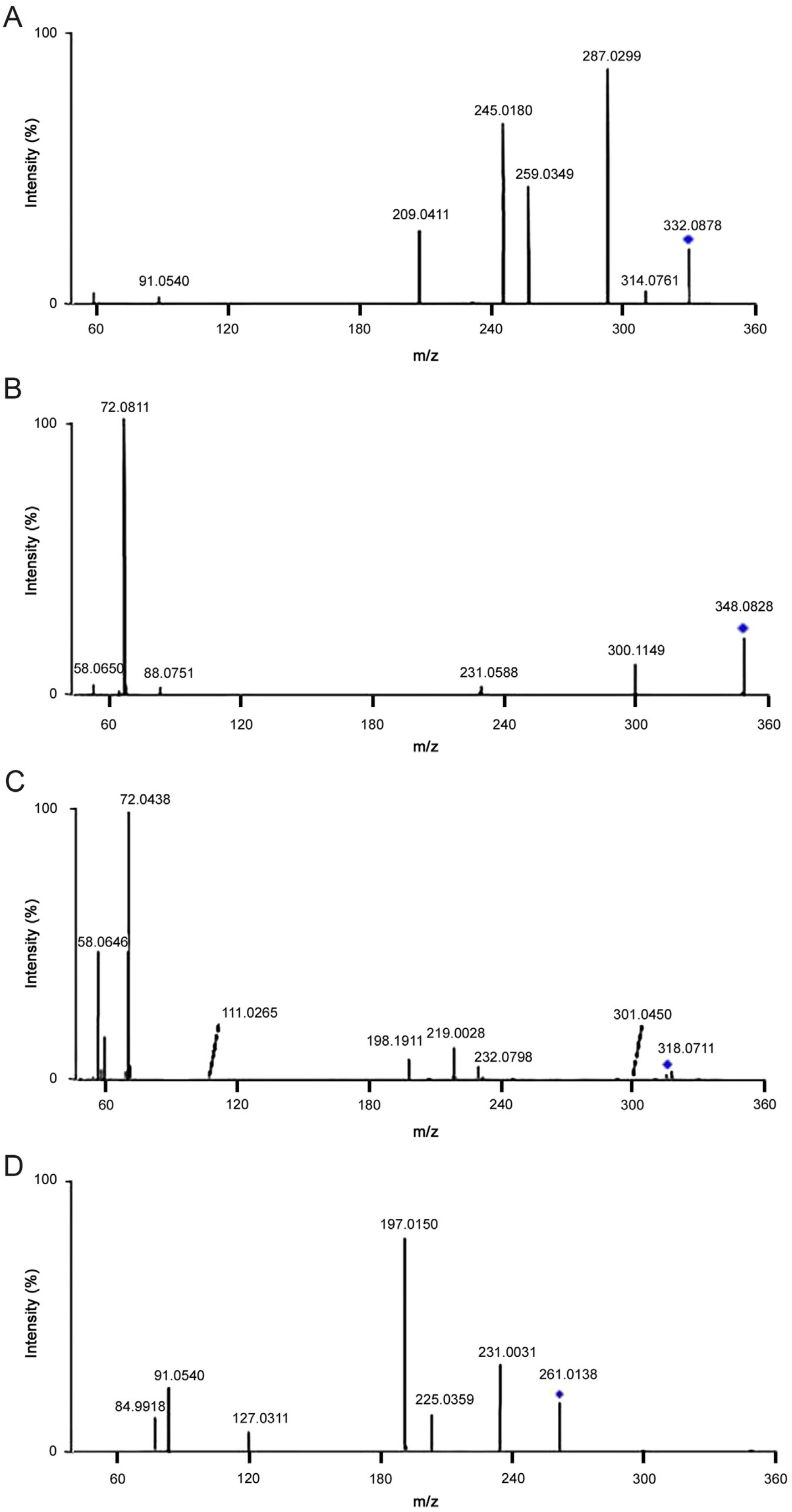


Fig. 3 LC–ESI–MS/MS spectra of (A) [M+H]$^+$ ions (*m/z* 332) of **ZTP** at 15 eV, (B) [M+H]$^+$ ions (*m/z* 348) of **Z1** at 15 eV, (**C**) [M+H]$^+$ ions (*m/z* 318) of **Z2** at 15 eV and (**D**) [M+H]$^+$ ions (*m/z* 261) of **Z3** at 15 eV.

Table 6 Elemental compositions for protonated **ZTP**, degradation products **Z1–Z3** and their product ions.

Drug/degradant	Molecular formula	Observed mass (Da)	Calculated mass (Da)	Error (ppm)	Neutral loss	Rt (min)
ZTP ($[M+H]^+$)	$C_{18}H_{19}ClNOS^+$	332.0878	332.0870	−1.81	–	9.5
	$C_{18}H_{17}ClNS^+$	314.0761	314.0765	2.31	H_2O	
	$C_{16}H_{12}ClOS^+$	287.0299	287.0292	−4.81	C_2H_7N	
Product ions	$C_{15}H_{12}ClS^+$	259.0349	259.0343	−3.16	CO	
	$C_{14}H_{10}ClS^+$	245.0180	245.0186	2.62	C_2H_2O	
	$C_{14}H_9S^+$	209.0411	209.0419	3.61	HCl	
Z1 ($[M+H]^+$)	$C_{18}H_{19}ClNO_2S^+$	348.0828	348.0820	−2.61	–	9.9
	$C_{18}H_{19}ClNO^+$	300.1149	300.1150	1.16	SO	
	$C_{14}H_{12}ClO^+$	231.0588	231.0571	−4.61	C_4H_7N	
Product ions	$C_4H_{10}ON^+$	88.0751	88.0757	2.16	$C_{14}H_9OSCl$	
	$C_4H_{10}N^+$	72.0811	72.0808	−3.12	$C_{14}H_9ClO_2S$	
	$C_3H_8N^+$	58.0650	58.0651	0.98	$C_{15}H_{11}ClO_2S$	
Z2 ($[M+H]^+$)	$C_{17}H_{17}ClNOS^+$	318.0711	318.0714	2.61	–	14.0
	$C_{17}H_{14}ClOS^+$	301.0450	301.0448	−2.68	NH_3	
	$C_{13}H_{14}NOS^+$	232.0798	232.0791	−3.16	C_4H_3Cl	
	$C_{12}H_8ClS^+$	219.0028	219.0030	3.26	C_5H_6NO	
Product ions	$C_{13}H_{12}NO^+$	198.1911	198.0913	1.18	H_2S	
	$C_6H_6S^+$	111.0625	110.185	2.61	$C_{11}H_{10}NOCl$	
	$C_3H_6NO^+$	72.0438	72.0444	4.98	$C_{14}H_{11}ClS$	
	$C_3H_8N^+$	58.0646	58.0651	3.16	$C_{14}H_9ClOS$	
Z3 ($[M+H]^+$)	$C_{14}H_{10}ClOS^+$	261.0138	261.0135	−2.61	–	18.8
	$C_{13}H_8ClS^+$	231.0031	231.0030	−1.01	CH_2O	
	$C_{14}H_9OS^+$	225.0359	225.0369	4.16	HCl	
Product ions	$C_{13}H_6Cl^+$	197.0150	197.0153	1.16	H_2S	
	$C_7H_8Cl^+$	127.0311	127.0309	−3.16	C_7H_2OS	
	$C_7H_7^+$	91.0540	91.0542	1.16	C_7H_3ClOS	
	$C_4H_2Cl^+$	84.9918	84.9840	−6.16	$C_{10}H_8OS$	

the pure drug to dry heat at 70 °C for 24 h but no degradation was observed. The drug was degraded at 70 °C temperature in 2 weeks time and only 3% thermal DP (**Z2**) was eluted at 14.0 min.

3.4. *Assay of zotepine in tablet formulations*

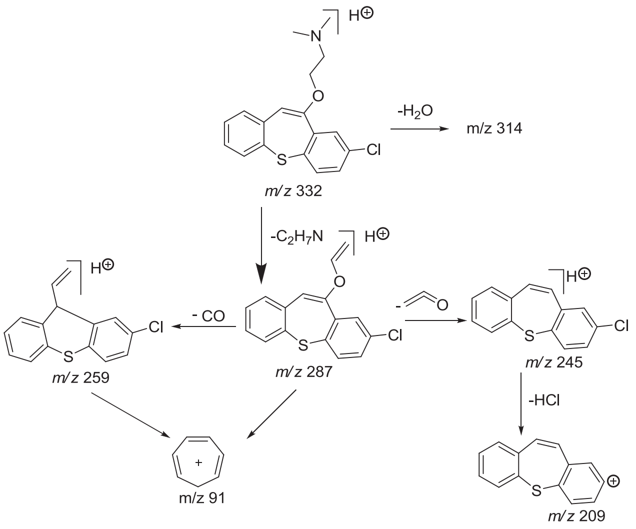
Twenty tablets are weighed and crushed in motor, the powder equivalent to 50 mg of ZTP was weighed and dissolved in MeOH: water (50:50) then it was sonicated, filtered and two series of dilutions are made in mobile phase to get a concentration of 100 μg/mL. These samples were subjected to HPLC analysis and the measured values were compared to those of labels (Table 4).

3.5. *Characterization of ZTP and its degradation products by LC–MS/MS*

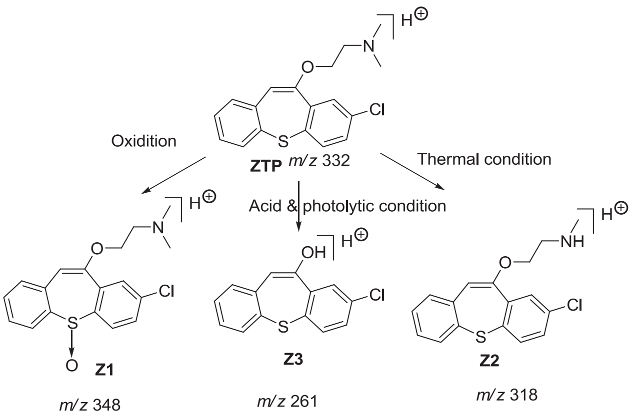
ZTP and other tricyclic class of pharmaceutical compounds have long been used for the treatment of mental disorders such as schizophrenia. Chlorpromazine and trimipramine contains carbon chains ending with a tertiary nitrogen atom, –(N–CH_2–CH_2–CH_2–N $(CH_3)_2$) and –(N–CH_2–$CHCH_3$)–CH_2–N$(CH_3)_2$), respectively. Smyth et al. [25] have established rules of fragmentation for such drugs with a carbon chain ending in a tertiary nitrogen atom with at least two methylene groups. These drugs eliminate the end nitrogen atom as the corresponding secondary amine in both in-source fragmentation (MS) and MS^2 mode. The deaminated ions lose the corresponding alkene from these two methylenes or substituted methylene groups. Chlorpromazine and Lignocaine yield a characteristic peak at *m/z* 86 due to $CH_2{=}CH$–$CH_2NH^+(CH_3)_2$ and $CH_2{=}N^+(C_2H_5)_2$, respectively. Flurazepam with side chain –N–CH_2–CH_2–N $(C_2H_5)_2$ yields a peak at *m/z* 73 due to loss of –NH $(C_2H_5)_2$ from the end-of chain. The phenothiazine and promethazine, with side chain –N–CH_2–CH (CH_3)–N$(CH_3)_2$, fragments at the C–C bond yielding a base peak at *m/z* 72 due to $CH_3CH{=}N^+(CH_3)_2$. Chlorpromazine and trimeprazine also yield a signal at *m/z* 45 due to loss of $(CH_3)_2NH$ from the end of chain. Promazine, with side chain –N–CH_2–CH_2–CH_2–N $(CH_3)_2$, fragments extensively to yield major signals at *m/z* 86 and 58 (base peak) [26]. These diagnostic peaks of low *m/z* values are observed in the EI-MS of phenothiazines, acepromzine and propionylpromazine which contain side chains with an amino group [27]. The electrospray ionization of pharmaceutically important 1, 4-benzodiazepines and their subsequent fragmentations were reported [28]. 1, 4-benzodiazepines generally eliminate 29u and 28u due to loss of COH and CO groups, respectively. This involves, ring contraction of seven membered heterocyclic rings to six membered rings [29]. The $[M+H]^+$ of amphetamine loses NH_3 by charge site initiated fragmentation with H atom transfer resulting in the corresponding alkene [30]. These studies on ESI-MS behavior of structurally similar drugs were helpful for interpretation of proposed structure of ZTP degradants.

3.5.1. *MS/MS collision-induced dissociation (CID) of ZTP*

The positive ion ESI-MS of ZTP shows a highly abundant $[M+H]^+$ and low abundance $[M+Na]^+$ ions. The CID spectrum (Fig. 3A) of protonated ZTP yields abundant product ions at *m/z* 287 (loss of N,N-dimethyl amine), *m/z* 259 (loss of CO from *m/z* 287), *m/z* 245 (loss of ketene from *m/z* 287) (base peak), *m/z* 209 (loss of HCl from *m/z* 245) and a low abundance ion at *m/z* 314



Scheme 1 Proposed fragmentation mechanism for protonated **ZTP** (*m/z* 332).



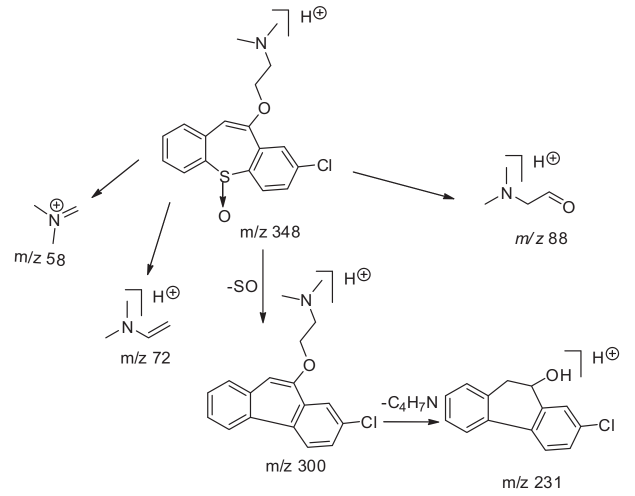
Scheme 2 Proposed structures of DPs (**Z1–Z3**) formed under various stress conditions.

(loss of H_2O) (Scheme 1). All these fragmentation pathways have been confirmed by accurate mass measurements (Table 6).

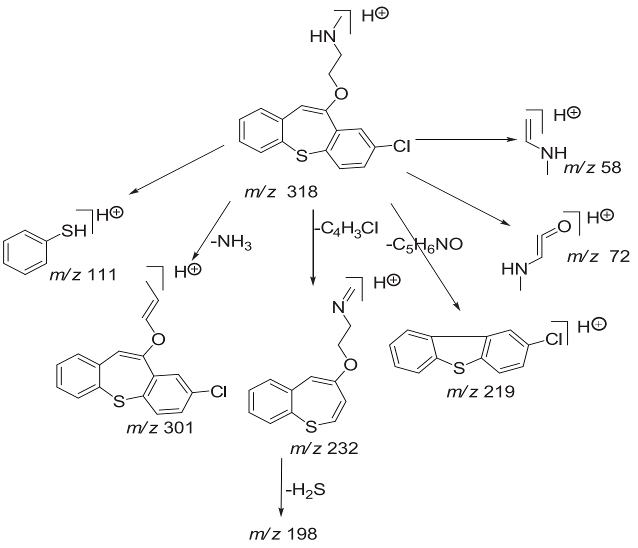
3.5.2. *MS/MS CID of degradation products (DPs)*

The three DPs, **Z1–Z3** were eluted within 20 min on C-18 column. The proposed structures of all the DPs with retention times are shown in Scheme 2 and Table 2. These structures have been further supported by LC-ESI-MS/MS experiments with accurate mass measurements of all the protonated DPs and their fragment ions (Table 6).

3.5.2.1. Z1 ($[M+H]^+$, m/z 348). The LC-ESI-MS/MS spectrum of $[M+H]^+$ions (*m/z* 348) of **Z1** (Rt = 9.9 min, oxidative degradation product), shows product ions at *m/z* 300 (loss of SO), *m/z* 231 (loss of N,N-dimethyl ethyl amine from *m/z* 300), *m/z* 88 (protonated 2-(dimethyl amino) acetaldehyde), *m/z* 72 (protonated N,N-dimethylethenamine) and *m/z* 58 (N-methyl-N-methylene-methanaminium) (Scheme 3, Fig. 3B). All these fragmentation pathways have been confirmed by accurate mass measurements (Table 6). As it can be seen from Scheme 3, the fragmentation pattern of protonated **Z1** is highly consistent with its proposed structure, 2-(8-chlorobenzo[b][1]benzothiepin-6-yl)oxy-N,N-dimethyl ethanamine-s-oxide. Zotepine-N-oxide [31] was not observed under optimized stress conditions and it may be formed during long storage conditions of liquid formulations [32].

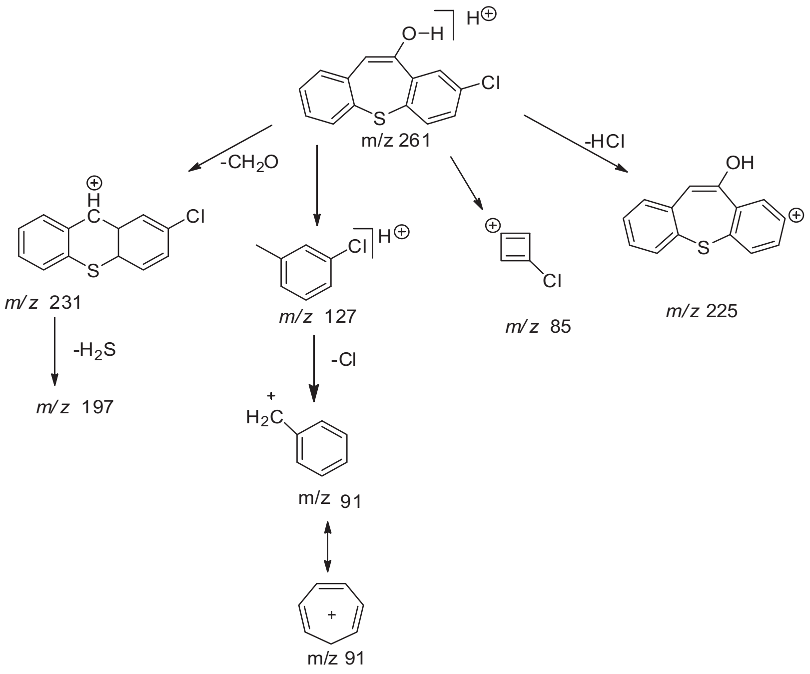


Scheme 3 Proposed fragmentation mechanism for degradation product **Z1**(*m/z* 348).



Scheme 4 Proposed fragmentation mechanism for degradation product **Z2** (*m/z* 318).

3.5.2.2. Z2 ($[M+H]^+$, m/z 318). The LC-ESI-MS/MS spectrum of $[M+H]^+$ions (*m/z* 318) of **Z2** (Rt = 14.0 min), which was observed under thermal degradation conditions, shows product ions at *m/z* 301 (loss of NH_3), *m/z* 232 (loss of C_4H_3Cl), *m/z* 198 (loss of H_2S from *m/z* 232), *m/z* 219 (loss of C_5H_9NO from *m/z* 318), *m/z* 72 (protonated 2-(methyl amino) ethenone), *m/z* 58 (protonated N-methyl ethenamine) (Fig. 3C). The fragmentation pathways have been confirmed by accurate mass measurements (Scheme 4 and Table 6). Based on MS/MS and accurate mass measurements, **Z2** was identified as 2-(8-chlorobenzo[b][1]benzothiepin-6-yl)oxy-N-methyl ethanamine cation.



Scheme 5 Proposed fragmentation mechanism for degradation product **Z3** (*m/z* 261).

3.5.2.3. Z3 ([M+H]$^+$, m/z 261). Fig. 3D shows the LC–ESI–MS/MS spectrum of [M+H]$^+$ions (*m/z* 261) of **Z3** (Rt 18.8 min), which was observed under acidic and photolytic conditions. The spectrum displays abundant product ions at *m/z* 231 (loss of HCHO), *m/z* 197 (loss of H_2S from *m/z* 231) and low abundance ions at *m/z* 225 (loss of HCl from *m/z* 261), *m/z* 127 (protonated 1-chloro-3-methylbenzene), *m/z* 91 ($C_7H_7^+$), and *m/z* 85 (2-chlorocyclobuta-1, 3-dien-1-ylium) (Scheme 5). The MS/MS experiments combined with accurate mass measurements (Table 6) have confirmed the proposed structures. All these data are highly compatible with the proposed structure (8-chlorodibenzo [b,f] thiepin-10-yl) oxonium for **Z3**.

4. Conclusions

A validated LC–MS/MS method for stability indicating assay of ZTP was developed. The degradation behavior of the drug was investigated under hydrolysis (acid, base and neutral), oxidation, photolysis and thermal stress conditions. The drug was found to be stable in basic, neutral conditions and unstable in oxidative conditions. The DPs were identified by [M+H]$^+$ ion and the proposed structures were supported by LC–MS/MS experiments combined with accurate mass measurements. The RP–HPLC method was validated as per ICH guidelines and finally applied to the marketed formulations.

Acknowledgments

The authors wish to thank Dr. Ahmed Kamal, Director, National Institute of Pharmaceutical Education and Research, Hyderabad for encouragement to communicate results. Mr. Naveen Reddy. Kandimalla thanks the Department of Pharmaceuticals (Ministry of chemicals and Fertilizers), New Delhi for grant of a MS (Pharma) fellowship.

References

[1] ICH, Stability Testing of New Drug Substances and Products Q1A (R2), in: Proceedings of the International Conference on Harmonization, IFPMA, Geneva, 2003.
[2] WHO, Draft Stability Testing of Active Pharmaceutical Ingredients and Pharmaceutical Products, World Health Organization, Geneva, 2007.
[3] CPMP, Note for Guidance on Stability Testing: Stability Testing of Existing Active Substances and Related Finished Products, Committee for Proprietary Medicinal Products, EMEA, London, 2002.
[4] TPD, Guidance for Industry Stability Testing of Existing Drug Substances and Products, Therapeutic Products Directorate, Health Canada, Ottawa, ON, 2003.
[5] S. Singh, M. Bakshi, Guidance on conduct of stress tests to determine inherent stability of drugs, Pharm. Tech. On-line 24 (2000) 1–14http://xa.yimg.com/kq/groups/2299115/609207339/name/Guidance_Stress_Tests.pdf.
[6] M. Bakshi, S. Singh, Development of validated stability-indicating assay methods-critical review, J. Pharm. Biomed. Anal. 28 (2002) 1011–1040.
[7] N.W Ali, S.S. Abbas, H-S. Zaaza, Validated stability indicating methods for determination of nitazoxanide in presence of its degradation products, J. Pharm. Anal 2 (2012) 105–116.
[8] S.M. Pawar, L.D. Khatal, S.Y Gabhe, et al., LC–UV and LC–MS evaluation of stress degradation behavior of desvenlafaxine, J. Pharm. Anal 2 (2011) 264–271.
[9] S Ulrich, F.P Meyer, B. Bogerts, A capillary gas-liquid chromatographic method for the assay of the neuroleptic drug zotepine in human serum or plasma, J. Pharm. Biomed. Anal 14 (1996) 441–449.
[10] B. Green, Zotepine: a clinical review, Expert Opin. Drug Metab. Toxicol. 5 (2009) 181–186.
[11] C. Kratzsch, F.T. Peters, T. Kraemer, et al., Screening, library-assisted identification and validated quantification of fifteen neuroleptics and three of their metabolites in plasma by liquid chromatography/mass spectrometry with atmospheric pressure chemical ionization, J. Mass Spectrom. 38 (2003) 283–295.
[12] H. Kirchherr, W.N. Kuhn-velten, Quantitative determination of forty-eight antidepressants and antipsychotics in human serum by HPLC

tandem mass spectrometry: a multi-level, single-sample approach, J. Chromatogr. B 843 (2006) 100–113.

[13] K. Nozaki, I. Osaka, H. Kawasaki, et al., Application of on-line electrochemistry/electrospray/tandem mass spectrometry to a quantification method for the antipsychotic drug zotepine in human serum, Anal. Sci. 25 (2009) 1197–1201.

[14] S. Broecker, F. Pragst, A. Bakdash, et al., Combined use of liquid chromatography-hybrid quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) and high performance liquid chromatography with photodiode array detector (HPLC-DAD) in systematic toxicological analysis, Forensic Sci. Int. 212 (2011) 215–226.

[15] K. Nozaki, H. Kitagawa, S. Kimura, et al., Investigation of the electrochemical oxidation products of zotepine and their fragmentation using on-line electrochemistry/electrospray ionization mass spectrometry, J. Mass Spectrom. 41 (2006) 606–612.

[16] Yu-Wei Chou, Wei-Shan Huang, Trace analysis of zotepine and its active metabolite in plasma by capillary electrophoresis with solid phase extraction and head-column field-amplified sample stacking, J. Chromatogr. A 1087 (2005) 189–196.

[17] O. Tanaka, T. Kondo, S. Kaneko, et al., A method for rapid determination of zotepine by gas chromatography–mass spectrometry, Ther. Drug Monit. 18 (1996) 294–296.

[18] J. Bickeboeller-Friedrich, H.H. Maurer, Screening for detection of new antidepressants, neuroleptics, hypnotics, and their metabolites in urine by GC–MS developed using rat liver microsomes, Ther. Drug Monit. 23 (2001) 61–70.

[19] ICH, Photostability testing of new drug substances and products Q1B, in: Proceedings of the International Conference on Harmonization, IFPMA, Geneva, 1996.

[20] B. Cai, J. Li, Evaluation of trifluoroacetic acid as an ion-pair reagent in the separation of small ionizable molecules by reversed-phase liquid chromatography, Anal. Chim. Acta 399 (1999) 249–258.

[21] R.M. Ornaf, M.W.Dong, Key concepts of HPLC in pharmaceutical analysis, in: S. Ahuja, M.W. Dong (Eds.), Handbook of Pharmaceutical Analysis by HPLC, vol. 6, Elsevier Ltd., Oxford OX5 1GB, UK, 2005, pp. 38–39.

[22] ICH, Validation of analytical procedures: text and methodology Q2 (R1), in: Proceedings of the International Conference on Harmonization, IFPMA, Geneva, 2005.

[23] M.V.N.K. Talluri, A. Kalyankar, S. Ragampeta, Synchronized separation of atorvastatin—an antihyperlipidemic drug with antihypertensive, antidiabetic, antithrombotic drugs by RP-LC for determination in combined formulations, J. Pharm. Anal. 2 (2012) 285–292.

[24] B.M.J. De Spiegeleer, M. D'Hondt, E. Vangheluwe, et al., Relative response factor determination of β-artemether degradants by a dry heat stress approach, J. Pharm. Biomed. Anal. 70 (2012) 111–116.

[25] W.F. Smyth, Recent studies on the electrospray ionisation mass spectrometric behavior of selected nitrogen-containing drug molecules and its application to drug analysis using liquid chromatography–electrospray ionisation mass spectrometry, J. Chromatogr. B 824 (2005) 1–20.

[26] W.F. Smyth, Electrospray ionisation mass spectrometric behavior of selected drugs and their metabolites, Anal. Chim. Acta 492 (2003) 1–16.

[27] H.J. Schulz, Analysis of Pharmaceutically Relevant Compounds using GC/MSD-EI/PCI/NCI, Agilent Technologies, Inc., USA, 2002.

[28] W.F. Smyth, S. McClean, V.N. Ramachandran, A study of the electrospray ionisation of pharmacologically significant 1,4-benzodiazepines and their subsequent fragmentation using an ion-trap mass spectrometer, Rapid Commun. Mass Spectrom. 15 (2000) 2061–2069.

[29] A.P. Bruins, Mechanistic aspects of electrospray ionization, J. Chromatogr. A. 794 (1998) 345–357.

[30] W.F. Smyth, C. Joyce, V.N. Ramachandran, et al., Characterisation of selected hypnoticdrugs and their metabolites using electrospray ionisation with ion trapmass spectrometry and with quadrupole time-of-flight mass spectrometry and their determination by liquid chromatography-electrospray ionisation–ion trap mass spectrometry, Anal. Chim. Acta 506 (2004) 203–214.

[31] Z. Tozuka, H. Kaneko, T. Shiraga, Strategy for structure elucidation of drug metabolites derived from protonated molecules and $(MS)^n$ fragmentation of zotepine, tiaramide and their metabolites, Drug Metab. Pharmacokinet. 17 (2002) 316–339.

[32] M.A. Dyer, A. Smith, K. Aktiengesellschaft, Liquid Pharmaceutical Formulation Containing Zotepine, EP1054666 B1, November 20, 2002.