Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 1274–1279
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

LC–UV and LC–MS evaluation of stress degradation behaviour of avizafone

D. Breton [a,*], D. Buret [a], A.C. Mendes-Oustric [a], P. Chaimbault [b], M. Lafosse [b], P. Clair [a]

[a] *Pharmacie Centrale des Armées, Département Laboratoire, BP 04, 45998 Orléans Armées, France*
[b] *Institut de Chimie Organique et Analytique, Université d'Orléans, BP 6759, 45067 Orléans Cedex 2, France*

Received 16 January 2006; received in revised form 18 March 2006; accepted 23 March 2006
Available online 27 April 2006

Abstract

It has been known for many years that benzodiazepine compounds effectively antagonize seizures induced by organophosphorous nerve agents. In the event of poisoning, a combination of three drugs is commonly used: an anticholinergic drug (e.g. atropine), an oxime used as cholinesterase reactivator (e.g. pralidoxime or HI-6) and an anticonvulsant (i.e. benzodiazepine). Most of anticholinergics and oximes are freely soluble in water, whereas many benzodiazepines are not. However, a water-soluble prodrug form of diazepam, avizafone, has been adopted by French armed forces for the immediate treatment of nerve agent seizure.

The degradation behaviour of this new drug was investigated under different stress degradation conditions (hydrolytic, oxidative, photolytic and thermal) as recommended by International Conference on Harmonization. Successful separation of the active pharmaceutical ingredient from decomposition products formed under stress conditions was achieved using liquid chromatography. The method was validated with respect to specificity, linearity, precision and accuracy.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Avizafone; Degradation; Stress studies; Liquid chromatography

1. Introduction

Organophosphate (OP) nerve agents (sarin, soman, tabun) are extremely toxic chemicals that were first developed by German chemist Gerhard Schrader before and during World War II. They pose potential neurotoxic exposure to both military and civilian population, as evidenced in armed conflict or terrorist attack.

Exposure to organophosphorous cholinesterase inhibitors causes a progression of toxic signs and symptoms, including hypersecretion, fasciculations, tremors, convulsions, coma, respiratory distress and should eventually lead to death. These toxic effects are due to hyperactivity of the cholinergic system as a result of inhibition of cholinesterase and the subsequent increase of the neurotransmitter acetylcholine at central and peripheral sites.

Benzodiazepines are effective against OP-induced symptoms, with strong synergistic effects when combined with cholinesterase reactivators and anticholinergic drugs [1]. Different benzodiazepines have been tested and a pronounced activity of diazepam was demonstrated [2]. Thus, diazepam has been recommended for standard treatment therapy of convulsions caused by nerve agents, along with cholinesterase reactivators (pralidoxime, HI-6) [3] and atropine that blocks muscarinic receptor overstimulation and reduces parasympathetic overstimulation [4].

Three-drug regimens are currently packaged in a single auto-injectors which must be used intramuscularly. However, diazepam is not water soluble, whereas cholinesterase reactivators and atropine are. This lack of solubility limits the pharmacological potency of diazepam for i.m. injection [5] and requires incorporation of organic solvent in the triple drug injectable formulation.

Therefore, a water-soluble prodrug of diazepam, avizafone (Fig. 1), was developed as a component of an aqueous drug mixture with atropine and a cholinesterase reactivator. Avizafone is effective in arresting soman-induced seizures but needs higher concentration than diazepam to reach the same plasma concentration [6].

To our knowledge, no study related to the stability-indicating behaviour of avizafone has been reported in the literature. Nonetheless, one of the International Conference on Harmonization (ICH) guidelines requires stress testing to be carried out

* Corresponding author. Tel.: +33 2 38607321; fax: +33 2 38607324.
E-mail address: dbreton.pca@noos.fr (D. Breton).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.03.025

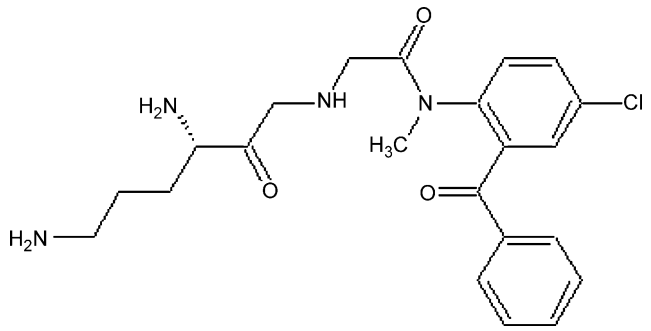

Fig. 1. Structure of avizafone.

to elucidate inherent stability characteristics of the active substance [7]. Susceptibility to hydrolysis, oxidation, light and heat are required tests. An ideal stability-indicating method should be useful to quantify the standard drug alone and also resolve its degradation products.

The aim of the present work was to perform stress studies on the drug in order to evaluate its intrinsic stability and to develop a validated liquid chromatography (LC) method.

2. Experimental

2.1. Materials and methods

Avizafone hydrochloride was purchased from NeoMPS (Strasbourg, France). Diazepam was obtained from Sigma–Aldrich (Saint Quentin Fallavier, France). 2-Amino-5-cholobenzophenone (ACB), 2-methylamino-5-chlorobenzophenone (MACB) and 3-amino-6-chloro-1-methyl-4-phenyl-2(1H)-quinolinone (MPQ) were procured from Promochem (Molsheim, France).

LC gradient-grade acetonitrile and methanol were obtained from VWR International (Fontenay sous Bois, France). Trifluoroacetic acid (TFAA) and ammonium acetate were supplied by Sigma–Aldrich (Saint Quentin Fallavier, France). Water was used after purification by Milli Q System (Millipore, Saint Quentin en Yvelines, France).

2.2. Instrumentation

The analytical system consisted of a Series 1100 LC–MS (Agilent Technologies, Massy, France) equipped with a vacuum degasser, a binary pump, an autosampler and a thermostated column compartment. Detection was performed using a Series 1100 DAD and a mass detector. The mass detector was a simple quadrupole mass spectrometer equipped with an electrospray ionisation (ESI) interface.

Chromatographic separation was achieved on a cyano-column (Phenomenex LUNA CN, 3 μm particle size). Analyses were performed at a flow rate of 300 μL min^{-1}. The column temperature was set at 35 °C. The injection volume was typically 20 μL. UV detection was carried out at 254 nm (DAD spectra 190–700 nm). MS spectra were obtained in the positive ion mode in the scan range between 100 and 700 Th.

The photochemical stability was carried out using the continuous irradiation source (300–800 nm) of a Suntest CPS+ (Atlas, Moussy le Neuf, France). This apparatus was equipped with a 1.1 kW xenon lamp with a minimum and a maximum effect of 250 and 765 W m^{-2}, respectively. The samples in the Suntest were exposed in borosilicate Petrie dishes for bulk powder or borosilicate vials for solutions.

2.3. Method development

2.3.1. Chromatographic conditions

To our knowledge, no method has been published on the separation of avizafone and its degradation products. Indeed, one of the first steps of degradation for this compound should be diazepam. Few publications deal with the decomposition of diazepam [8–10]. Some methods were developed for these studies [11–13], but none have been associated with mass spectrometry. Numerous LC–MS methods have been reported previously concerning diazepam and its metabolite (desmethyldiazepam) in biological samples [14–16].

Harsh condition studies on diazepam showed that this benzodiazepine is hydrolysed to many degradation compounds [10]. At least three of them appear when this active pharmaceutical ingredient is exposed to intense light (Fig. 2). There are two main degradation products: MACB, which is a yellow product and MPQ. MACB may degrade further, giving rise to ACB. MPQ and ACB make their appearance in particular stress conditions [10].

In acidic solution, avizafone has two positive charges in it structure. Thus the hydrophilic part of the molecule leads to a shorter retention time when using an apolar column (C18). On the other hand, MACB, a degradation product of diazepam is really apolar (Log $P = 4.33$). Therefore, moderately polar stationary phase (CN) was preferred, and short analysis time was obtained by using a column of 2 mm internal diameter.

For use in mass spectrometry, volatile buffer composed of TFAA (10 mM) and ammonium acetate (5 mM) was employed in which ammonia was used to set the pH to the correct value (pH 4.7). Two organic modifiers were required to achieve a good resolution between the five compounds in a single run. Without methanol, some analytes were not separated (ACB and MACB). But lack of acetonitrile was associated with deformation in peaks, especially of avizafone. Thus mobile phase was composed of three parts: aqueous solution (70%), methanol (10%) and acetonitrile (20%). Columns of lenghts between 20 and 150 mm were tested during the development. A length of 50 mm allowed a good resolution within 15 min. A diode array detector operated at 254 nm was used to quantify all known molecules.

2.3.2. Mass detection

The single quadrupole spectrometer gave molecular weights of potential unknown degradation products. For the purpose, ESI source parameters were optimised to reduce the background and to minimized fragmentation reactions. After the optimisation of the LC–MS system, ionisation of analytes was carried out using different settings. MACB and ACB showed a poor detection, probably linked with their high apolarity. On the other hand, diazepam and MPQ were well detected. The molecular mass

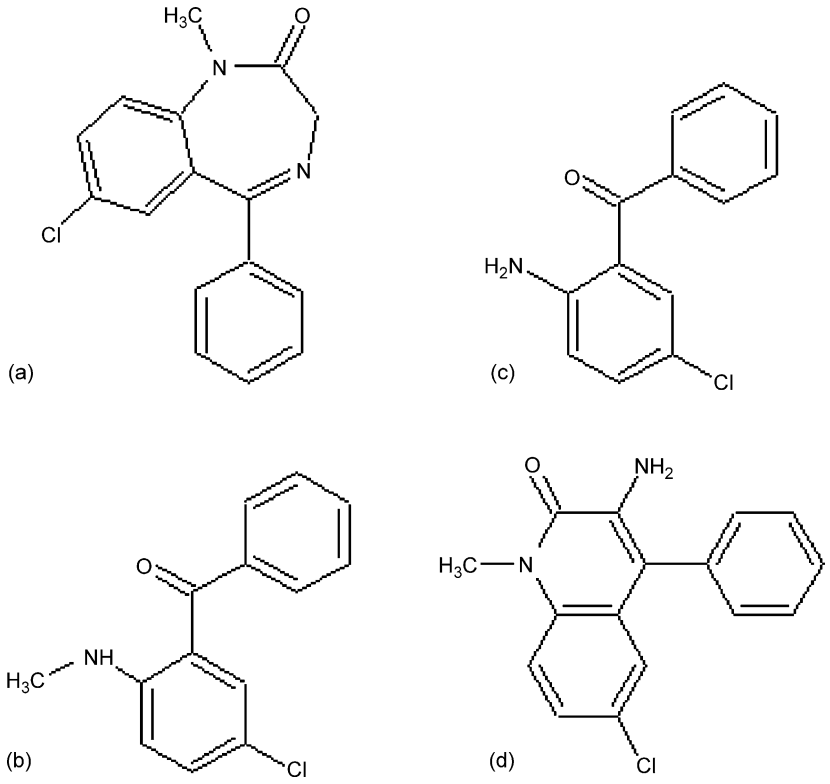

Fig. 2. Structure of diazepam and qualified degradation products: (a) diazepam; (b) MACB; (c) ACB; (d) MPQ.

value of these two last products was equivalent (284.5) resulting in a single $[M + H^+]$ peak in the mass spectrum.

2.4. Procedure validation

Specificity of the method was established by verifying purity of each compound in a mixture of the drug and it known degradation products. Precision was evaluated on two different concentrations for degradation products. Precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day) and was expressed as a R.S.D.% of a series of measurements.

Accuracy was studied at the same concentration as precision. Limits of detection (LOD) and quantitation (LOQ) were calculated using the standard method [17].

2.5. Stress testing

2.5.1. Exposure to artificial light

Irradiation of samples was carried out according to the ICH guidelines for photostability testing drug substances and drug products [18]. Irradiation (300–800 nm) was achieved using the Suntest CPS+ as describe above. Irradiance was set to 765 W m^{-2}, which is the maximum irradiance of the apparatus. In these conditions, the end criteria of 200 W h m^{-2} and 1.2 million lxh correspond to 2.8 and 7.1 h, respectively.

2.5.2. Exposure to hydrolytic conditions

Acid decomposition studies were performed by dissolving the drug in HCl solution (pH 1). The studies in basic media were done in NaOH media (pH 12). Oxidative studies were done initially in 1 and 10% H_2O_2. All these forced degradation studies were performed at room temperature (22 °C) and in the dark in order to exclude the possible effect of light. The duration of exposure was 24 h.

2.5.3. Exposure to dry heat

Susceptibility of the drug to dry heat was studied by exposing the drug to 60, 80 and 100 °C for 2 weeks. Powder and aqueous solution (without pH adjustment) were tested.

2.6. Sample preparation

Bulk powder (1 batch) was placed in borosilicate Petrie dishes. Powder was spread across the dish to give a thickness not more than 3 millimetres. Solutions were studied at a concentration of 1 mg mL^{-1} in water (or media of the study for hydrolytic conditions) and were diluted with mobile phase prior to analysis. They were placed in borosilicate vials.

For photolytic studies, protected samples, wrapped in aluminium foil, were placed alongside authentic samples. They were added to exclude thermal effects.

3. Results and discussion

3.1. Method validation

The absence of any co-eluting peak (Fig. 3) indicates a good specificity for the method with UV detection. The resolution factor for the five known products was better than 1.8 from the nearest resolving peak.

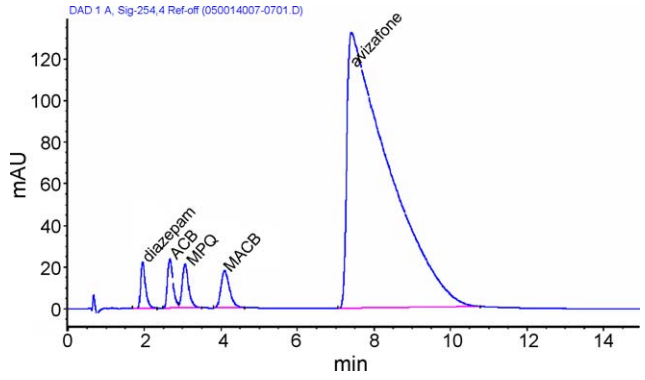

Fig. 3. Representative LC–UV chromatogram of avizafone and its known degradation products.

Table 1
Precision study ($n = 6$ for repeatability; $n = 3$ for intermediate precision)

Compound	Concentration ($\mu g\,mL^{-1}$)	Repeatability (%)	Intermediate precision (%)
Avizafone hydrochlorhyde	100	2.9	5.0
Diazepam	2.5	2.1	2.6
	5.0	1.9	4.4
MACB	1.0	3.2	3.4
	2.0	2.2	3.3
ACB	1.0	1.4	3.2
	2.0	1.6	2.2
MPQ	1.0	2.4	2.9
	2.0	1.4	1.5

The linearity of the method was validated over the concentration range of 5–125 $\mu g\,mL^{-1}$ for avizafone hydrochloride, 0.125–10 $\mu g\,mL^{-1}$ for diazepam and 0.05–5 $\mu g\,mL^{-1}$ for other known decomposition products. The responses were found to be linear in the investigated concentration range. The correlation coefficient values for calibration curve ($n = 3$) were >0.99 for each compound.

Data obtained from precision experiments for repeatability and intermediate precision are given in Table 1. Results confirm that the method was sufficiently precise. As shown from the data in Table 2, good recoveries of the solutes were obtained.

Table 2
Accuracy study ($n = 6$)

Compound	Concentration ($\mu g\,mL^{-1}$)	Recovery (%)	Confidence interval ($\alpha = 5\%$) (%)
Avizafone hydrochloride	100	99.4	[97.1–101.7]
Diazepam	2.5	101.1	[100.0–102.3]
	5.0	98.1	[96.3–99.8]
MACB	1.0	101.4	[99.9–103.0]
	2.0	99.4	[98.0–100.7]
ACB	1.0	102.4	[101.1–103.7]
	2.0	100.0	[99.1–100.9]
MPQ	1.0	101.2	[99.9–102.4]
	2.0	99.6	[98.9–100.2]

Table 3
Limits of detection and quantitation (UV detection)

Compound	LOD	LOQ
Avizafone hydrochloride	3.0 $\mu g\,mL^{-1}$	6.5 $\mu g\,mL^{-1}$
Diazepam	40 $ng\,mL^{-1}$	90 $ng\,mL^{-1}$
MACB	20 $ng\,mL^{-1}$	40 $ng\,mL^{-1}$
ACB	30 $ng\,mL^{-1}$	50 $ng\,mL^{-1}$
MPQ	40 $ng\,mL^{-1}$	70 $ng\,mL^{-1}$

LOQ and LOD calculations are depicted in Table 3. LOQ and LOD were then verified by injecting an adapted concentration of each compound.

3.2. Degradation behaviour of avizafone

LC studies of samples (powder and solution) obtained on stress testing (photolytic, oxidative, hydrolytic and thermal conditions) suggested the following degradation behaviour.

3.2.1. Photostability studies

Organoleptic evaluation shown that powder and solution rapidly became yellow (2 h for powder and 1 h for solution). This colouration could be due to formation of MACB or ACB, which are of this colour at high purity. LC–UV studies of light exposed samples revealed that degradation behaviour of avizafone in powder and solution were quite different.

The compound in powder indicated to be rather stable. Only 5% degradation was seen on exposure to light for 7.1 h (765 $W\,m^{-2}$). The extent increased to 10% after 21 h. In total, four minor degradation products were identified: diazepam, ACB, MPQ and MACB.

The drug in solution was less stable when exposed to light. Almost 70% drug degraded on light exposure for 7.1 h. The major product was MACB. The same precipitated from the solution and had to be dissolved using methanol. Diazepam appeared in the chromatogram between 0.5 and 2 h of reaction and disappeared later (Fig. 4). LC–MS was used to confirm these results. After 21 h exposition, avizafone had quasi-totally disappeared (less than 10% was left). The major degradation product was MACB and a minor degradation product was ACB. No other decomposition product was seen during this study by LC–UV or LC–MS.

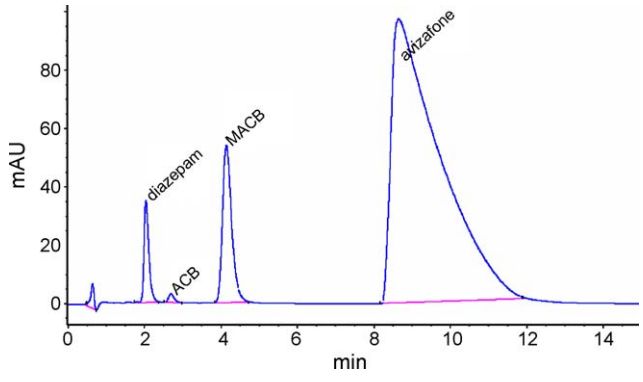

Fig. 4. Chromatogram of sample subjected to light (0.5 h, solution).

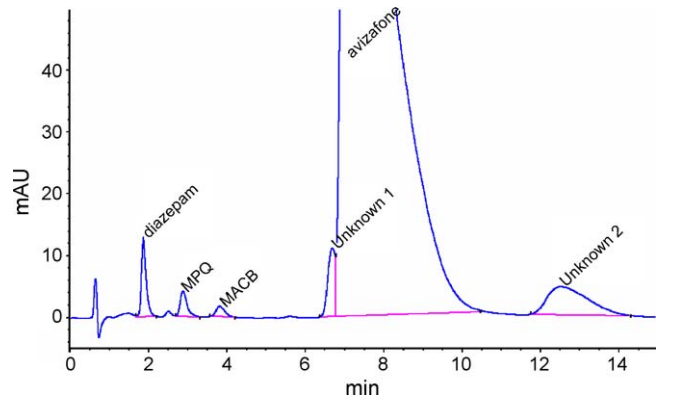

Fig. 5. Chromatogram of sample subjected to dry heat (80 °C, 14 days, solution).

3.2.2. Studies under acidic and basic conditions

After 24 h exposure to the two media at room temperature, no degradation of the drug was observed. The concentration of solutions remained stable. Moreover, no peak was observed in UV or mass chromatogram. Avizafone seemed to be really insensitive to pH variation.

3.2.3. Oxidative stress studies

Avizafone was stable in 1% hydrogen peroxide at room temperature after 24 h. It degraded slightly even in 10% peroxide solution in the same period. Almost, no drop was seen in the solution concentration of the drug. Only one degradation product (MACB) was observed, which increased to 0.1% (w/w) at the end of the studies.

3.2.4. Dry heat studies

Avizafone powder was found to be stable to dry heat. Only negligible degradation (<5%) was observed after exposure to 100 °C for 2 weeks. No additional peak was observed in the chromatogram on UV- or MS detection.

The decomposition behaviour at high temperature was quite different in solution while at 60 °C, only 1–2% degradation was obtained after 2 weeks exposure. The extent of degradation rose to 80% in the same period at 100 °C. Moreover, many decomposition products appeared in the chromatogram (UV and/or MS). The major degradation product was MPQ, which was formed via diazepam whose peak was seen during the course of the reaction. Two others minor degradation products were MACB and ACB (1–2% each). Two additional peaks appeared (Fig. 5). The first one (no full resolution with avizafone) may be a rearrangement product of the drug, as the mass value of this compound was equal to that of avizafone. The most retained compound showed a peak at *m/z* 395 in LC–MS. Isotopic distribution on the mass spectrum was in favour of the presence of chlorine in the structure of this degradation product (Fig. 6). This new decomposition product is now under investigation in our laboratory. However, it seemed that the high retention time should be linked with the glycine part of avizafone. So this chemical function should be present in this product.

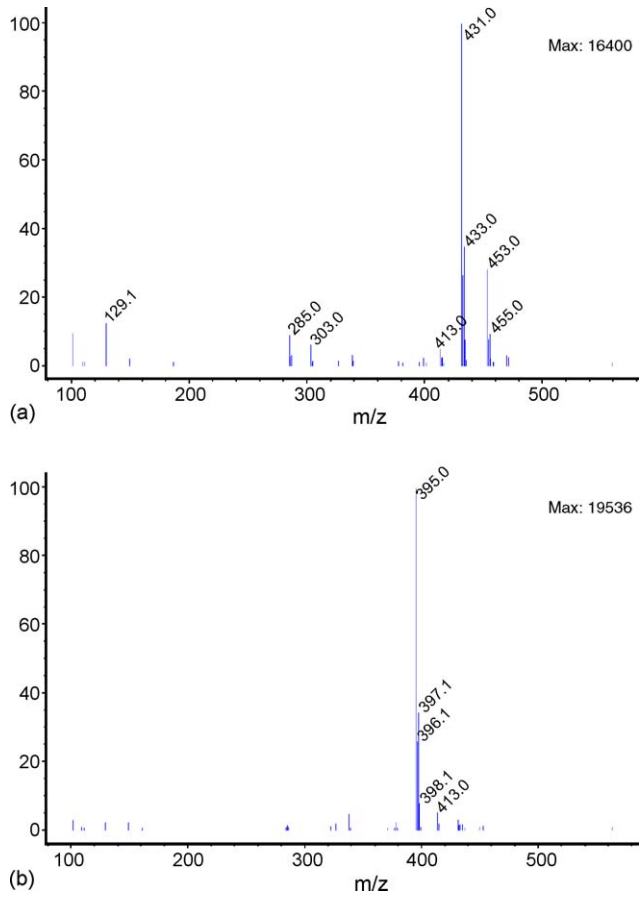

Fig. 6. Mass spectra of unexpected heat decomposition products: (a) unknown 1; (b) unknown 2.

4. Conclusion

An original LC method was developed and validated for stability-indicating studies of avizafone. UV detection allowed an accurate quantitation of known chromophoric compounds, while MS detection was used to find the mass value of unexpected degradation product.

In this study, intrinsic stability of avizafone was established using various ICH recommended stress conditions. The drug as such was very stable. However, it was unstable in an aqueous solution. In the latter case, unknown decomposition products were formed under harsh conditions.

This active pharmaceutical ingredient was associated with atropine and a reactivator (pralidoxime or HI-6) in a multicomponent auto-injector. Because of the better stability of avizafone in powder, a lyophilized form of the antidote combination is now under evaluation. In this last case, drugs are thought to be more stable and then allow to improve the shelf-life of the antidote. The first generation of this original packaging contained avizafone and atropine as a liquid mixture with preservative [19].

Acknowledgment

The authors thank to M. Koltunski for her skillful technical assistance.

References

[1] J. Bajgar, Adv. Clin. Chem. 38 (2004) 151–216.
[2] J. McDoough, J. McMonagle, T. Copeland, D. Zoeffel, T.M. Shih, Arch. Toxicol. 73 (1999) 473–478.
[3] F. Woreck, R. Widmann, O. Knopff, L. Szincz, Arch. Toxicol. 72 (1998) 237–243.
[4] B.R. Capacio, T.M. Shih, Epilepsia 32 (1991) 604–615.
[5] M. Maidment, D. Upshall, J. Biopharm. Sci. 1 (1990) 19–32.
[6] G. Lallement, F. Renault, D. Baubichon, M. Peoc'h, M.F. Urckhart, M. Galonnier, D. Clarençon, N. Jourdil, Arch. Toxicol. 74 (2000) 480–486.
[7] ICH Topic Q1A (R2): stability testing of new drug substances and products, in: Proceedings of the International Conference on Harmonisation, EMEA, London, 2003.
[8] J.T. Carstensen, K.S. Su, P. Maddrel, J.B. Johnson, H.N. Newmark, Bull. Parenteral Drug Ass. 25 (1971) 193–202.
[9] W. Han, G. Yakatan, D. Maness, J. Pharm. Sci. 66 (1977) 573–577.
[10] N.S. Nudelman, R.G. Waibaum, J. Pharm. Sci. 84 (1995) 208–211.
[11] M. Emery, J. Kowto, J. Pharm. Sci. 68 (1979) 1185–1187.
[12] G. Fyllingen, F. Kristiansen, P.O. Roksvaag, Pharm. Acta Helv. 66 (1991) 44–46.
[13] C. Manucci, J. Bertini, A. Cocchini, A. Perico, F. Salvagani, A. Triolo, J. Pharm. Sci. 82 (1993) 367–370.
[14] S. McClean, E. O'Kane, J. Hillis, W.F. Smyth, J. Chromatogr. A 838 (1999) 273–291.
[15] J. Wang, X. Shen, J. Fenyk-Melody, J.V. Pivnichny, X. Tong, Rapid. Commun. Mass Spectrom. 17 (2003) 519–525.
[16] M. Walles, W.M. Mullet, J. Pawliszin, J. Chromatogr. A. 1025 (2004) 85–92.
[17] J. Mocak, A.M. Bond, S. Mitchell, G. Scollary, Pure Appl. Chem. 69 (1997) 297–328.
[18] ICH Topic Q1B: photostability testing of new drug substances and products, in: Proceedings of the International Conference on Harmonisation, EMEA, London, 1996.
[19] P. Clair, K. Wiberg, I. Granelli, I. Carlsson Bratt, G. Blanchet, Eur. J. Pharm. Sci. 9 (2000) 259–263.