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Mass spectrometry study of increased breakdown of an anticonvulsivant drug substance: Avizafone

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Abstract

The French Military Health Service (SSA) developed a new pharmaceutic speciality as a treatment against neurotoxic organophosphate poisoning (NSP), as a substitute for existing therapeutics. The Armed Forces Central Pharmacy (PCA) is in charge of the development of therapeutic formulation and stability studies. This product includes three drug substances: atropine, pralidoxime and avizafone, an amine prodrug of diazepam, soluble in water. The PCA performed a stability study of this formulation according to the International Conference on Harmonization (ICH) recommendations: it was used to display interaction between the molecules and the plastic of the cartridge (the container turned yellow). Since no degradation product of atropine and pralidoxime was observed, a complementary evaluation of avizafone and its main known degradation products (diazepam, carbostyril and methylaminobenzochlorophenone [MACB]) was initiated. The results were used to determine the degradation products obtained under different conditions and the kind of mechanisms, which may occur as the formulation ages: adsorption or absorption by the bulk and/or increasing degradation products. The analytical methods developed here are a direct sample analysis by mass spectrometry (MS) using different ionization modes and liquid chromatography (LC) with UV detection to confirm the results obtain with MS.

Keywords: Mass spectrometry; Ionization mode; Avizafone; Degradation products

1. Introduction

Fighting against chemical weapons, which may be military vectors and/or used by terrorists has now become a priority for governments. Among these agents, organophosphates play a particularly important part.

For this purpose, the French Military Health Service (SSA) has developed a method of rapid administration of a new antidote combination. The treatment is composed of three active pharmaceutical products: atropine, a molecule with anticholinergic properties [1], pralidoxime, as a cholinesterase reactivator [2], and avizafone, a diazepam amine prodrug, soluble in water, which has anticonvulsant properties [3]. These therapeutics, administered via the intramuscular route by an auto-injector, are capable of coping with the emergency situation created by intoxication with chemical organophosphate agents. The stability of such therapeutic combinations has been already demonstrated [4].

Three drug substances are in the injection system in the form of an aqueous solution or in freeze-dried form, in a polypropylene cartridge. In accordance with its legal position, Armed Forces Central Pharmacy (PCA) performed a stability study of this formulation in its final packaging. The study procedure applied the International Conference on Harmonization (ICH) recommended conditions [5,6]. Under accelerated (40 °C, 75% HR) and long-term study conditions (25 °C, 60% HR), yellowing of the cartridge plastic was observed [7]. This change in colour is not obvious at 5 °C, under the reference conditions.

No degradation products of atropine and pralidoxime were found during the stability study. We then decided to perform a complementary study using avizafone, a molecule less well known than other drug substances; a study of increased break-

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down applying ICH recommended conditions [5,6] was performed on avizafone and its main known degradation products: diazepam, carbostyril and MACB. These analyses were used to finalize the complete breakdown process of this molecule and determine the final degradation product, which could interact with the plastic container holding the medicinal product. For this study, we used direct sample analysis by mass spectrometry (MS) with different modes of ionization and liquid chromatography (LC) with UV detection to confirm the results obtain with mass spectrometry.

2. Experiment

2.1. Chemicals

Avizafone (pro-diazepam or 2-benzoyl-4-chloro-*N*-methyl-*N*-lysylglycin anilide), MACB (2-methyl-amino-5-chlorobenzophenone) and carbostyril (3-amino-6-chloro-1-methyl-4-phenyl carbostyril), i.e., Roche (London, England); diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4 benzodiazepine-2-one), i.e., European Council (Strasbourg, France); methanol and acetonitrile of LC grade, i.e., VWR International (Fontenay-sous-Bois, France). The water used was purified with a Milli Q System (Millipore, St. Quentin-en-Yvelines, France). All the other chemicals used such as sulfuric acid, sodium hydrogenphosphate and heptansulfonic acid were analytical grade and purchased from Sigma.

2.2. Stability study programme

The stability study was performed over 1 month, under ICH conditions:

- 25 °C 60% relative humidity (RH),
- 40 °C 75% RH,
- acidic aqueous solution (HCl 0.1 M), ambient temperature,
- basic aqueous solution (NaOH 0.1 M), ambient temperature,
- oxidizing aqueous solution (H_2O_2 10%), ambient temperature,
- intensive exposure to light (SUNTEST ATLAS display, Cergy-Pontoise, France),
- reducing aqueous solution (mixture of 0.1 M HCl and Zn shots to obtain hygrogen),
- ambient temperature.

The reducing aqueous solution does not follow ICH recommendations, but we decided to test this condition, because the incipient formation of hydrogen can reduce all or some of these double-bonded molecules.

Tests were performed at the following times: T0, T0 + 2 h, T0 + 4 h, T0 + 6 h, T0 + 24 h, T0 + 48 h, T0 + 72 h, T0 + 15 days and T0 + 1 month for all environments. In the case of intensive exposure to light, the duration was reduced because, according to the calculations provided by the ATLAS company, 1 month of exposure under natural conditions is equivalent to 4 h of intensive exposure using the SUNTEST. It was then decided to perform an analysis of light exposure at T0, T0 + 1 h, T0 + 3 h and T0 + 5 h.

2.3. Mass spectrometry

Two MS were used. The mass detector was a single quadrupole MS (Thermo Electron MD 1000, France). A sample of about 1 μg (10 μl methanolic solution at 10 mg/100 ml) was inserted through a direct inlet probe. Spectra were obtained by electron ionization (EI), under positive (CI^+) and negative (CI^-) chemical ionization conditions. The source conditions for EI were 70 eV electron energy and the temperature was programmed from 60 to 600 °C at a rate of 65 °C/min. The reference temperature was 300 °C. Data were collected over a mass range of 15–500 uma using a 0.90 s scan time and a 0.10 s interscan delay. For CI, methane was used as the reagent gas and typical source conditions were the same as EI. Probe spectra were obtained using both EI mode and CI conditions. Samples were analysed with a direct inlet probe. The software used on the Thermo Electron instrument for data processing is Mass Lab version 1.4.

An electrospray (ESI) was performed on Sciex API 300 apparatus. The method used was ion spray mode. The flow-rates of N_2 as nebulization gas and curtain gas were 1.46 and 0.95 l/min, respectively. Counter-electrode and skimmer voltages were set at 0 and 200 V, respectively.

2.4. Liquid chromatography

LC analyses were carried out using a conventional LC system consisting of a 1100 series liquid chromatograph equipped with a vacuum degasser, G1322A, a binary pump G1311A, a thermostated column compartment G1316A, an autosampler G1313A with an injection volume of 20 μl and a diode array detector G1314, all from Agilent Technologies (Massy, France).

For the determination of avizafone and its main known degradation products the column used was Zorbax SB CN (150 mm × 4.6 mm), the flow-rate was 1.5 ml/min and the detector wavelength was 230 nm. All chromatographic experiments were carried out in the gradient mode (Table 1).

The aqueous phase was phosphate buffer, pH 3.0, was prepared by mixing 6.9 g of sodium hydrogenophosphate with 3.843 g of heptansulfonic acid and water (Milli-Q System) and adjusting to 3.0 with 1 M phosphoric acid.

Table 1
Gradient mode

Time (min)	% aqueous phase	% acetonitrile
0	72	28
4.5	72	28
7	60	40
16	60	40
18	72	28
23	72	28

3. Results

3.1. Spectra comparison

Mass spectrometry with direct sample insertion was chosen as an informative analysis method because of its rapidity and specificity. To determine which ionization technique is the most appropriate for monitoring the evolution of the different molecules studied, T0 analyses (Fig. 1) were performed by EI^+/MS, CI^+/MS and CI^-/MS. The same study was performed concurrently by ESI/MS.

The analysis of avizafone at T0 performed in EI^+/MS revealed the evolution of the mass spectra during the rise in temperature and the systematic absence of the molecular ion (*m/z* 430) as shown in Fig. 2a (150 °C) and b (250 °C). The increase in temperature involves the appearance of peaks at higher values of *m/z*; ions formed in this way more frequently result from pyrolysis with combination reactions than from molecular ion fragmentation.

In return, the spectra of known degradation products are always steady during the rise in temperature, and each compound (diazepam, carbostyril and MACB) has a specific response temperature for its characteristic ions: 250, 150 and 200 °C, respectively. Their EI^+/MS spectra are specific and informative (Fig. 2c: diazepam, d: carbostyril and e: MACB).

Thus, it is possible, during the analysis of avizafone, to underline the spectrum of each degradation product formed when the temperature is programmed and characterize it by comparison with a spectra library. If other components are formed, their spectra complicate the interpretation; the following results at the time of increased breakdown attempt to illustrate this.

Generally, a CI^+/MS spectrum must show a simplified spectrum giving the MH^+ ion. Under the conditions used, methane appears to be unadapted because it gives little characteristic spectra of avizafone with no signal at M + 1, and only fragmentation signals for carbostyril and MACB.

In return, the study in CI^-/MS (Fig. 3) can be used to obtain a mass spectrum for avizafone with only a few peaks but in the presence of the molecular ion (*m/z* 412 and 430, respectively).

The CI^-/MS analysis of diazepam, carbostyril and MACB also produced mass spectra showing the molecular mass of the component studied. These peaks were: *m/z* 284, 268 and 282 and 245, respectively. As these signals are not in the avizafone spectrum, this method (CI^-/MS) seems appropriate for displaying the formation of these derivatives and confirming the observation made in EI^+/MS.

In the same way, ESI analyses (Fig. 4) also produce very easily interpretable, much easier mass spectra than positive chemical ionization does. Indeed, only the molecular mass of each component appears for avizafone (*m/z* 431), diazepam (*m/z* 285) and carbostyril (*m/z* 285). For MACB, a little fragmentation is observed since the spectrum is composed of two peaks highly pertaining to the majority (*m/z* 102 and 246). Nevertheless, it must be remembered that this technique cannot be used to differentiate between the two isobaric components, diazepam and carbostyril. It is therefore possible to split these two components by collision induced dissociation (CID), and in this case, characteristic fragments can be obtained: *m/z* 165 and 193 for diazepam, and *m/z* 234 and 249 for carbostyril. In spite of everything, although the spectra obtained by CID produce an interesting image, they cannot be included in a spectra library, because they depend largely on experimental conditions and the installation used. In conclusion, in this

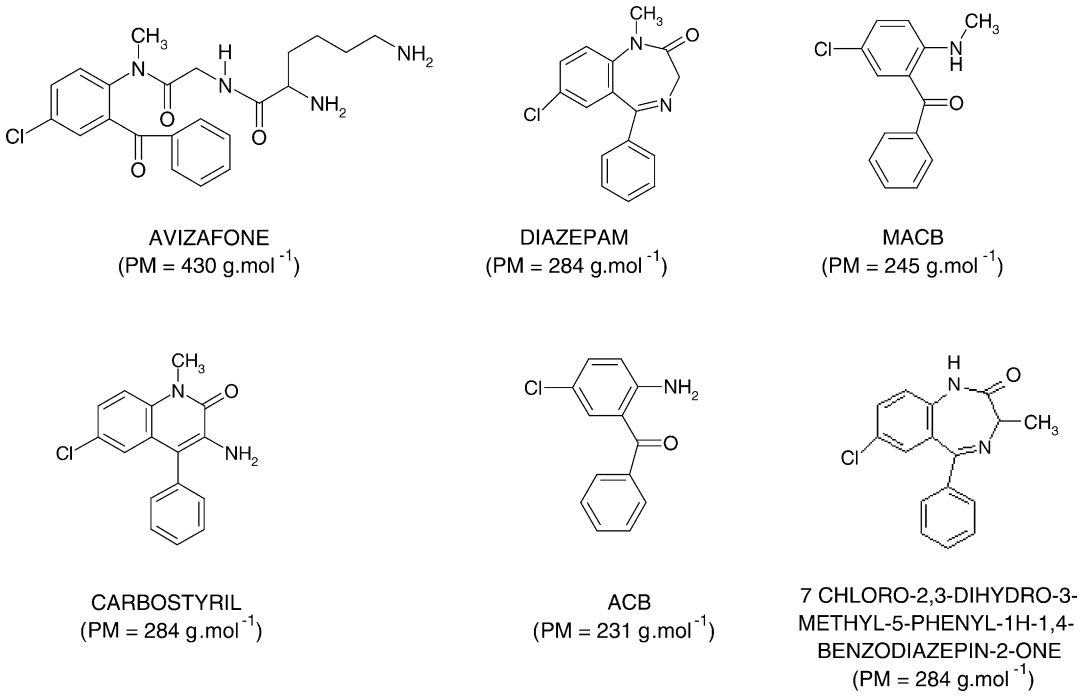

Fig. 1. Compound formulae.

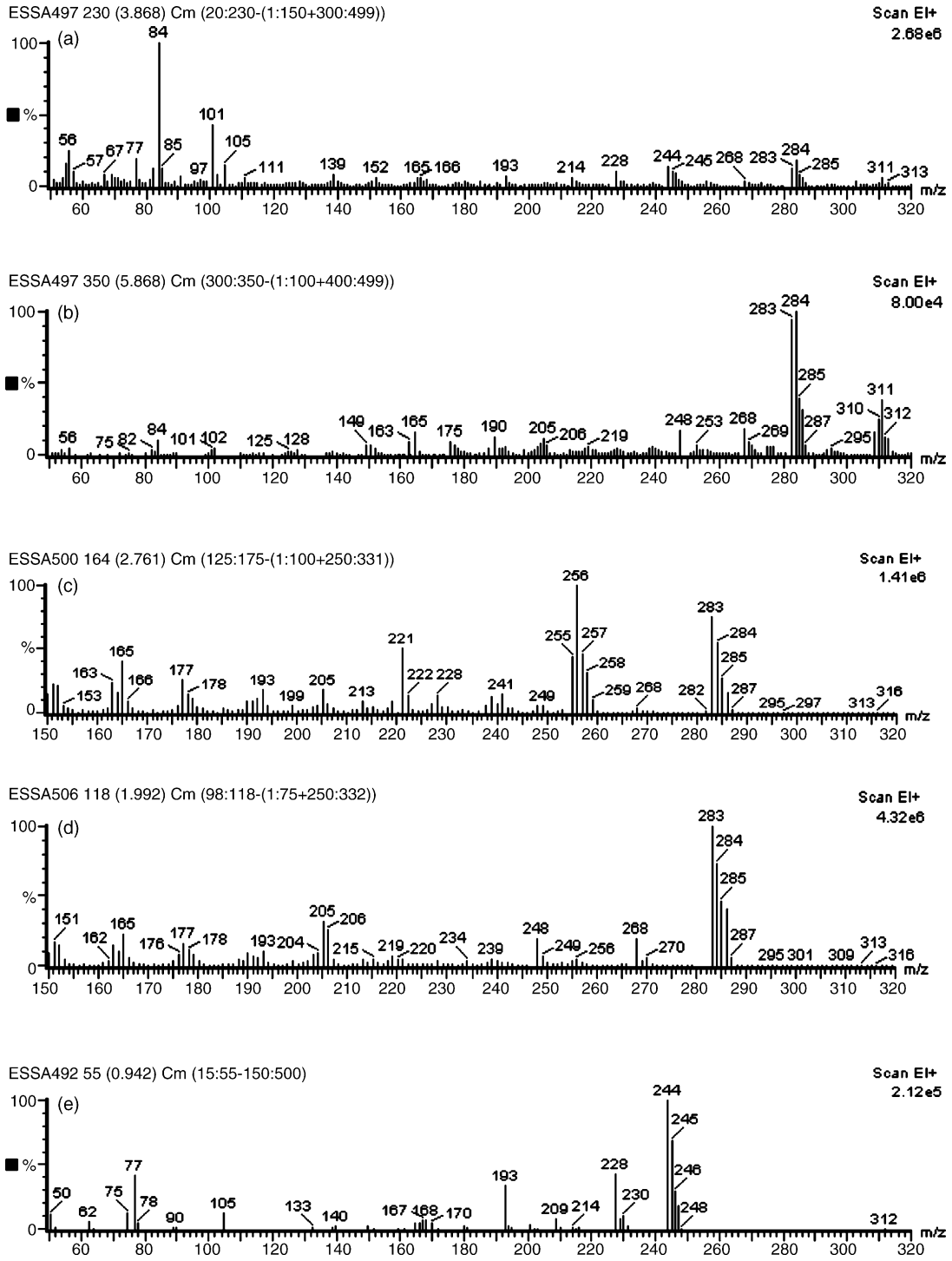

Fig. 2. Mass spectrometry in EI^+ mode: (a) avizafone at 250 °C, (b) avizafone at 300 °C, (c) diazepam at 200 °C, (d) carbostyril at 250 °C and (e) MACB at 150 °C.

study only EI^+/MS, CI^-/MS and ESI/MS will be taken into account.

3.2. Application to the study of increased breakdown

In EI^+/MS mode, the study of breakdown away from light at 25 °C and 60% HR or 40 °C and 75% HR gives similar results. Avizafone breaks down into diazepam (*m/z* 221, 255–257, 283–285) and MACB (*m/z* 193, 228–230, 244–246) after 24 h, but after 15 days, peaks at *m/z* 205–207 and 249–250 cannot be identified in a library.

In acidic and basic aqueous solutions, evolution takes place just after insertion into the solution, with development of the peaks already observed, *m/z* 205–207 and 249–250. If searches in an EI^+/MS library do not lead to identification, we notice that the spectrum remains similar enough, leading to the conclusion that this degradation evolves little in time in basic environments. On the other hand, after 1 month in an acid solution, the presence

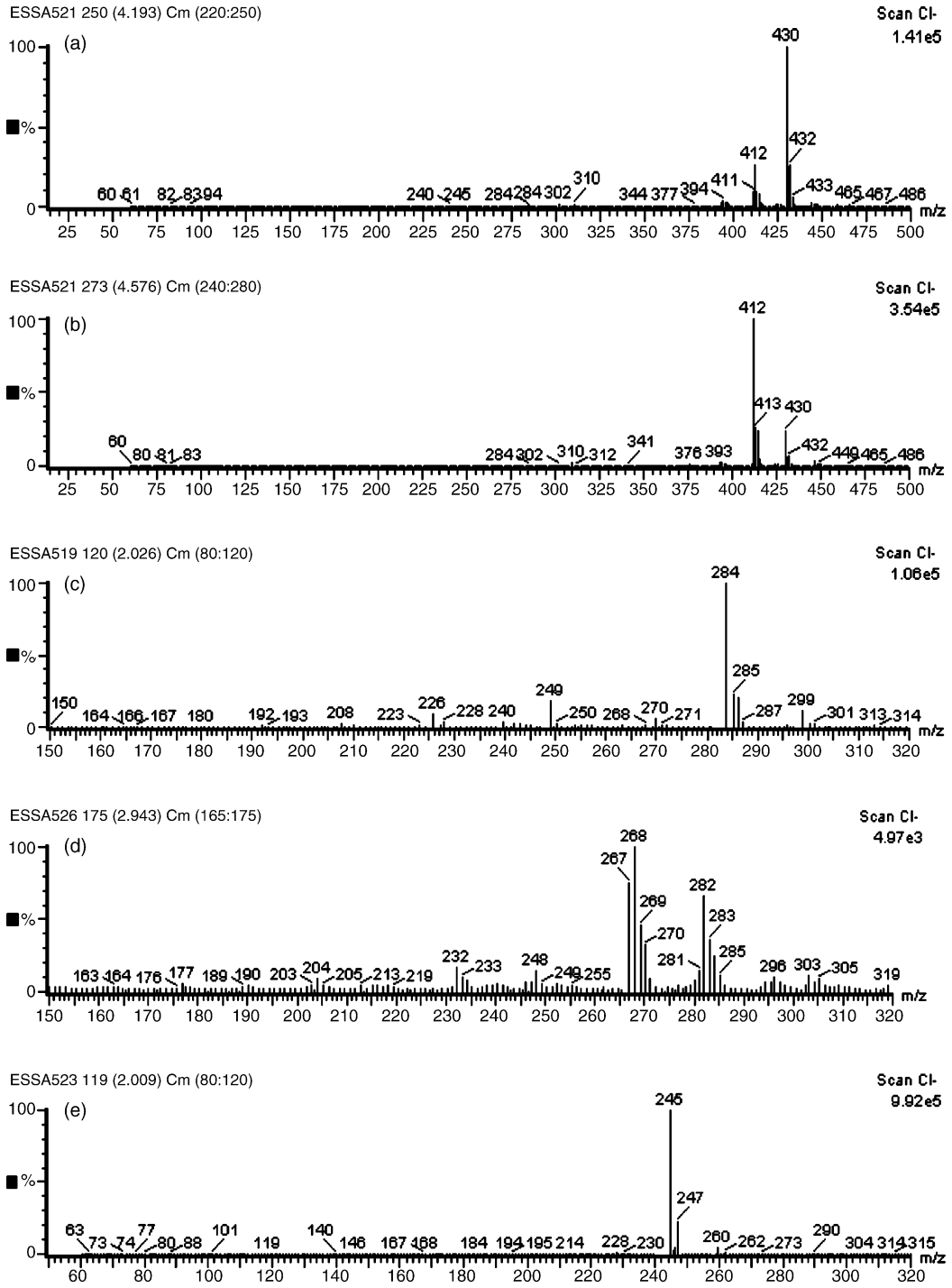

Fig. 3. Mass spectrometry in CI^- mode: (a) avizafone at 300 °C, (b) avizafone at 350 °C, (c) diazepam at 200 °C, (d) carbostyril at 250 °C and (e) MACB at 150 °C.

of characteristic MACB ions (*m*/*z* 193, 228–230, 244–246) leads to breakdown of diazepam observed in small quantities.

As the study of breakdown in a reducing aqueous solution was performed in an HCl environment, we noted the breakdown already observed since this acid solution was first put into solution, yet by 15 days, the spectrum is very different: spectra made at different temperatures were different and complex (peaks up to *m*/*z* 134/136/138/140 but their relative intensities did not lead to the conclusion that chlorine was present in the fragments) and could not be used for identification. On the other hand, these spectra have the same profile in time for a given temperature, which indicates a limited evolution of 15 days to 1 month.

For studies in oxidizing aqueous solution, spectrum stability was observed for 15 days with the persistent presence of avizafone in the form of its spectrum (Fig. 2b). Evolution of the EI^+/MS spectrum shows the formation of diazepam starting from T0 + 24 h, then, after 4 days, MACB peaks appear. In spite of everything, avizafone is not completely broken down after 1 month because in CI^-/MS, it was observed at 350 °C (Fig. 5b). This type of ionization also proves that MACB (*m*/*z* 245–247)

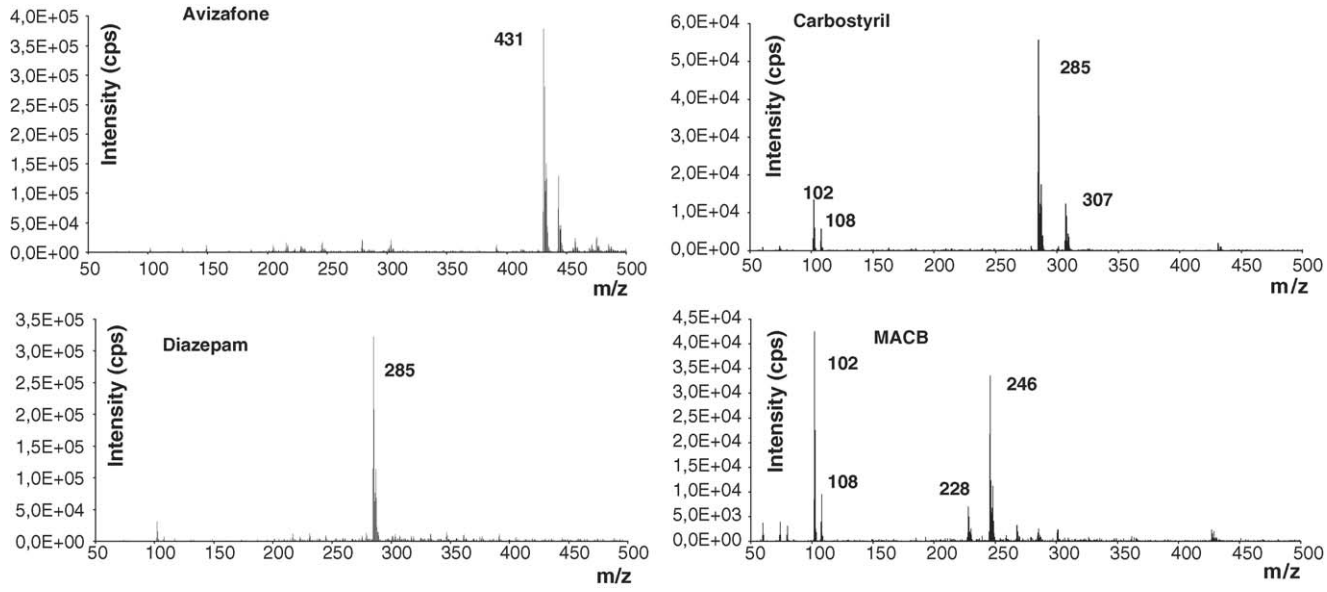

Fig. 4. Mass spectrometry in ESI mode.

and ACB (*m*/*z* 231–233) are present at 300 °C (Fig. 5a). This scheme also shows the advantage of analysing at two different temperatures, in order to see both MACB and ACB at 300 °C and avizafone at 350 °C. In return, we did not find peaks likely to correspond to diazepam.

For intensive light exposure tests, the avizafone spectrum was unchanged after 1 h of exposure. Then, at T0 + 3 h, spectra made at two different temperatures revealed the presence of diazepam (Fig. 6a; 300 °C) and 7-chloro-2,3-dihydro-3-methyl-5-phenyl-1H-1,4-benzodiazepine-2-one (Fig. 6b; 350 °C), iden-

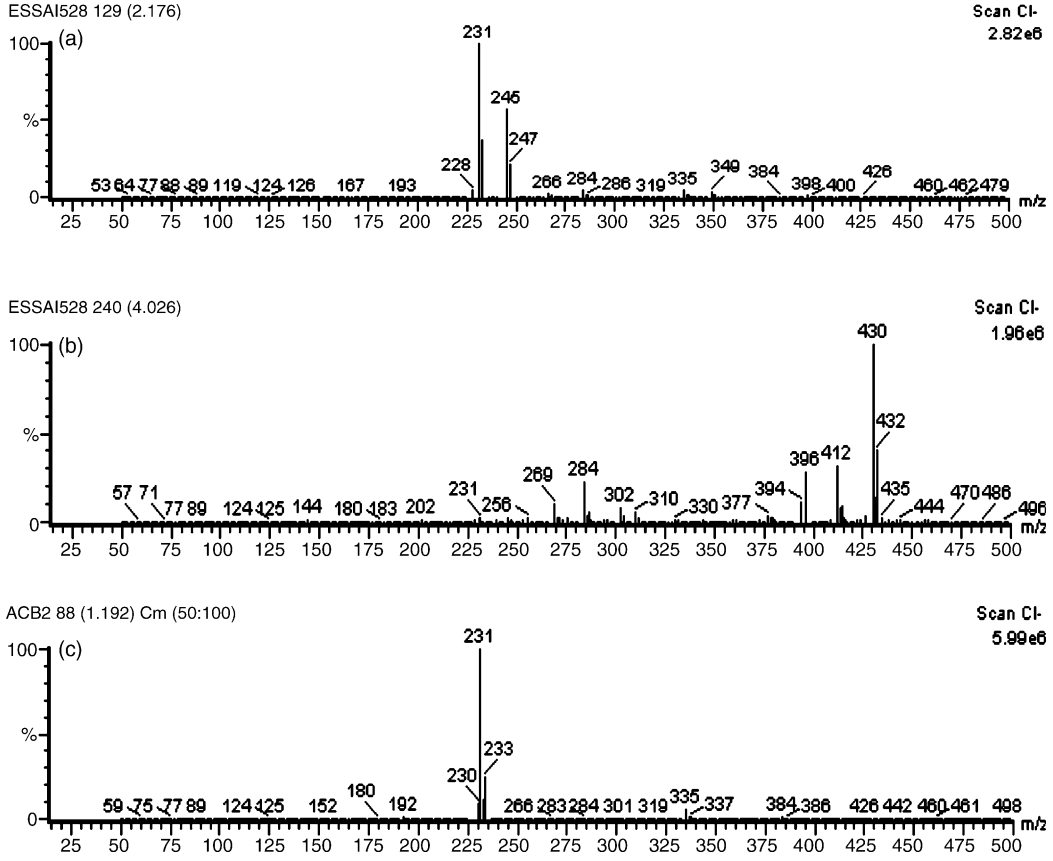

Fig. 5. Mass spectrometry in CI⁻ mode. Avizafone after 1 month in an oxidative mixture: (a) spectrum at 300 °C, (b) spectrum at 350 °C and (c) ACB spectrum from library.

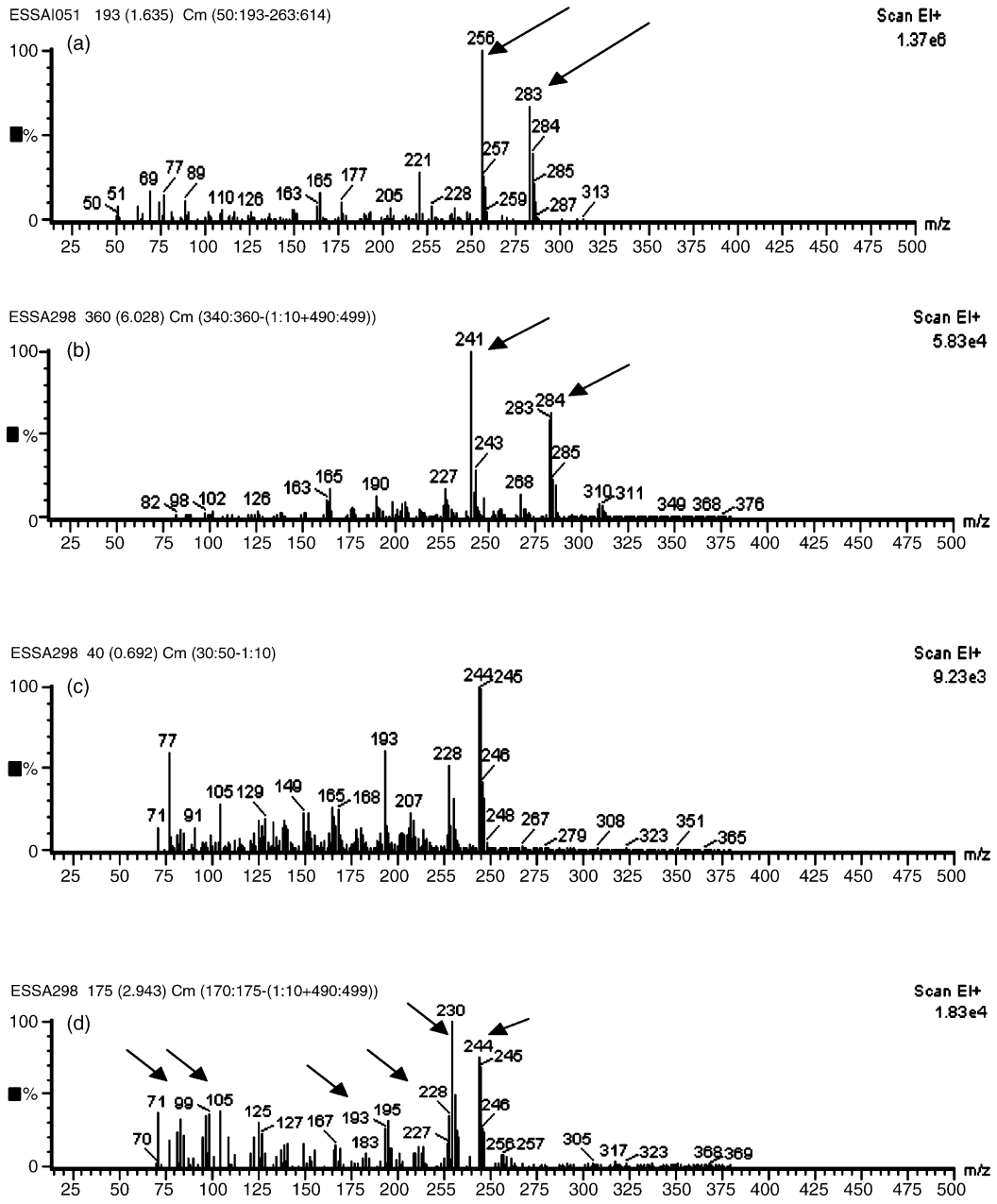

Fig. 6. Avizafone mass spectrum under Suntest conditions: (a) after 3 h Suntest, spectrum at 300 °C (diazepam), (b) after 3 h Suntest, spectrum at 350 °C, (c) after 7 h Suntest spectrum at 150 °C MACB and (d) after 7 h Suntest spectrum at 250 °C ACB.

tified by the library (Fig. 7). Then, at T0 + 7 h, MACB (spectrum at 150 °C; Fig. 6c), ACB (spectrum at 250 °C; Fig. 6d) appeared and 7-chloro-2,3-dihydro-3-methyl-5-phenyl-1H-1,4-benzodiazepine-2-one and diazepam were still present.

The ESI spectrum (Fig. 8) showed that avizafone disappeared after 5 h under Suntest, confirming the presence of diazepam (*m*/*z* 285–287), and MACB (*m*/*z* 246–248/102) and ACB (*m*/*z* 231). We must remember that the *m*/*z* 285–287 peak can also represent 7-chloro-2,3-dihydro-3-methyl-5-phenyl-1H-1,4-benzodiazepine-2-one and carbostyril. The ambiguity between isobaric compounds can be eliminated by CID.

In the same way, the MS–MS method in ESI (Fig. 9) can be used to distinguish isobaric compounds such as diazepam and

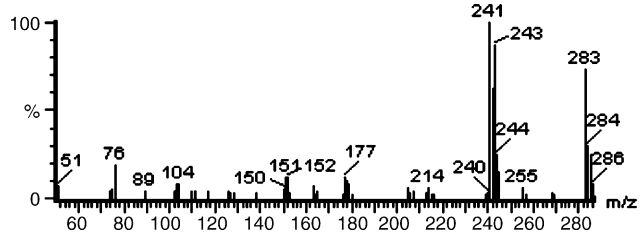

Fig. 7. Library mass spectrum of 7-chloro-2,3-dihydro-3-methyl-5-phenyl-1H-1,4-benzodiazepine-2-one.

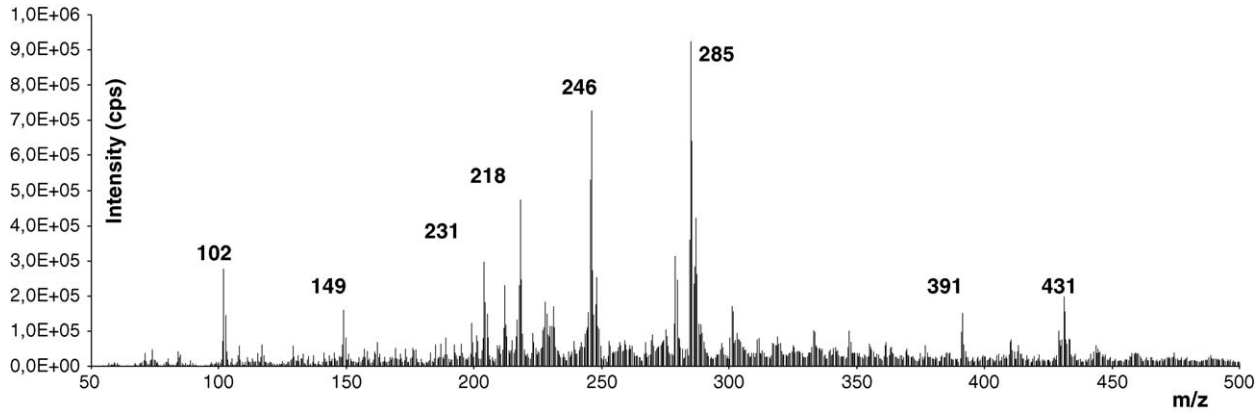

Fig. 8. Mass spectrum of avizafone in ESI mode after 5 h under Suntest conditions.

carbostyril; fragmentation of the *m/z* 285 ion gives 154, 193, 222, 257 ions for diazepam and *m/z* 269 and 249 ions for carbostyril. In the same way, the avizafone spectrum at T0 + 5 h of exposure to light, gives a signal that we attribute to MACB. To confirm this, you only have to split the 246 ions in this spectrum and in the MACB spectrum: the identity of the two fragmentation spectra confirms the presence of MACB.

3.3. Confirmation by liquid chromatography

A study of stability on an original combination of antidotes against the poisonings by the neurotoxic organophosphate consisted of atropine, HI-6 and avizafone [4] led to the individual analysis of each of the compounds by LC.

Through various publications [8–10], it seems that the reverse phase LC is the analytical method, mostly used for the study of avizafone and diazepam and that the analysis of the MACB and the carbostyril is not mentioned. It is necessary to notice that the composition of the mobile phase is always constituted by a mixture of acidified ACN/water, in pH allowing the analysis of compounds in the ion pair form.

Compounds are detected in UV in a wavelength varying from 230 to 254 nm. Until now, no analytical method allowed the simultaneous revealing of avizafone, diazepam, carbostyril and MACB. So, it was thus tried to obtain these four compounds on the same chromatogram by reverse phase LC and to optimize the experimental conditions. Once this technique was optimized, she will allow to verify the results obtained in MS.

By referring to the results obtained in MS, in the ease and in the speed of stake in work of analyses, it was chosen to follow the evolution between T0 and T0 + 7 h of avizafone, diazepam, MACB and carbostyril under the influence of Suntest. These analyses of LC/UV have to allow us to confirm the following results: stability of diazepam, MACB and carbostyril until

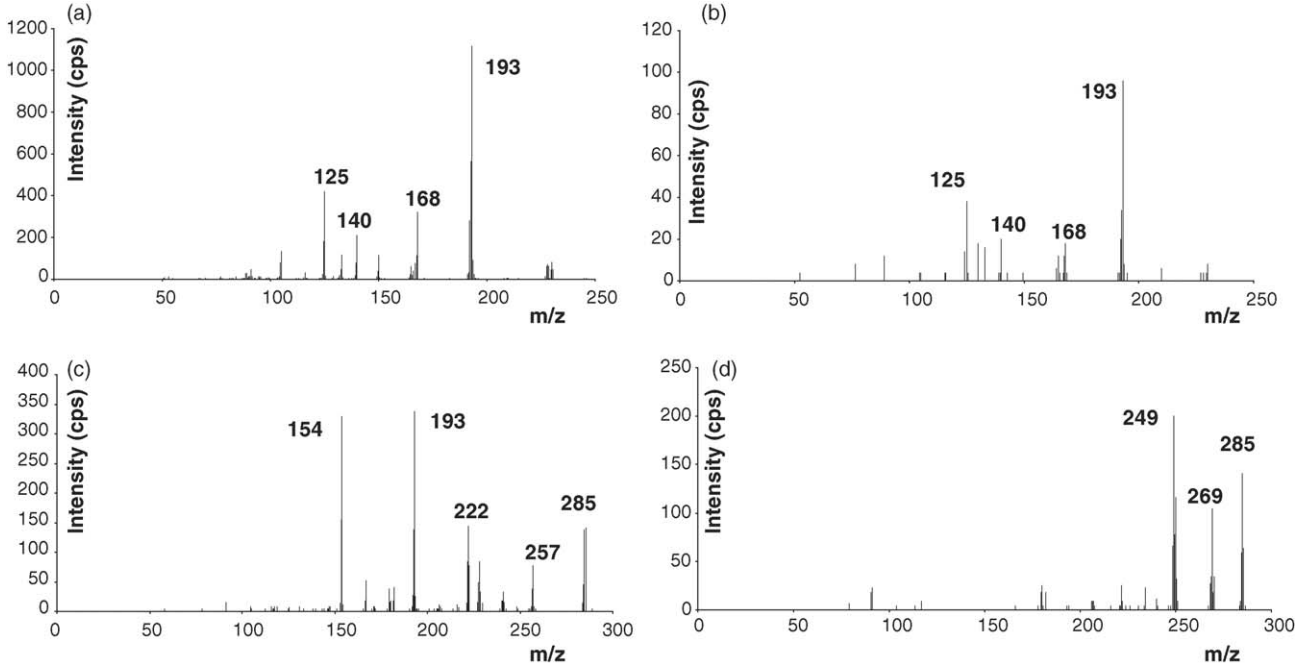

Fig. 9. MS–MS spectrum of avizafone *m/z* 246 ion (a) after 5 h under Suntest conditions (see Fig. 7 MS spectrum) and MS–MS spectrum of *m/z* 246 ion of MACB (b) and MS–MS spectrum of *m/z* 285 ion of diazepam (c) and carbostyril (d).

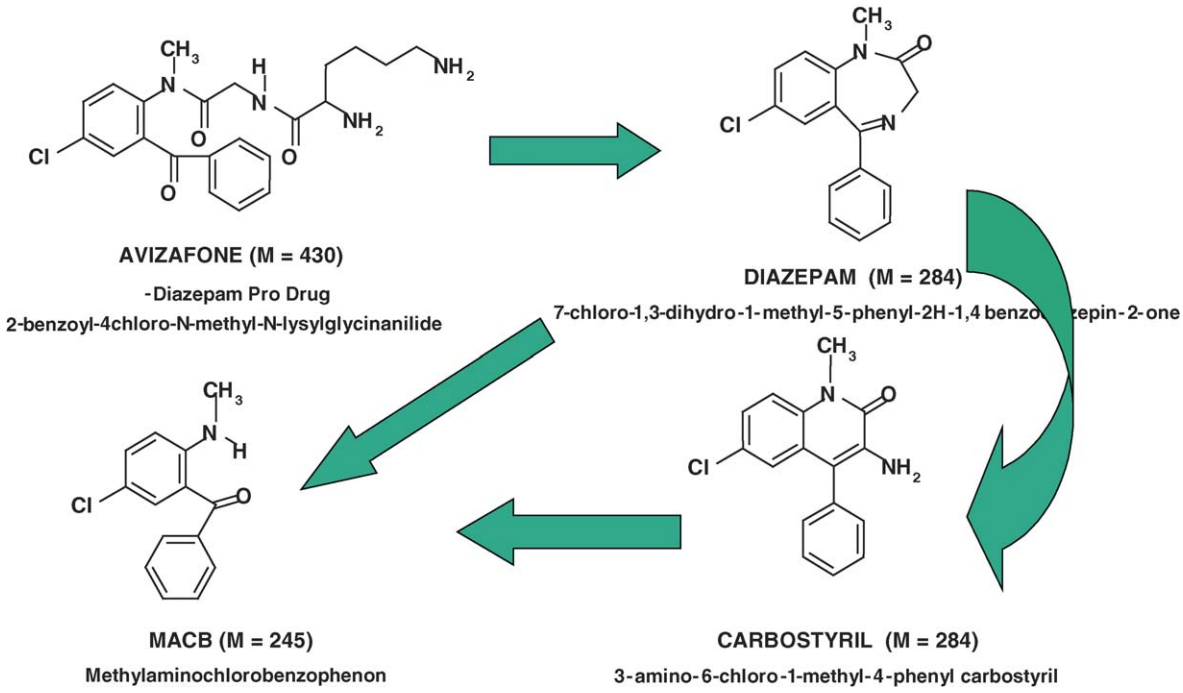

Fig. 10. Proposed avizafone breakdown scheme.

T0 + 7 h of extensive light exposure and the degradation of avizafone first of all in diazepam and 7-chloro-2,3-dihydro-3-methyl-5-phenyl-1H-1,4-benzodiazepin-2-one then the appearance of ACB and MACB.

To T0 and after an extensive light exposure of 1, 3, 5 and 7 h avizafone, diazepam, carbostyril and MACB are put in solution in some chromatographic quality methanol in a concentration of 20 ppm and are preserved at +4 °C not to alter them before chromatographic analysis (Fig. 10).

The chromatograms of diazepam, MACB and carbostyril to T0 and after 7 h from extensive light exposure is presented in Fig. 11. By comparing chromatograms obtained to T0 and T0 + 7 h in Suntest for three molecules, we can notice that molecules are stable. Indeed, no new peak appears on the chromatogram after extensive light exposure with regard to that obtained to T0. Furthermore, if we follow the evolution of the height and the area of the peaks of these three molecules to T0, T0 + 1 h, T0 + 3 h, T0 + 5 h and T0 + 7 h, the obtained values do not almost evolve (Fig. 12).

Avizafone begins to degrade from T0 + 3 h in Suntest (Fig. 13a). Indeed, the chromatogram realized after this time (weather) of exposure shows the appearance of two new peaks having time of keeping back (t_R) of 3.48 and 8.32 min. By referring to the previous analyses realized on the main products of degradation known for avizafone and in the results obtained in MS, we can say that the first peak (t_R = 3.48 min) does not correspond to an already studied molecule and that the second peak (t_R = 8.32 min) corresponds to diazepam.

The chromatogram obtained after 5 h from extensive light exposure (Fig. 13 b) does not show the appearance of new peaks but the degradation of avizafone seems to continue. Indeed, an increase in terms of height and area of peaks corresponding to the unknown molecule and to the diazepam and a decrease of the same values for the peak corresponding to avizafone are constated. All these results are completely in agreement with the observations made after the analyses of mass spectrometry: the unknown peak could thus correspond to 7-chloro-2,3-dihydro-3-methyl-5-phenyl-1H-1,4-benzodiazepin-2-one. The chromatogram obtained after 7 h from extensive light exposure (Fig. 14a) shows, besides the presence of the unknow molecule, of avizafone and diazepam, two new peaks having t_R 11.22 and 14.59 min. By referring to analyses realized on the main products of degradation known for avizafone, it would be possible to conclude that the first peak (t_R = 11.22 min) corresponding to carbostyril and the second (t_R = 14.59 min) to MACB.

Nevertheless, the observations made in MS reminded us that the first peak would be rather of ACB and not carbostyril. A solution of ACB of 20 mg/l was thus realized and analyzed to T0 by this technique of LC. The obtained chromatogram (Fig. 14 b) shows that ACB has a t_R very near to that of carbostyril (11.22 min for the ACB and 11.35 for carbostyril). This allows us to conclude that the first peak corresponds credibly to ACB and not to carbostyril. On the other hand, for the second peak, the observations made in MS consolidate the fact that it is about MACB.

4. Discussion

The presence of the degradation products mentioned (diazepam, carbostyril and MACB) during accelerated breakdown means that the stability of these compounds must be checked under the same degradation conditions as those applied to avizafone. Table 2 gives a summary of these results. The results are in general agreement with those obtained for avizafone. Indeed, MACB was steady in all the methods used. This compound is the final degradation product since avizafone is the

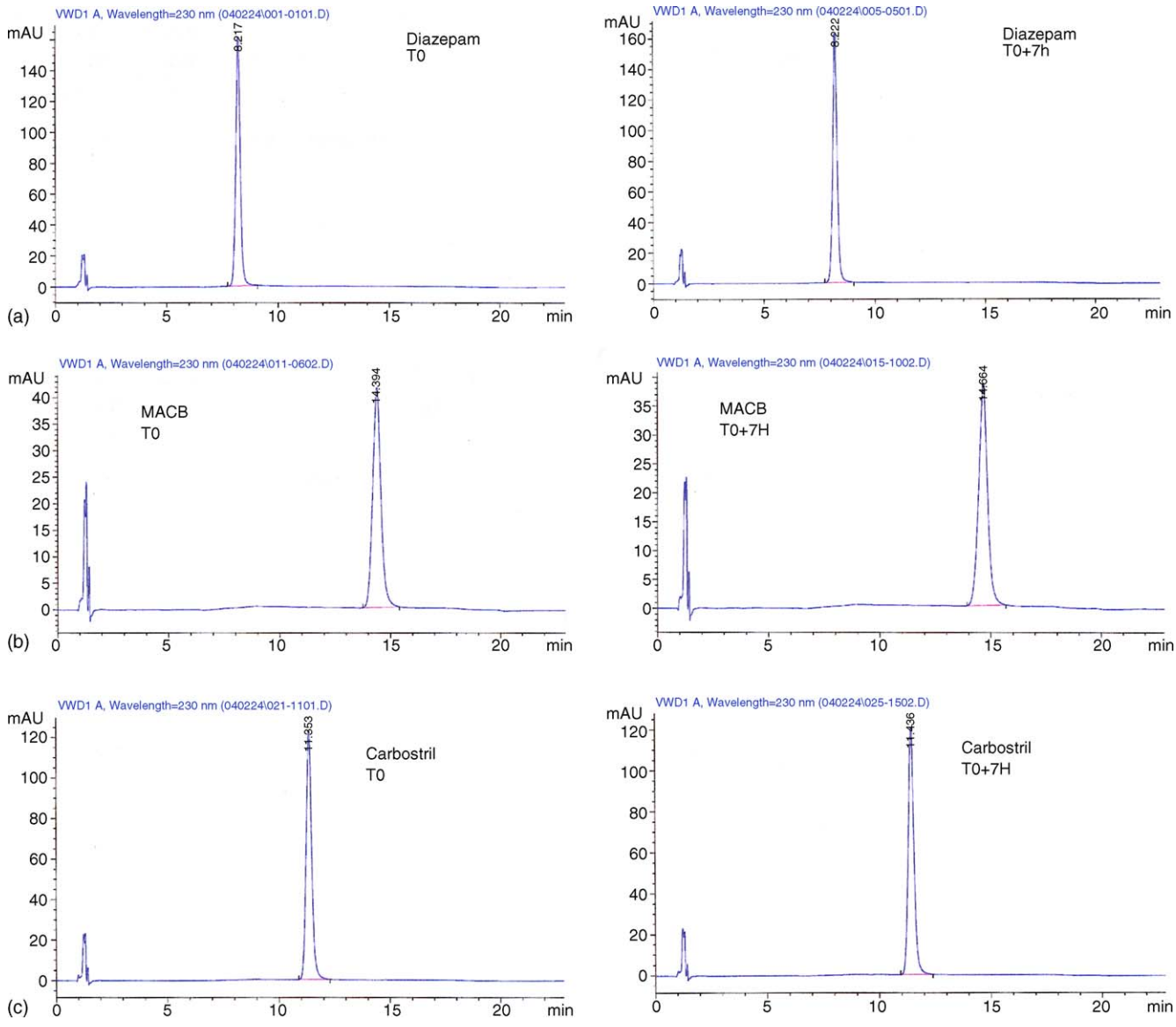

Fig. 11. Comparison chromatograms between T0 and after 7 h under Suntest of diazepam, MACB and carbostyril.

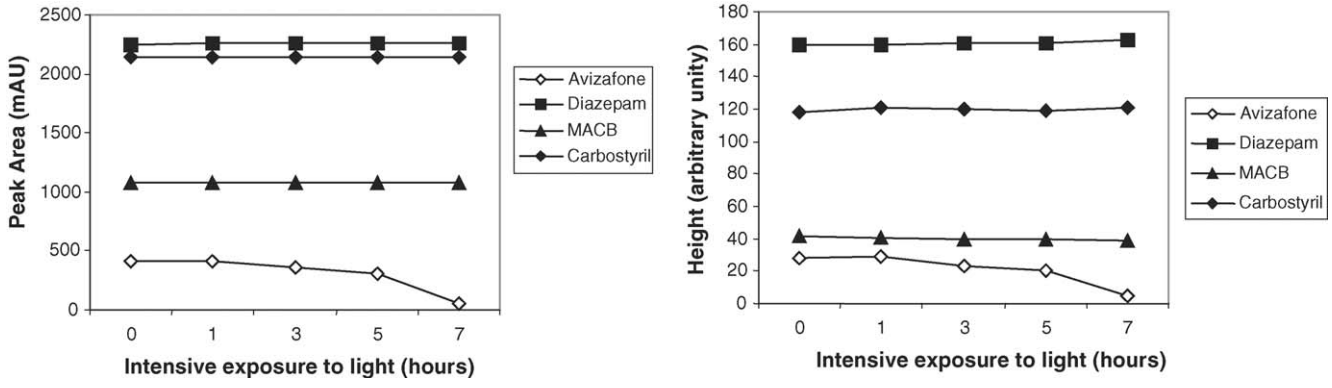

Fig. 12. Stability of diazepam, MACB and carbostyril under Suntest.

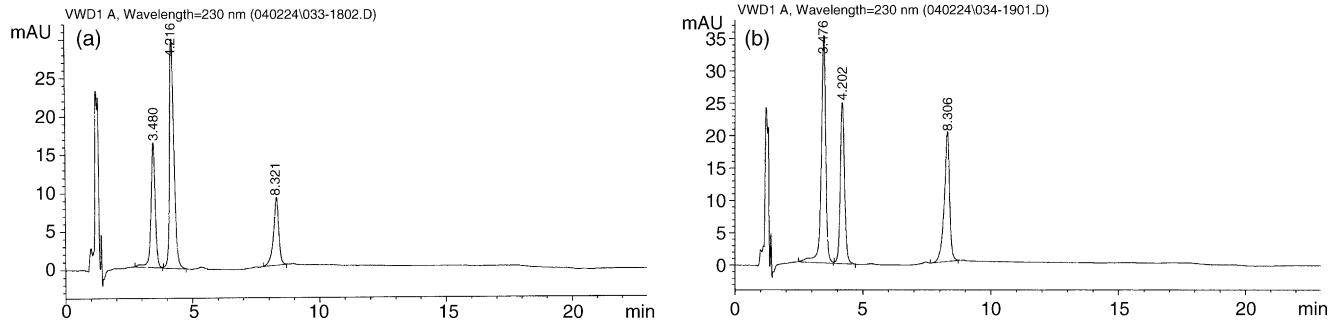

Fig. 13. Chromatograms of avizafone after 3 h (a) and 5 h (b) under Suntest.

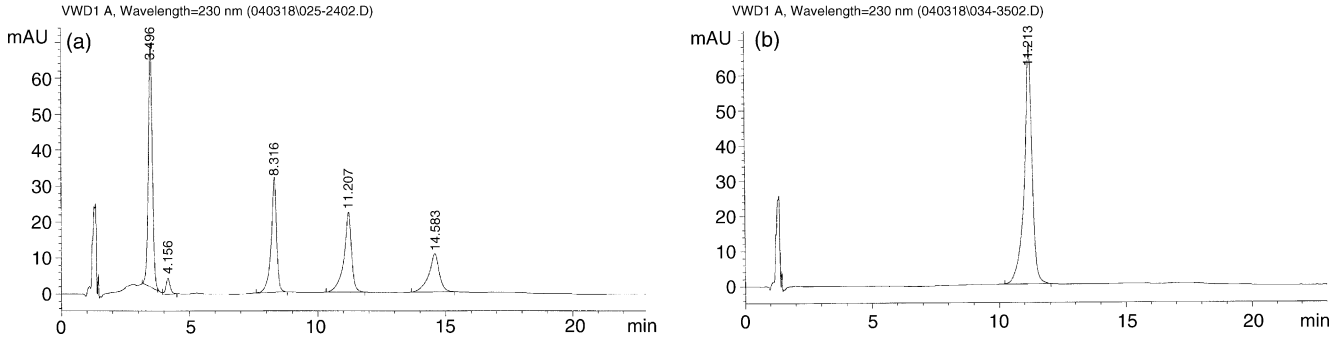

Fig. 14. Chromatograms of avizafone after 7 h under Suntest (a) and ACB at T0 (b).

Table 2
Results of avizafone breakdown and main degradation products

	25 °C 60% HR	40 °C 75% HR	NaOH 0.1 M	HCl 0.1 M	H_2O_2 10%	Suntest	Reducing
Diazepam	Stable	Stable	Formation of MACB	Stable	Formation of MACB and ACB	Stable	Formation of MACB and other compounds requiring careful study
MACB	Stable	Stable	Stable	Stable	No detection after T0 + 96 h	Stable	Stable
Carbostyril	Stable	Stable	Stable	Formation of diazepam	No detection after T0 + 72 h	Stable	Formation of other compounds requiring careful study
Avizafone	Formation of diazepam and MACB	Formation of diazepam and MACB	Formation of other compounds requiring a careful study	Formation of diazepam and MACB	Formation of MACB and ACB	Formation of diazepam, MACB and ACB	Formation of other compounds requiring careful study

starting material. Diazepam breaks down into MACB, but never into carbostyril, and carbostyril breaks down into diazepam or other molecules, which are difficult to identify, but never into MACB.

The direct insertion method cannot be used for quantitative analysis of the results obtained. However, it is clear that under ICH stability test conditions (25 °C 60% HR and 40 °C 75% HR), avizafone, the final degradation product is MACB. These results were also used to perfect the breakdown scheme (Fig. 9). Yet, this scheme does not exactly match the breakdown obtained in each environment studied, but it appears to be the most probable on examining our storage conditions for the medicinal product.

Furthermore, chromatographic analysis consolidate this plan of degradation because the results obtained in LC are similar to those observed in MS for the factor of study of extensive light exposure ending in the training of several products of degradation.

5. Conclusion

This study of increased breakdown enabled us to finalize a general breakdown scheme for avizafone, in which, under the standard conditions of the stability studies performed (25 °C 60% HR and 40 °C 75% HR), MACB appears to be the final degradation product. Since the mixture of the three drug sub-

stances is at pH 2.3, and analysis in aqueous acid solution shows a certain instability of avizafone (formation of diazepam then MACB), it is probable that this mixture will not be stable. The auto-injector should therefore be stored at 5 °C, and/or we should slightly increase the pH of the compartment containing the drug substances to improve this stability.

This study of accelerated breakdown also underlined the advantages and disadvantages of the different methods of ionization applied to the molecules studied.

This leads us to conclude that all three techniques are fast and complementary for identifying molecules, but none of them, used alone, identifies the molecules observed. Nevertheless, the most importing is that the express sample analysis by MS allows to obtain exploitable qualitative results very quickly because these are similar to those obtained by LC with UV detection.

Finally, the electrospray method will be coupled to separation by liquid chromatography for a more extensive study of the impurities not identified in this work.

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