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Study of thiorphan degradation

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Summary

The degradation of the enkephalinase inhibitor thiorphan was studied. Liquid chromatography UV and electrochemical detections and several techniques of spectrometry showed that the degradation was oxidative and generates diastereoisomers of the corresponding disulfide. The degradation kinetics of thiorphan under different conditions of storage have been investigated by high performance liquid chromatography with UV detection. The influence of air, temperature, light and pH was studied. The combination of these factors enabled us to determine the optimal conditions of conservation and to propose a stable injectable form.

Introduction

Thiorphan, (R,S)-mercapto-3-benzylpropanoyl-2-glycine, is an inhibitor of enkephalinase (Schwartz et al., 1985; Chipkin, 1986; Chipkin et al. 1982a), a physiological dipeptidylcarboxypeptidase (Hudgin et al., 1981; Schwartz et al., 1981; Llorens et al., 1982), involved in the degradation of endogenous enkephalins. Pharmacological studies, performed on animals, showed antinociceptive activities after parenteral administration (Roques et al., 1980; Chipkin et al., 1982b and c; Fournie Zaluski et al., 1985). Nevertheless, thiorphan is quickly degraded in aqueous solution, which complicates preparation of a stable parenteral form.

Two kinds of degradation in aqueous solution were shown:

- a non-oxidative degradation, shown at a very high temperature
- an oxidative degradation, at room temperature. We studied this kind of degradation, because it takes place in the usual conditions of storage.

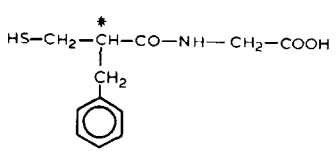

Fig. 1. Structure of thiorphan.

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It is essential to establish the degradation mechanism to elucidate the structure of the oxidation products. This identification is necessary to study degradation kinetics in order to determine the optimal conditions of conservation and to perform a stable pharmaceutical preparation.

Materials and Methods

Materials

Thiorphan, (Roques, patent 800861).

Degradation conditions

Thiorphan is degraded if an aqueous solution of thiorphan is in contact with the air for several days. This process can be immediately obtained by addition of a 0.1 N iodine solution or by bubbling oxygen into an aqueous solution of thiorphan.

Preparation of the degradation products

The easier and more efficient way to oxidize thiorphan is to add an iodine solution until stable coloration of the aqueous solution. Degradation products can be obtained after precipitation in an acid pH and extraction by ethyl ether.

Crystallisation or preparative liquid chromatography performed on an oxidized thiorphan aqueous solution did not enable us to separate the two degradation products.

Chromatography

HPLC analysis. The liquid chromatographic system consisted of a C18 microbondapak Waters column (30 cm × 4.6 mm i.d. packed with 5 μm particle size) used at ambient temperature and a Knauer pump. Samples were injected using a Rheodyne injection valve with a 20 μl loop.

The UV detection was performed with a variable-wavelength UV detector operated at 210 nm. Chromatographs were recorded with a Shimadzu CR3A computer integrator.

The electrochemical detection was performed with a Metrohm 656 detector and chromatograms were recorded with a Metrohm labograph E586.

The mobile phase consisted of: ammonium acetate 0.01 M (pH 4.2 adjusted with acetic acid) – acetonitrile (70 : 30, v/v). The flow rate was 1 ml/min.

Quantitative TLC analysis. Separation was performed on Kieselgel GF253 TLC plates with fluorescent indicator (20 × 20 cm). TLC were performed with a Camag apparatus that consisted of a Camag linomat for application of samples, a Camag TLC scanner II spectrodensitometer and a Camag TLC integrator.

The mobile phase consisted of isopropanol, water and ethyl acetate (40 : 20 : 40, v/v/v).

Spectrometry

Spectrometry with multichannel detection. The analysis was performed with a HP 8451A spectrophotometer with photodiode array and the detection with a HP 85 computer. The solvent associated water and methanol (70 : 30 v/v).

Mass spectrometry. Direct application was performed with a quadrupolar Nermag 10–10 spectrometer equipped with a Sidar computer system. Ionisation was made by electron impact at 70 eV.

Gas chromatography. Mass spectrometry: GC used a Hewlett Packard gas chromatograph with a SE 30 column (10 m × 0.22 mm) and MS was performed with a HP 5992B spectrometer.

Conditions of conservation

Different factors were studied: air (oxygen), temperature (−20 °C, +4 °C and +40 °C), light and pH. An aqueous solution of thiorphan was prepared as 50 mg/liter. This solution was stored under different conditions:

Influence of air. A part of the solution of thiorphan was left in contact with the air. Another part was filled into flasks sheltered from the air.

Influence of temperature. Flasks were partly filled and stored at different temperatures: +4 °C (refrigerator), −20 °C (freezer) and incubated at +40 °C.

Influence of light. Flasks were partly filled and covered with aluminium foil.

Influence of pH. Three solutions of thiorphan were prepared with 3 aqueous buffer solutions at pH 5, 7 and 8. The interval between the 3 pHs was chosen narrow because thiorphan must be administred intrathecally.

Results and Discussion

Characterization of thiophan degradation products

Oxidative degradation of thiorphan in solution

Analysis by HPLC with UV detection and TLC with spectrophotodensitometric detection of a thiorphan solution left several days in contact with the air or partially oxidized by an iodine solution showed a decrease of the thiorphan peak and two further peaks placed after thiorphan if HPLC (Fig. 2) and on both sides if TLC (Fig. 3).

The oxidative mechanism of the degradation was identified by HPLC analysis with electrochemical detection. In the same conditions of degradation, it showed the decrease of thiorphan peak but did not detect the further two peaks.

Identification of the degradation products

As we did not succeed in separating the 2 degradation products, we analysed the mixture.

According to the thiorphan formula, we could make some hypotheses. Thiorphan shows in its formula a functional group thiol SH. This group is very reductive and can be easily oxidized to give a disulfide (S–S), a sulfonic acid (SO_3H) or a sulfon (SO).

Two of the 3 suppositions could be ruled out according to the stoichiometry of the oxidation reaction, the elementary analysis and the molecular absorption spectrophotometry with multichannel detection.

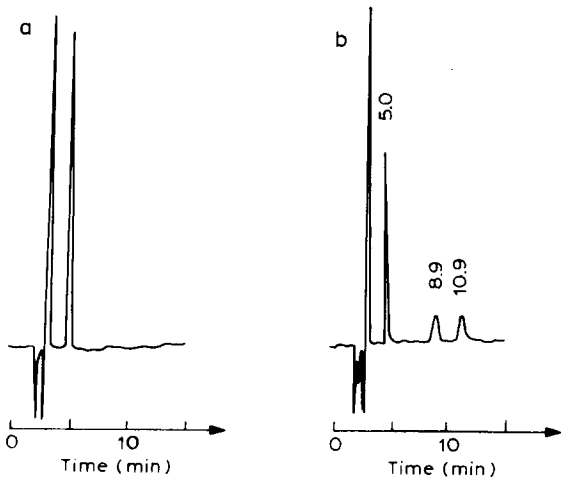

Fig. 2. a: aqueous solution of thiorphan. b: aqueous solution of partially oxidized thiorphan.

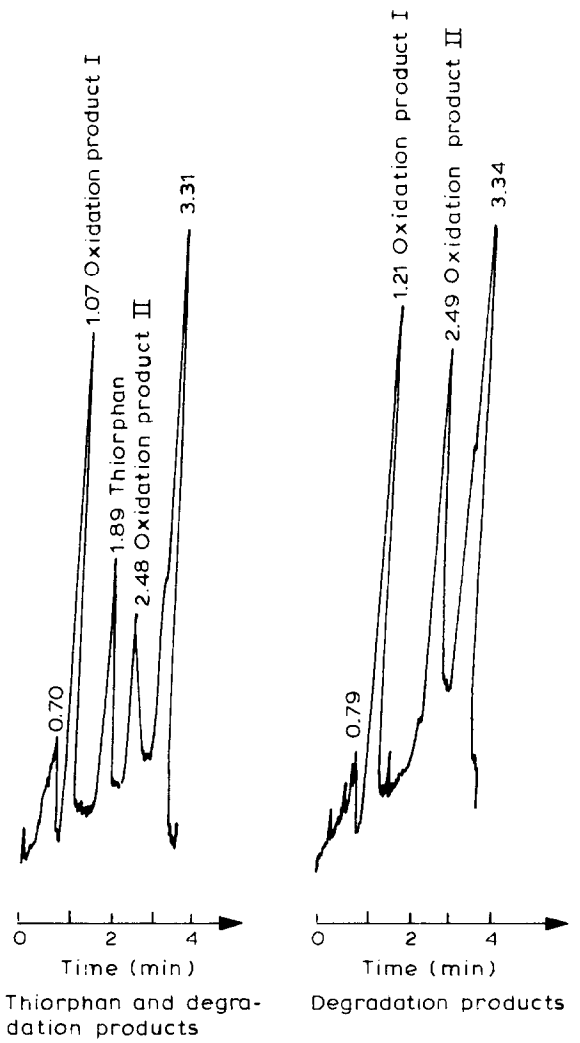

Fig. 3. TLC of thiorphan solutions.

Elementary analysis. The elementary analysis of carbon C, hydrogen H, oxygen O, nitrogen N and sulfur S gave very similar results for thiorphan and the degradation products (Table 1).

Molecular absorption spectrophotometry with multichannel detection. The molecular absorption spectrophotometry with multichannel detection after HPLC enabled us to perform separately an UV analysis of the different products. It gave

TABLE 1

Elementary analysis of thiorphan

	Thiorphan	Oxidized thiorphan
C %	56.61	56.63
H %	5.91	5.88
N %	5.23	5.57
O %	18.99	19.02
S %	12.66	12.68

similar UV spectra for thiorphan and the two degradation products. The fact that we found also very similar first and second derivatives (Fig. 4) suggests similar structures for thiorphan and its degradation products.

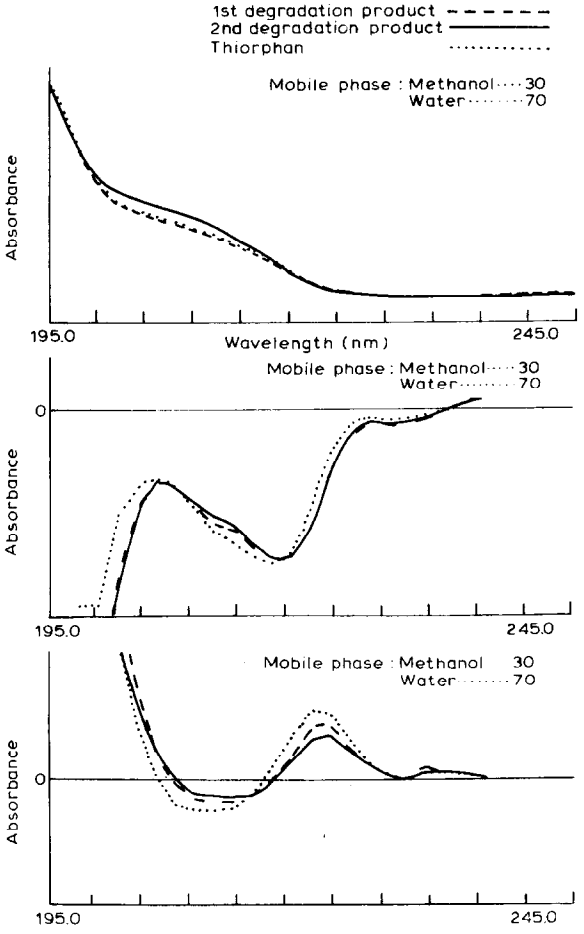

Fig. 4. UV spectrophotometry of thiorphan and degradation products.

Mass spectrometry. Direct application. After direct application and 70 eV electron impact (EI), thiorphan spectrum showed a molecular ion at $m/z = 253$ (Fig. 5). The degradation products' spectrum revealed a molecular ion at $m/z = 504$ (Fig. 6), related to the mass of the disulfide, a hypothesis we made before. Masses between $m/z = 253$ and $m/z = 504$ were identified as fragments of the disulfide.

Mass spectrometry with direct application enabled us to determine that one of the two degradation products was the disulfide. To confirm and complete these results, we used combined techniques: gas chromatography–mass spectrometry (GC-MS), liquid chromatography–mass spectrometry (LC-MS) and mass spectrometry–mass spectrometry (MS-MS).

Gas chromatography–mass spectrometry. To be analysed with GC, thiorphan must be methylated by diazomethane.

After GC analysis, methylated thiorphan revealed two peaks with similar height ratio. Detection with mass spectrometry gave two corresponding spectra:

The first spectrum showed a molecular ion $m/z = 267$, related to the monomethylated thiorphan. Study of the different fragments enabled us to place the methyl substituent on the carboxyl COOH.

The second spectrum showed a molecular ion $m/z = 281$, related to the dimethylated derivative. Double methylation took place on the SH and COOH of thiorphan.

GC-MS performed on oxidized and methylated thiorphan showed the same two peaks but with a large preponderance for peak I ($m/z = 267$) over peak II ($m/z = 281$).

These results can be explained by the fact that degradation of thiorphan gave disulfide. This disulfide was methylated on the 2 COOH and was undoubtedly reduced in the chromatograph where it gave the monomethylated compound of thiorphan. The small peak II ($m/z = 281$) can be explained by reduction of thiorphan during methylation.

Liquid chromatography–mass spectrometry. Combination LC-MS did not result in mass spectra of the two separated degradation products.

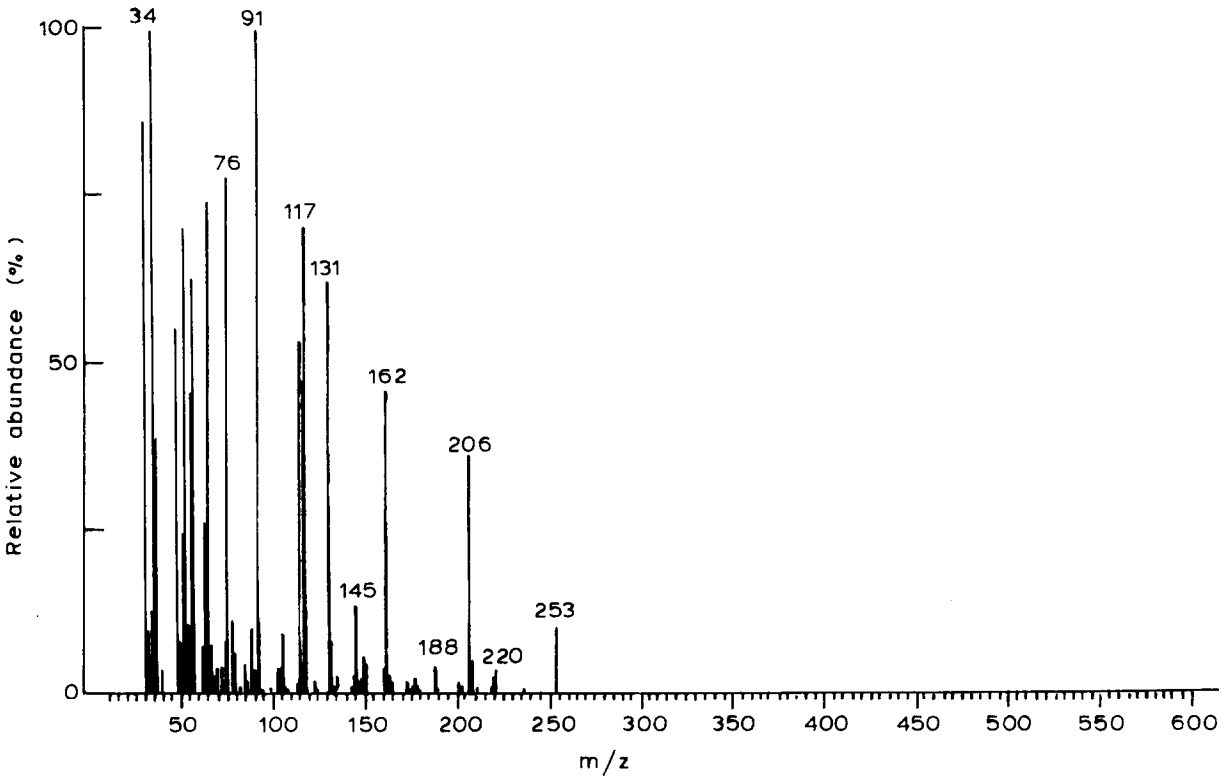

Fig. 5. Mass spectrum of thiorphan.

Ionisation performed in the mass spectrometer after the liquid chromatography was too drastic and caused an important destruction of the molecule, giving fragments with very low molecular masses.

Mass spectrometry–mass spectrometry. MS-MS was performed on thiorphan degradation products with negative chemical ionisation NCI (NH_3). Study of parent ions and the resulting daughter ions showed that there was only one molecular ion $m/z = 504$ for the oxidized thiorphan (under publication).

Stereoisomery of thiorphan and its disulfide

These results drew us to the conclusion that the two degradation products had the same molecular mass but had different chromatographic behaviors. The only possible explanation was the presence of diastereoisomers of the disulfide.

Actually, thiorphan has in its formula an asymmetrical carbon and can show two configurations: R or S. The thiorphan we used was a mixture of these configurations (Fig. 7) (Scott et al., 1985; Evans et al., 1985).

After degradation, thiorphan was transformed into its disulfide with two asymmetrical carbons, that has 4 isomers (Fig. 8): Chromatography isolated (1) compounds A (R,S) and D (S,R) that could be superposed in space and were identical; (2) compounds B (R,R) and C (S,S), related to one another as mirror images, representing the optical antipodes.

Study of the degradation kinetics of thiorphan in aqueous solution

All the results given are expressed as percentages of unoxidized thiorphan (Table 2).

Influence of oxygen

The results show an important influence of the oxygen in the air, which was suggested by the

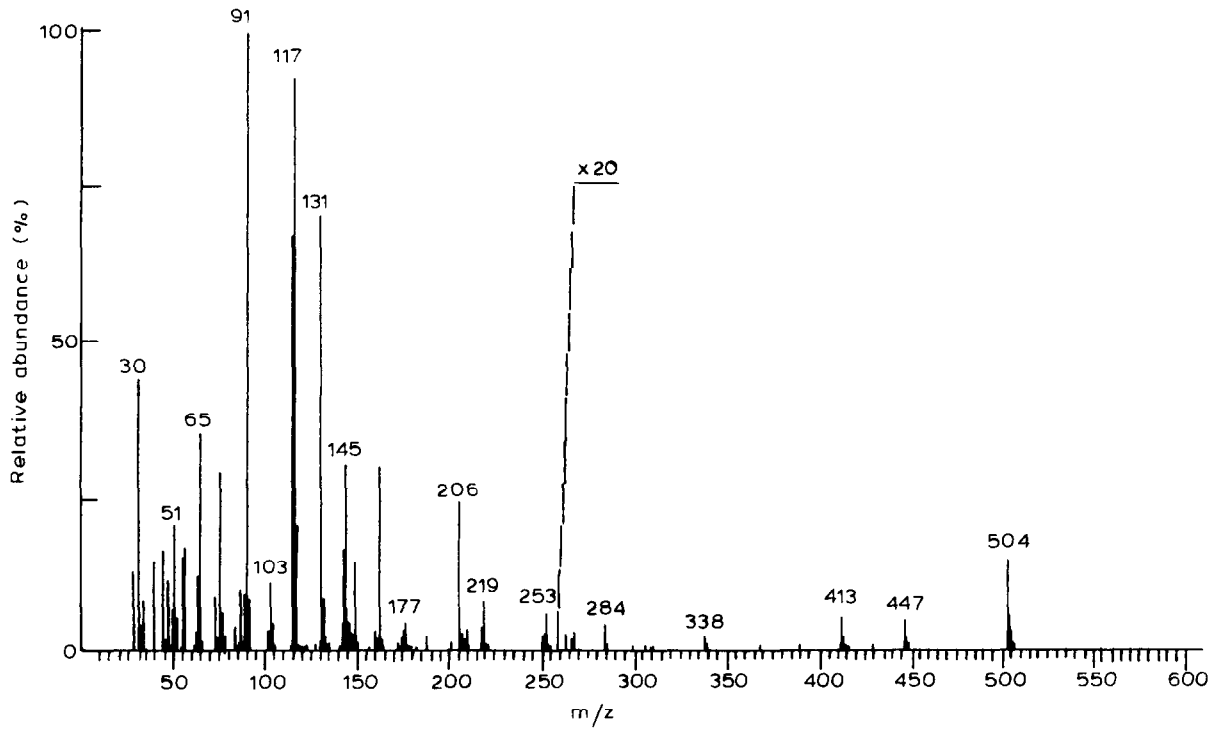

Fig. 6. Mass spectrum of disulfide.

oxidizing mechanism of the degradation of thiorphan. The slight degradation noted without air can be explained by oxygen dissolved in the solution. A purge with nitrogen should improve the stability of thiorphan in solution.

Influence of temperature

The results show that congelation at $-20\,^{\circ}C$ and cold at $+4\,^{\circ}C$ are two efficient ways to protect thiorphan.

Heat ($+40\,^{\circ}C$) greatly accelerates thiorphan degradation.

Influence of light

Like heat, but to a lesser extent, light accelerates the oxidative degradation of thiorphan.

Influence of pH

The degradation of thiorphan is accelerated by basic and neutral pH. Therefore, thiorphan must be preserved at an acid pH.

Fig. 7. A: configuration S. B: configuration R.

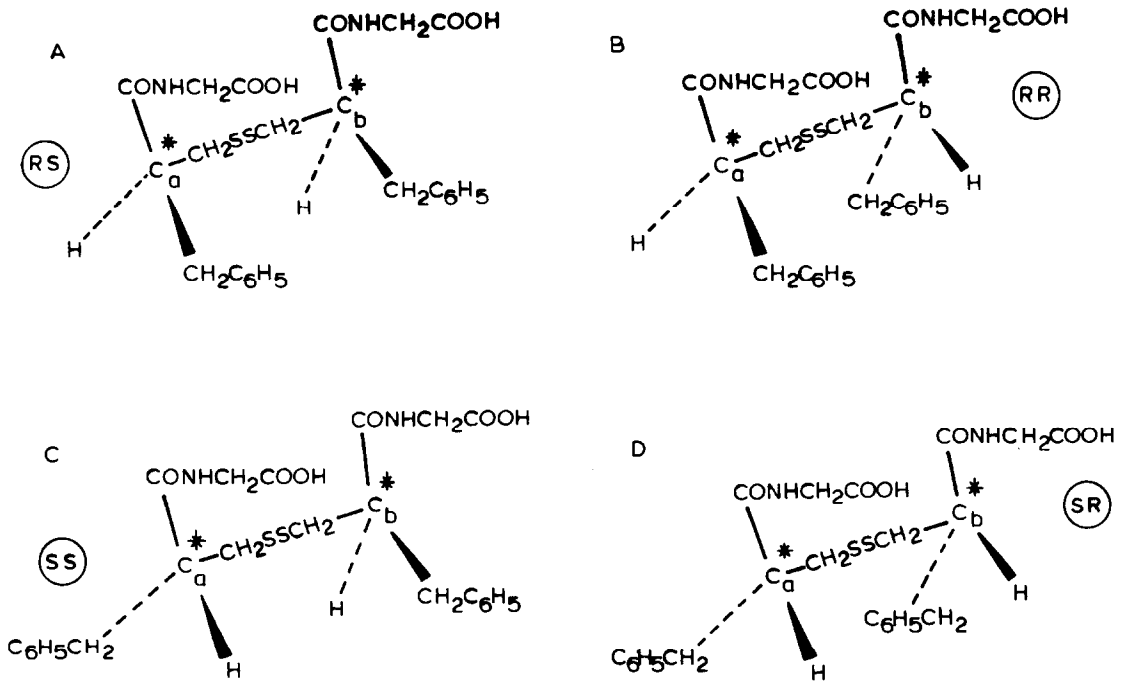

Fig. 8. A: C_a, R; C_b, S. B: C_a, R; C_b, R. C: C_a, S; C_b, S. D: C_a, S; C_b, R.

TABLE 2

Influence of oxygen, temperature, light and pH on thiorphan solution stability (%)

	D1	D2	D3	D4	D7	D9	D11	D30	D45
In air	98	92	91	84	65	54	36	25	0
Sheltered	92	91	78	77	72	71	60	59	53
+40 °C	77	57	46	44	28	7	0	0	0
Room temp.	98	92	91	84	65	54	36	25	0
+4 °C	96	92	91	90	90	81	75	55	50
−20 °C	92	90	90	89	88	85	78	76	75
Light	98	92	91	84	65	54	36	25	0
Sheltered	92	89	87	86	84	82	57	44	38
pH 5	97	93	92	90	83	78	–	–	–
pH 7	93	90	58	42	31	0	–	–	–
pH 8	94	83	56	38	29	0	–	–	–

D = day

TABLE 3

Optimal conditions of conservation for thiorphan solution (%)

	W1	W2	W3	W5	W6	W8	W9	W10
Cond. 1	100	99	95	100	93	93	97	97
Cond. 2	100	98	99	95	97	94	97	98

W = week

Optimal conditions of conservation

Considering the previous results (best conservation if sheltered from air and light, in cold state and at an acid pH). Two conditions of conservation have been considered (Table III). Condition 1: non-buffered aqueous solution of thiorphan (pH 4.5) sparged with nitrogen, covered with aluminium paper and stored at +4°C. Condition 2: solution of thiorphan in dextrose 5% (pH 5), sparged with nitrogen, spiked, covered with aluminium paper and stored at +4°C.

The percentages of un-oxidized thiorphan were determined during 10 weeks.

Conclusion

Thiorphan oxidative degradation generated disulfide diastereoisomers that were separated by chromatography. Knowledge of the origin and identity of the oxidation product will enable us to define a method that will quantify thiorphan in aqueous solution and show degradation products in order to perform an oxidative degradation kinetics study. This method will give the opportunity to investigate influence of usual factors that can be involved in the degradation of a product in solution to determine optimal conditions of conservation for preparation of a pharmaceutical parenteral solution for therapeutic use.

These results led us to the following discussions: (1) Considering that we had a very small quantity of product, we had to work at the limits of the method sensibility. Therefore, we can consider that, referring to these results, thiorphan conservation in these last two conditions is good during at least 2 months. (2) Freeze-drying is not essential. Therefore, we can propose, for an injectable form, ampoules or flasks with opaque packing with a slightly acid solution of thiorphan, sparged with nitrogen and stored at +4°C.

A complete study of the degradation kinetics of a thiorphan solution in the long run is necessary to assess the validity period of such a solution for therapeutical use.

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