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Journal of Chromatography A, 846 (1999) 207–216

JOURNAL OF CHROMATOGRAPHY A

Comparison of analytical methods for investigating the photostability of isoxicam

H. Bartsch, A. Eiper, K. Habiger, H. Kopelent-Frank*

Department of Drug Analysis, Institute of Pharmaceutical Chemistry, University of Vienna – Centre of Pharmacy, Althanstrasse 14, A-1090 Vienna, Austria

Abstract

A comparison of three different methods (HPTLC–densitometry, HPLC, CE) developed for the photostability testing of isoxicam is presented. The stability indicating capability of the assays is proved using forced degradation by exposing a sample solution to artificial irradiation from a xenon source. The chromatograms and electropherogram of the resulting solution show isoxicam well resolved from the degradation products. For quantitation, external calibration is employed, all calibration curves being linear in the respective concentration range of interest. Photodegradation of isoxicam is studied with special emphasis on the investigation of the correlation between concentration of the sample solution and the stability. Isoxicam solutions of three different concentrations (2 mg ml^{-1}; 250 μg ml^{-1}; 40 μg ml^{-1}) are subjected to simulated sunlight for 480 min and tested for stability. The methods are compared in respect of performance, precision, detection and quantitation limits.

Keywords: Stability studies; Photodegradation; Validation; Pharmaceutical analysis; Isoxicam

1. Introduction

The results of photostability studies of drugs are influenced by manifold factors which often seem to be not sufficiently taken into account. The dependency of the concentration of the sample solution on its photostability for instance is frequently underestimated. To exemplary illustrate this, the class of oxicam compounds belonging to the group of non-steroidal antirheumatic drugs is well suited since publications dealing with photostability of oxicams mention quite diverse results. Accordingly, contradictory data concerning the photostability of oxicam derivatives can be found in the literature. Piroxicam solutions (1% in aqueous media) exposed to sunlight were found to be stable for 72 h [1]. On the other hand aqueous solutions (~2.3 μg ml^{-1}) of tenoxicam, a thienothiazine oxicam derivative, showed about 50% loss of the drug when exposed to sunlight for 3 h [2]. These diverse results promoted interest to carry out a comprehensive study on the photostability of oxicams. Special emphasis was laid on the potential correlation between the sample concentration and the photostability of the oxicams. The influence of different light sources was considered as well. We found the stability of tenoxicam and piroxicam to be dependent of the nature of light, a marked concentration dependency was observed as well [3,4]. This concentration dependency of the photodegradation of the oxicams might help explaining the different results published [1,2], and empha-

*Corresponding author. Tel.: +43-1-31336-8317; fax: +43-1-31336-771.

E-mail address: kopelent@speedy.pch.univie.ac.at (H. Kopelent-Frank)

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00134-X

sises the importance to consider manifold factors in photostability tests of drugs.

Isoxicam is a potent long acting anti-inflammatory agent, highly effective in relieving the symptoms of rheumatoid arthritis and degenerative joint disease [5]. As a side effect it has been reported to lead to serious skin diseases [6]. Chromatographic systems for determination of isoxicam in human plasma and urine have previously been published [5,7].

In this paper a study on the stability of isoxicam (Fig. 1) including comparison of three different stability indicating methods [high-performance thin-layer chromatography (HPTLC)–densitometry, high-performance liquid chromatography (HPLC), capillary electrophoresis (CE)] developed for the photostability testing of isoxicam in simulated sunlight is presented. New assays had to be developed since up to now stability indicating HPTLC and CE separations had not been described. The HPLC systems proposed previously [5,7] had not been established for stability purposes but exclusively for the determination of isoxicam in biological matrices (blood, serum, urine) and mainly used eluents containing acetonitrile. In our study we intended to utilize the less toxic methanol as organic component of the eluent, in addition we wanted to prove if other methods like CE and HPTLC were equally useful for stability test evaluations. Especially smaller laboratories like hospital pharmacies are often faced with drug stability problems and should be encouraged to carry out their own investigations. Since they mostly have limited equipment the comparison of HPLC with HPTLC should show that though HPTLC is a classic method which was pushed into the background by HPLC, it still can be widely used leading to comparable results. CE which is recently gaining in popularity for drug analysis up to now has not frequently been applied to stability investigations so including this method in our study seemed advantageous. In the present study the stability was investigated by quantitation of isoxicam by the methods mentioned. Moreover, the methods are compared in respect of performance, precision, detection and quantitation limits.

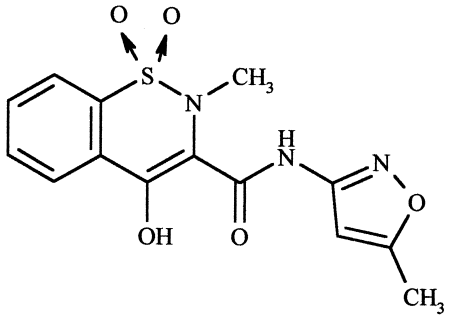

Fig. 1. Isoxicam.

2. Experimental

2.1. Materials

Isoxicam was obtained from Sigma (Vienna, Austria) and stored at room temperature. A 25% NH_4OH solution analytical grade was obtained from Riedel-de Haen (Seelze, Germany). For HPTLC chloroform, 1-propanol and 96% acetic acid were of analytical grade and obtained from Merck (Darmstadt, Germany). Methanol HPLC reagent and water HPLC reagent were obtained from J.T. Baker (Deventer, Netherlands). Sodium acetate and conc. acetic acid for the preparation of acetate buffer, pH 4.6 were of analytical grade. A 20 m*M* sodium phosphate buffer solution, pH 8.0 for HPCE was obtained from Fluka (Buchs, Switzerland).

2.2. Sample preparation

Solutions containing isoxicam at three different concentrations (2 mg ml^{-1}, 250 μg ml^{-1} and 40 μg ml^{-1}) in 2.5% NH_4OH solution (pH~11.8) were prepared. For each concentration three sample solutions were prepared, for HPTLC–densitometry and CE each tested in triplicate and for HPLC each tested in duplicate for exposure to irradiation in the Suntest. For HPTLC and CE the solutions were used as described, samples for HPLC were diluted with the eluent before injection (solutions of 2 mg ml^{-1} were diluted 1:50, v/v; of 250 μg ml^{-1} were diluted 1:10, v/v, and those of 40 μg ml^{-1} were diluted 1:2, v/v).

2.3. HPTLC–densitometry

2.3.1. Equipment

Analyses were carried out using a Shimadzu (Kyoto, Japan) CS-9301 PC dual-wavelength flying spot scanner (P/N 206-80625). Separation was

Table 1
Intra-day precision of proposed HPTLC–densitometry method

Concentration (μg ml^{-1})	*n*	Found mean concentration±SD (μg ml^{-1})	RSD (%)
2000	9	1816.6±35.94	1.98
2000	9	1831.1±41.32	2.26
2000	9	1824.1±42.82	2.35
250	9	256.5±5.72	2.23
250	9	256.3±8.18	3.19
250	9	257.6±7.14	2.77
40	9	42.1±1.34	3.18
40	9	42.1±0.97	2.31
40	9	42.7±1.42	3.33

achieved on Merck HPTLC plates 10×10 cm, silica gel 60 F_{254} using a Camag (Berlin, Germany) horizontal developing chamber for 10×10 cm chromatographic plates. Plates are prewashed before use with methanol–dichloromethane (1:1, v/v). Sample application was performed with a Camag Linomat IV (Hamilton syringe 100 μl).

2.3.2. Analytical conditions

The samples were applied on the plate bandwise 1 cm above the plate-ledge. The volume of the sample solution introduced to the HPTLC plate was chosen according to the concentration of each solution (1 μl for 2 mg ml^{-1}; 3 μl for 250 μg ml^{-1}; 10 μl for 40 μg ml^{-1}). The HPTLC mobile phase was chloroform–1-propanol–96% acetic acid (9:0.5:0.5, v/v/v). The plates were developed using chamber saturation at room temperature.

The densitometric measurements were made at λ=280 nm using zig-zag scan with a swing width of 3 mm and a beam size of 0.4 mm. The comparison of the remission spectra of the isoxicam peak of freshly prepared as well as stressed solutions proved that no degradation products overlap the peak of isoxicam. For quantitation external calibration was carried out. For each concentration range five standard solutions were prepared. Linear calibration curves were obtained for each concentration range of the samples [concentration 40 μg ml^{-1}: 44.0, 35.2, 26.4, 17.6 and 8.8 μg ml^{-1} (r≥0.997), concentration 250 μg ml^{-1}: 300, 240, 180, 120 and 60 μg ml^{-1} (r≥0.997) and concentration 2 mg ml^{-1}: 2.20, 1.76, 1.32, 0.88 and 0.44 mg ml^{-1} (r≥0.996)].

The method was validated by evaluation of the intra- and inter-day precision (Tables 1 and 2). The relative standard deviations (RSDs) on the basis of quantitative results by external calibration for nine replicate spotted points were found to be between 1.98% and 2.35% (2 mg ml^{-1}), 2.23% and 3.19% (250 μg ml^{-1}) and 2.31% and 3.33% (40 μg ml^{-1}) in the intra-day assay. The RSDs in the inter-day assays (three days, n=27) were 2.14% for 2 mg ml^{-1}, 2.66% for 250 μg ml^{-1} and 2.94% for 40 μg ml^{-1}. The detection limit (LOD) of the method was determined to be 5 μg ml^{-1} and the quantitation limit (LOQ) 10 μg ml^{-1} (LOD 3:1 S/N; LOQ 10:1 S/N; top to top).

2.4. HPLC

2.4.1. Equipment

Analyses were carried out using a Shimadzu HPLC system [pumps: Shimadzu LC 10 AS; diode-array detector: Shimadzu SPD-M10A; column oven: Shimadzu CTO-10AC (20°C); Rheodyne injection

Table 2
Inter-day precision of proposed HPTLC–densitometry method

Concentration (μg ml^{-1})	Days	Found mean concentration±SD (μg ml^{-1})	RSD (%)
2000	3	1823.9±39.03	2.14
250	3	256.8±6.83	2.66
40	3	42.3±1.24	2.94

Table 3
Intra-day precision of proposed HPLC method

Concentration (μg ml^{-1})	*n*	Found mean concentration±SD (μg ml^{-1})	RSD (%)
2000	9	2035.4±43.65	2.14
2000	9	1967.3±33.79	1.72
2000	9	1936.2±29.14	1.50
250	9	249.9±5.86	2.34
250	9	244.2±4.28	1.75
250	9	250.8±6.46	2.57
40	9	42.5±0.73	1.72
40	9	40.5±0.66	1.62
40	9	42.1±0.97	2.30

valve with a 20-μl loop]. Separation was achieved on a Merck LiChrospher 100 RP 18 endcapped column, 5 μm, 119 mm×3 mm I.D.

2.4.2. Analytical conditions

The HPLC mobile phase was prepared using methanol–acetate buffer, pH 4.6. The mobile phase was filtered and degassed before use. Isocratic elution at 20°C and a flow-rate of 0.8 ml/min was employed with methanol–acetate buffer (pH 4.6, 0.4 *M*) (40:60, v/v).

Diode array detection was used wavelengths set at an absorption maximum of the substance (λ=280 nm) and at 254 nm the universal wavelength used for aromatic compounds. The peak purity index for the drug substance was investigated and found to be better than 0.9996 in chromatograms of the standard compounds as well as in the chromatograms of the stressed solutions. For quantitation external calibration was carried out. For each concentration range five standard solutions were prepared considering the dilution of the samples before injection as well. Linear calibration curves were obtained in all cases [concentration 40 μg ml^{-1}: 44.0, 35.2, 26.4, 17.6 and 8.8 μg ml^{-1} (r≥0.9992), concentration 250 μg ml^{-1}: 300, 240, 180, 120 and 60 μg ml^{-1} (r≥0.9974) and concentration 2 mg ml^{-1}: 2.20, 1.76, 1.32, 0.88 and 0.44 mg ml^{-1} (r≥0.9992)].

The method was validated by evaluation of the intra- and inter-day precision (Tables 3 and 4). The RSDs on the basis of quantitative results by external calibration for nine replicate injections were found to be between 1.50% and 2.14% (2 mg ml^{-1}), 1.75% and 2.57% (250 μg ml^{-1}) and 1.62% and 2.30% (40 μg ml^{-1}) in the intra-day assay. The RSDs in the inter-day assay (three days, n=27) were 2.76% for 2 mg ml^{-1}, 2.48% for 250 μg ml^{-1} and 2.78% for 40 μg ml^{-1}. The detection limit of the method was determined to be 0.32 μg ml^{-1} and the quantitation limit 1.25 μg ml^{-1} (LOD 3:1 S/N; LOQ 10:1 S/N; top to top).

2.5. CE

2.5.1. Equipment

Analyses were carried out using a Hewlett-Packard (Waldbronn, Germany) ^{3D}CE system equipped with an uncoated capillary (HP), 40 cm effective length×50 μm I.D.

2.5.2. Analytical conditions

A new capillary was flushed with 1 *M* and 0.1 *M*

Table 4
Inter-day precision of proposed HPLC method

Concentration (μg ml^{-1})	Days	Found mean concentration±SD (μg ml^{-1})	RSD (%)
2000	3	1979.6±54.62	2.76
250	3	248.3±6.16	2.48
40	3	41.7±1.16	2.78

Table 5
Intra-day precision of proposed CE method

Concentration (μg ml^{-1})	*n*	Found mean concentration±SD (μg ml^{-1})	RSD (%)
2000	9	2141.4±30.39	1.42
2000	9	2087.6±69.37	3.32
2000	9	2140.8±39.76	1.86
250	9	223.6±4.76	2.13
250	9	231.3±6.82	2.95
250	9	234.2±8.04	3.43
40	9	42.0±0.91	2.16
40	9	41.0±1.42	3.47
40	9	39.6±1.02	2.56

sodium hydroxide (15 min each) and water (at least 30 min) before use. Before each injection the capillary was preconditioned by flushing with run buffer for 5 min. Samples were injected by pressure of 50 mbar for 3.00 s. For separation a 20 m*M* sodium phosphate buffer solution, pH 8.0 was used, a voltage of 25 kV was applied at a temperature of 25°C.

Diode array detection was used wavelengths set at an absorption maximum of the substance (λ=280 nm) and at 254 nm the universal wavelength used for aromatic compounds and at 214 nm. The peak purity index for the drug substance was investigated and found to be better than 0.990 in electropherograms of the standard compounds as well as in the electropherograms of the stressed solutions. For quantitation external calibration was used. For each concentration range five standard solutions were prepared. Linear calibration curves were obtained in all cases [concentration 40 μg ml^{-1}: 44.0, 35.2, 26.4, 17.6 and 8.8 μg ml^{-1} ($r \geq 0.998$), concentration 250 μg ml^{-1}: 300, 240, 180, 120 and 60 μg ml^{-1} ($r \geq 0.998$) and concentration 2 mg ml^{-1}: 2.20, 1.76, 1.32, 0.88 and 0.44 mg ml^{-1} ($r \geq 0.997$)].

The method was validated by evaluation of the intra- and inter-day precision (Tables 5 and 6). The RSDs on the basis of quantitative results by external calibration for nine replicate injections were found to be between 1.42% and 3.32% (2 mg ml^{-1}), 2.13% and 3.43% (250 μg ml^{-1}) and 2.16% and 3.47% (40 μg ml^{-1}) in the intra-day assay. The RSDs in the inter-day assay (three days, n=9) were 2.54% for 2 mg ml^{-1}, 3.42% for 250 μg ml^{-1} and 3.60% for 40 μg ml^{-1}. The detection limit of the method was determined to be 1 μg ml^{-1} and the quantitation limit 5 μg ml^{-1} (LOD 3:1 *S*/*N*; LOQ 10:1 *S*/*N*; top to top).

2.6. Light conditions

The sample solutions (10 ml each in a 10-ml volumetric flask) were exposed to forced irradiation using a Suntest CPS accelerated exposure machine (Heraeus, Hanau, Germany): xenon burner, black panel temperature: 49°C at maximum radiation intensity; windowglass filter; time factor: 15 (1 min Suntest ≅ 15 min natural sunlight).

3. Results and discussion

Three different analytical assays (HPTLC–densitometry, HPLC and CE) were developed to allow

Table 6
Inter-day precision of proposed CE method

Concentration (μg ml^{-1})	Days	Found mean concentration±SD (μg ml^{-1})	RSD (%)
2000	3	2123.3±53.98	2.54
250	3	229.7±7.86	3.42
40	3	40.8±1.47	3.60

the selective quantitation of isoxicam in presence of its degradation products. HPLC of course is the most frequently used method in drug analysis. A comparison of the results obtained with this method with different other methods seemed of interest. The applicability of CE for drug determination is documented by the highly increasing number of papers on this subject, but only few papers up to now dealt with stability indicating separations. TLC was pushed into the background by the increasing success of HPLC though this method seems well suited especially for occasional applications since it is rapid, can economically be employed for routine use, is versatile and needs less system maintenance than e.g., HPLC. The stability indicating capability of the assays for the determination of isoxicam was proved using sample solutions subjected to forced degradation by exposing them to artificial irradiation from a xenon source in a Suntest. This accelerated exposure machine is rated at 15-times the intensity of sunlight thus leading to reduced testing time. It provides radiation distribution similar to natural sunlight and reproducible conditions giving repeatable level of irradiation which is not guaranteed when reliance is placed on varying intensities of natural sunlight. Different exposure times were chosen for the respective concentrations.

The resulting chromatograms and the electropherogram of the stressed solutions are shown in Figs. 2–4. In all cases the degradation products are well resolved from the peak of isoxicam. The different number of degradation products shown in the chromatograms and electropherogram are due to the different detection limits of the methods, CE moreover showing the highest number of products since detection in contrast to HPLC is possible at short wavelengths. No degradation products overlap the peak of isoxicam which was proved by assessing the peak purity (comparison of remission spectra in HPTLC, peak purity index in HPLC in all cases found to be better than 0.9996, peak purity index in CE better than 0.990).

For the photostability testing isoxicam solutions of three different concentrations (2 mg ml^{-1}; 250 μg ml^{-1}; 40 μg ml^{-1}) were subjected to simulated sunlight for 480 min. Samples were removed at

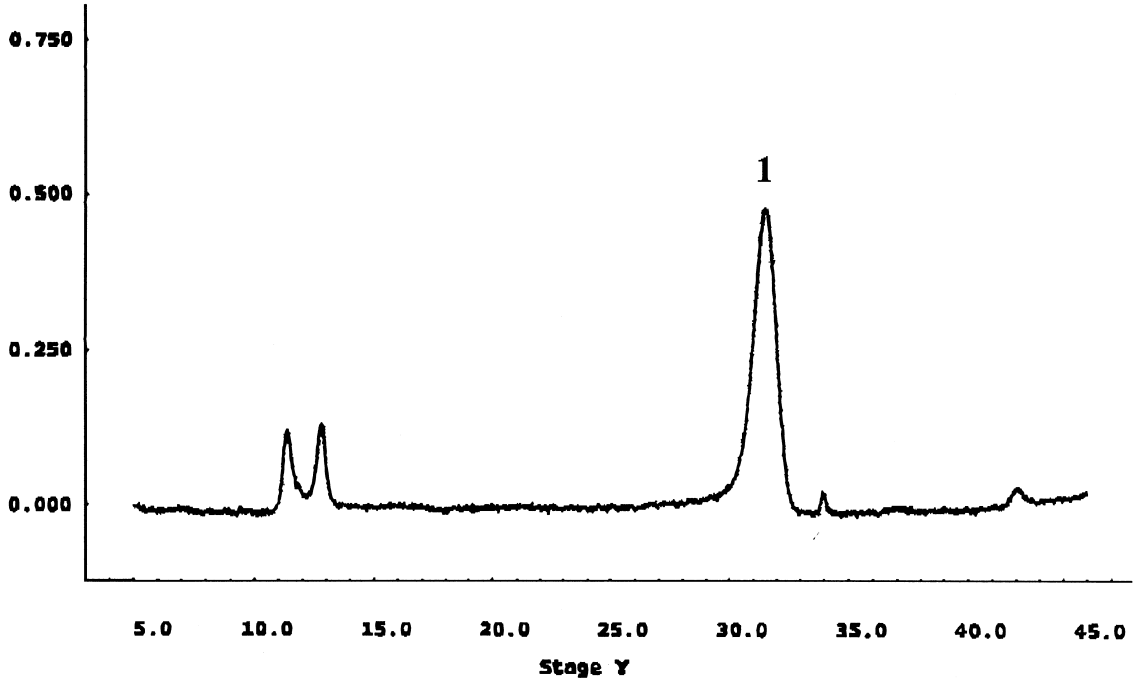

Fig. 2. HPTLC chromatogram of isoxicam (2 mg ml^{-1}) irradiated for 5 h in the Suntest.

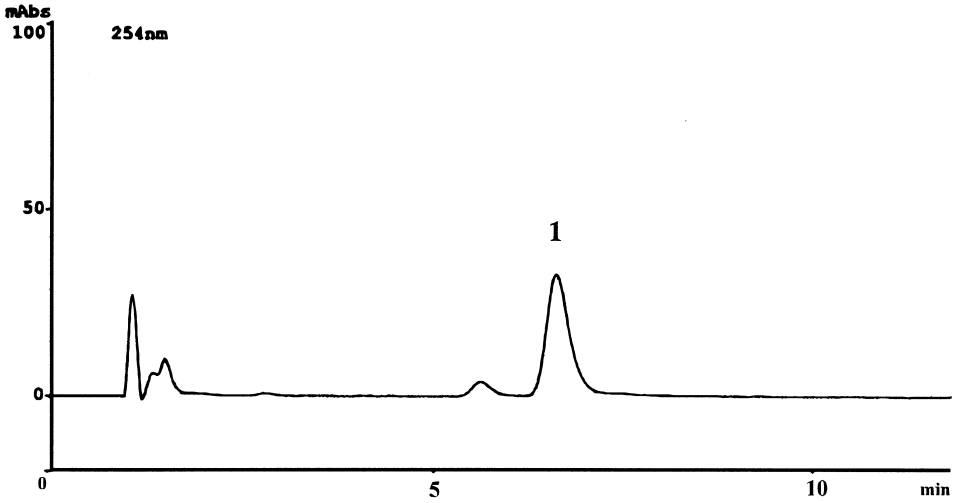

Fig. 3. HPLC chromatogram of isoxicam (40 μg ml^{-1}) irradiated for 3 h in the Suntest.

certain times and tested for the amount of isoxicam remaining in the solution. The results are given in Tables 7–9. Light exposure leads to degradation of different extent strongly dependent on the respective concentration of the drug substance. The results of the three different analytical methods correspond very well. The concentration dependency of the photodegradation is distinctly shown (Fig. 5).

The RSD of the three methods at each concentration (Tables 1–6) as could be expected gave the best results for quantitation utilizing HPLC. HPTLC–densitometry and CE showed higher standard deviations, which nevertheless are within appropriate levels for stability tests. Since migration time reproducibility might lead to problems in CE, a migration time validation was carried out ($n=9$), the migration times found to be 2.799 ± 0.083 (RSD 0.30%). HPLC showed the lowest and HPTLC the highest detection and quantitation limits of the three used methods (Table 10).

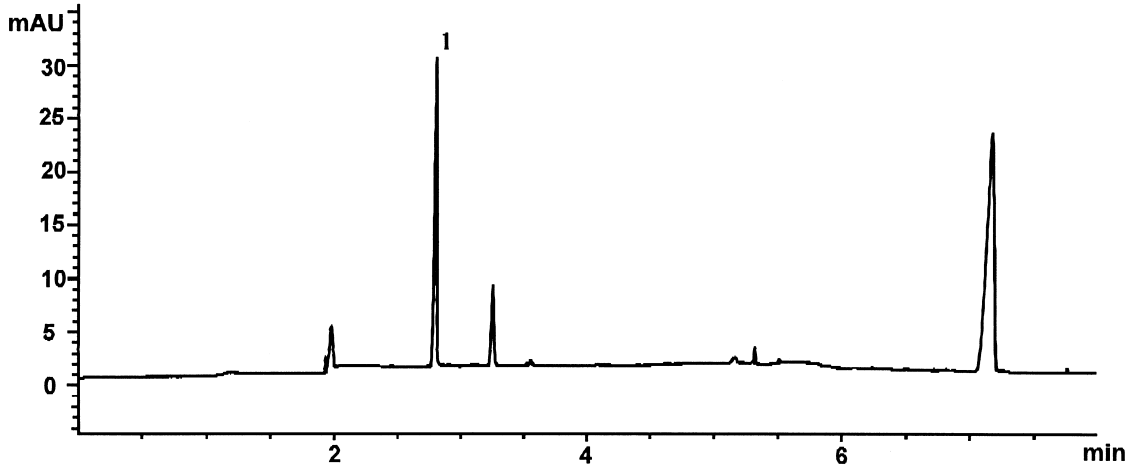

Fig. 4. CE electropherogram of isoxicam (250 μg ml^{-1}) irradiated for 5 h in the Suntest.

Table 7
Degradation of isoxicam in solutions of 2 mg ml^{-1} (100% at $t=0$) (mean $n=9$ for HPTLC and CE) (mean $n=6$ for HPLC) (irradiation in the Suntest)

Time (min)	HPTLC (% of initial concentration)	HPLC (% of initial concentration)	CE (% of initial concentration)
0	100.00	100.00	100.00
24	95.01	97.86	99.07
48	94.81	98.65	98.62
96	91.25	96.87	96.05
144	88.43	93.43	92.80
192	82.73	91.49	91.03
288	78.52	86.64	87.43
384	74.76	80.91	81.91
480	70.63	73.80	75.42

Table 8
Degradation of isoxicam in solutions of 250 μg ml^{-1} (100% at $t=0$) (mean $n=9$ for HPTLC and CE) (mean $n=6$ for HPLC) (irradiation in the Suntest)

Time (min)	HPTLC (% of initial concentration)	HPLC (% of initial concentration)	CE (% of initial concentration)
0	100.00	100.00	100.00
24	98.69	98.60	93.88
48	94.19	93.53	90.08
96	84.09	92.22	79.44
144	71.73	71.75	68.39
192	60.82	62.92	59.87
288	46.97	39.86	32.48
384	19.57	20.96	15.33
480	3.17	5.50	5.16

Table 9
Degradation of isoxicam in solutions of 40 μg ml^{-1} (100% at $t=0$) (mean $n=9$ for HPTLC and CE) (mean $n=6$ for HPLC) (irradiation in the Suntest)

Time (min)	HPTLC (% of initial concentration)	HPLC (% of initial concentration)	CE (% of initial concentration)
0	100.00	100.00	100.00
24	88.13	88.00	83.62
48	74.49	74.30	71.10
96	40.83	46.00	39.60
144	19.41	24.29	22.63
192	9.59	10.96	10.96
288	–[a]	–	–
384	–	–	–
480	–	–	–

[a] –=Not detectable.

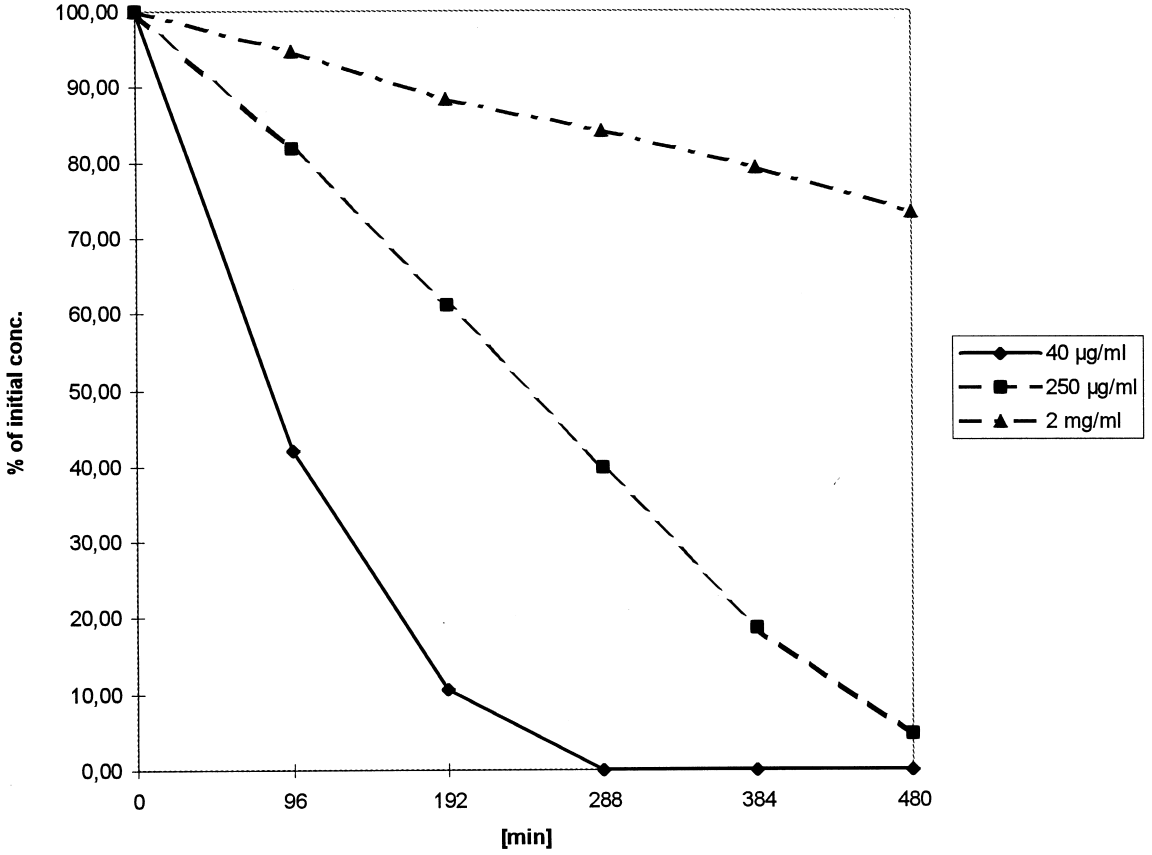

Fig. 5. Degradation of isoxicam in solution at different concentrations.

Table 10
Comparison of detection and quantitation limits of the used methods

Method	LOD (µg ml^{-1})	LOQ (µg ml^{-1})
HPTLC	5	10
HPLC	0.32	1.25
CE	1	5

4. Conclusions

The photostability of isoxicam in solution was tested using irradiation from a xenon source which yields light corresponding to natural sunlight with regard to wavelength distribution and relative intensities at the respective wavelengths. The photodegradation of isoxicam was found to be highly dependent on the concentration of the sample solution which is proved by the quantitative results. Elucidation of the structure of the degradation products will be subject to a further project. The three assay methods proposed for selective quantitation of isoxicam in presence of its degradation products proved to be well suitable thus stability tests of oxicams can be evaluated with either assay according to the equipment available. Since generally stability tests utilizing CE have not been frequently reported up to now, the comparison of the results obtained by the employed CE and HPLC methods was of special interest.

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