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High-performance liquid chromatographic determination of chlordiazepoxide, its metabolites and oxaziridines generated after UV irradiation

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Abstract

A reversed-phase high-performance liquid chromatographic (HPLC) procedure for the determination of chlordiazepoxide, its four metabolites and three unstable photoproducts (oxaziridines) generated by UV irradiation is reported. The influence of pH and acetonitrile (CH_3CN) and tetrahydrofuran (THF) percentages in the mobile phase was investigated. The optimum method uses a Nucleosil C_{18} column (5 μm) with a mobile phase consisting of CH_3CN–THF–0.06 *M* phosphate buffer (pH 5.8) (22:2:76). Eight drugs covering a wide range of lipophilicity and polarity were separated in 50 min. The absolute detection and quantification limits of benzodiazepines were less than 1 and 5 ng, respectively. The HPLC method alone or coupled with the UV irradiation procedure showed good precision (R.S.D. 1.7–4.0%) and excellent linearity in the range of 1.5–43 μg/ml ($r \geq 0.999$).

1. Introduction

Chlordiazepoxide (CDZ), used as a tranquillizer, undergoes extensive chain metabolization in man. The major metabolites are desmethylchlordiazepoxide (DES-CDZ) and demoxepam (DEM) and the minor metabolites are desmethyldiazepam (DES-DIAZ) and oxazepam (OXAZ), which are generally detected during chronic administration of CDZ [1] (Fig. 1).

Under UV irradiation, CDZ [2,3] and its two main metabolites isomerize to give photoproducts implicated in phototoxic effects (Fig. 1). Oxaziridine (OX) appeared to be the first and main drug formed if CDZ is irradiated with long-wavelength UV radiation. The N-oxide function of the parental 1,4-benzodiazepine is then transformed into an epoxy group [4–7]. When exposed to UV radiation, oxaziridine is transformed into quinoxaline and benzoxadiazocine [4] and when subjected to high temperature it is converted into the parent drug [8].

Reversed-phase high-performance liquid chromatographic (RP-HPLC) procedures have often been used to determine CDZ and its metabolites in biological fluids [9–16]. The determination of

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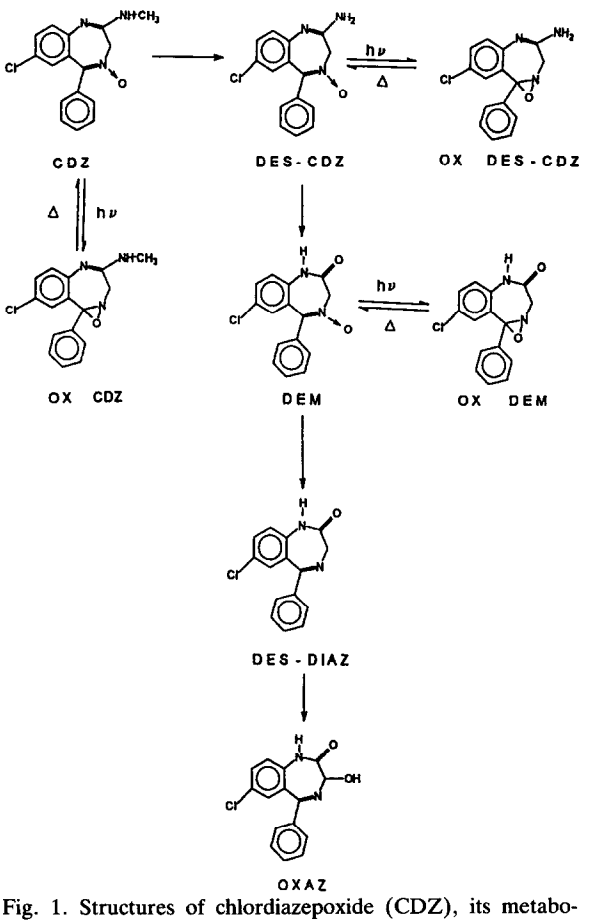

Fig. 1. Structures of chlordiazepoxide (CDZ), its metabolites (DES-CDZ, desmethylchlordiazepoxide; DEM, demoxepam; DES-DIAZ, desmethyldiazepam; OXAZ, oxazepam) and its photoproducts (OX CDZ, oxaziridine of chlordiazepoxide; OX DES-CDZ, oxaziridine of desmethylchlordiazepoxide; OX DEM, oxaziridine of demoxepam).

main decomposition products, namely demoxepam and 2-amino-5-chlorobenzophenone, in CDZ pharmaceutical formulations [17,18] and benzophenones in biological materials [19–21] have also been reported.

In this study, we developed and validated an RP-HPLC method for the separation and determination of CDZ, its four metabolites and three oxaziridines obtained after UV irradiation of CDZ, DES-CDZ and DEM or a mixture thereof. The production and separation of these drugs were the main objectives of this work for further investigations of their *in vitro* cytotoxicity mechanism and *in vivo* pharmacokinetics.

2. Experimental

2.1. Reagents and solutions

Chlordiazepoxide (CDZ) was kindly supplied by Hoffman-La Roche (Brussels, Belgium). Demoxepam (DEM) and desmethylchlordiazepoxide (DES-CDZ) were synthesized according to published methods [22–24] and their purity was monitored by IR and NMR spectrometry. Desmethyldiazepam (DES-DIAS) and oxazepam (OXAZ) were purchased from Sigma (St. Louis, MO, USA) and Federa (Brussels, Belgium), respectively.

Disodium hydrogenphosphate dihydrate and potassium monohydrogenphosphate were of analytical-reagent grade from Merck (Darmstadt, Germany). Acetonitrile (CH_3CN) and tetrahydrofuran (THF) were of HPLC grade from Lab-Scan Analytical Science (Dublin, Ireland) and Merck, respectively. Laboratory-purified HPLC-grade water was used in all mobile phases. The mobile phases were filtered through 0.2-μm membranes (Sartorius, Göttingen, Germany) and sonicated before use. Drugs were dissolved in acetonitrile.

2.2. HPLC system

The HPLC system consisted of a Gilson (Villiers le Bel, France) Model 305 pump, a Rheodyne (Cotati, CA, USA) Model 7125 loop injector and an HM holochrome UV monitor (Gilson) connected to a recorder (Kipp and Zonen, Brussels, Belgium). The reversed-phase chromatographic column (100 × 4.6 mm I.D.) was packed with Nucleosil C_{18} (5 μm) (Chrompack, Antwerp, Belgium) and operated at 20°C and a mobile phase flow-rate of 1 ml/min. The mobile phases tested to optimize the separation of CDZ, its metabolites and its photoproducts consist of CH_3CN, THF and phosphate buffer in various proportions, at different pH values and at different molarities. Volumes of 20–100 μl of sample solutions were injected; detection was performed at 265 nm. The selectivity of the method and the purity of the three photoproducts obtained were investigated with a

photodiode-array detector [Beckman (Fullerton, CA, USA) Model 168 System Gold and System Gold chromatography software (ASW 2)] connected to the HPLC system.

2.3. Irradiation system

The irradiation system was constructed in our laboratory. The materials (Oriel, Stratford, CT, USA) used were a Model 68806 arc lamp power supply which operates with a 150-W xenon lamp, a Photomax 60.100 lamp housing with the xenon lamp in the vertical position, a reflector provided with a demineralized water circulation cooling system, an RAL-UV mirror and lens system and a monochromator with a band pass of 6 nm and set at 350 nm.

2.4. Conditions for UV irradiation of CDZ and its metabolites

Irradiation was carried out at 350 nm to avoid subsequent photoisomerization of oxaziridine [4] (power supply of the lamp set at 6.5 A for a tension of 18 V). The optimum CDZ irradiation conditions to produce pure OX quantitatively were previously determined for concentrations up to 152 μg/ml: CH_3CN as irradiation solvent, a temperature of 10°C, a volume of 0.7 ml for the irradiated solutions and an irradiation time of 70–90 min for the CDZ solutions [25]. The optimum DES-CDZ and DEM irradiation conditions were the same as those for CDZ.

3. Results and discussion

3.1. Organic modifier concentration

In order to select a mobile phase able to separate CDZ and its metabolites, we studied the variation of the retention time (t_R), the peak resolution (R_s) and the separation factor (α) between drugs as a function of the decreasing percentage of CH_3CN and addition of THF. The first mobile phase tested [CH_3CN–0.06 *M* phosphate buffer (pH 5.4)] was previously used to study the kinetics of the photoisomerization of CDZ into oxaziridine and the determination of hydrolysis impurities, namely demoxepam, its oxaziridine and 2-amino-5-chlorobenzophenone, after UV irradiation [25]. The results showed that DES-CDZ and OXAZ are not separated ($R_s = 0.75$; $\alpha = 1.20$) with this mobile phase. Further, slight tailing of the peak of the oxaziridine derived from CDZ was observed (t_R = 15.50 min). Therefore, we first added THF, which works as a tailing suppressor but strongly decreases the retention time of CDZ and its oxaziridine (t_R of OX CDZ = 2.50 min). Therefore, we progressively decreased the proportion of CH_3CN to 23% to obtain a good separation and resolution of CDZ and its four metabolites in less than 20 min. However this mobile phase composition [CH_3CN–THF–0.06 *M* phosphate buffer (pH 5.4) (23:1:76)] was not able to separate OX CDZ and OX DEM after UV irradiation ($R_s = 0$; $\alpha = 1.03$).

3.2. Influence of pH

With a constant proportion of CH_3CN (23%), the influence of pH was tested (Table 1). In addition, for each pH tested, at least two percentages of THF were also investigated. pH was found to be a key parameter for the separation of CDZ, its metabolites and its photoproducts, as illustrated in Fig. 2. When the pH is in the range 5.4–6.0, the order of elution of the drugs in Table 1 is DEM, DES-CDZ, OXAZ, CDZ, DES-DIAZ, OX DES-CDZ, OX DEM and OX CDZ. A decrease in pH to 5.0 results in a reversed elution between the peaks of OX DEM and OX CDZ and also a weak resolution and separation of the two following peaks, DES-DIAZ and OX DES-CDZ, without any further changes (Table 1). At pH 4.5, other major modifications occur, namely, DEM and DES-CDZ are partly or not separated, OXAZ and CDZ co-elute, the four following peaks are separated but the retention times of OX DES-CDZ and OX CDZ have drastically decreased, whilst the retention times of DES-DIAZ and OX DEM do not vary (Table 1).

Mobile phases tested at pH 5.8 and 6.0 separated of all the drugs. We selected the following

Table 1
Influence of pH and THF percentage on the separation of CDZ, its metabolites and its photoproducts

Mobile phase	Parameter	Drug							
		DEM	DES-CDZ	OXAZ	CDZ	DES-DIAZ	OX DES-CDZ	OX DEM	OX CDZ
pH 6.0									
1% THF,									
23% CH_3CN	t_R	5.50	7.75	9.50	12.50	19.00	45.00	49.50	67.00
	R_s	2.86	2.00	3.00	5.27	8.96	1.67	4.97	
	α	1.50	1.26	1.35	1.57	2.44	1.10	1.36	
2% THF,									
23% CH_3CN	t_R	4.75	6.50	8.00	10.25	15.75	35.75	40.25	54.25
	R_s	3.50	2.80	3.00	6.00	7.73	1.48	4.46	
	α	1.47	1.27	1.32	1.59	2.36	1.13	1.36	
3% THF,									
23% CH_3CN	t_R	3.75	5.00	6.50	8.00	12.00	28.00	31.25	42.00
	R_s	1.50	1.58	1.33	3.00	6.29	1.09	3.75	
	α	1.45	1.38	1.27	1.57	2.45	1.12	1.36	
pH 5.8									
1% THF,									
21% CH_3CN	t_R	6.75	10.25	12.50	16.25	27.00	55.75	67.00	78.75
	R_s	2.33	1.43	2.27	4.20	7.74	2.40	2.08	
	α	1.61	1.24	1.33	1.70	2.11	1.21	1.18	
1% THF,									
22% CH_3CN	t_R	5.50	8.00	10.00	13.25	20.25	43.75	52.25	63.00
	R_s	1.83	1.08	2.00	3.88	6.43	2.05	2.00	
	α	1.56	1.29	1.36	1.57	2.22	1.20	1.21	
1% THF,									
23% CH_3CN	t_R	4.75	6.50	8.25	10.75	16.25	34.00	40.50	49.50
	R_s	2.00	1.09	1.67	3.57	10.14	2.07	2.48	
	α	1.47	1.32	1.34	1.56	2.16	1.20	1.23	
2% THF,									
22% CH_3CN	t_R	4.75	6.50	8.25	10.25	16.25	35.00	41.75	51.00
	R_s	1.60	1.40	2.00	3.57	5.90	1.94	2.32	
	α	1.47	1.32	1.28	1.65	2.23	1.20	1.23	
2% THF,									
23% CH_3CN	t_R	4.50	6.25	8.00	10.00	15.00	31.75	38.00	47.00
	R_s	2.00	1.56	1.64	3.38	6.80	2.00	2.55	
	α	1.50	1.33	1.29	1.56	2.20	1.20	1.24	
pH 5.0									
1% THF,									
23% CH_3CN	t_R	5.25	7.00	9.50	11.00	18.50	21.75	47.50	28.75
	R_s	1.40	1.50	1.50	3.88	0.81	2.07[a]	4.94	
	α	1.41	1.42	1.18	1.75	1.19	1.34[a]	1.68	
2% THF,									
23% CH_3CN	t_R	4.50	6.00	8.00	9.50	15.00	17.75	37.75	24.00
	R_s	1.25	2.25	1.00	3.57	1.05	2.47[a]	5.27	
	α	1.43	1.40	1.21	1.65	1.20	1.37[a]	1.60	

[a] Values between OX DES-CDZ and OX CDZ. Mobile phase composition: CH_3CN (21–23%)–THF (1–3%)–0.06 *M* phosphate buffer (pH 4.5–6.0) (78–74%).

Table 1 *(continued)*

Mobile phase	Parameter	Drug							
		DEM	DES-CDZ	OXAZ	CDZ	OX DES-CDZ	OX CDZ	DES-DIAZ	OX DEM
pH 4.5									
1% THF,									
23% CH_3CN	t_R	5.00	6.00	9.25	9.25	13.75	16.50	19.00	49.25
	R_s	0.40	3.00	0.00	2.43	2.00	1.50	11.27	
	α	1.25	1.65	1.00	1.55	1.22	1.16	2.68	
2% THF,									
23% CH_3CN	t_R	4.75	4.75	7.75	7.75	11.00	13.25	16.00	41.00
	R_s	0.00	2.67	0.00	1.29	1.29	1.87	9.27	
	α	1.00	1.69	1.00	1.48	1.23	1.22	2.67	

mobile phase composition to validate the method: CH_3CN–THF–0.06 *M* phosphate buffer (pH 5.8) (22:2:76). It was demonstrated that it maintained a good resolution and separation even if deterioration of the column or ambient temperature variations occurred. As our main objective was to determine low parent benzodiazepine concentrations after irradiation, we need chromatographic conditions that allow a very good separation between these peaks (DEM, DES-CDZ and CDZ) and those of drugs which have retention times close to them and which undergo no degradation (OXAZ and DES-DIAZ). The mobile phase chosen is suitable to resolve this problem and particularly to obtain a good resolution between CDZ and OXAZ. Moreover, these conditions provided a reasonable analysis time.

Fig. 3 illustrates the separation of CDZ and its metabolites before UV irradiation and Fig. 4

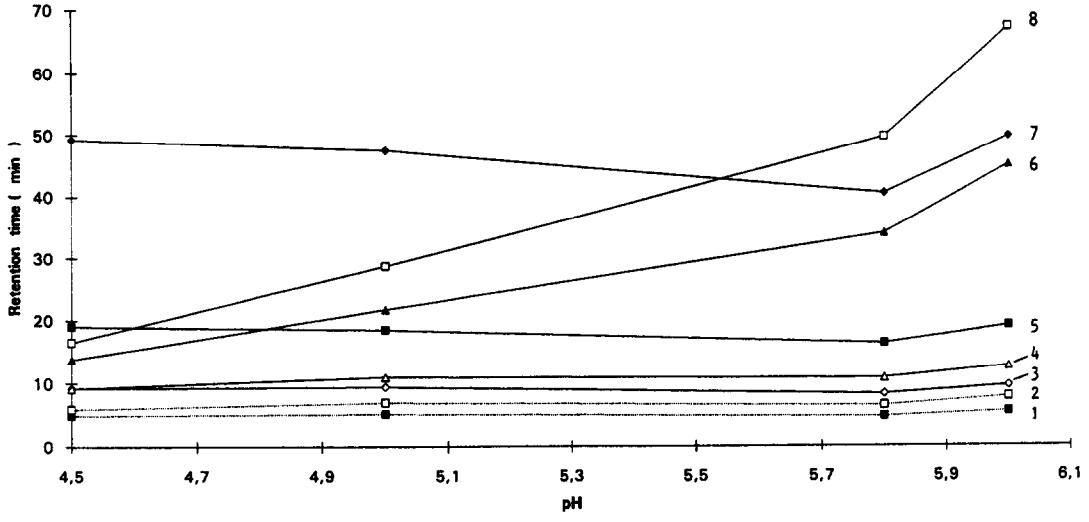

Fig. 2. Variation of retention times of CDZ, its metabolites and its photoproducts as a function of pH. Mobile phase composition: CH_3CN–THF–0.06 *M* phosphate buffer (pH 4.5–6.0) (23:1:76). 1 = DEM; 2 = DES-CDZ; 3 = OXAZ; 4 = CDZ; 5 = DES-DIAZ; 6 = OX DES-CDZ; 7 = OX DEM; 8 = OX CDZ.

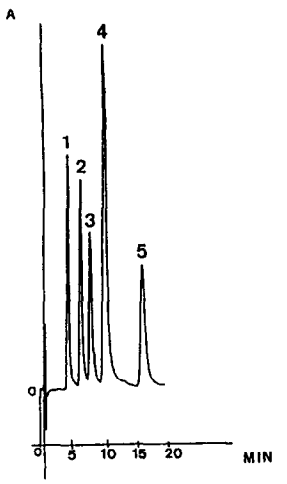

Fig. 3. HPLC separation of chlordiazepoxide (4) and its metabolites, namely desmethylCDZ (2), demoxepam (1), desmethyldiazepam (5) and oxazepam (3). Column temperature, 20°C; flow-rate, 1 ml/min; UV detection at 265 nm. Amounts injected: (1) 310; (2) 230; (3) 480; (4) 420; (5) 670 ng.

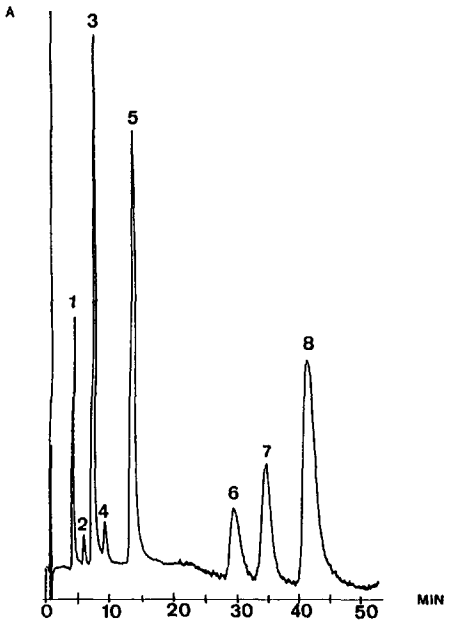

Fig. 4. HPLC separation of chlordiazepoxide (4), its metabolites, namely desmethylCDZ (2), demoxepam (1), desmethyldiazepam (5) and oxazepam (3), and its photoproducts after UV irradiation for 70 min at <10°C, namely oxaziridine of desmethylCDZ (6), oxaziridine of demoxepam (7) and oxaziridine of CDZ (8). Column temperature, 20°C; flow-rate, 1 ml/min; UV detection at 265 nm. Amounts injected: (1) 150; (2) 16; (3) 480; (4) 19; (5) 670 ng; (6–8) unknown.

shows the same separation but after UV irradiation and hence in presence of the three photoproducts.

We also tested different molarities of the phosphate buffer, *viz.*, 0.06, 0.10 and 0.20 *M*, but no advantages accrued. Indeed, the increase in the ionic strength induces a decrease in resolution. Moreover, it would decrease the column lifetime owing to phosphate crystallization.

3.3. Validation of the method

The results were linear over the following ranges: DEM, 0.2–31.8 μg/ml; DES-CDZ, 0.15–23.20 μg/ml; OXAZ, 0.31–49.10 μg/ml; CDZ, 0.27–43.00 μg/ml; and DES-DIAZ, 0.43–68.40 μg/ml. The equations of the calibration graphs and the regression coefficients are given in Table 2. The linearities were verified on three different days with the five drugs in a mixture dissolved in acetonitrile. The absolute detection and quantification limits for the five drugs were all less than 1 ng (for concentrations yielding a signal-to-noise ratio of 2) and less than 5 ng (for concentrations yielding a signal-to-noise ratio of 10), respectively, as illustrated in Table 3. These were determined using a diode-array detector, which also verified the specificity of the method and the purity of the eight substances present after UV irradiation.

The precision was determined by six replicate injections of a solution that contained CDZ and its four metabolites. Table 4 shows the results of the intra- and inter-day determination of these five drugs. There is a good intra-day precision with a mean R.S.D. lower than 2.8% for all the drugs and also a good inter-day precision with a mean R.S.D. lower than 4.1% (determined on four days).

Fig. 5 shows the linear response between the CDZ concentration before irradiation and the peak height of undecomposed CDZ for a constant irradiation time of 70 min. Therefore, the percentage of CDZ remaining is independent of

Table 2
Calibration graphs for CDZ and its metabolites

Compound	Range (μg/ml)	Slope (m)	Intercept (c)	r*
DEM	0.2–31.8	0.2082	0.0075	0.9990
		0.2221	−0.0677	0.9995
		0.2191	−0.0781	0.9995
DES-CDZ	0.15–23.20	0.1665	0.0757	0.9986
		0.1777	−0.0053	0.9991
		0.1800	−0.0347	0.9996
OXAZ	0.31–49.10	0.5177	0.0388	0.9989
		0.5574	−0.1353	0.9992
		0.5538	−0.1479	0.9997
CDZ	0.27–43.00	0.1955	0.0113	0.9989
		0.2084	−0.0577	0.9994
		0.2080	−0.0893	0.9993
DES-DIAZ	0.43–68.40	0.8915	−0.0995	0.9989
		0.9500	−0.2101	0.9996
		0.9494	−0.2857	0.9993

$y = mx + c$, where y = concentration of the injected substance (μg/ml) and x = peak height of the substance (cm). r^* = regression coefficient.

Table 3
Absolute limits of detection and quantification of CDZ and its metabolites

Drug	Injected amount (ng)	
	Limit of detection	Limit of quantification
DEM	0.4	2.0
DES-CDZ	0.3	1.5
OXAZ	0.6	3.0
CDZ	0.5	2.5
DES-DIAZ	0.9	4.5

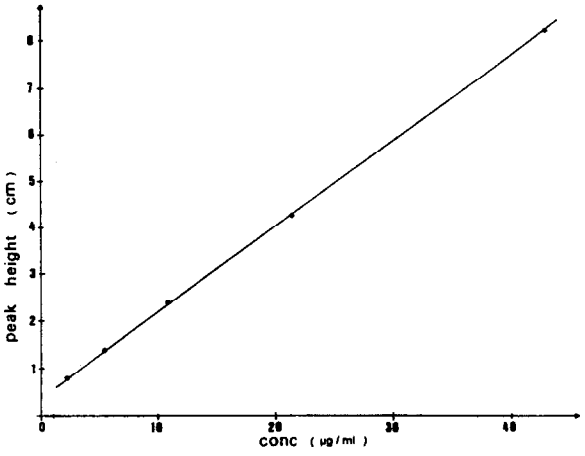

Fig. 5. Variation of the CDZ peak height after irradiation for 70 min at <10°C as a function of initial CDZ concentration dissolved in acetonitrile.

the initial CDZ concentration. These results confirm that the CDZ photoisomerization follows a first-order reaction [25]. Moreover, DES-CDZ and DEM were found to show the same kinetic behaviour. It is reasonable to consider that each mole of oxaziridine is stoichiometrically generated from 1 mol of the parent benzodiazepine as only one photoproduct was detected after irradiation of N-oxide derivatives and as the results show a linear response between the parent benzodiazepine concentration before irradiation and the peak height of OX generated during 70 min of irradiation.

The increase in the retention times of the drugs caused by modifications of the mobile phase did not change the photodegradation kinetics of the 1,4-benzodiazepines. This result shows that the unstable oxaziridines did not re-

Table 4
Within-assay reproducibility and day-to-day precision

Validation[a]	Drug[b]				
	DEM	DES-CDZ	OXAZ	CDZ	DES-DIAZ
Intra-assay R.S.D. (%) ($n = 6$)	1.7	2.6	2.2	2.0	2.7
Inter-assay R.S.D. (%) ($n = 4$)	3.5	3.0	4.0	2.7	4.0

[a] n = Number of injections.
[b] Concentrations: DEM 31.8, DES-CDZ 23.2, OXAZ 49.1, CDZ 43.0, DES-DIAZ 68.4 μg/ml.

form the parent drug on the column during the HPLC separation. The stability of the photoproducts was followed during their conservation in CH_3CN at −25°C in the dark. The results demonstrated that no degradation occurred during a 10-day period.

In conclusion, the reported and validated HPLC method separates CDZ, its metabolites and its photoproducts in spite of their wide ranges of lipophilicity and polarity. Further, the development of this assay (HPLC and irradiation systems) is a major step for further investigations of the production, stability, cytototoxicity mechanisms and pharmacokinetics of the oxaziridines.

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