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Fully automated high-performance liquid chromatographic method for the determination of carzelesin (U-80,244) and metabolites (U-76,073 and U-76,074) in human plasma

Olaf van Tellingen[a,*], Esther M. Pels[a], Roland E.C. Henrar[b], Lawrence J. Schaaf[c], Guy E. Padbury[c], Jos H. Beijnen[d], Willem J. Nooijen[a]

[a]*Department of Clinical Chemistry, Netherlands Cancer Institute, Antoni van Leeuwenhoekhuis, Plesmanlaan 121, 1066 CX Amsterdam, Netherlands*

[b]*European Organisation for Research and Treatment of Cancer, New Drug Development Office, Free University, Amsterdam, Netherlands*

[c]*Upjohn Company, Kalamazoo, MI, USA*

[d]*Slotervaart Hospital, Louwesweg 6, Amsterdam, Netherlands*

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Abstract

Carzelesin (U80,244, I) is a cyclopropylpyrroloindole prodrug analog. The compound exerts its cytotoxic activity after conversion, via U-76,073 (II) to U-76,074 (III) by binding to the DNA minor groove in a sequence selective fashion. In pre-clinical investigations the drug displayed a broad spectrum activity against human xenografts in mice. To enable pharmacokinetic monitoring during the phase I clinical trials, we have developed a selective and sensitive assay for the parent compound and its two metabolites. Sample pre-treatment has been automated by using the ASPEC system and involves solid-phase extraction of the diluted plasma sample (1:3 in 20% v/v acetic acid) on an SPE-C_{18} precolumn followed by two consecutive washings with water and acetonitrile. The compounds are eluted with 600 μl of dimethylacetamide and 500 μl is injected on a Spherisorb-CN column. The sample is chromatographed using a linear gradient from 24% to 60% (v/v) acetonitrile in 20 m*M* phosphate buffer (pH 6.5). The eluate fraction containing the three compounds is heart-cutted in a 2-ml sample loop, switched onto a Spherisorb-ODS column and separation is accomplished using a mobile phase of acetonitrile–20 m*M* phosphate buffer pH 6.5 (64:36, v/v). UV detection is used with absorbance monitored at 360 nm. This highly selective method offers a lower limit of detection of less than 1 ng/ml for each of the compounds using 1000 μl of plasma and enables the quantification of the analytes with an acceptable precision and accuracy over a range of 1 to 50 ng/ml. The assay has been implemented in a phase I clinical trial.

1. Introduction

The unique DNA-interactive properties of the potent cytotoxic antibiotic CC-1065 (Fig. 1) has directed research to the development of other cyclopropylpyrroloindole (CPI) analogs, sharing this novel mechanism of DNA binding but lacking its characteristic delayed toxicity [1]. The CPI analogues are DNA minor groove binders containing a cyclopropyl group, which mediates N^3-adenine covalent adduct formation in a sequence-selective fashion with no intercalation.

* Corresponding author.

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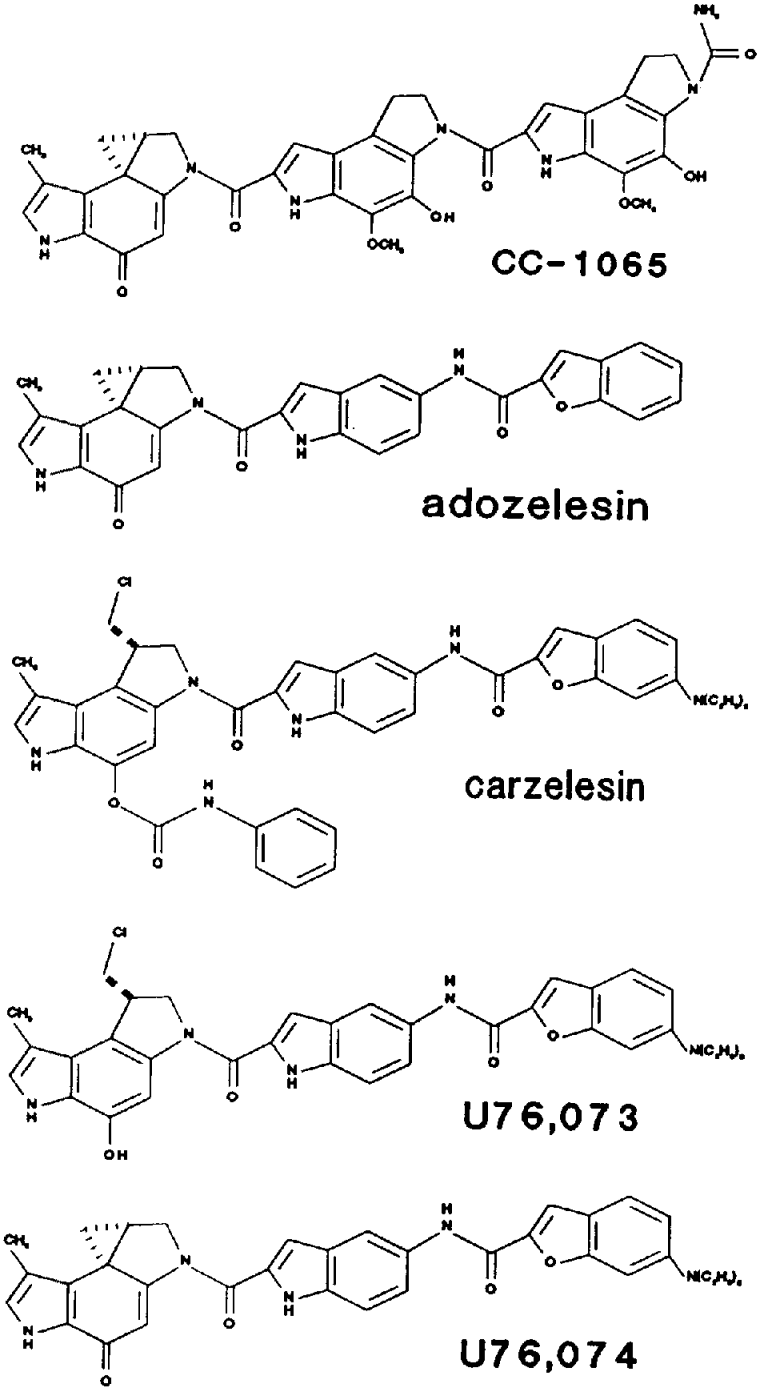

Fig. 1. Molecular structures of CC-1065, carzelesin, adozelesin, II and III.

Unlike CC-1065 or adozelesin, which is the first CPI analogue being tested in clinical trial [2], carzelesin (U-80,244, I) was designed to be an inactive prodrug lacking the cyclopropyl group. Activation of carzelesin requires two steps, *i.e.* (1) hydrolysis of the phenylurethane substituent to form U-76,073 (II), and (2) ring closure to form the cyclopropyl-containing DNA-reactive U-76,074 (III).

In comparison to adozelesin or III, carzelesin proved to be more efficacious against a broad panel of murine and human tumor xenografts [1]. It was postulated that these favorable antitumor properties may be related to the pharmacokinetic behavior of the inactive prodrug and its active metabolite.

Based on the promising results in preclinical investigations, carzelesin has been selected for clinical trial. In order to study the pharmacokinetics of this new chemical entity, we have developed a high-performance liquid chromatographic assay capable of determining carzelesin and both II and III in human plasma. It was essential for the analytical method to possess a high sensitivity as the safe starting dose for the phase I clinical trial was defined to be as a low as 24 $\mu g/m^2$. The reported method fulfills these requirements and will be used for the pharmacokinetic study during the phase I trials.

2. Experimental

2.1. Reagents

Carzelesin (batch 22711-IG-141), II (batch 26098-IG-114) and III (batch 23899-IG-4) were obtained from The Upjohn Company (Kalamazoo, MI, USA). Stock solutions of 100 $\mu g/ml$ of each compound were made in ice-cold dimethylacetamide (DMA) and stored at −70°C.

All other reagents were purchased from E. Merck (Darmstadt, Germany) and were of analytical grade, except for acetonitrile which was of Lichrosolv quality. Blank human plasma was obtained from healthy donors. Water was purified by the milli-Q plus system (Millipore, Milford, MA, USA). Bakerbond solid-phase extraction (SPE) C_{18} columns (batches D40502 and G05083) were from J.T. Baker, Phillipsburg, NJ, USA.

2.2. Instrumentation

The sample robot (automated sample preparation with extraction columns; ASPEC) is from Gilson Medical (Middleton, WI, USA). The Spectroflow SF400 pumps, SF757 detectors are from Kratos (Ramsey, NJ, USA). Integration was done using an SP4270 integrator coupled to a WINner/286 data station (Spectra Physics, San Jose, CA, USA). A model 5140 solvent programmer (Kipp en Zn, Delft, Netherlands) controlled the gradient, the start signals for the integrator and the Multiport Streamswitch (MUST) (Spark, Emmen, Netherlands). A Julabo Paratherm thermostat pump (Juchheim Labortechnik, Seelbach, Germany) operating at −10°C and pumping an ethanol–water mixture (1:1, v/v) was used for cooling the ASPEC sample tray. The actual temperature of the plasma samples in the sample tray was 3–4°C.

2.3. Sample preparation

All sample preparation steps are automated by the ASPEC system. SPE C_{18} columns are preconditioned with 1500 μl of acetonitrile and 2000 μl water, successively. The sample is mixed with 3000 μl of 20% (v/v) acetic acid in water and then loaded on the SPE column. The SPE column is washed with 2000 μl of water and 2000 μl of acetonitrile and dried between each wash step by flushing with 2000 μl of air. The compounds are eluted from the SPE column with 600 μl of DMA. A volume of 500 μl is injected in the HPLC system.

2.4. High-performance liquid chromatography

A schematic diagram of the HPLC system is depicted in Fig. 2. The program of the solvent programmer controlling pumps A and B is shown in Table 1. Mobile phase A consists of 20 m*M* dipotassium phosphate buffer adjusted to pH 6.5 with 5 *M* hydrochloric acid; a stock

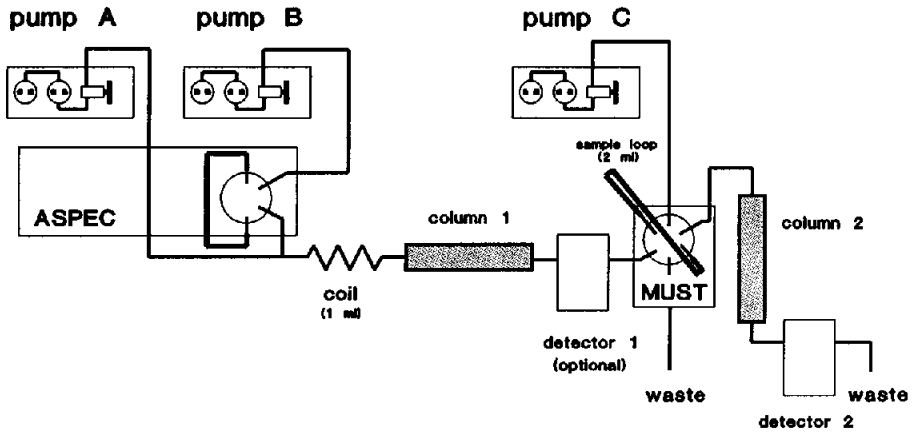

Fig. 2. Schematic diagram of the HPLC system in combination with the ASPEC module.

solution is stored at 4°C and stable for at least 3 weeks. To avoid bacterial growth a fresh aliquot is used for one day only. The solution is degassed by vacuum just prior to use.

Mobile phase B is a mixture of acetonitrile–water (90:10, v/v) whereas mobile phase C is composed of a mixture of acetonitrile–20 m*M* phosphate buffer pH 6.5 (64:36, v/v). Both mobile phase B and C are degassed by sonication for 5 min and, if kept well-sealed to prevent the evaporation of acetonitrile, they are stable for at least 1 week. The flow-rate of mobile phase C is maintained at 0.7 ml/min. Columns 1 and 2 are both glass columns (100 × 3.0 mm I.D.) packed with 5-μm Spherisorb CN material and 5-μm Sperisorb-ODS2 material, respectively (Chrompack, Middelburg, Netherlands). Column 1 is protected by a guard column (10 × 3.0 mm I.D.), which is packed with pellicular polar bonded phase material (Chrompack) and which is replaced when the back-pressure increases. The UV detector operates at a wavelength of 360 nm with the time constant set at 2 s.

Table 1
Scheme of the gradient controler

Step	Time	Flow	%B	Auxiliary[a]		
				1	2	3[b,c]
0	0.0	0.8	27	–	–	+
1	0.1	0.6	27	+	–	+
2	0.2	0.6	27	–	–	+
3	5.0	0.6	27	–	–	+
4	6.8	0.8	27	–	–	+
5	7.0	0.8	27	–	–	–
6	10.0	0.8	66	–	–	–
7	11.0	0.8	66	–	–	–
8	11.5	0.8	66	–	+	+
9	12.0	0.8	95	–	–	+
10	15.5	0.8	95	–	–	+
11	16.0	0.8	27	–	–	+

[a] Auxiliary 1: timed event for start integrator 1 (from optional UV detector 1); auxiliary 2: timed event for start integrator 2 (from UV detector 2); auxiliary 3: connected with MUST 6-way valve.
[b] –: sample loop is placed between UV detector 1 and waste.
[c] +: sample loop is placed between pump C and column 2.

The purity of the reference compounds has been tested with a chromatographic system composed of a glass column (100 × 3 mm I.D.) packed with 5-μm Spherisorb-ODS2 material, a mobile phase of acetonitrile–20 m*M* phosphate buffer pH 6.5 (64:36, v/v) pumped at a flow-rate of 0.6 ml/min and a 1000S UV-photodiode array detector (Kratos, Ramsey, NJ, USA). A Rheodyne 7010 injector with a 20-μl sample loop was used. Reference standards (100 μg/ml dissolved in DMA) were mixed with water (1:1, v/v) and injected within 1 min to prevent adsorption to the wall of the vial.

2.5. Preparation of calibration standards

All steps are carried out with solvents and tubes cooled in ice-water. A standard mixture of 10 μg/ml is prepared by mixing volumes of 50 μl

of each stock solutions of carzelesin, II and III and 350 μl DMA. This standard mixture is diluted further with DMA to 0, 20, 40, 100, 200, 400 and 1000 ng/ml. A calibration curve in plasma is prepared by adding 50 μl of each standard to 1000 μl of blank human plasma. Final concentrations in plasma are 0, 1, 2, 5, 10, 20, 50 ng/ml.

2.6. Calibration

Peak areas were used for quantitative computations. Calibration curves were fitted by performing unweighted linear regression analysis after log–log transformation of the abscissa (concentration) and ordinate (peak area).

2.7. Validation

The validation included a calibration curve determined in duplicate and three control levels spiked at 2, 10 and 50 ng/ml assayed in quadruplicate at three separate days.

The accuracy of the method at each standard concentration or control level was calculated from the percentage deviation (%Dev), which is defined as $\%Dev = 100(RC - NC)/NC$, where RC and NC represent the interpolated and nominal concentrations, respectively. The within-run relative standard deviations (%R.S.D.) were calculated from the compound variance of the calibration standards and of the control samples.

The recovery of the sample pretreatment procedure was estimated at 2, 10 and 50 ng/ml. Volumes of 50 μl of the respective standard in DMA (40, 200 and 1000 ng/ml) were completed to 600 μl with DMA and injected immediately.

The selectivity of the assay was investigated by the analysis of plasma samples from 15 randomly selected cancer patients, who did not receive carzelesin. The lower limit of detection (LOD) is defined as the concentration giving a response equal to a signal-to-noise ratio of 3. The lower limit of quantitation (LLQ) is defined as the lowest standard in the analytical run which is quantitated with a %Dev and %R.S.D. of less than 20%.

Two quality control specimens for use during routine analyses, containing 15 ng/ml of either carzelesin and III, or II were stored at −20°C. With each run these specimens were analyzed in duplicate.

2.8. Collection of samples for pharmacokinetic studies

A volume of 8 to 10 ml of blood is collected in EDTA containing tubes and immersed in ice-water, immediately, for a period of at least 5 min. Within 45 min plasma must be separated by centrifugation (5 min, 2500 *g*) in a cooled (4°C) centrifuge and snap-frozen in ethanol–solid carbon dioxide. If stored at −20°C the samples are stable for at least 6 weeks. Samples are thawed in ice-water prior to analysis.

2.9. Pharmacokinetic study

Blood samples taken at selected time points from a patient who received a 10-min intravenous infusion of 24 $\mu g/m^2$ carzelesin as part of a phase I clinical trial were used to demonstrate the applicability of the assay.

3. Results and discussion

3.1. Physicochemical properties and purity of the reference compounds

The test compounds are virtually insoluble in all aqueous and most organic solvents tested. They are soluble in dimethylformamide and DMA. If dissolved in DMA and stored at −70°C, they are stable for at least 6 months. A rapid conversion of carzelesin and II dissolved in DMA occurs at room temperature or higher. Judged from the chromatographic trace, the standard solutions of carzelesin and III were over 99% pure. Only a minor impurity (less than 0.5%) of a peak co-eluting with II was found in both carzelesin and III reference standards. Several batches of II were checked for impurities. The batch of II used for this study was the purest available and contained 1.6% of a peak co-eluting with III.

Although the compounds exhibit native fluo-

rescence properties, the fluorescence yield was insufficient for the development of an assay, which met the desired sensitivity. Furthermore, the compounds do not show electrochemical activity and, therefore, the choice for a suitable detection technique was restricted to UV detection. The absorption maxima of the compounds are situated between 360 and 380 nm.

3.2. Sample preparation

Apparently, the test compounds are tightly bound to plasma proteins, as retention on SPE C_{18} columns during sample loading could only be achieved if the sample was first diluted (1 + 3) with acetic acid of sufficient molar strength (20%, v/v). A high selectivity with liquid–solid extraction was possible due to the low solubility of the compounds in acetonitrile. Whereas washing the SPE column with 2 ml of acetonitrile is very effective for elution of the majority of potentially interfering endogenous compounds, it did not result in any significant loss of the test compounds. Quantitative elution was accomplished with 600 μl of DMA. However, the use of DMA impeded a concentration step of the sample because, even under relatively mild conditions such as vacuum concentration, significant conversion of carzelesin and II occurred.

It is important to note that marked differences between different batches of SPE columns occurred and it should be stressed that if a new batch is to be used for the analyses, this should be selected carefully. The absolute recoveries of the test compounds using an appropriate batch of SPE columns should range between 85 and 90% over the complete calibration range. Loss of analytes, mainly during the sample loading step may result in lower and highly variable recoveries. Upon selecting a potentially useful batch, the assay should be revalidated.

3.3. High-performance liquid chromatography

With a simple isocratic HPLC system, as used for checking the purity of the test compounds, only a maximum of 50 μl of DMA diluted 1:1 with water could be injected without rigorous peak distortion, resulting in unacceptable values for LLQ. To enable the injection of a substantial fraction of the SPE eluate for the HPLC analysis an on-line concentration step was developed.

The injection valve of the ASPEC is placed in the flow stream from pump B pumping a mobile phase of acetonitrile–water (90:10, v/v). Mixing of mobile phase B with mobile phase A takes place after the injector in a mixing coil of 1 ml. When the SPE-eluate is injected in the HPLC system, it is diluted with mobile phase A (Steps 1–5, Table 1). The DMA in the sample is eluted in the solvent front, while the test compounds are retained on column 1 (Spherisorb-CN). The flow-rate is reduced to compensate the higher back-pressure of the more viscous DMA/water mixture. Next, the test compounds are eluted with a linear gradient (Steps 5–8, Table 1). Carzelesin as well as II and III elute as broad and partly overlapping peaks in a band of approximately 1.6 ml. By means of the MUST switching valve, provided with a 2-ml volume sample loop, the part of the gradient eluting from column 1, containing all compounds, is directed on to column 2. Due to the presence of the gradient, the compounds are concentrated on top of column 2 and then separated isocratically yielding sharp chromatographic peaks (Fig. 3).

3.4. Stability of the test compounds during the analytical procedures

The chromatographic traces obtained from a set of plasma samples each spiked with 50 ng/ml of a test compound revealed that no significant conversion of carzelesin, II or III occurs during the analytical procedures.

The stability of the test compounds was also determined under conditions occurring during sample handling after the collection for pharmacokinetic study. From these studies it can be concluded that the temperature of the sample is the most critical factor (Fig. 4). At elevated temperatures (room temperature or 37°C) both carzelesin and II dissolved in blood or plasma are rapidly converted, whereas this process is

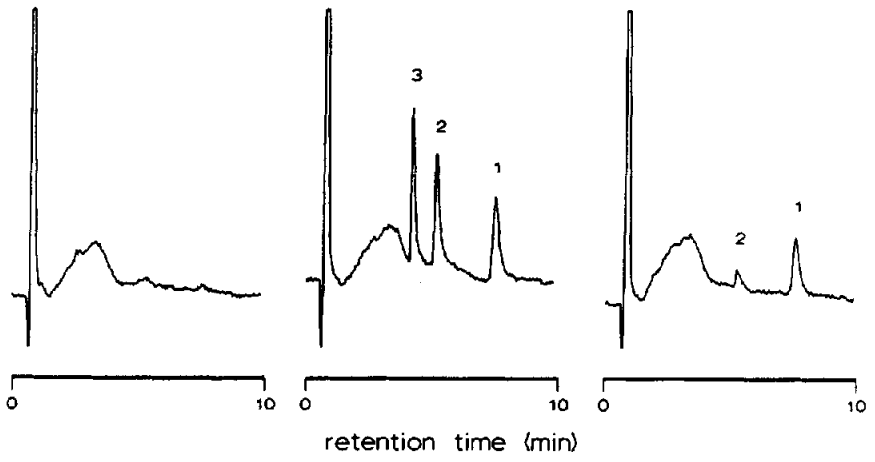

Fig. 3. Representative chromatograms from a blank plasma sample (left), a sample spiked with 5 ng/ml of each test compound (middle) and a patient sample taken 15 min after cessation of the infusion (right). (1 = carzelesin, 2 = II, 3 = III).

inhibited at 0–4°C. Under these conditions, the compounds are stable for at least 1 h. Although III is the supposed end-product, the concentration in plasma diminishes slowly upon incubation at 37°C.

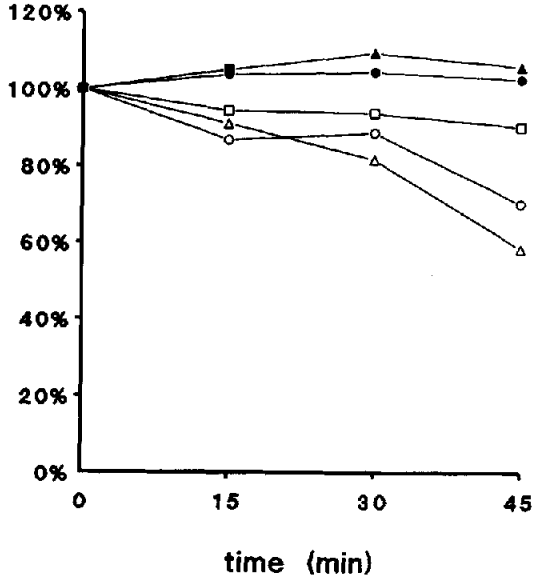

Fig. 4. Stability of carzelesin (●,○), II (▲, △) and III (■,□) upon incubation in human blood at 37°C (solid symbols) and at 4°C (open symbols).

3.5. Calibration

The mathematical models used for calculating the calibration curves included weighted ($1/Y$) linear regression, log–log linear regression and quadratic regression analysis. The slight deviation of linearity, occurring predominantly in the lower part of the calibration curve is best described by the log–log linear regression model.

A typical set of parameters of the equation $\log(\text{response}) = a\log(\text{concentration}) + b$ for carzelesin is: $a = 1.131$ and $b = 3.464$ ($r = 0.9996$). Note that a would have matched 1.000 if no deviation from linearity would be present.

3.6. Validation

No interferences were present in any of the blank patient samples. The LOD was approximately 0.5, 0.6 and 0.8 ng/ml for III, II and carzelesin, respectively. The absence of a suitable candidate precluded the use of an internal standard. Despite the relative complexity of the analytical methodology and the lack of a suitable internal standard, acceptable results for the accuracy and precision were achieved (Table 2). The LLQ is 1 ng/ml for the three compounds. The day-to-day precision, calculated from the quality control specimens analyzed during

Table 2
Validation

Sample	Concentration	Carzelesin		II		III	
	(ng/ml)	%R.C.R.	%R.S.D.	%R.C.R.	%R.S.D.	%R.C.R.	%R.S.D.
Standard[a]	1	−6.3	8.5	−2.5	7.3	−4.7	1.6
	2	9.2	7.6	0.3	10.5	2.2	3.8
	5	0.7	3.3	5.0	7.2	1.9	2.0
	10	1.2	1.5	2.7	1.8	4.7	1.3
	20	−3.9	10.9	−3.5	11.1	0.2	3.9
	50	0.8	4.2	−0.3	3.1	−3.8	1.2
Control[b]	2	2.5	19.2	1.9	11.4	3.7	5.2
	10	1.8	3.9	3.2	14.3	5.1	3.7
	50	−1.2	5.0	1.3	5.5	−2.5	2.7

[a] Assayed in duplicate.
[b] Assayed in quadruplicate.

routine analyses were 9.1%, 13.4% and 8.5% for carzelesin, II and III, respectively.

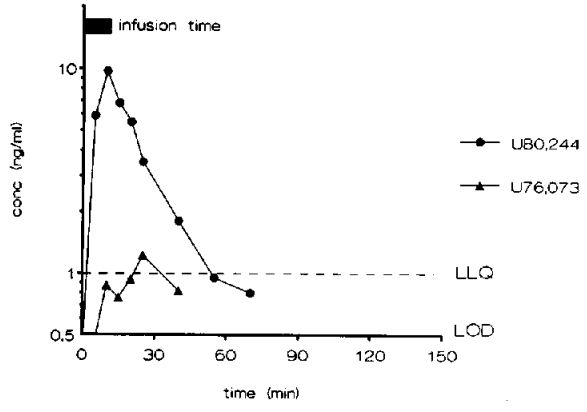

Fig. 5. Log plasma concentration–time curves of carzelesin (●) and II (▲) of a patient receiving 24 $\mu g/m^2$ of the drug as a 10-min intravenous infusion.

3.7. Applicability of the assay

In spite of the low dosage being administered at the first dose step of the clinical phase I trial, the sensitivity of the assay was sufficient to detect carzelesin and II until approximately 1 h after the cessation of the infusion (Fig. 5). No III was detected in any of the samples.

4. References

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