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# Determination of polyoxyethyleneglycerol triricinoleate 35 (Cremophor EL) in plasma by pre-column derivatization and reversed-phase high-performance liquid chromatography

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**Abstract**

A sensitive and selective reversed-phase high-performance liquid chromatographic (HPLC) method for the determination of polyoxyethyleneglycerol triricinoleate 35 (Cremophor EL; CrEL), which requires only microvolumes (20 $\mu$l) of plasma, has been developed and validated. The procedure is based on saponification of CrEL in alcoholic KOH, followed by extraction of the released fatty acid ricinoleic acid with chloroform and derivatization with 1-naphthylamine. Margaric acid was used as the internal standard. The products are separated using an HPLC system consisting of an analytical column packed with Spherisorb ODS-1 material and a mobile phase of methanol–acetonitrile–10 m*M* potassium phosphate buffer pH 7.0 (72:13:15, v/v). Detection was executed by UV absorption at 280 nm. The lower limit of quantitation and the lower limit of detection in plasma are 0.01 and 0.005% (v/v) of CrEL, respectively. The percentage deviation and precision of the procedure, over the validated concentration range of 0.01 to 1.0% (v/v) of CrEL in plasma, are ≤8.0% and ≤6.6%, respectively. Compared to the previously described bioassay, the presented HPLC method possesses superior sensitivity and reliability. Preliminary pharmacokinetic studies of CrEL in mice and patients receiving paclitaxel formulated in CrEL have demonstrated the applicability of the presented assay.

*Keywords:* Polyoxyethyleneglycerol triricinoleate 35; Cremophor EL

## 1. Introduction

Polyoxyethyleneglycerol triricinoleate 35 (Cremophor® EL: CrEL) (Fig. 1) is a liquid product formed by the reaction of ethylene oxide with castor oil at molar ratio of 35:1 [1,2]. The major components of CrEL are triglycerides which contain about 87% of ricinoleic acid. CrEL is being used as a vehicle in pharmaceutical preparations for the solubilization of a variety of hydrophobic drugs, including cyclosporin A and vitamins. During the last years, CrEL has attracted the attention of many investigators in cancer chemotherapy, because of its use as a vehicle in the clinical formulation of the new antitumor agent paclitaxel. The pharmaceutical formulation (Taxol®) contains 30 mg paclitaxel dissolved in 5.00 ml of a mixture of CrEL and dehydrated ethanol USP (1:1, v/v). Although CrEL

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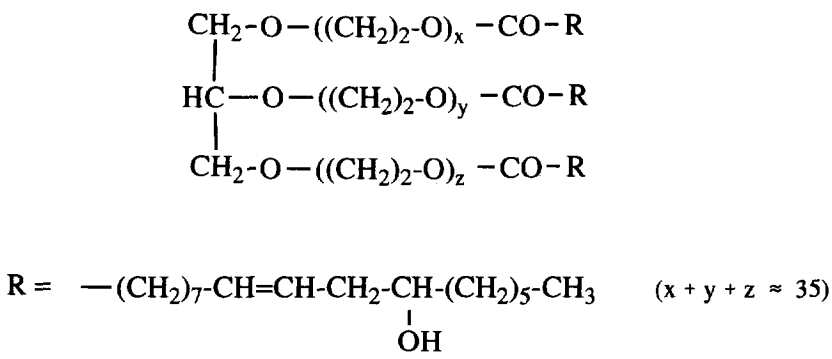

Fig. 1. Chemical structure of the major component of CrEL.

has already been used for the formulation of other anti-cancer agents (e.g., teniposide) the amount of CrEL administered concomitantly with paclitaxel is much higher. Patients treated with 250 mg/m$^2$ of the drug may receive up to 40 ml of CrEL. Recent studies have provided evidence that CrEL has a major impact on the pharmacology of paclitaxel, such as modulation of the multidrug resistance P-glycoprotein pump [3–7]. However, pivotal information on the pharmacokinetics of CrEL is scarce, which is due to the absence of a suitable analytical methodology. Webster et al. have developed a bioassay for CrEL in human plasma, which is based on the ability of CrEL to act as a modulator of P-glycoprotein [8]. Major drawbacks of this laborious procedure are the relatively large sample volume (1.5 ml), the need for a pretreatment plasma sample from each individual patient for the construction of a standard curve and the relatively poor sensitivity and precision of the assay. We present a novel assay for the analysis of CrEL in plasma based on quantitation of the major component of this triglyceride, ricinoleic acid, after saponification of CrEL followed by extraction and derivatization of the analyte with 1-naphthylamine. The *N*-ricinoleoyl-1-naphthylamine product is separated from the reaction products of the internal standard (margaric acid) and derivatized endogenous fatty acids by reversed-phase high-performance liquid chromatography (HPLC) with UV detection. The procedure requires only microvolumes of sample and is now successfully used in studies on the pharmacology of CrEL in laboratory animals and patients receiving Taxol.

## 2. Experimental

### 2.1. Chemicals and materials

CrEL [lot 32H0925; specific gravity (25°C/25°C)=1.05–1.06], ricinoleic acid, margaric acid and 1-naphthylamine were purchased from Sigma (St. Louis, MO, USA). Paclitaxel was supplied by Bristol-Myers Squibb (Princeton, NJ, USA) as a concentrated sterile solution containing 6 mg/ml in a 5-ml vial in CrEL and dehydrated ethanol USP (1:1, v/v) (Taxol). All other chemicals and solvents were of analytical or LiChrosolv grade and were obtained from Merck (Darmstadt, Germany). Drug-free human plasma originated from the Central Laboratory of the Blood Transfusion Service (Amsterdam, Netherlands). Drug-free mouse plasma was collected from 12 female FVB mice (age 10–14 weeks). Plasma was separated by centrifugation at 3000 *g* for 10 min at 0°C. Water purified by the Milli-Q Plus system (Millipore, Milford, MA, USA) was used throughout.

### 2.2. Stock solutions

Standard stock solutions of 1.0% (v/v) of CrEL were prepared in drug-free human plasma by addition of 50 $\mu$l of CrEL to 4950 $\mu$l of plasma, followed by vigorous mixing. Stock solutions of 2 mg/ml of margaric acid (internal standard) were prepared in methanol. All stock solutions were stored at −20°C.

### 2.3. Calibration standards and quality control samples

Spiked plasma samples used as calibration standards were prepared with each run at concentrations of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1.0% (v/v) of CrEL by serial dilutions of the 1.0% (v/v) standard stock solution. Quality control samples were prepared at three levels, i.e., 0.05, 0.1 and 0.5% (v/v) of CrEL, in blank mouse and human plasma. The quality control samples were stored in polypropylene tubes at −20°C until analysis.

### 2.4. Sample pre-treatment

A volume of 10 μl of the internal standard stock solution and 200 μl of alcoholic KOH USP were added to 20 μl of plasma in a 2.0-ml microtube (Eppendorf, Hamburg, Germany). The mixture was deproteinized by vortex-mixing for 30 s followed by centrifugation at 2500 *g* for 10 min in a Model J-6B/P centrifuge (Beckman, Palo Alto, CA, USA). The supernatant was transferred to a 10-ml glass tube with PTFE-covered screw-cap, and saponification was performed by heating at 100°C for 30 min in a water-bath (Heto Lab Equipment). After cooling, 200 μl of 1.0 *M* aqueous HCl and 2 ml of chloroform were added and the reaction solutions were mixed vigorously for 5 min, followed by centrifugation at 460 *g* for 5 min. Next, the aqueous layer was removed by suction, and the organic layer was decanted into a clean 4.6-ml glass tube. The solvent was evaporated in vacuo in a Speed-Vac Plus SC210A apparatus (Savant, Fariningdale, NY, USA) at 43°C, and the residue was reconstituted in 500 μl of benzene by sonication for 1 min. To each sample 500 μl of 2.0% (v/v) of oxalylchloride in benzene was added, and the mixture was incubated at 70°C for 30 min in a thermostatically controlled waterbath (Thermolyne Dri-Bath, Dubuque, IO, USA). The organic solvent was evaporated by vacuum concentration and the residue was reconstituted in 100 μl of benzene, followed by adding of 100 μl of 40 m*M* 1-naphthylamine in benzene and 10 μl of 5.6% (v/v) triethylamine in benzene. The mixture was vortex-mixed for 30 s, and incubated for 30 min at 37°C. Next, the sample was again evaporated to dryness, redissolved in 400 μl of methanol–acetonitrile–water (72:13:15, v/v) by vortex-mixing for 20 s, transferred to a 1.5-ml microtube (Eppendorf), and an aliquot of 20 μl was injected onto the HPLC system.

### 2.5. HPLC analysis

The HPLC equipment consisted of a Model 300C pump (Gynkotek, Germering, Germany), a Marathon HPLC autosampler equipped with a 20-μl sample loop (Spark Holland, Emmen, Netherlands), and a Spectroflow SF 757 UV–Vis variable-wavelength detector (Kratos, Ramsey, NJ, USA). Chromatographic separations were achieved at ambient temperature using two coupled chromsep glass columns (100×3 mm each) packed with 5 μm Spherisorb ODS-I material. The mobile phase was methanol–acetonitrile–10 m*M* potassium phosphate buffer pH 7.0 (72:13:15, v/v), and was delivered at a flow-rate of 0.4 ml/min. Detection was carried out at 280 nm with a filter rise time of 1 s. Peak recording and integration was performed with a Spectra Physics SP4600 DataJet integrator connected to a WINner/286 chromatography work station (San Jose, CA, USA). Ratios of peak areas of *N*-ricinoleoyl-1-naphthylamine to the internal standard versus concentrations of the standard were used for quantitative computations. Calibration graphs were fitted by using second order polynomial (quadratic) regression analysis.

### 2.6. Validation

#### 2.6.1. Precision and accuracy

A validation run included a set of calibration samples assayed in duplicate and quality control samples at three levels in quadruplicate and was performed on three separate occasions. Control samples were analyzed on several days with repeated freezing and thawing of the samples. Statistical computations were performed with the software package Number Cruncher Statistical System (NCSS version 5.0; J.L. Hintze, East Kaysville, UT, USA, 1991) on an IBM compatible computer. An estimate of the precision was obtained by one-way analysis of variance for each test concentration, using the run day as the classification variable. The between-day precision (BDP) was calculated by:

$$BDP = \frac{\left(\frac{DayMS - ErrMS}{n}\right)^{0.5}}{GM \times 100} \quad (1)$$

where DayMS is day mean square, ErrMS is error mean square, $n$ is number of replicates within each day and GM is grand mean. The within-day precision (WDP) was calculated by:

$$WDP = (ErrMS)^{0.5}/GM \times 100 \quad (2)$$

The accuracy or percentage deviation (%DEV) was calculated as:

$$\%DEV = \frac{(\text{observed concentration} - \text{nominal concentration})}{\text{nominalconcentration}} \times 100 \quad (3)$$

*2.6.2. Detection and quantitation limits*

To determine the limit of detection (LOD) and the lower limit of quantitation (LLQ), blank plasma samples from 6 mice and individuals were spiked at 0.001, 0.005 and 0.01% (v/v) of CrEL. The LOD was established at the level which was significantly different from zero by a paired Student's $t$-test ($P < 0.05$). The concentration which could be quantitated with a %DEV and WDP less than 20%, was accepted as the LLQ.

*2.6.3. Stability*

The stability of *N*-ricinoleoyl-1-naphthylamine in the processed specimens kept at room temperature was established with samples spiked at a concentration of 0.1% (v/v) of CrEL. Samples in quadruplicate were injected onto the HPLC system at each of the following time points: 0, 6, 12, 24, 48, 72, 96 and 120 h.

*2.7. Mouse samples*

To demonstrate the applicability of the assay, blood samples of female FVB mice (10–14 weeks, 23–25 g), withdrawn 60 min after administration of an intravenous bolus dose of 20 mg/kg of paclitaxel formulated in CrEL–ethanol–0.9% NaCl in water (25:25:50, v/v), were analyzed. Blood was collected in heparin containing microtubes, and the plasma was separated by centrifugation at 3000 *g* for 10 min at 0°C. Samples were then stored at −20°C until the time of analysis.

## 3. Results

Several chromatographic peaks are detected in a processed sample of CrEL in ethanol (Fig. 2). Apart from two large peaks, which eluted near the solvent front, a third major peak with a retention time of approximately 10.0 min was detected. The retention time of this peak corresponded to that observed for *N*-ricinoleoyl-1-naphthylamine, after injection of a processed sample of pure ricinoleic acid. This compound was well separated from the reaction products of the internal standard and of all other major endogenous fatty acids (Table 1). The detection limit, estimated at a signal-to-noise ratio of 5, corresponded to the amount injected after processing of a 20-μl sample of pure ricinoleic acid (0.00001%, v/v) diluted in ethanol. At this sensitivity, however, interfering peaks were observed in processed samples of mouse or human plasma (Fig. 3 and Fig. 4).

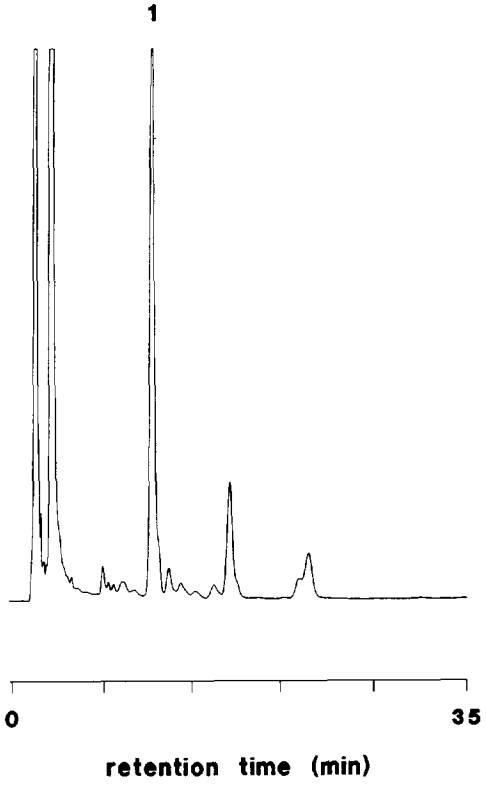


Fig. 2. HPLC chromatogram of a processed sample of crude CrEL in ethanol. The peak labeled (1) correspond to the 1-naphthylamine derivative of ricinoleic acid.

Table 1
HPLC retention times of 1-naphthylamine derivatives of ricinoleic acid endogenous free fatty acids and margaric acid (internal standard)

| Compound | Retention time (min) |
|---|---|
| Ricinoleic acid | 10.0 |
| Linolenic acid | 11.6 |
| Myristic acid | 12.5 |
| Palmitoleic acid | 13.3 |
| Arachidonic acid | 14.4 |
| Linoleic acid | 15.2 |
| Palmitic acid | 21.0 |
| Oleic acid | 22.0 |
| Margaric acid | 27.8 |
| Stearic acid | 37.0 |

By comparing *N*-ricinoleoyl-1-naphthylamine peak areas from water and plasma spiked with CrEL after sample pre-treatment, the recovery of the alcoholic potassium hydroxide protein precipitation was found to be 100.0%. The LOD and the LLQ of CrEL in plasma of mice and humans were 0.005% (v/v) and 0.01% (v/v), respectively. Calibration curves of CrEL constructed in human plasma over the concentration range 0.01–1.0% (v/v) were best fitted by second-order polynomial (quadratic) regression analysis (Fig. 5). The correlation coefficients were higher than 0.993 in all three validation runs. The assay has been validated in terms of precision and accuracy (Table 2). Both accuracy (%DEV) and precision (BDP and WDP) were within the acceptable ±15% range, and results were similar for plasma samples from mice and humans. Repeated freezing and thawing had no effect on the accuracy. Processed samples, maintained at room temperature, were stable for at least 120 h. The applicability of the assay for the determination of CrEL in in vivo samples was tested in mice. Apart from a number of peaks that were also found in blank mouse plasma, an extra peak with a retention time corresponding to

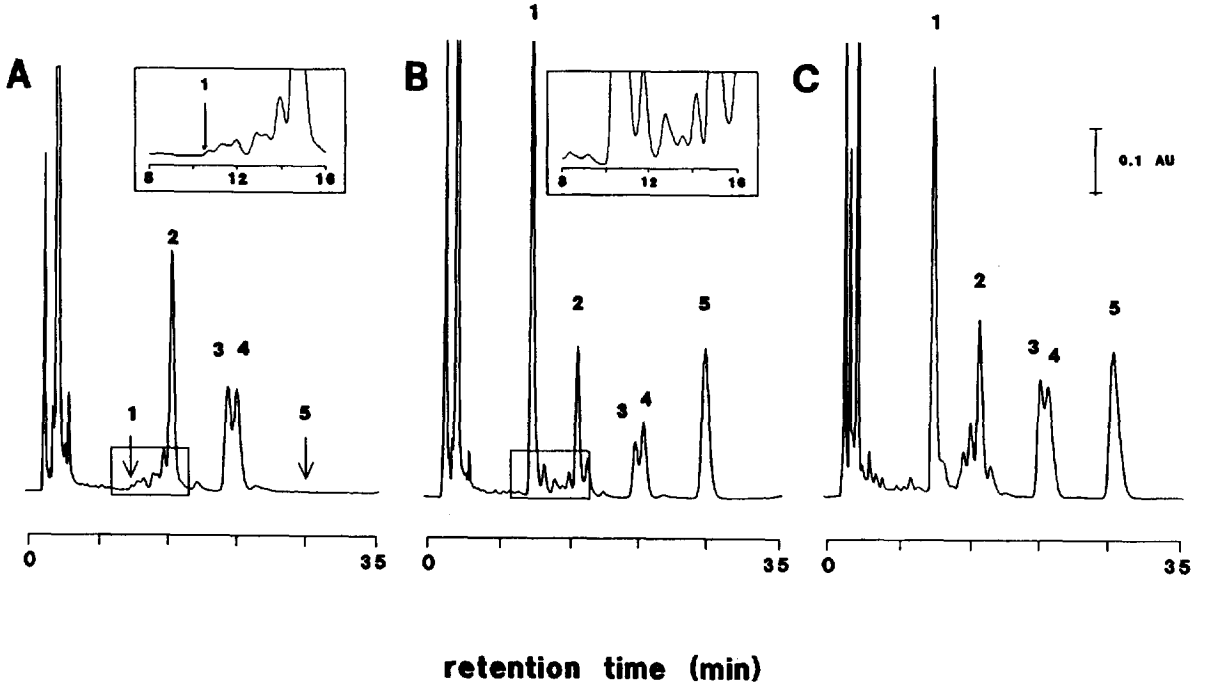


Fig. 3. HPLC chromatograms of a blank mouse plasma sample (A), a mouse plasma sample spiked with 1.0% (v/v) of CrEL (B) and a mouse plasma sample obtained 60 min after the administration of 20 mg/kg of paclitaxel, formulated in CrEL–ethanol–0.9% NaCl in water (25:25:50, v/v) (C). The labeled peaks correspond to the 1-naphthylamine derivatives of ricinoleic acid (1), the endogenous free fatty acids linoleic acid (2), palmitic acid (3) and oleic acid (4) and the internal standard margaric acid (5). Endogenous fatty acids in plasma were identified and confirmed by analysis of reference standards.

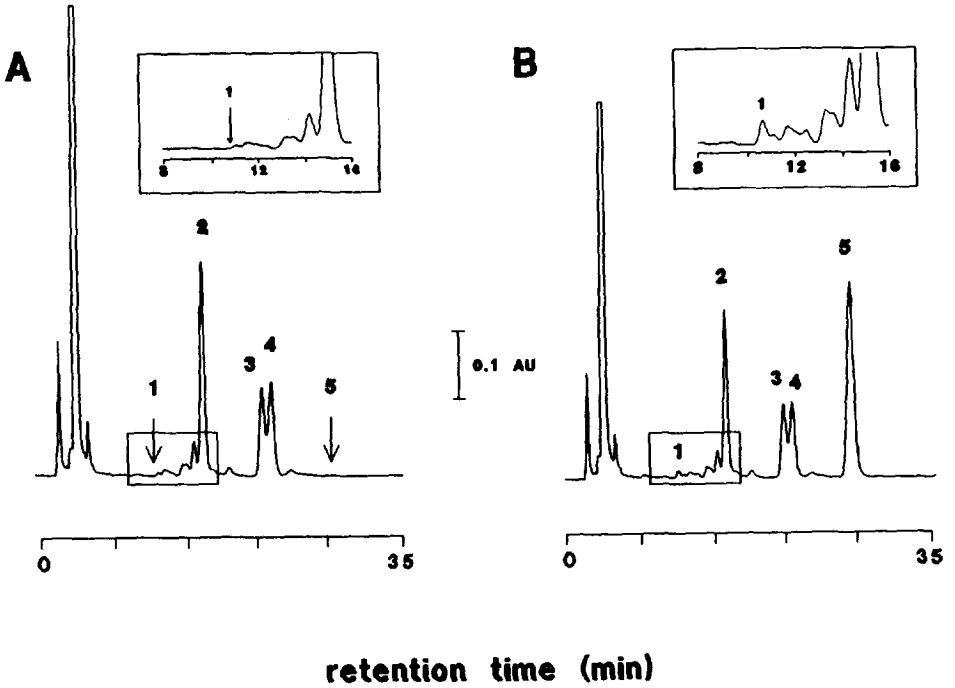


Fig. 4. HPLC chromatograms of a blank human plasma sample (A), and a human plasma sample spiked with 0.01% (v/v) of CrEL (B). The labeled peaks correspond to the 1-naphthylamine derivatives of ricinoleic acid (1), the endogenous free fatty acids linoleic acid (2), palmitic acid (3) and oleic acid (4), and the internal standard margaric acid (5). Endogenous fatty acids in plasma were identified and confirmed by analysis of reference standards.

*N*-ricinoleoyl-1-naphthylamine was detected. This peak was absent if the saponification step was omitted from the procedure.

## 4. Discussion

We have developed a sensitive and accurate analytical method for CrEL in plasma, which is based on the determination of ricinoleic acid, the major fatty acid in this triglyceride. The procedure involves precipitation of plasma proteins with alcohol, saponification of the ester bonds with alkaline ethanol according to USP XXI (1985), extraction with chloroform after acidification, derivatization with 1-naphthylamine and HPLC separation with UV detection of the reaction products. Like for other common triglycerides, the direct determination of CrEL in biological samples is hampered due to the variability in the composition of the fatty acids substituted, and the absence of a suitable chromophor. Extraction of triglycerides from biological matrices with a mixture of chloroform and methanol followed by saponification of the ester bonds has been utilized over the past decades as a routine procedure [9]. By this approach, however, the recovery of CrEL is very low due to the relatively hydrophilic nature of the substance. However, protein precipitation with alcoholic KOH provided a good recovery and the protein-free supernatant could merely be used in the subsequent saponification step and further processing. The potassium salts were converted to the fatty acids by acidification of the sample. After acidification and extraction with chloroform, the fatty acids are converted to their respective acylchlorides, which in a subsequent step react with 1-naphthylamine and form products that contain a naphthyl moiety, enabling UV detection at 280 nm. By using this procedure each fatty acid was converted into its 1-naphthylamine derivative in a quantitative manner only when using benzene solutions. The reaction products are very

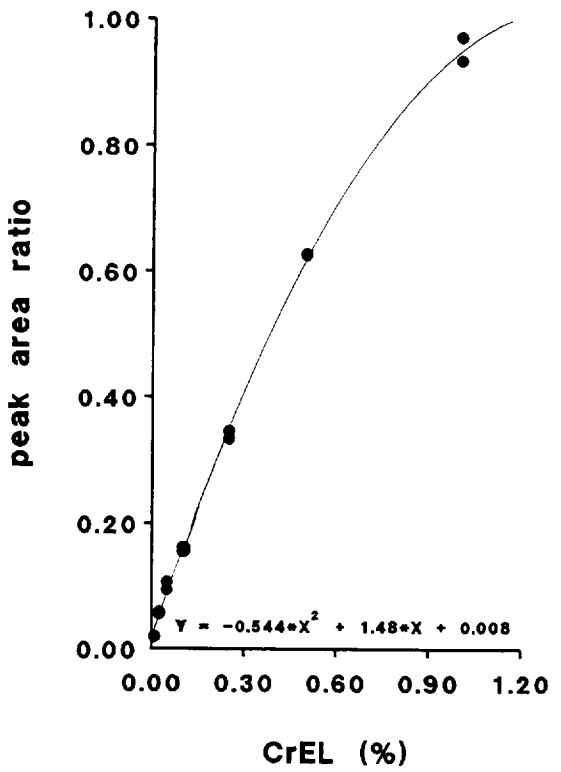


Fig. 5. Typical concentration–response curve of CrEL in plasma.

stable at room temperature, allowing the pre-treatment of a large number of samples in each analytical run, and the use of an autosampler for injection. The *N*-ricinoleoyl-1-naphthylamine compound eluted before many other reaction products of endogenous fatty acids. Although the detection limit of the HPLC system in terms of signal to noise is extremely low, the LLQ in plasma was about 1000-fold higher due to the presence of endogenous interferences. However, the LLQ of 0.01% (v/v) in plasma is sufficient for monitoring plasma levels of CrEL in samples obtained from mice and patients receiving paclitaxel formulated in CrEL. The contents of ricinoleic acid in CrEL was highly uniform throughout different batches, allowing the use of one batch of CrEL for the preparation of calibration standards. In addition, the level of ricinoleic acid appears to be a reliable measure of the CrEL concentration, since in vivo degradation of CrEL yielding this product was not observed. The determination of endogenous fatty acids in plasma, using a slightly modified procedure as described earlier by Ikeda et al. [10], has been utilized in our laboratory for several years and is now successfully applied for the quantitation of ricinoleic acid.

In conclusion, a sensitive assay for the determination of CrEL in microvolumes of plasma has been developed and validated. The procedure is now implemented in pharmacokinetic studies in patients and mice receiving the anti-tumor drug paclitaxel formulated in CrEL.

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Table 2
HPLC validation characteristics of CrEL in mouse and human plasma ($n=3$)

| Nominal concentration (%, v/v) | Mean measured concentration (%, v/v) | Deviation (%) | Precision (%) | |
|---|---|---|---|---|
| | | | Within-day | Between-day |
| *Mouse* | | | | |
| 0.025 | 0.027 | +8.0 | 6.6 | 4.4 |
| 0.100 | 0.104 | +4.0 | 2.6 | 2.3 |
| 0.500 | 0.502 | +0.4 | 1.2 | 1.7 |
| *Human* | | | | |
| 0.025 | 0.026 | +4.0 | 5.8 | 5.1 |
| 0.100 | 0.103 | +3.0 | 3.2 | 3.1 |
| 0.500 | 0.508 | +1.6 | 2.0 | 1.9 |

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