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# High-performance liquid chromatographic assay for the determination of the novel podophyllotoxin derivative dimethylaminoetoposide (NK611) in human plasma

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**Abstract**

A simple, rapid and reproducible plasma assay for the determination of the novel epipodophyllotoxin derivative, dimethylaminoetoposide (NK611, I) and its N-demethyl metabolite (II) is reported. The method involves solid-phase extraction using an isolute $C_{18}$ cartridge and HPLC separation on a reduced-activity $C_{18}$ column (8 cm long) with a mobile phase of acetonitrile–water–0.1 *M* phosphoric acid (23:76:1, v/v/v); peaks are detected at 205 nm. The intra- and inter-day precision and accuracy are within 5 and 4% for I and II, respectively. The sensitivity is 20 ng/ml for both I and II. The assay is applicable to clinical pharmacokinetic studies. In one cancer patient who received both an oral and an intravenous dose of 10 mg of I the bioavailability was 82% and the clearance 20.8 ml/min.

## 1. Introduction

Etoposide is an epipodophyllotoxin derivative which has shown good antitumour activity in a variety of human neoplasms [1]. It has recently been used in many protocols via the oral route since there is evidence that a chronic daily oral treatment may be equally effective as or even more effective than i.v. regimens and has an acceptable toxicity [2–4]. However, an important drawback of oral dosing of etoposide is the inter-patient variability of bioavailability [5,6].

Dimethylaminoetoposide (I, NK611, Fig. 1) is a novel water-soluble oral podophyllotoxin derivative with a dimethylamino group substituted in the D-glucose moiety of etoposide. Studies in murine transplantable tumours and human xenografts have demonstrated that I has anti-tumour activity superior or at least equivalent to that of etoposide [7,8]. In comparison to etoposide, the most relevant feature of I is the higher oral absorption with a bioavailability value of ≥80% in dogs [9]. This finding makes I a compound worthy of further investigation. On the basis of this premise, a rational clinical development of I would of necessity start with a pharmacokinetic study to evaluate the bioavailability of this compound in humans and to decide

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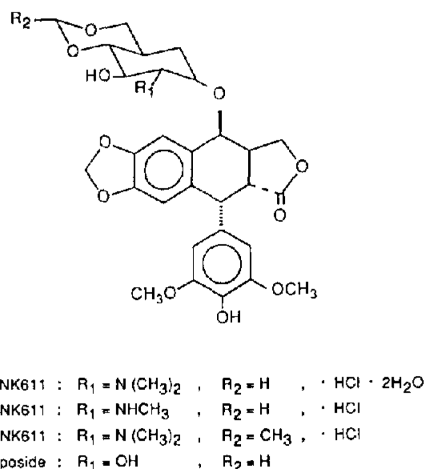


Fig. 1. Chemical structure of I (NK611), II (demethyl-NK611), III (Pr-NK611) and etoposide.

which schedules are to be tested in subsequent trials.

The phase I study presently being performed at the Ospedale San Giovanni, Bellinzona on a daily × 5 schedule has therefore been designed as a randomized cross-over comparative trial, with groups of six patients treated at three different dose levels, in order to determine the bioavailability of oral dosing of I compared with intravenous administration of I in the same patient and to define the pharmacokinetic profile of a single dose of I after different doses. Especially to be defined is the pharmacokinetic profile of I after administration of very low doses, suitable for chronic daily treatments, which are likely to be studied in the future.

In the present report we describe a new HPLC assay following solid-phase extraction (SPE), and show its applicability in the investigation of the clinical pharmacokinetics of I.

## 2. Experimental

### 2.1. Reagents and chemicals

Compound I (5*R*, 5a*R*, 8a*R*, 9*S*)-9-[[2-deoxy-2-(dimethylamino)-4,6,-0-(*R*)-ethylidene-β-D-glucopyranosyl]oxy]-5,8,8a,9-tetrahydro-5-(4-hydroxy-3,5-dimethoxyphenyl)furo[3′4′:6,7]naphto[2,3-*d*]-1,3-dioxol-6[5aH]-one hydrochloride dihydrate, its potential N-demethyl metabolite (II) and Pr-NK611 (III) (see the structures in Fig. 1) were supplied by ASTA Medica (Frankfurt, Germany).

Phosphoric acid ($H_3PO_4$) 89% and potassium dihydrogeno phosphate anhydrous ($KH_2PO_4$) of analytical grade were purchased from Merck (Darmstadt, Germany). Methanol ($CH_3OH$) and acetonitrile ($CH_3CN$) were of HPLC grade and were purchased from J.T. Baker B.V. (Deventer, Netherlands) and Carlo Erba (Milan, Italy), respectively.

### 2.2. Instrumentation

The HPLC system consisted of a Model 717 WISP autosampler and a Model 6000A pump from Millipore-Waters (Milford, MA, USA). The detector used was a spectrophotometer Model LC290 with variable-wavelength UV-Vis from Perkin-Elmer (Norwalk, CT, USA). SPE was performed using a Bench Mate Workstations system from Zymark (Hopkinton, MA, USA).

### 2.3. Extraction procedure

Compound I was extracted as follows: frozen plasma (0.5 ml) was rapidly thawed, supplemented with 100 μl of III (5 μg/ml in $CH_3OH$) as internal standard (I.S.) and 0.4 ml of cold 0.02 *M* $KH_2PO_4$ pH 3.0. The acid buffer had to be added as quickly as possible in order to avoid isomerization of I to picro-I. This isomerization may occur in aqueous solution at $pH > 4$, as already reported for the parent compound etoposide [10]. After vortex-mixing for 5 s and brief centrifugation at 1200 *g* for 3 min the samples were processed using a Bench Mate Workstation for SPE. The extraction was carried out with an isolute $C_{18}$ cartridge (STEP-BIO, Bologna, Italy, manufactured by International Sorbent Technology, Mid. Glamoran, UK), preconditioned at a flow-rate of 0.5 ml/s with 6 ml of $CH_3OH$–$CH_3CN$ (1:1, v/v) and 3 ml of water. Then 0.95 ml of plasma were loaded (flow-rate 0.04 ml/s) and the cartridge was washed at a flow-rate of 0.07 ml/s with water (1 ml), 25% $CH_3CN$ (2 ml) and water (1 ml). The

final elution (flow-rate 0.05 ml/s) was done with 3 ml of $CH_3CN$–$CH_3OH$ (1:1, v/v) after drying the cartridge 3 min with air. The eluted solution was evaporated under nitrogen, the residue was dissolved in 100 $\mu$l of $CH_3OH$–0.02 *M* $KH_2PO_4$ pH 3.0 (1:9, v/v) and 20 $\mu$l were injected onto the HPLC system with the WISP autosampler.

### 2.4. Chromatographic conditions

HPLC analyses were carried out with a reduced-activity $C_{18}$ column (8 cm × 3 mm I.D., Perkin-Elmer) and a mobile phase of $CH_3CN$–$H_2O$–0.1 *M* $H_3PO_4$ (23:76:1, v/v/v) previously filtered through 0.45 $\mu$m filters and degassed. The flow-rate was 1.4 ml/min and peaks were detected at 205 nm (*i.e.* $\lambda_{max}$). At the end of the daily analyses the HPLC column was washed with 20% $CH_3OH$ for 15 min and with 50% $CH_3CN$ for another 15 min at a flow-rate of 1.0 ml/min. Stability in the WISP autosampler was determined by periodic injection during the night of an aliquot of standard solution (in $CH_3OH$–0.02 *M* $KH_2PO_4$ pH 3.0 (1:9, v/v)) of I, II and I.S.

### 2.5. Validation study

Precision and accuracy were evaluated by determining I and II in two quality control (QC) samples (prepared at the nominal concentration of 50 and 700 ng/0.5 ml in quintuplicate) on three different days. To assay the QC, three different calibration curves of five plasma concentrations (20, 50, 100, 500, and 1000 ng/0.5 ml) of I and II were performed in duplicate.

The precision of the method at each concentration was expressed as a coefficient of variation (C.V.) by calculating the standard deviation as a percentage of the mean calculated concentration, while the accuracy of the procedure was determined by expressing the mean calculated concentration as percentage of the added concentration.

The percentage extraction recovery of I and II at three different plasma concentrations in triplicate (20, 100, 1000 ng/0.5 ml) was determined by comparing the peak-height ratios of I and I.S. from chromatograms of extracted plasma samples, where the I.S. was added after the elution of I from the $C_{18}$ cartridge, with those of the unextracted external standards prepared in $CH_3OH$–$CH_3CN$ (1:1, v/v) solution. The detection limit was defined as the concentration at which the signal-to-noise ratio was 3. The quantitation limit was defined as the lowest concentration with an intra-day C.V. $\leq$ 10%.

### 2.6. Drug formulation

Compound I for clinical use was provided by ASTA Medica as 5-mg tablets for oral use and vials containing 20 mg on an anhydrous basis. A 5-ml volume of a 5% glucose solution is added to each vial to give a solution of 4 mg/ml I. The appropriate amount of drug is then diluted in 250 ml of 5% glucose solution and administered as an i.v. infusion of 30 min.

### 2.7. Biological samples

Blood samples were collected from the first patient who entered the phase I study of I on the daily ×5 schedule. The patient was sampled after administration of 10 mg of I, given orally on day 1 of the first cycle, and intravenously on day 1 of the subsequent cycle, which was repeated after 4 weeks. The patient fasted overnight before the oral administration and for 4 h afterward. Samples were obtained before and at 12 different times after drug administration. Blood samples were immediately centrifuged (5 min, 800 *g*) at 4°C and plasma stored frozen until analysis.

## 3. Results and discussion

### 3.1. Chromatography

Fig. 2A shows a typical chromatogram of an extracted patient plasma sample before drug administration. No interfering substances were present at the retention time of I, II and I.S. Fig. 2B shows a plasma blank (0.5 ml) spiked with 200 ng of I, 200 ng of II and 500 ng of I.S. The metabolite and the parent compound were

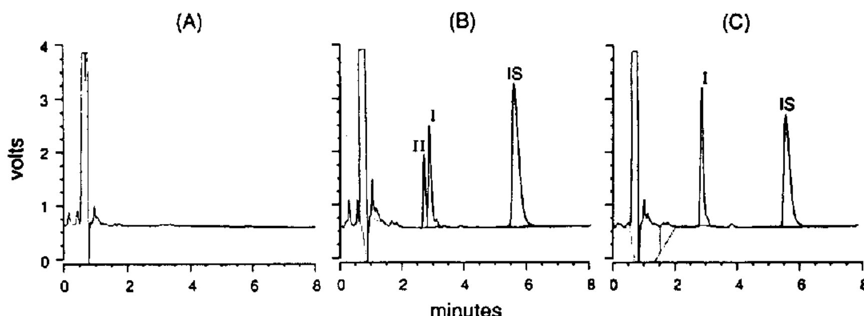


Fig. 2. Chromatograms of a blank plasma sample (A), a plasma sample added with II, I and I.S., (B) Part C shows a plasma sample taken 1.5 h after the administration of I.

eluted at 2.6 and 2.8 min respectively and appeared resolved from the plasma matrix. The retention time of I.S. was 5.5 min. The choice of an acid pH of the mobile phase is critical for obtaining a good resolution of I and II. The last chromatogram (Fig. 2C) corresponds to a plasma sample obtained 1.5 h after an oral dose of 10 mg of I, *i.e.* the peak level. The identity of the peak was checked by evaluating the absorbance spectra using a diode array detector.

No traces of II were detected in this or in the other chromatograms of extracted plasma samples.

A column life of at least 600 injections was achieved by washing the column for 15 min with 20% $CH_3OH$ and for 15 min with 50% $CH_3CN$ at the end of every daily analysis.

No chromatographic interferences from drugs that could be co-administered to cancer patients such as etoposide, teniposide, doxorubicin, methotrexate, allopurinol, Ara-c, prednisone and aspirin were found.

Compound I, II and I.S. were found to be stable after one night at room temperature in the WISP autosampler.

### 3.2. Accuracy and precision

The reproducibility of the method was evaluated by analyzing 5 replicates of two QC samples containing I and II at nominal concentrations of 50 and 700 ng/0.5 ml on three different days. Extracted standard curves made in the range from 20 to 1000 ng/0.5 ml showed a great linearity, with a coefficient of correlation always higher than 0.999. Fig. 3 shows the regression curves obtained between peak response and the added concentrations for I (A) and II (B).

The intra- and inter-day precision and accuracy for both I and II are reported in Tables 1 and 2, respectively. The method was found to be highly precise with a C.V. ≤ 5%.

As shown in Table 3, the mean extraction

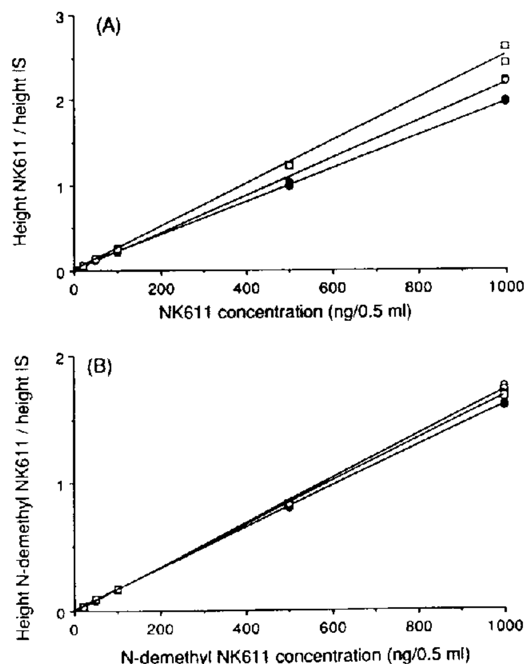


Fig. 3. Regression curves obtained in three different days (□, day 1; ○, day 2; ●, day 3) between peak response and the added concentrations for I (A) and II (B).

Table 1
Summary of intra-assay accuracy and precision data for I

| Day | Mean observed concentration (ng/0.5 ml) | *n* | C.V. (%) | Accuracy (% of nominal concentration[a]) |
|---|---|---|---|---|
| 1 | 54.203 | 5 | 1.25 | 108.4 |
| | 701.517 | 5 | 0.52 | 100.2 |
| 2 | 50.063 | 5 | 5.03 | 100.1 |
| | 712.883 | 5 | 1.52 | 101.8 |
| 3 | 50.466 | 5 | 2.04 | 100.9 |
| | 707.919 | 5 | 1.14 | 101.1 |
| Overall | 51.577 | 15 | 4.73 | 103.2 |
| | 707.440 | 15 | 1.28 | 101.1 |

[a] QC nominal concentrations were 50 ng/0.5 ml and 700 ng/0.5 ml.

Table 2
Summary of intra-assay accuracy and precision data for II

| Day | Mean observed concentration (ng/0.5 ml) | *n* | C.V. (%) | Accuracy (% of nominal concentration[a]) |
|---|---|---|---|---|
| 1 | 50.600 | 5 | 1.07 | 101.2 |
| | 705.389 | 5 | 0.84 | 100.8 |
| 2 | 48.681 | 5 | 2.22 | 97.4 |
| | 702.335 | 5 | 0.53 | 100.3 |
| 3 | 49.258 | 5 | 3.66 | 98.5 |
| | 692.694 | 5 | 1.28 | 98.9 |
| Overall | 49.513 | 15 | 2.88 | 99.0 |
| | 700.139 | 15 | 1.13 | 100.0 |

[a] QC nominal concentrations were 50 ng/0.5 ml and 700 ng/0.5 ml.

recovery from the plasma matrix for I is >70% with a S.D. <8% at each of the three concentrations tested. The extraction recovery for II is in line with that found for the parent compound but with a lower recovery (61.4%) at a concentration of 20 ng/0.5 ml.

Table 3
Recovery of I and II from plasma

| Added concentration (ng/0.5 ml) | I | | II | |
|---|---|---|---|---|
| | Mean (*n* = 3) recovery (%) | C.V. (%) | Mean (*n* = 3) recovery (%) | C.V. (%) |
| 20 | 70.1 | 7.6 | 61.4 | 2.5 |
| 100 | 74.4 | 2.6 | 71.7 | 1.4 |
| 1000 | 71.7 | 3.2 | 74.2 | 2.1 |

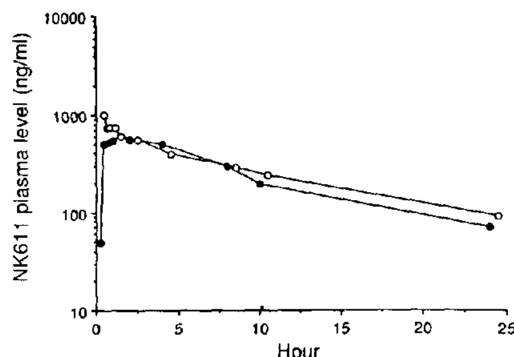

Fig. 4. Plasma decay curve of I after an oral (●) and intravenous (○) dose of 10 mg.

The quantitation limit of the assay for both substances was fixed at 10 ng/0.5 ml; at this concentration the within-day C.V. was 7.2% for I and 3.4% for II, respectively.

### 3.3. Clinical application

Fig. 4 shows the plasma decay curves of the level of I measured with the present method in a patient who entered a phase I study. Compound I was administered at the same total dose of 10 mg on day 1 of the first cycle, when it was given orally, and of the second cycle, when it was given intravenously. After oral administration, plasma levels of I could be detected already at 15 min (being 49 ng/ml) and up to 24 h, being 68 ng/ml. At the peak time, 1.5 h after administration, the concentration was 595 ng/ml; the drug disappeared with a terminal half life of 7.2 h. After 30 min i.v. infusion, the drug plasma level was 1016 ng/ml, the terminal half life 9.5 h, and the clearance 20.8 ml/min.

This patient showed a good absorption of the oral formulation of I with a bioavailability (AUC p.o./AUC i.v.) of 82%.

## 4. Conclusion

The present report describes an analytical method to measure the level of I in human

plasma. In addition to the high degree of selectivity, sensitivity, precision and accuracy, it should be noted that the method is rapid, requiring only a single extraction procedure and a rapid HPLC analysis. The method is suitable for the determination of the drug plasma levels in patients undergoing clinical investigations and will prove useful for evaluating the bioavailability and pharmacokinetic/pharmacodynamic properties of this novel antitumour agent.

## 5. Acknowledgements

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