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High-performance liquid chromatographic assay for the determination of the novel etoposide derivative dimethylaminoetoposide (NK611) and its metabolites in urine of cancer patients

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Abstract

A simple, reproducible and specific urine assay for the novel epipodophyllotoxin derivative dimethylaminoetoposide (NK611, **I**) its picro form (**III**), the N-demethyl metabolite (**II**) and its picro form (**IV**) is reported. The method involves the addition of Pr-NK611 as internal standard, chloroform extraction and HPLC separation on a Nova-Pak C_{18} column with a mobile phase of acetonitrile–0.05 *M* KH_2PO_4 (pH 6.4) (23:77, v/v). UV detection was used with absorbance monitored at 205 nm and the limit of quantification was 100 ng/ml. The intra- and inter-day precisions were within the ranges 1.1–3.4% and 1.9–2.4% for all analytes and the accuracy was 101–107%. The extraction recovery was more than 88% for **I**, **II** and **IV** and more than 83% for **III**. The assay is applicable to the urinary monitoring of **I**–**IV** in clinical pharmacokinetic investigations.

1. Introduction

NK611 (**I**, Fig. 1) is a novel water-soluble podophyllotoxin derivative with a dimethylamino group substituted in the D-glucose moiety of etoposide. Studies in murine transplantable tumours and human xenografts indicate that it has antitumor activity superior or at least equivalent to that of etoposide [1,2]. In comparison with etoposide, the main feature of **I** is its higher oral absorption with a bioavailability of ≥80% in dogs [3].

Hüttmann et al. [4] reported an assay for **I** in plasma and, more recently, this laboratory also developed an assay for plasma [5], currently used to study the plasma pharmacokinetics of **I** and **II** in patients in phase I clinical studies. Neither method, however, is suitable for the determination of **I**, its metabolite N-demethyl-NK611 (**II**) or the isomerization products picro-NK611 (**III**) and picro-N-demethyl-NK611 (**IV**) (see the structures in Fig. 1) in urine because of insufficient recovery and cleaning of the samples.

In order to evaluate the urinary elimination of **I**, its metabolite and isomerization products, we developed an HPLC assay with sufficient sen-

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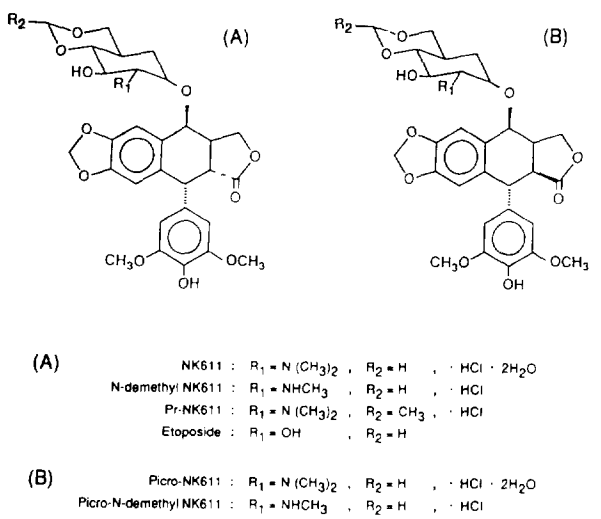

Fig. 1. Structures of **I** (NK611), **II** (N-demethyl NK611), **III** (picro-NK611), **IV** (picro-N-demethyl-NK611), the I.S. (Pr-NK611) and etoposide.

sitivity, precision and accuracy. Here, we describe the procedure, the validation of the assay and the initial results of its application to determine **I–IV** in the urine of patients receiving **I**.

2. Experimental

2.1. Chemicals

Compound **I** (5*R*,5a*R*,8a*R*,9*S*)-9-{[2-deoxy-2-(dimethylamino)-4,6-O-(*R*)-ethylidene-β-D-glucopyranosyl]oxy}-5,8,8a,9-tetrahydro-5-(4-hydroxy-3,5-dimethoxyphenyl)furo[3′4′:6,7]-naphtho[2,3-*d*]-1,3-dioxol-6[5a*H*]one hydrochloride dihydrate, its picro form (**III**), its N-demethyl metabolite (**II**), the picro form of the metabolite (**IV**) and the internal standard (I.S.) Pr-NK611 (see the structure in Fig. 1) were supplied by ASTA Medica (Frankfurt, Germany).

Diisopropyl ether, chloroform ($CHCl_3$), anhydrous potassium dihydrogenphosphate (KH_2PO_4), ammonium acetate and acetic acid, all of analytical-reagent grade, were purchased from Merck (Darmstadt, Germany). Acetonitrile (CH_3CN) and methanol (CH_3OH) were of HPLC grade and were purchased from Carlo Erba (Milan, Italy). No further purification of solvents and reagents were performed.

2.2. Instrumentation

The HPLC system consisted of a WISP 717 autosampler, a Model 510 pump, a Nova-Pak C_{18} HPLC column (150 × 3.9 mm I.D.), 4 μm and a Maxima 820 Workstation system for chromatography, all from Waters-Millipore (Milford, MA, USA). A Model LC 290 variable-wavelength UV–Vis detector(Perkin-Elmer, Norwalk, CT, USA) was used.

2.3. Extraction procedure

The four analytes were extracted as follows. Frozen urine (0.5 ml) was rapidly thawed, supplemented with 100 μl of Pr-NK611 (15 μg/ml in MeOH) as internal standard (I.S.) and 0.5 ml of cold 0.5 *M* ammonium acetate buffer (pH 3.5). The acid buffer had to be added as quickly as possible in order to avoid the isomerization of **I** and **II** into the picro forms **III** and **IV**. This can occur in aqueous solution at pH >4, as already reported for etoposide [6]. After vortex mixing for 5 s, 5 ml of diisopropyl ether were added to the samples, which were then mixed for 20 min and centrifuged for 10 min at 1200 *g*. The upper organic phase was discarded and the residue was extracted (20 min) with 8 ml of chloroform. After centrifugation (10 min at 1200 *g*), the aqueous phase was discarded and the chloroform was dried under nitrogen at room temperature. The residue was dissolved in 100 μl of methanol–0.02 *M* KH_2PO_4 (pH 3.0) (1:9) and 25 μl were injected into the HPLC system with the WISP autosampler.

2.4. Chromatographic conditions

HPLC analyses were carried out with a Nova-Pak C_{18} column (150 × 3.9 mm I.D. 4 μm) and a mobile phase of acetonitrile–0.05 *M* KH_2PO_4 (pH 6.4) (23:77, v/v) previously filtered through 0.45-μm filters and degassed. The flow-rate was 2.0 ml/min and peaks were detected at 205 nm

(i.e., λ_{max}). Chromatograms were evaluated from peak heights.

External standard samples of **I** and **II** in methanol–0.02 *M* KH_2PO_4 (pH 3.0) (1:9) were injected to assess the potential isomerization of the two compounds during HPLC separation.

At the end of the daily analyses, the HPLC column was washed with 20% acetonitrile for 15 min and with acetonitrile for another 15 min at a flow-rate of 1.0 ml/min.

2.5. Validation study

Precision and accuracy were evaluated by determining **I–IV** in ten replicates of one quality control (QC) sample, prepared at a nominal concentration of 1000 ng/ml, on three different days. To assay the QC sample, calibration graphs for four urine concentrations (100, 200, 1000 and 2000 ng/ml) of the four compounds were obtained in duplicate on each day. The precision of the method at each concentration was expressed as the coefficient of variation (C.V.) by calculating the standard deviation as a percentage of the mean calculated concentration; accuracy was determined by expressing the mean calculated concentration as a percentage of the added concentration.

The extraction recoveries of **I–IV** at two different urine concentrations (200 and 2000 ng/ml) were determined in quintuplicate by comparing the peak-height ratios of each analyte and the I.S. from chromatograms of extracted urine samples, where the I.S. was added after the extraction procedure, with those of the unextracted external standards prepared in chloroform.

The detection limit was defined as the concentration at which the signal-to-noise ratio was 3. The quantification limit was defined as the concentration with an intra- and inter-day C.V. $<10\%$.

2.6. Urine samples

Urine samples were collected from two patients who participated in phase I clinical studies with NK611 currently in progress at the Ospedale San Giovanni, Bellinzona (Switzerland), and at the Technical University, Munich (Germany).

Urine was collected at intervals of 0–6, 6–12, 12–24, 24–48 and 48–72 h after an i.v. and an oral administration of 10 mg of NK611. To prevent isomerization of **I** and **II** to the picro forms **III** and **IV**, 100 ml of 0.5 *M* ammonium acetate buffer (pH 3.5), were added to the containers used for urine collection. From the urine volume collected during the above intervals, 10-ml aliquots were taken and kept frozen at −20°C until analysis.

3. Results and discussion

3.1. Chromatography

No sign of the formation of **III** and **IV** was seen when external standard samples of **I** and **II** were injected under the described chromatographic conditions.

Fig. 2A shows a chromatogram of a urine sample extracted before drug administration. No interfering peaks were present at retention times corresponding to those of **I–IV** and the I.S. Fig. 2B shows the chromatogram of an extracted sample of 0.5 ml of urine spiked with 500ng of the four analytes and 1500 ng of I.S. The chromatographic separation takes a long time, but the conditions described achieve complete separation and good resolution of the peaks of the five compounds from the urine matrix. The N-demethyl metabolite, its picro form, NK611 and picro-NK611 were eluted at 4.2, 6.4, 10.8 and 12.8 min, respectively. The retention time of the I.S. was 26.0 min. The chromatogram in Fig. 2C is of a urine sample from the 6–12 h period after the last daily oral doses of 10 mg of **I** on day 21; the concentrations of **I** and **II** were 454 and 736 ng/ml, respectively. The two picro forms, **III** and **IV**, were present at concentrations near the limit of detection, 142 and 128 ng/ml, respectively. The identities of the peaks were checked by evaluating the absorbance spectra using a diode-array detector.

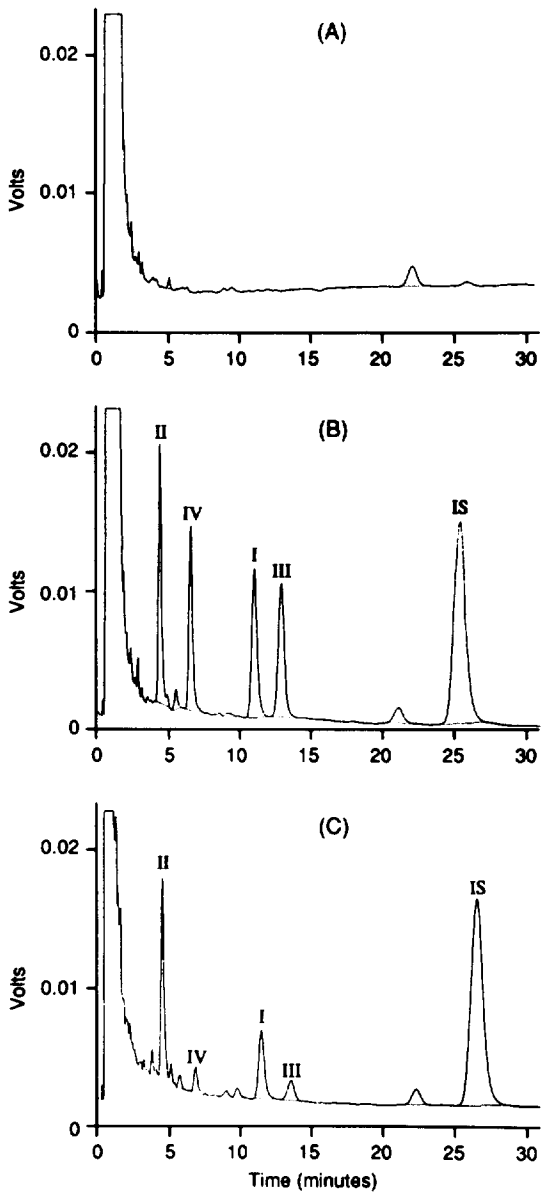

Fig. 2. Chromatograms of (A) a blank urine sample and (B) a urine sample with **I**, **II**, **III**, **IV** and I.S. added. (C) Chromatogram of a urine sample from the 6–12 h period after daily oral doses of **I** for 21 days.

A column lifetime of at least 800 injections was achieved by washing the column for 15 min with 20% acetonitrile and 15 min with acetonitrile at the end of every day's analyses. No chromatographic interferences were found from drugs that might be administered to cancer patients, such as etoposide, teniposide, doxorubicin, methotrexate, allopurinol, Ara-c and aspirin.

3.2. Accuracy and precision

The reproducibility of the method was evaluated by analysing ten replicates of one QC sample containing compounds **I–IV** at nominal concentrations of 1000 ng/ml over three different days. Calibration graphs obtained in the range 100–2000 ng/ml showed good linearity, with a correlation coefficient higher than 0.999. The intra-day and inter-day precision and accuracy for the four analytes are reported in Tables 1 and 2. The method was highly precise, with a C.V. $\leq$3.4% and accuracy within the range 101–107%.

As shown in Table 3, the mean extraction recovery from the urine matrix for the four compounds at the two concentrations tested, 200 and 2000 ng/ml, was always >83% with a maximum C.V. of 11.2% for **II** at 2000 ng/ml.

The quantification limit of the assay for the four compounds was fixed at 100 ng/ml. At this concentration the within-day C.V.s were 1.8, 3.6, 4.5 and 3.8% for **I**, **II**, **III** and **IV**, respectively.

3.3. Clinical application

Fig. 3 reports the percentage excretion of **I**, **II**, **III** and **IV** in 72-h urine measured in two patients, I.R. and F.S., taking part in a phase I clinical study of **I**. For patient I.R., who received both oral and intravenous (30-min infusion) doses of 10 mg of **I**, the total proportions of the dose recovered in the urine were, respectively, 18.6% (11.0% **I**, 7.3% **II**, 0.3% **III**, **IV** not detectable) and 21.6% (14.5% **I**, 6.3% **II**, 0.8% **III**, **IV** not detectable) after the two routes. For patient F.S., who received daily oral doses of 10 mg of **I** for 21 days, the recoveries of the excreted dose after the last dose were 12.1, 14.6, 2.3 and 1.4% for **I**, **II**, **III** and **IV**, respectively.

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

Day	Mean observed[a] concentration (ng/ml)		C.V. (%)		Accuracy (% of nominal concentration)[b]	
	I	**II**	**I**	**II**	**I**	**II**
1	1019.6	1013.4	2.1	2.7	102.0	101.3
2	1016.6	1024.8	2.1	2.0	101.7	102.5
3	1033.4	1017.2	1.1	1.2	103.3	101.7
Overall	1022.8	1018.4	1.9	2.0	102.3	101.8

[a] Each value is the mean of ten determinations.
[b] QC nominal concentration 1000 ng/ml.

Table 2
Summary of intra- and inter-assay precision and accuracy data for **III** and **IV**

Day	Mean observed[a] concentration (ng/ml)		C.V. (%)		Accuracy (% of nominal concentration)[b]	
	III	**IV**	**III**	**IV**	**III**	**IV**
1	1067.2	1014.8	1.4	3.4	106.7	101.5
2	1043.6	1016.4	1.5	1.9	104.4	101.6
3	1033.2	1008.6	1.8	1.1	103.3	100.9
Overall	1048.6	1012.8	2.0	2.4	104.9	101.3

[a] Each value is the mean of 10 determinations.
[b] QC nominal concentration 1000 ng/ml.

4. Conclusion

A method was developed to measure NK611, N-demethyl-NK611 and the two picro forms in human urine. The method is based on double solvent extraction and HPLC analysis, and gives good sensitivity, a high degree of selectivity and high precision and accuracy. The assay is suitable for the determination of **I**, its metabolite and isomerization products in patients undergoing

Table 3
Recovery of the four analytes from urine

Added concentration (ng/ml)	Mean recovery ($n = 5$) (%)[a]			
	I	**II**	**III**	**IV**
200	90.8 (3.6)	88.9 (4.2)	83.2 (3.8)	97.5 (6.1)
2000	93.8 (6.6)	95.3 (11.2)	90.7 (7.1)	94.1 (4.1)

[a] Values in parentheses are coefficients of variation (%).

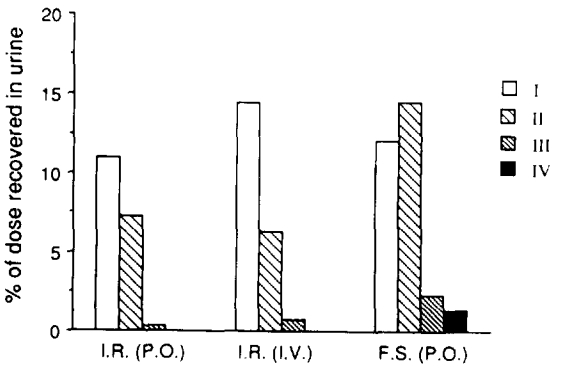

Fig. 3. 72-h urinary recoveries of I and its metabolites in two patients.

clinical investigations and will be useful in completing the pharmacokinetic/pharmacodynamic studies of this novel antitumour agent.

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