Journal of Chromatography, 620 (1993) 233–238
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7082

Determination of the new podophyllotoxin derivative NK 611 in plasma by high-performance liquid chromatography with ultraviolet detection

A. Hüttmann, K. Mross* and D. K. Hossfeld

Department of Medicine, Division of Oncology and Hematology, University Hospital Eppendorf, Martinistrasse 52, D-20251 Hamburg (Germany)

(First received May 11th, 1993; revised manuscript received August 3rd, 1993)

ABSTRACT

A high-performance liquid chromatographic method has been developed for the determination of the new podophyllotoxin derivative NK 611 in plasma samples. A solid–liquid extraction procedure with C_{18} extraction columns was used for extraction of plasma samples containing NK 611. The adsorbed NK 611 was eluted from the extraction columns with methanol–acetonitrile (50:50, v/v). The elution liquid was injected into a reversed-phase system consisting of a Chrompack C_{18} column. The mobile phase was acetonitrile–20 m*M* phosphate buffer, pH 7 (30:70, v/v). The UV detection mode allows sensitive determination of NK 611 in plasma within phase I trials. The limit of detection was 10 ng/ml, the limit of quantitation 35 ng/ml (for 1 ml of extracted plasma and 20-μl injection volume). The calibration curve is linear within the concentration range 100–1000 ng/ml. The recovery of NK 611 from spiked plasma samples was approximately 80%.

INTRODUCTION

NK 611, a new podophyllotoxin derivative, is investigated in a clinical phase I trial in Germany. The only chemical difference to the well known cytostatic agent etoposide is the introduction of an amino group to the sugar moiety. The chemical structures of etoposide (VP-16), NK 611 and the internal standard PrNK 611 are shown in Fig. 1. Pre-clinical results showed, that NK 611 is expected to have good anti-tumor activity at much lower doses than etoposide [1]. The most important difference between the drugs is the 120–170 times higher water solubility of NK 611 [2]. A highly probable advantage is its higher potency as well as a better oral bioavailability compared with etoposide.

Several methods have been published for the high-performance liquid chromatographic (HPLC) determination of etoposide in plasma using ultraviolet, electrochemical or fluorescence detection [3–5]. However, the substance-specific characteristics, *e.g.* the pK_a-value of 7.7 (etoposide: pK_a-value 9.8) made development and evaluation according to the guidelines of "Good Laboratory Practice" (GLP) necessary. These guidelines were developed by the members of the OECD (Organization for Economic Cooperation and Development) with the aim to improve the quality of studies and to guarrantee international approval of the concession of a drug.

This paper describes a simple and rapid method for the reversed-phase determination of NK 611 in plasma samples.

* Corresponding author.

0378-4347/93/$06.00

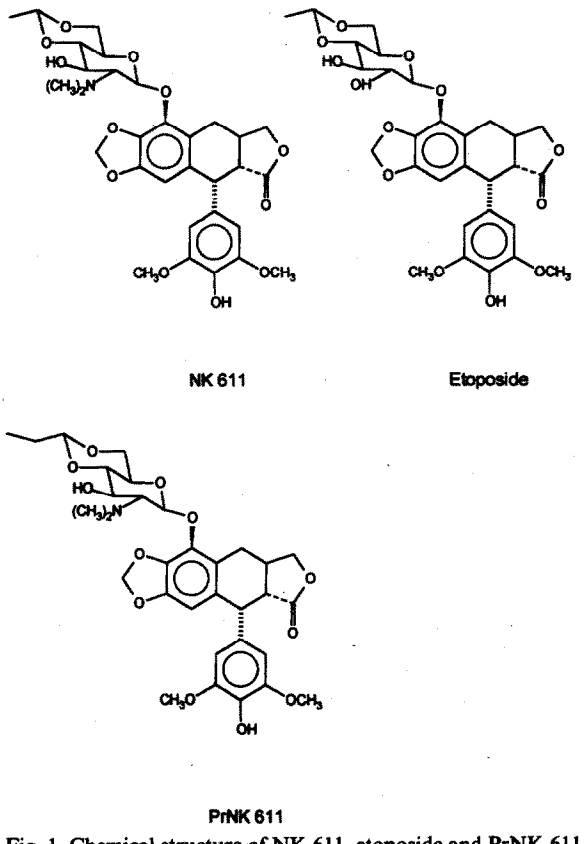

Fig. 1. Chemical structure of NK 611, etoposide and PrNK 611.

EXPERIMENTAL

Chemicals and reagents

NK 611 analytical standard and propylidene-NK 611 (PrNK 611) internal standard were obtained from Asta Medica (Frankfurt, Germany). Both were kept in powder form at 4°C or in aqueous 20 m*M* KH_2PO_4 (pH 3.5) solutions at −80°C. Under these conditions, the powder and the solutions were stable for at least 18 months. Water (for HPLC) was obtained from Baker (Deventer, The Netherlands). Methanol, acetonitrile, sodium hydroxide and phosphoric acid were obtained from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate was obtained from Serva (Heidelberg, Germany).

Plasma samples

Blood was collected in polypropylene tubes containing ammonium-heparinate as anticoagulant. Samples were centrifuged at 3100 *g* for 10 min at room temperature. The plasma layer was stored frozen at −80°C until analysis.

During the phase I study, twelve blood samples were taken over a period of 48 h after infusion.

Extraction of NK 611 from plasma samples

For solid-phase extraction C_{18} sample preparation columns, size 3CC, obtained from Varian (Harbor City, USA) and a sample preparation unit (SPU), obtained from Merck (Darmstadt, Germany) were used.

After thawing, the samples were vortex-mixed and diluted (depending on sampling time after infusion of NK 611) 1:20, 1:10 or 1:5 in plasma provided by the blood donor centre. The last sample collected 48 h after infusion remained undiluted. From each sample, as well as from the standards used for the calibration curve and from the samples used for accuracy and precision tests, aliquots of 900 μl were mixed with 100 μl PrNK 611 (internal standard). The samples were centrifuged at 16 000 *g* for 2 min. All samples were processed in polypropylene tubes to minimize a possible loss of NK 611 by adsorption.

The sample preparation columns were conditioned successively with 2 ml of methanol and 4 ml of distilled water, subsequently 900 μl of each prepared sample were pipetted on the columns. Then the extraction columns were washed with 4 ml of 20 m*M* phosphate buffer (pH 7) and allowed to dry for 10 min. Elution of NK 611 and PrNK 611 (internal standard) was done by adding a solution of 4 ml of methanol–acetonitrile (50:50, v/v). The samples eluted from the columns were transferred to a metal heating block (48°C) and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 200 μl of mobile phase and sonicated for 10 min. After centrifugation at 16 000 *g* for 2 min, samples were split into 2 fractions and 20 μl of each were injected into the HPLC unit.

Chromatography

The chromatographic system consisted of a Merck-Hitachi L-6200A pump, a Merck-Hitachi

AS-2000 auto sampler and a Merck-Hitachi L-4250 UV-detector, set at a wavelength of 215 nm. Chromatograms were recorded and peaks were integrated using a Merck-Hitachi D-2500 integrator. Seperations were performed with a Microspher C_{18} guard column connected to a Microspher C_{18} column (200 mm × 3 mm I.D.) (all Chrompack, The Netherlands).

The mobile phase was acetonitrile–20 m*M* phosphate buffer (adjusted to pH 7 with sodium hydroxide) (30:70, v/v). The flow-rate was 1 ml/min.

Calibration curves

Plasma NK 611 concentrations were determined from calibration curves obtained with five standard plasma samples of known concentrations: 100, 250, 500, 750 and 1000 ng/ml NK 611 dissolved in plasma provided by the blood donor centre; extraction was done as described above. After HPLC analysis, peak-height ratios (NK 611 / PrNK 611 internal standard) were plotted against the corresponding NK 611 amount in each standard solution.

Extraction recovery

To determine the NK 611 extraction recovery, experiments were performed on 6 successive days over the entire calibration range defined above. The procedure was to compare the height of the peaks of NK 611 detected in extracted plasma and in directly injected analytical-standard solutions containing the same amount of NK 611 as 900 μl of plasma. Extraction and HPLC determination were performed as described above.

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ determination was performed for NK 611 analytical-standard solutions in 20 m*M* phosphate buffer and for plasma samples spiked with NK 611.

Concentrations were 25–40 ng/ml in steps of 1 ng/ml for NK 611 in phosphate buffer solutions and 5–40 ng/ml in steps of 5 ng/ml for NK 611 in plasma samples. Twenty microliter of each phosphate-buffer solutions was injected directly into the HPLC unit, the plasma samples were processed as described in Experimental.

RESULTS

Chromatography

The column used resolved and separated NK 611 and PrNK 611 (internal standard) sufficiently. Metabolites were not observed. Fig. 2 shows chromatograms obtained from blank plasma, plasma spiked with analyte, plasma spiked with analyte and internal standard and a sample obtained 4 h after infusion of 80 mg/m^2 NK 611. The retention times were approximately 14 min for NK 611 and 28 min for PrNK 611. The "plasma" peak was eluted from *ca.* 0.5–8 min.

Linearity

The linearity of the method was studied using the same concentrations and method as for the calibration curves. Peak-height ratios (NK 611/internal standard) were plotted against NK 611 concentration in the standard solutions. The weighting function was $w(y_i) = 1$ (absolute (= no) weighting). Calculation was done by the least squares method. The mean correlation coefficient (r^2) is 0.9996, the standard deviation (S.D.) is ±0.0003. The equation of the mean regression line was $y = 0.002(x) - 0.025$.

Accuracy and precision

For the accuracy and precision test plasma samples containing 118 ng/ml and 955 ng/ml NK 611 were prepared. Extraction and HPLC determination were done as described above. For the within-day accuracy and precision six samples of each concentration were prepared. For the day-to-day accuracy and precision nineteen determinations over a period of 63 days were evaluated. The results are shown in Table I. Most values were within the first standard deviation, all within the second.

Extraction recoveries and detection limits

The extraction recoveries determined as described in Experimental were as follows: Analyzing replicate samples ($n = 6$) with concentrations of 100, 250, 500, 750 and 1000 ng/ml yielded recoveries in the 74–83% range with standard deviations in the 2.7–4.9 range.

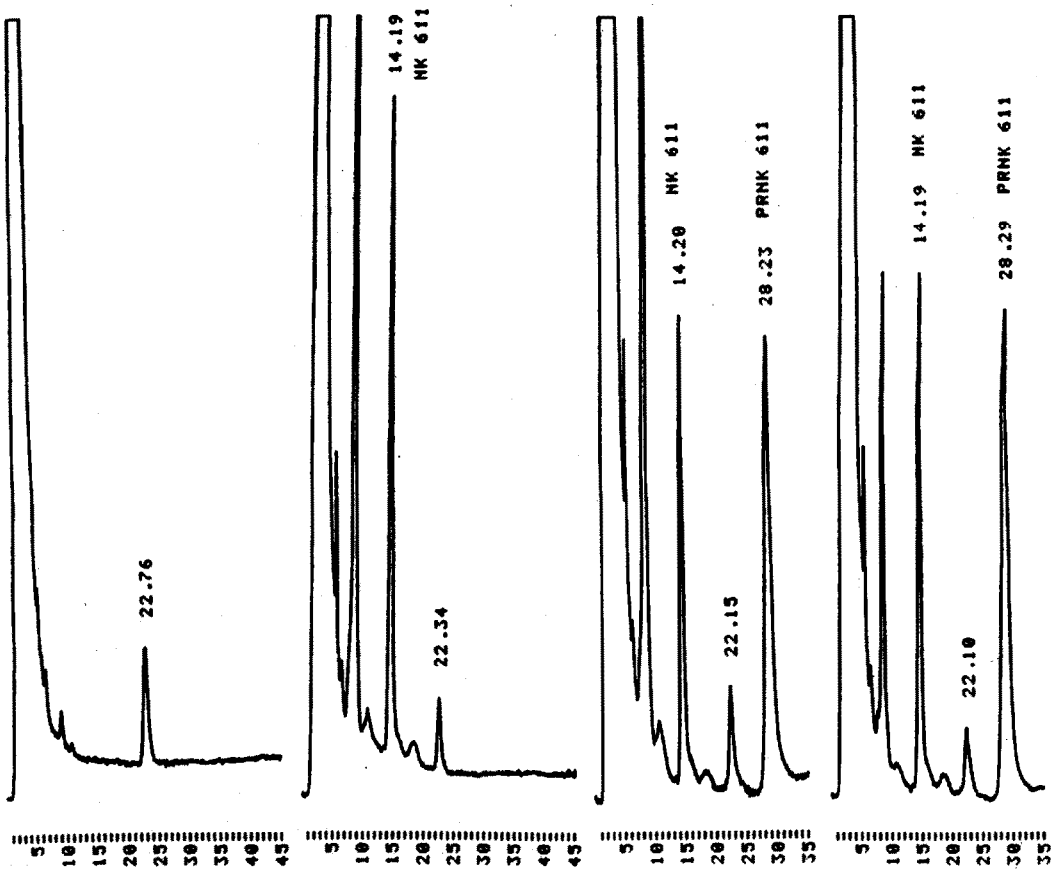

Fig. 2. Chromatograms obtained from blank plasma, plasma spiked with 1000 ng/ml analyte, plasma spiked with 750 ng/ml analyte and internal standard, and a chromatogram obtained 4 h after infusion of 80 mg/m^2 body surface. The sample was diluted 1:10 and represents a concentration of 7.7 μg/ml.

TABLE I

ACCURACY AND PRECISION OF NK 611 PLASMA DETERMINATION

Concentration spiked (ng/ml)	Concentration calculated (mean ± S.D.) (ng/ml)	Relative error (mean ± S.D.) (%)
Within-day (n = 6)		
118	125.9 ± 6.4	6.8 ± 5.3
955	955.4 ± 38.8	3.8 ± 1.5
Day-to-day (n = 19)		
118	119.3 ± 9.4	6.6 ± 4.7
955	974.3 ± 50.7	3.2 ± 4.6

The LOD for NK 611 in phosphate buffer solutions was 33 ng/ml, the LOQ was 40 ng/ml. LOD and LOQ for extracted plasma samples were 10 ng/ml, respectively 35 ng/ml. The different LOD and LOQ for samples in phosphate buffer solutions and for extracted samples is explained by the 45 times higher absolute amount of NK 611 in the 900 μl spiked plasma samples compared to the absolute amount of NK 611 in the 20 μl phosphate buffer solution.

Interferences

A phase I trial with a cytostatic drug implicates that patients are sometimes treated with various

drugs during the sampling period. Such drugs were *e.g.* analgetics, hormones and antiemetics. A review on chromatograms of 20 patients showed, that there was no interference between NK 611, PrNK 611 and other drugs, excipients, impurities or metabolites.

Analyte stability

The analyte stability was tested under different conditions. Plasma samples containing 1000 ng/ml were kept at 4°C, room temperature and 37°C over a period of 24 h and afterwards processed as described before. HPLC analysis showed that there was no decomposition of the drug.

General results

Fig. 3 shows a concentration–time plot of a patient treated with 80 mg NK 611/m^2 body surface. Calculation of the area under the curve by integration allows the determination of plasma clearence, volume of distribution, mean residence time, half life and other values which are important for later clinical use of the drug.

DISCUSSION

The described HPLC assay allows accurate quantitative analysis of NK 611 in plasma samples. Generally, the assay is not very laborious due to the use of a sample preparation unit (SPU) with a vacuum device enabling the simultaneous processing of 24 samples. This method was developed in consideration of the requirements of a validated assay [6], which means that statistical data and reproducibility must be adequate according to the guidelines for GLP. The quality of the assay is proved by the good results of the tests for accuracy and precision and by the linearity of calibration curves. The disadvantage of this assay is that runs were quite long because of the retention time of the internal standard, but PrNK 611 was the only internal standard available. Other podophyllotoxin derivatives, such as etoposide or teniposide, are inappropriate as internal standard because their retention time is similar to that of NK 611 or because the compound "disappeared" in the "plasma" peak. We have not observed metabolites with the described method. Several postulated metabolites of VP-16-213 have been reported [7]. It is possible, that NK 611 also has a metabolic pathway. However, we did not observe any peak in the chromatograms suggesting metabolization.

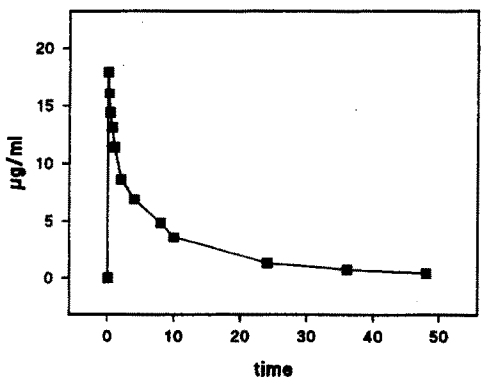

Fig. 3. Concentration–time plot of a patient treated with 80 mg NK 611/m^2 body surface.

The extraction recoveries were satisfactory. They could not be improved by using chloroform–methanol or chloroform–acetonitrile mixtures as a eluent. More than 50% methanol in the eluent broadened the "plasma" peak, causing the NK 611 peak to disappear. The same problem was encountered when the washing buffer was adjusted at pH < 7.

The intention of the clinical phase I study is to determine the dose limiting toxicity (DLT), the maximum tolerable dose (MTD) and the pharmacokinetics (PK). In this phase I trial we found that even at an initial dose of 60 mg/m^2 body surface the samples obtained 48 h after infusion did not reach the lowest calibration curve concentration of 100 ng/ml. Considering the fact that a phase I trial necessarily implicates increase of the intravenously administered dose (in our study the MTD was 120 mg/m^2 body surface) it may be expected that this assay is adequate for clinical use.

In future, a lower LOD and LOQ might be desirable, especially for planned oral bioavailability studies. This can easily be done by injecting more than the small amount of 20 µl used in this study into the HPLC unit or/and by enlarging the

quantity of plasma loaded onto the sample preparation columns. An improvement by a factor of 5 might be feasible. Yet, when investigating the pharmacokinetics after intravenous administration the problem is not the LOQ but the plasma concentrations that are too high to be detected without dilution. Whether plasma concentrations after oral administration will be lower than after intravenous injection is an empiric problem that can not be predicted. If so, the lowest calibration curve concentration can be set to the LOQ instead of 100 ng/ml. If this is not sufficient, a modification as suggested above can be performed.

We think that the assay covers all existing clinical needs and may easily be adapted to all clinical needs which might probably appear.

ACKNOWLEDGEMENT

We acknowledge the excellent technical assistance by K. Vehrs and K. Herbst. The development of this assay was supported by the Dep. Cancer Research of ASTA Medica AG (Frankfurt a. M., Germany) and the Erich und Gertrud Roggenbruck, Stiftung zur Förderung der Krebsforschung in Hamburg. We appreciate the administrative support by Mrs. Fröhlich and Mrs. Gerbig. This publication includes part of the thesis of A. Hüttmann.

REFERENCES

1 H. Ekimoto, K. Okamato, K. Kusama, H. Mashiba and T. Takeuchi, *Proc. Ann. Meeting Am. Assoc. Cancer Res.*, 33 (1992) A2606.
2 H. Ekimoto, K. Okamato, K. Nakamoria, K. Takahashi and T. Takeuchi, *Proc. Ann. Meeting Am. Assoc. Cancer Res.*, 33 (1990) A2668.
3 R. J. Strife, I. Jardine and M. Colvin, *J. Chromatogr.*, 182 (1980) 211–220.
4 T. J. Littlewood, A. L. Hutchings, D. P. Bentley and B.P. Spragg, *J. Chromatogr.*, 336 (1984) 434–437.
5 D. D. Stiff, T. L. Schwinghammer and S. E. Corey, *J. Liq. Chromatogr.*, 15(5) (1992) 863–873.
6 V. P. Shah, K. K. Midha, S. Dighe, I. J. McGilveray, J. P. Skelly, A. Yacobi, T. Layloff, C. T. Viswanathan, C. E. Cook, R. D. McDowall, K. A. Pittman and S. Spector, *Eur. J. Drug. Metab. Pharmacokin.*, 16(4) (1991) 249–255.
7 R. A. Bender, E. Hamel and K. R. Hande, in B. A. Chabner and J. M. Collins (Editors), *Cancer Chemotherapy: Principles and Practice*, J. B. Lippincot, Philadelphia, PA, 1990, Ch. 10, pp. 262–275.