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Determination of intoplicine, a new antitumour drug, in human whole blood and plasma by normal-phase high-performance liquid chromatography with fluorescence detection

R. van Gijn* and H. Rosing

Slotervaart Hospital, Louwesweg 6, 1066 EC Amsterdam (Netherlands)

W. W. ten Bokkel Huinink and O. van Tellingen

Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam (Netherlands)

J. B. Vermorken and A. J. M. Liefting

Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam (Netherlands)

R. Bruno

Rhône-Poulenc Rorer, 20 Avenue Raymond Aron, 92165 Antony Cedex (France)

H. M. Pinedo

Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam (Netherlands) and Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam (Netherlands)

J. H. Beijnen

Slotervaart Hospital, Louwesweg 6, 1066 EC Amsterdam (Netherlands) and Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam (Netherlands)

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ABSTRACT

Intoplicine, a benzopyrido-indole derivative, is a novel anticancer agent currently under phase I clinical evaluation. A selective, sensitive normal-phase high-performance liquid chromatographic (HPLC) assay with fluorescence detection, suitable for the determination of intoplicine in human plasma and whole blood, is described. The sample pretreatment involves a protein precipitation step with 2-propanol. The reported assay was validated, and the stability of the analyte in plasma, in whole blood and in the extraction fluid was investigated. The method has been implemented in a pharmacokinetic phase I clinical trial with intoplicine given as a 24-h intravenous infusion.

* Corresponding author.

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INTRODUCTION

Intoplicine (RP60475F; NSC D645008; 11-(3-dimethylaminopropyl-amino)-3-hydroxy-8-methyl-7H-benzo[*e*]pyrido[4,3-*b*]-indole, dimethanesulphonate; Fig. 1) is a novel, potential, antitumour drug selected for development through a collaborative study between the manufacturer and the Curie Institute [1]. It belongs to a new class of antitumour drugs that interact with the nuclear DNA-modifying enzymes topoisomerase I and II [2]. The cytotoxicity of intoplicine, mediated by inhibition of topoisomerases, is induced by the stabilization of the cleavable enzyme–DNA complexes which, ultimately, leads to cell death. In *in vitro* studies intoplicine was found to be a potent inhibitor of DNA, RNA and, to a lesser extent, protein synthesis. *In vivo* the drug has demonstrated a broad spectrum of activity against a variety of transplantable murine tumours [3]. On the basis of these interesting properties intoplicine was selected for clinical phase I evaluation. Determination of the pharmacokinetics of a drug under investigation is one of the objectives of a phase I study. From investigations with radiolabelled drug it has been concluded that intoplicine is more than 70% bound to erythrocytes, which necessitated the development of a drug assay for both plasma and whole blood.

We here describe a selective and sensitive assay for the determination of intoplicine in human plasma and whole blood. The method has been implemented in a pharmacokinetic phase I clinical trial with intoplicine given as a 24-h intravenous infusion once every three weeks. Preliminary pharmacokinetic results at the starting dose of 12 mg/m^2 are given. The reported assay was validated, and the stability of the analyte in plasma, whole blood and the extraction fluid (2-propanol) was investigated. This is the first report in the literature describing the analysis of intoplicine in biological fluids.

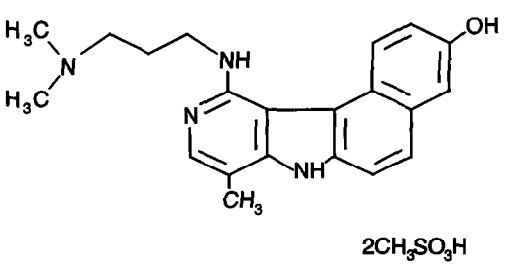

Fig. 1. Structure of intoplicine dimethanesulphonate.

EXPERIMENTAL

Equipment

The HPLC system consisted of a Type 510 solvent-delivery system (Waters Assoc., Milford, MA, USA), an LS 40 fluorescence detector (Perkin Elmer, Norwalk, CT, USA), an SP 8880 automatic sample injection device (Spectra Physics, Santa Clara, CA, USA) and an SP 4600 integrator (Spectra Physics). The analytical column (100 mm × 3.0 mm I.D.) was filled with ChromSpher silica material (particle size 5 μm) (Chrompack, Bergen op Zoom, Netherlands) and was protected by a Chromspher silica (10 mm × 3.0 mm I.D.; particle size 5 μm)(Chrompack) guard column. UV spectra of intoplicine were recorded with an SP8-400 UV–VIS spectrophotometer (Pye Unicam, Cambridge, UK). Fluorescence emission and excitation spectra of intoplicine were recorded on-line with an HPLC LS-40 fluorescence detector (Perkin Elmer).

Chemicals

Intoplicine (as dimethanesulphonate) originated from Rhône-Poulenc Rorer (Antony, France). Dichloromethane was obtained from Mallinckrodt (Paris, KY, USA), ammonia p.a., sodium hydroxide p.a., hydrochloric acid p.a. (37%) and LiChrosolv-grade 2-propanol were from Merck (Darmstadt, Germany). Home-made distilled water was used throughout. Drug-free heparinized human plasma was obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, Netherlands). Whole blood was provided by healthy volunteers.

Stock solution

Stock solutions of intoplicine (10 μg/ml) were prepared by dissolving the appropriate amount of the drug, accurately weighed, in water. The stock solution was stored, prior to analysis, at

−30°C and was found to be stable for at least two months under these conditions.

Calibration samples

Plasma. The stock solution was diluted with 2-propanol to give standard solutions with concentrations of 100, 10 and 1 ng/ml. Calibration samples were prepared by adding 0, 100, 250, 500, 750 and 1000 μl of a standard solution to 100 μl of drug-free plasma in polypropylene Eppendorf cups. The total volume of 2-propanol of each standard was brought to 1000 μl. Calibration samples were prepared in the ranges 100–1000, 10–100 and 1–10 ng/ml in plasma. The samples were vortex-mixed for 30 s and centrifuged at 9500 *g* for 3 min. The clear supernatant was then transferred to an autosampler vial, and 100 μl were injected onto the HPLC column.

Whole blood. The stock solution was diluted with 2-propanol to give standard solutions with concentrations of 1000–100 and 10 ng/ml. Calibration samples were prepared by adding 10, 25, 50, 75 and 100 μl of a standard solution in polypropylene Eppendorf cups. The solvent was evaporated under nitrogen (40°C) and 100 μl of drug-free whole blood were added. The sample was vortex-mixed for 15 s and equilibrated for 30 min at ambient temperature. Next, 1.0 ml of 2-propanol was added and the sample was vortex-mixed for 30 s and centrifuged for 3 min at 9500 *g*. The clear supernatant was then transferred to an autosampler vial, and 100 μl were injected onto the HPLC column.

Calibration curves

The wide concentration range of interest (1 ng/ml to 1 μg/ml) was divided into three decades, and each decade was described by an equation, calculated by using the "unweighted" least-squares method.

Sample extraction procedure

To a 100-μl sample (plasma or whole blood) in a polypropylene Eppendorf cup, 1.0 ml of 2-propanol was added. The sample was vortex-mixed for 30 s and centrifuged for 3 min at 9500 *g*. The clear supernatant was then transferred to an autosampler vial and 100 μl were injected into the HPLC system.

Chromatography

Chromatographic analyses were performed at ambient temperature with a mobile phase composed of dichloromethane–2-propanol–10% ammonia (40:40:1, v/v/v). Prior to use dichloromethane and 2-propanol were filtered through a 0.22-μm filter. The fluorescence was monitored at wavelengths set at 375 nm for emission and 425 nm for excitation. The flow-rate was maintained at 0.6 ml/min. Aliquots of 100 μl were injected into the chromatograph.

Determination of recovery, accuracy, precision, detection limit and lower limit of quantitation

Recoveries of intoplicine from plasma and whole blood were calculated by comparing the analytical results with 2-propanolic solutions containing known quantities of the analyte. The accuracy and precision of the method were determined by replicate analysis of known concentrations in the middle of each calibration curve. Linearity was investigated by least-squares regression analysis of the analytical results from spiked plasma and whole-blood samples. The detection limit of the HPLC assay is the drug concentration at which the fluorescence response is equal to three times the average noise signal. The lower limit of quantitation is defined as the concentration of the lowest standard in the analytical run which is quantitated with a definite level of certainty.

Determination of stability

The chemical stability of intoplicine in plasma and whole blood was investigated by adding known amounts of the drug. The spiked samples were stored at −30°C and the residual drug concentration was determined periodically. Stability of intoplicine in 2-propanol was studied at −30°C and at ambient temperature.

Pharmacokinetics

A 57-year-old male patient suffering from a tumour of unknown origin was administered 22.8

mg (12 mg/m^2) of intoplicine dissolved in 500 ml of a 0.9% sodium chloride solution by continuous intravenous infusion over 22 h. Serial blood samples (5 ml) were collected in heparinized tubes from an indwelling intravenous cannula placed in the arm contralateral to that receiving the drug. Samples were taken prior to the start of the infusion, during the infusion, at the end of the infusion and at specific times after the infusion. An aliquot of whole blood (1 ml) was immediately taken out of the collection tube and stored in a polypropylene tube at −30°C prior to analysis. The remaining fraction was centrifuged at 3000 *g* for 15 min and plasma was transferred to a polypropylene tube and stored at −30°C prior to analysis.

RESULTS AND DISCUSSION

Chromatography and detection

Several HPLC systems have been tested with the aim of finding the optimal conditions for the determination of intoplicine. With reversed-phase chromatography on phenyl, C_8 or C_{18} columns, the analyte exhibited broad tailing peaks probably because of the basic character of the tertiary amine in the aliphatic side-chain of the molecule (Fig. 1). Also, the use of non-modified aluminium oxide as stationary phase with an aqueous solvent mixture gave poor results, while these systems have been useful for the analysis of basic drugs [4]. On the other hand, normal-phase chromatography using a silica gel column gave acceptable results. The influence of each component of the mobile phase (dichloromethane–2-propanol–ammonia) on the chromatographic behaviour of the drug has been studied. Optimum results were established with a mobile phase composition of dichloromethane–2-propanol–10% ammonia (40:40:1, v/v/v). In the absence of ammonia the drug shows very strong affinity for the silica phase with retention times >60 min. The amount of ammonia was, however, restricted by the working pH range of the silica stationary phase between 2 and 10. The lifetime of the used column (stored in the mobile phase) was established to be at least three months.

In aqueous and organic solutions the UV absorption spectrum of intoplicine shows a maximum at 270 nm (molar absorptivity 29 700 for aqueous solutions and 34 900 for organic solutions). However, at this wavelength there are many endogenous interferences in the chromatograms of plasma and whole-blood extracts that preclude UV absorption as a sensitive detection method. In a mixture of organic solvent and ammonia (the pH of the mixture should be more than 7.5) intoplicine is highly fluorescent. This property appeared very suitable for sensitive and selective detection and was a major reason for choosing normal-phase chromatography, allowing the analysis to be performed at alkaline pH. The selection of fluorescence wavelengths was based on scanning experiments with the drug in the mobile phase, and optimum excitation and emission wavelengths were found at 375 and 425 nm, respectively. The native fluorescence properties of intoplicine are mainly a function of pH, which should be more than 7, indicating that the compound only fluoresces in its deprotonated form. No interfering peaks were observed in any of the biological samples used in our studies, or in any plasma/whole-blood samples obtained from healthy volunteers. Representative HPLC chromatograms for the analysis of patient plasma and whole blood are shown in Fig. 2.

Sample pretreatment

Protein precipitation with 2-propanol is a simple procedure yielding high recoveries (Table I). Good precision and accuracy in plasma and whole blood were achieved without the need for an internal standard (Table II). Although the recovery for whole blood is relatively low, the accuracy and precision data justify the use of the 2-propanol deproteination method as a sample pretreatment procedure.

Calibration samples

Intoplicine has a high affinity for erythrocytes [5]. The time required to bind to red blood cells was investigated by adding known amounts of the drug to whole-blood samples. The spiked samples were kept at 20°C and the recovery of

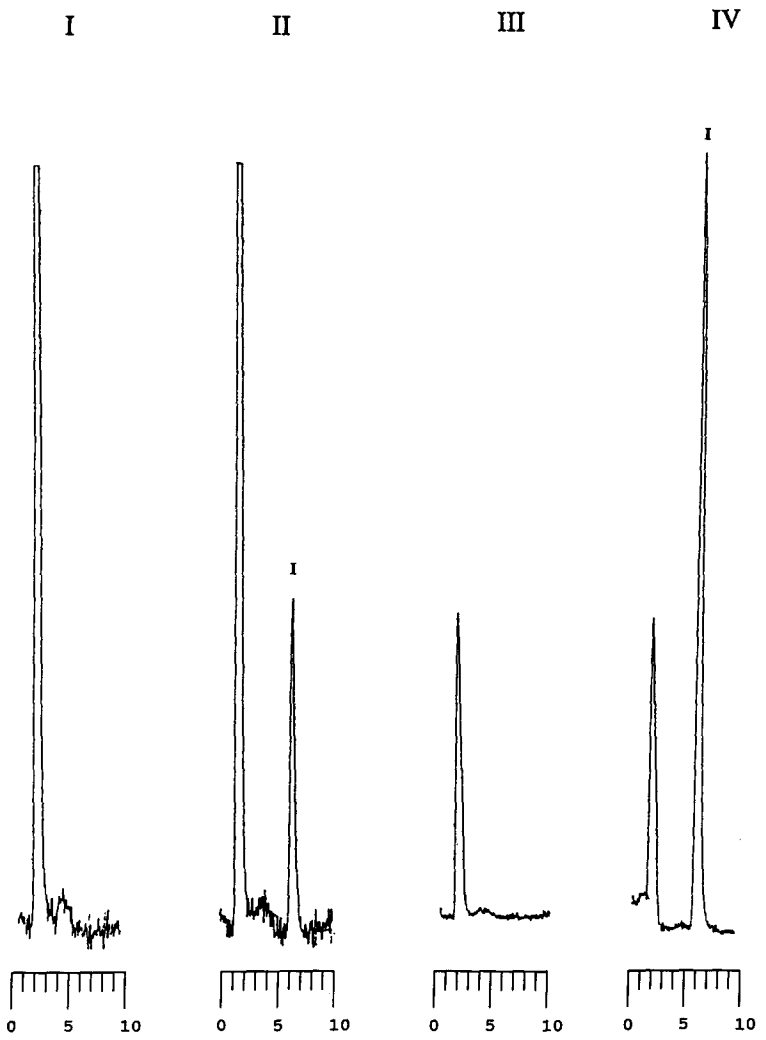

Fig. 2. HPLC of blank plasma (I), a plasma sample (II) (concentration of intoplicine 13.8 ng/ml), blank whole blood (III) and and a whole blood sample (IV) (concentration of intoplicine 113.6 ng/ml) of a patient receiving 12 mg/m^2 intoplicine as a 22-h intravenous infusion. The retention time of intoplicine (I) is 6.5 min.

intoplicine was determined periodically. The results of these tests are shown in Fig. 3 and demonstrate that after 0.5 h a constant recovery of about 65% is obtained, indicating that the binding process of intoplicine has been completed. Experiments at 37°C also showed a constant recovery of about 65%, which was reached after 10 min. It can be assumed that this state has been reached in the patient samples during sampling. Therefore, the whole-blood calibration samples were allowed to reach equilibrium by incubating them for 0.5 h at 20°C before extraction with 2-propanol. With this procedure calibration samples and patient samples are adjusted maximally to the same condition. In contrast to the whole-blood samples there was no indication that intoplicine bound to plasma components (recoveries >95%). The relatively low recoveries from whole blood are apparently due to the high degree of binding to blood cells. However, the good accuracy and precision data from the validation of the assay in whole blood justify the use of the presented methodology.

TABLE I

RECOVERIES OF INTOPLICINE FROM PLASMA AND WHOLE BLOOD

Concentration (ng/ml)	Recovery[a] (%)	C.V[b] (%)	n[c]
Plasma			
5.010	97.8	7.8	4
50.10	105.4	9.3	4
501.0	105.6	7.0	4
Whole blood			
5.010	60.1	14.0	3
50.10	60.1	10.8	4
501.0	61.2	5.2	4

[a] Recoveries of intoplicine from plasma and whole blood were calculated by comparison with 2-propanolic solutions containing known quantities of intoplicine.
[b] C.V = coefficient of variation.
[c] n = number of determinations (each determination consisted of five replicates).

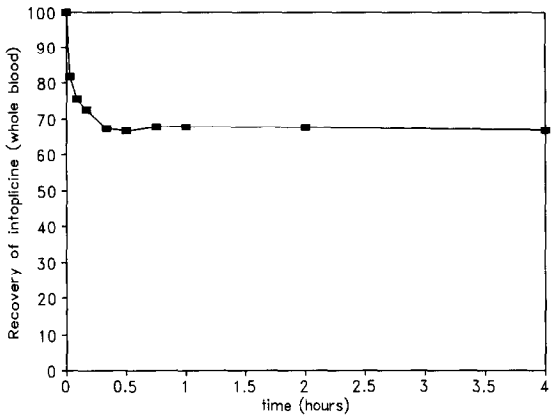

Fig. 3. Time–recovery plot, obtained after spiking whole blood with intoplicine (concentration 100 ng/ml) with sampling at indicated time points.

Validation of the assay

The analytical methodology was validated in terms of recovery, detection limit, precision, accuracy and linearity. Overall mean recoveries of intoplicine were 102.9 ± 4.4 and 60.5 ± 0.6% for plasma and whole blood, respectively (Table I).

The detection limit of the presented assay is 0.5 ng/ml using a 100-μl sample with a 100-μl injection of the extract onto the HPLC column. The lower limit of quantitation is 1.0 ng/ml (accuracy 103 ± 9.2 and 109 ± 14.2% for plasma and whole blood samples, respectively; n = 5) using a 100-μl sample with a 100-μl injection of the ex-

TABLE II

ACCURACY AND PRECISION (C.V.) FOR THE BIO-ANALYSIS OF INTOPLICINE IN PLASMA AND WHOLE BLOOD

Theoretical concentration (ng/ml)	Measured concentration (ng/ml)	Accuracy (%)	C.V.[a] (%)	n[b]
Plasma				
1.002	1.032	103.0	9.2	5
5.010	4.870	97.2	4.9	5
50.10	50.05	100.8	1.7	5
501.0	514.0	102.6	1.4	5
1002	983.3	98.1	1.7	5
Whole blood				
1.002	1.092	109.0	14.2	5
5.010	4.920	98.2	5.0	5
50.10	49.40	98.6	3.9	5
501.0	492.5	98.3	4.7	5
1002	1002	100.0	4.2	5

[a] C.V. = coefficient of variation.
[b] n = number of replicates.

TABLE III

EQUATIONS OF CALIBRATION LINES FOR THE ANALYSIS OF INTOPLICINE IN PLASMA AND WHOLE BLOOD

Concentration range (ng/ml)	Equation[a]	r^2	n^b
Plasma			
1–10	$y = 59321(\pm 3466)x - 9648\ (\pm 30090)$	0.9931	5
10–100	$y = 61384(\pm 1644)x - 163843(\pm 119632)$	0.9979	5
100–1000	$y = 62509(\pm 645)x - 350750(\pm 469661)$	0.9997	5
Whole blood			
1–10	$y = 48245(\pm 1519)x - 7531\ (\pm 13801)$	0.9957	5
10–100	$y = 51925(\pm 1064)x - 83790\ (\pm 92867)$	0.9983	5
100–1000	$y = 43917(\pm 1241)x - 1060936(\pm 1082670)$	0.9968	5

[a] x is the concentration of intoplicine in ng/ml and y is integrator peak area units. S.D. is given in parentheses.
[b] Number of replicates.

tract onto the HPLC columnn. Accuracy and precision characteristics of the assay have been tabulated (Table II). The calibration curves in plasma and whole blood were linear from 1 ng/ml to 1 μg/ml using 100-μl samples (Table III).

Possible co-medication drugs (acetylsalicylic acid, dexamethasone, acetaminophen, ibuprofen, megestrol acetate, procainamide and ranitidine), each at its own therapeutic concentration, were extracted with 2-propanol and injected into the HPLC system; no interferences were found.

Stability

Intoplicine (concentration 50 ng/ml) is stable in plasma, whole blood and 2-propanol at −30°C for at least two months (Table IV).

The extracted drug is also stable (1–1000 ng/ml) in 2-propanol at ambient temperature for at least 24 h, which enables the use of an autosampler for HPLC injection.

Clinical pharmacokinetics

Log concentration–time curves of intoplicine in plasma and whole blood of a patient receiving 12 mg/m^2 as a 22-h intravenous infusion are shown in Fig. 4. The curves demonstrate an extremely long half-life of the drug in whole blood (>60 h). At this dose level the peak levels of intoplicine were 13.8 ng/ml in plasma and 113.6 ng/ml in whole blood. The topoisomerase I and II inhibitory activity of intoplicine *in vitro* is maximal about 1 μ*M* (540 ng/ml) [6]. This concentra-

TABLE IV

STABILITY OF INTOPLICINE IN PLASMA, WHOLE BLOOD AND 2-PROPANOL AT −30°C, INITIAL CONCENTRATION: 50 ng/ml

Matrix	Percentage of initial concentration (± C.V.) at the indicated time[a]				
	$t = 0$	One day	One week	One month	Two months
Plasma	100	99.4 ± 1.6	99.3 ± 2.3	98.0 ± 2.5	100.0 ± 1.3
Whole blood	100	98.1 ± 1.6	96.8 ± 2.5	99.4 ± 1.2	99.8 ± 1.8
2-Propanol	100	99.0 ± 1.5	97.5 ± 2.0	98.6 ± 1.4	98.1 ± 1.5

[a] Results are reported as mean ± coefficient of variation (C.V.) of three determinations.

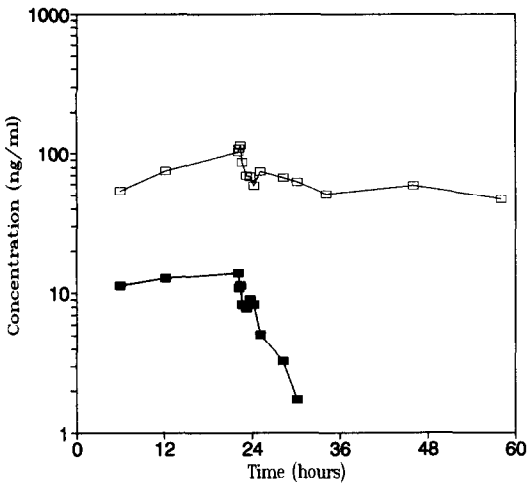

Fig. 4. Log concentration–time curves of intoplicine in plasma (■) and whole blood (□) of a patient receiving 12 mg/m^2 as a 22-h intravenous infusion.

tion is clearly not reached at the first dose step of the phase I study. Metabolic products of intoplicine have not been identified so far.

CONCLUSION

In conclusion, a simple, sensitive, selective and validated HPLC method for the analysis of the new investigational cytotoxic drug intoplicine in plasma and in whole blood has been developed and requires only 100 μl of sample. The assay is suitable for human pharmacokinetic studies.

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