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Note

Determination of vinblastine in MO_4 mouse fibrosarcoma cells by high-performance liquid chromatography

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Vinblastine is an antitumour drug widely used for the treatment of neoplastic diseases. The anti-invasive effect of this drug and other microtubule inhibitors on MO_4 cells in vitro and in vivo have been studied by different authors [1–4]. It seems that the anti-invasive activity in vitro is obtained with levels that are achievable in current clinical practice [4]. It would be interesting to determine the pharmacokinetics at the cellular level, especially in tumour cells, in order to elucidate the correlation between the plasma kinetic levels and the cellular kinetic behaviour.

A previously described high-performance liquid chromatography (HPLC) method has been developed in our laboratory that allows the determination of

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vinblastine and vindesine in plasma and urine with vincristine used as internal standard (I.S.) [5]. This method has already been used to study the pharmacokinetics of the vinca alkaloids administered to patients according to different dose profiles [6].

This paper describes an adaption of the method for the determination of vinblastine in MO_4 cells, as well as in the cell culture medium. The method is applied to the determination of vinblastine in cells that have been exposed for seven days to the drug in different concentrations.

EXPERIMENTAL

Apparatus

A Varian 5060 liquid chromatograph, equipped with a Rheodyne loop injector (100-μl sample loop) and a Varian UV-100 variable-wavelength detector operating at 220 nm, was used. The chromatograms were recorded and integrated with a Varian CDS 401 data system. The column was stainless steel (250×4 mm I.D.) packed with LiChrosorb CN, particle size 5 μm. A guard column (30×4 mm I.D.) filled with the same material was used to protect the analytical column. The mobile phase was acetonitrile–phosphate buffer pH 3 (60:40, v/v). All experiments were carried out at ambient temperature. All laboratory glassware was silanized with Surfasil (Pierce, Rockford, IL, U.S.A.) diluted with acetone.

Standards and reagents

Vinblastine sulphate (Velbe®) and vincristine sulphate (Oncovin®) were of pharmaceutical purity and were obtained from Eli Lilly (Brussels, Belgium). Chloroform was pro analysis quality, and acetonitrile and dichloromethane were of liquid chromatographic grade. All these solvents were obtained from Merck (Darmstadt, F.R.G.). Doubly distilled water, further purified with a Water-I system (Gelman Sciences, Ann Arbor, MI, U.S.A.), was used for the buffer solutions. Sodium octylsulphate, phosphoric acid, sodium dihydrogen phosphate monohydrate and potassium chloride were pro analysis quality (Merck).

A phosphate buffer (pH 3) with an ionic strength (μ) of 0.4 containing $5 \cdot 10^{-2}$ *M* octyl sulphate was used for the extraction. For the mobile phase a phosphate buffer (pH 3) with $\mu = 0.08$ was used.

Cell cultures

MO_4 cells are virally transformed fibrosarcoma-like C_3H mouse cells, and were maintained in monolayer culture using Eagle's Minimum Essential Medium Modified (Modified MEM, Gibco Europe, Paisley, U.K.) supplemented with 10% foetal calf serum (Gibco Europe) and 0.05% L-glutamine (Difco Labs., Pasture, Brussels, Belgium). Penicillin G (100 E/ml) and streptomycine sulphate (100 μg/ml) were added to the culture medium. Hereafter the supplemented culture medium will be called MEM 10. The cells were placed in culture vessels of area 75 cm^2 (Nunc, Gibco Europe) and kept in a CO_2 incubator (Forma Scientific, Brugman TTO, Brussels, Belgium).

Vinblastine was added to MEM 10 in different concentrations: 0.1 μg/ml (series

1), 1 μg/ml (series 2) and 10 μg/ml (series 3). Since all these concentrations had a reversible effect on MO_4 spheroids for at least six days [4], the uptake of vinblastine by the MO_4 cells was followed for seven days. The concentration of vinblastine in the MO_4 cells and in the corresponding MEM 10 was determined at different time intervals: 30 min (D_0), 48 h (D_2), 96 h (D_4) and 168 h (D_7).

Extraction and recovery of vinblastine from MEM 10 and the MO_4 cells

The MO_4 cells were lysed before the extraction procedure was carried out. The cells were first scraped from the bottom of the culture vessel with a policeman, and single cells were obtained by means of a syringe. The cell suspension was then placed in a centrifuge tube and centrifugated for 15 min at 700 *g*. The supernatant was discarded, and 4 ml of potassium chloride (0.075 *M*) were added to perform the cell lysis in hypotonic medium at 37 °C for 30 min. The solution was then put in an ultrasonic bath for 1 min to ensure complete cell lysis. In each series of experiments, on the different days of sample collection, a supplementary culture vessel was treated in the same way except that the cells were trypsinized for cell count. The number of non-vital cells was evaluated using the tryptan blue exclusion test. The number of cells was 10^7. The number of dead cells was not taken into account in calculating the vinblastine concentration in the cells.

The following extraction procedure was applied: to 4 ml of MEM 10 or to 4 ml of potassium chloride (containing the lysed cells), 100μl of vincristine (I.S.) were added. Deproteinization was carried out by adding acetonitrile dropwise under continuous vortexing. After centrifugation for 30 min the acetonitrile was evaporated under a gentle nitrogen stream at 60 °C, and 10 ml of phosphate buffer (pH 3, $\mu = 0.4$) containing $5 \cdot 10^{-2}$ *M* sodium octylsulphate and 5 ml of chloroform were added. The mixture was shaken for 30 min. After centrifugation, 4 ml of the organic phase were evaporated at 30 °C under a nitrogen stream and reconstituted in 200 μl of dichloromethane. A 100-μl aliquot was injected into the HPLC system. The concentration of the I.S. solution was different for the three experiments: for series 1, 200 ng/ml; for series 2, 1 μg/ml; for series 3, 10 μg/ml. Different volumes of acetonitrile for deproteinization were tested: 0, 0.8, 2, 4 and 8 ml.

Quantitation was carried out by intrapolation on an extracted standard curve in MEM 10 (peak area of drug/peak area of I.S. versus concentration of drug). For the determination of the extraction recovery, quantitation was performed by comparison with a standard curve obtained from an aqueous solution.

Preparation of standard curves in MEM 10

A stock solution of vinblastine was prepared in water in a concentration of 200 μg/ml, and from this standard solutions to spike MEM 10 were prepared in the following final concentrations of vinblastine in MEM 10: 0.025, 0.05, 0.1 and 0.2 μg/ml for series 1, 0.1, 0.5, 1 and 2 μg/ml for series 2, and 0.5, 5, 10 and 20 μg/ml for series 3. The vinblastine concentration in the MO_4 cells and in the corresponding MEM 10 was determined by intrapolation on these standard curves.

RESULTS AND DISCUSSION

Extraction and chromatographic procedure

The extraction procedure is an ion-pair extraction at pH 3 with octylsulphate as counter-ion, which has been successfully applied to the isolation of basic drugs

in biological material [7]. This procedure has also been used for the extraction of vinblastine, vindesine and vincristine from plasma and urine [5]. Since interactions occur between proteins, drugs and counter-ion, resulting in a poorer extraction recovery, it is necessary to perform a deproteinization step for plasma by adding acetonitrile [5]. A volume of acetonitrile equal to twice the volume of plasma is needed to obtain an effective deproteinization [7]. Before the addition of the buffer solution and the organic solvent the acetonitrile must be evaporated, in what is the most time-consuming step in the whole extraction procedure [7]. Since MEM 10 only contains 10% calf serum, it is obviously necessary to investigate first if deproteinization is needed and, if it is, to determine the optimal volume acetonitrile in order to shorten the analysis. The extraction recoveries with different amounts of acetonitrile were determined with 100 ng/ml vinblastine and 200 ng/ml vincristine (I.S.). Without deproteinization the extraction recovery was $41 \pm 6.7\%$ ($n=6$) for vinblastine and $74.2 \pm 3.8\%$ ($n=6$) for vincristine. With 0.8 or 2 ml of acetonitrile, the recovery was ca. 90% for vinblastine and 80% for vincristine. With 4 ml of acetonitrile the recovery was $101.3 \pm 8.8\%$ ($n=6$) for vinblastine and $90.6 \pm 6.4\%$ ($n=6$) for vincristine. With 8 ml of acetonitrile, the same extraction recovery is obtained for vinblastine and the extraction recovery of vincristine does not increase.

The amount of acetonitrile used has also an influence on the blank chromatogram. With 0.8 or 2 ml some endogenous compounds interfere with the peaks of interest. This does not happen when 4 or 8 ml of acetonitrile is used. Hence, 4 ml of acetonitrile was used for deproteinization. The same procedure has been evaluated for the MO_4 cells with the same results as described for MEM 10.

Fig. 1 shows the chromatograms of a blank MEM 10 sample (a), a spiked MEM 10 sample (b) and a sample of MO_4 cells (c). The HPLC system previously described [5] was used, with a small change in the mobile phase composition because another CN column was used: the same mobile phase solvents were used, acetonitrile–phosphate buffer (pH 3), but in a different ratio (60:40 instead of 65:35). The ionic strength of the buffer was 0.08 instead of 006. With this HPLC system vinblastine and vincristine were baseline-resolved within 10 min, and no interferences from endogenous compounds were observed.

Cellular uptake of vinblastine

In a first experiment, we exposed the MO_4 cells to vinblastine for seven days at 37°C. At different times (D_0, D_2, D_4 and D_7) vinblastine was determined in the cells after lysis and in the corresponding MEM 10. Since the experiments were carried out at 37°C, the stability of vinblastine (100 ng/ml) in MEM 10 at this temperature over seven days was investigated (Table I). Vincristine was added on the day of analysis to serve as a control. Vinblastine degrades at 37°C so that, after seven days, 80–85% of the initial vinblastine concentration remains.

In the first experiment the cells were placed in MEM 10 supplemented with 100 ng/ml vinblastine. Fig. 2a shows the ratio of the vinblastine concentration in the cells to the vinblastine concentration in MEM 10 (mean value of six samples) as a function of time. In the first 48 h the cells accumulated vinblastine, whereafter a steady state was observed. The number of dead cells increased as a func-

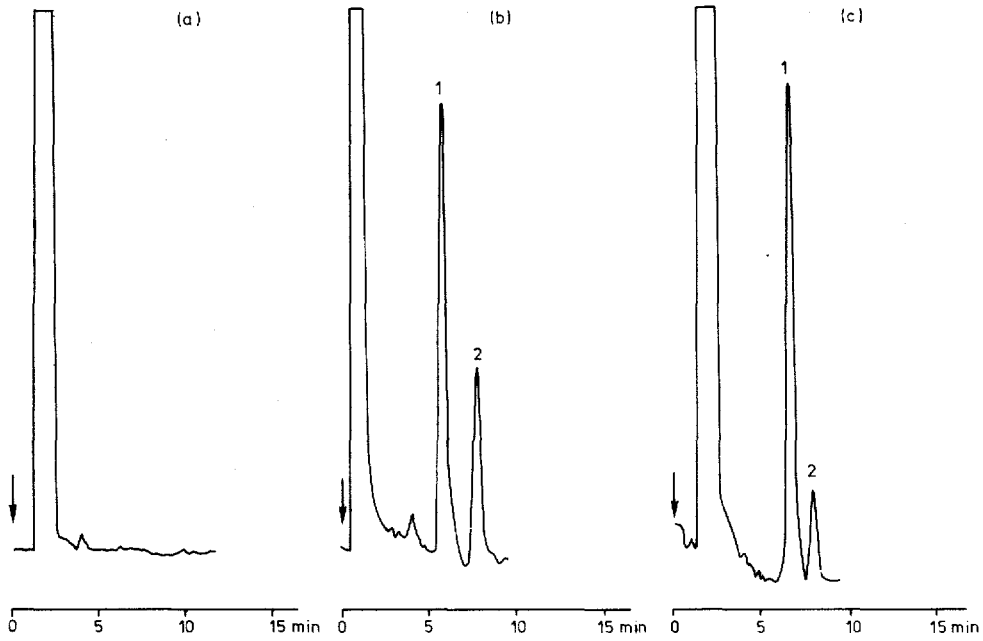

Fig. 1. Chromatograms of (a) a blank MEM 10 sample, (b) a MEM 10 sample spiked with vinblastine (0.1 μg/ml) and vincristine (0.2 μg/ml) and (c) an extract of MO_4 cells. Mobile phase, acetonitrile–phosphate buffer pH 3 (60:40, v/v); flow-rate, 1.5 ml/min; detection, 220 nm; a.u.f.s., 0.01. Peaks: 1 = vincristine (I.S.); 2 = vinblastine.

tion of time with increasing concentration of the drug. However, it never exceeded 20% after D_7.

Before the experiment was repeated with a 10- and a 100-fold higher concentration of vinblastine (1 and 10 μg/ml) to investigate if the cells are able to accumulate the drug to a greater extent, the extraction recoveries in these concentration ranges, using 4 ml of acetonitrile for deproteinization, were determined. At 1 μg/ml, the extraction recoveries of vinblastine and vincristine were 92.9 ± 6.1%

TABLE I

STABILITY OF VINBLASTINE AND VINCRISTINE (100 ng/ml) IN MEM 10 AT 37 °C

Compound	Time	Recovery (%)	Coefficient of variation ($n=6$) (%)
Vinblastine	D_0	100	8.3
	D_2	97.0	3.3
	D_4	87.0	10.0
	D_7	82.0	8.3
Vincristine	D_0	90.0	5.6
	D_2	97.0	4.2
	D_4	92.0	6.5
	D_7	89.2	5.7

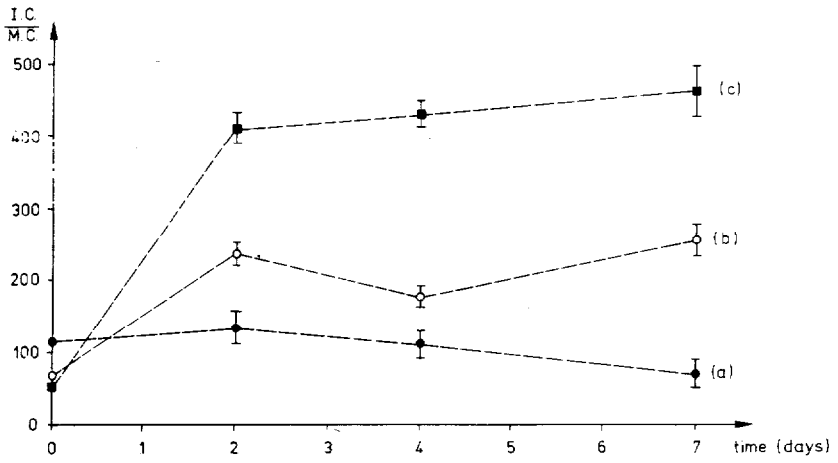

Fig. 2. Plots representing the cellular uptake of vinblastine as a fucntion of time. Different concentrations of vinblastine were tested: (a) 0.1 μg/ml; (b) 1 μg/ml; (c) 10 μg/ml. I.C./M.C. = intracellular concentration/medium concentration.

and 87.9 ± 10.4%, respectively, and at 10 μg/ml, a recovery of 80.9 ± 5.1% was obtained for vinblastine and 77.9 ± 5.6% for vincristine ($n=6$). The results obtained with these two concentrations are given in Fig. 2b and c. It seems that, in both cases, the cells cannot accumulate vinblastine to the same ratio level after 30 min ($=D_0$), when a 10- or 100-fold higher concentration of drug is administered. However, after 48 h a large increase is observed, which would indicate that the cells are stimulated and able to accumulate vinblastine in a greater extent. After 48 h the curves exhibit a similar profile. One can state that the accumulation ratio of vinblastine by MO_4 cells is a dose-related phenomenon. A possible explanation for the observed behaviour in the first 48 h is that an active transport mechanism is responsible for the uptake of vinblastine into the MO_4 cells [8]. However, this is only a supposition, which needs further experiments for confirmation. Nevertheless the method described here is suitable for these experiments.

CONCLUSION

A method has been developed that permits the determination of vinblastine in MO_4 mouse fibrosarcoma cells and in the corresponding culture medium, using vincristine as I.S. This method involves ion-pair extraction in combination with an HPLC technique for quantitative determination. The usefulness of the method is tested for the uptake of vinblastine by MO_4 cells with different concentrations of the drug. The procedure seems to be suitable for the elucidation of the cellular pharmacokinetics of vinblastine.

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