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Simultaneous determination of cytotoxic (adriamycin, vincristine) and modulator of resistance (verapamil, S 9788) drugs in human cells by high-performance liquid chromatography and ultraviolet detection

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Abstract

Multidrug resistance (MDR), which was described for structurally and mechanistically unrelated anticancer agents, was modulated in vitro by a series of compounds which were of different chemical origin. In this situation, the selection of a correct assay dosage to study the MDR modulation mechanism was a problem. We developed a high-performance liquid chomatography (HPLC) method which enabled the simultaneous determination of three major cytotoxins (adriamycin, daunorubicin, vincristine) and two well-known modulators (S 9788, verapamil). This assay was fully validated and was used to follow, for the first time, the uptake and accumulation behaviour of adriamycin and S 9788 co-incubated with resistant and sensitive cell lines (KB-3-1; KB-A1).

Keywords: Daunorubicin; Adriamycin; Vincristine; Verapamil; S 9788

1. Introduction

The development of anticancer drug resistance remains a major drawback of chemotherapy in clinical oncology. One important mechanism drug resistance in cancer cells is the multidrug resistance (MDR) phenotype [1,2]. A causal link between MDR and membrane P-glycoprotein (P-gp) hyperexpression is well established [3,4]. Characteristically, MDR cell lines show extensive cross-resistance to structurally and mechanistically unrelated anticancer agents, which tend to be amphipatic and relatively high molecular weight natural products [5–7]. The P-gp acts as an ATP-dependent efflux pump, which decreases the intracellular concentration of the cytotoxic agent and, thus, its efficacy [8,9]. Different compounds have been shown to reverse MDR by decreasing the efflux of cytotoxic drugs [4]. The mechanism of action of P-gp and reversal compounds is not well understood yet. All published studies quantified the efflux rate or the intracellular accumulation of the cytotoxic drug without taking

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into consideration the behaviour of the modulator itself [10]. Most of the methods developed are based on fluorescence or radioactivity measurements and are poorly validated. These techniques of dosage are insufficient as they can not discriminate between the signals coming from different xenobiotics. To overcome the lack of specificity, we developed and validated an original HPLC method suitable for the simultaneous quantification of three cytotoxic and two modulator drugs, which have been well documented in scientific literature, to improve the knowledge about acquired resistance. The HPLC assay was used to compare the initial rate of uptake for S 9788 and verapamil and to study the interactions between doxorubicin and S 9788 accumulation in KB sensitive and resistant cell lines.

2. Experimental

2.1. Chemicals

Adriamycin (ADR) and daunorubicin (DNR) were kindly provided by Farmitalia (Carlo Erba, Milan, Italy). 6-[4-(2,2-Di-(4-fluorophenyl)ethylamino)-1-piperidinyl]-N,N'-di-2-propenyl-1,3,5-triazine-2,4-diamine (S 9788) and hexamethylmelamine (HMM) were generous gifts of the Servier International Research Institute (Courbevoie, France) and the National Cancer Institute (Bethesda MD, USA), respectively. Verapamil (VRP) and vincristine (VCR) were obtained from Sigma–Aldrich (Bornem, Belgium). All reagents were of analytical grade namely: acetonitrile (Lab-Scan Analytical Sciences, Ireland), potassium dihydrogenphosphate, phosphoric acid, silver nitrate, sodium chloride, 2-propanol, chloroform (Merck Darmstadt, Germany), deionised water was prepared on site and further purified using a Milli Q system (Millipore, Brussels, Belgium).

2.2. Preparation of standards

Stock solutions (0.01 *M*) were prepared in acetonitrile and secondary solutions were obtained by dilution, either in mobile phase to be used as chromatographic standards or in water, to spike biological samples for recovery studies.

The concentration ranges of these solutions were: ADR and DNR, 0.5–5.0 μ*M*; S 9788, 10–100 μ*M*; HMM, 1.0–10 μ*M*; VCR, 2.5–25 μ*M*; VRP, 8.0–80 μ*M*.

2.3. Cell line and culture medium

The KB-3-1 cell line human epidermoid carcinoma [11] and its counterpart, KB-A1, which was 340-fold resistant to ADR, were kindly provided by Dr. M. Gottesman (Bethesda, MD, USA) via Dr. J.Y. Charcosset (Toulouse, France). These cell line were grown in DMEM medium supplemented with 10% fetal calf serum, 2 m*M* l -glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10 m*M* HEPES buffer, pH 7.4 at 37°C in a humidified 5% CO_2 air mixture. The KB-A1 cell line was cultured in the presence of 2 μ*M* ADR until one week prior to the beginning of the experiments.

2.4. Uptake and accumulation studies

The KB-3-1 ($1.25 \cdot 10^6$ cells) and KB-A1 ($1.60 \cdot 10^6$ cells) cell lines were plated for 24 h at 37°C in a 25 cm^2 flask containing 10 ml of growth medium. After that period, the culture medium was removed and replaced by 10 ml of fresh medium containing the xenobiotic at the concentration to be tested. The concentration ranges were 50–1000 μ*M* for verapamil and 5–60 μ*M* for S 9788, respectively. The uptake rate was determined from a 5-min contact time. For the accumulation studies, cells were exposed to the drugs at a concentration of 5 μ*M* for different times ranging from 5 to 240 min. After incubation, medium was sucked up and cells were quickly washed twice in ice-cold phosphate-buffer saline (PBS). The cells were detached with trypsin [12] and the intracellular amount of drug was determined by HPLC.

2.5. Extraction of drugs from KB cells

Samples of $2 \cdot 10^6$ cells were prepared in plugged polypropylene tubes and extracted according the following steps: (1) addition of 20 μl $AgNO_3$ (33%, w/v) and agitation for 10 s, (2) ultrasonication for 20 min (Bransonic 52, Vel, Belgium), (3) addition of 140 μl of CH_3CN, vortexed for 5 min, (4) cooling of

tubes at 4°C for 30 min (5) centrifugation for 30 s at 10 000 *g*, (6) addition of 200 μl of phosphate buffer (pH 3; 200 m*M*) and (7) injection of 50 μl of the supernatant in the HPLC system.

2.6. Chromatographic system

The HPLC system consisted of a Rheodyne 7125 injector (Analis, Namur, Belgium) equipped with a 100 μl injector loop, a Gilson 307 solvent delivery system (Analis). Separations were carried out under isocratic conditions (1.00 ml/min) on a RP18 Hibar LiChrocart column, 250×4.6 mm I.D., 7 μm particle size (Merck). The mobile phase was comprised of 0.2 *M* potassium dihydrogenphosphate, 2 ml/l triethylamine adjusted to pH 3 with 0.2 *M* orthophosphoric acid (650 ml) and acetonitrile (350 ml). Detection was by UV absorbance at 237 nm using an HP 1050 variable wavelength detector (Hewlett–Packard, Brussels, Belgium) linked to an analysis chromatography software (Borwin 1.20, JMBS Developments, Paris, France), and a photodiode array detector (Beckman Instrument: Module 168 System Gold Chromatography software ASW 2, Analis).

2.7. Validation study

2.7.1. Intra-assay, inter-assay precision and recoveries

Intra-assay variability was assessed from cell samples (6 replicates) spiked with standards (20 μl) at six different concentrations. The experiments were performed on three different days to determine the between day reproducibility. The relative standard deviation (R.S.D.) was used as an indicator of precision and was calculated from the peak area ratios of analyte/internal standard (I.S.).

2.7.2. Linearity, limits of detection and limits of quantification

The homogeneity of variances was checked by Bartlett's test [13] and regression analysis was used to assess the linearity between the areas ratios (analyte/I.S.) and the concentrations of drugs. The lowest limit of detection (LOD) was defined as the concentration which had a signal-to-noise ratio of 3:1. For limit of quantification (LOQ), the ratios considered were 10:1 with a R.S.D. value less than 10%.

2.7.3. Specificity

The specificity of the method was assessed by analysis of biological samples on a Beckman Model 168 diode array system. The detector output was analysed by the System Gold (Beckman Instruments). The algorithm performs purity analysis in real time, as the compounds elute and indicates how many compounds reside under an impure peak. The algorithm analyses information using a technique based on principal component analysis (PCA) [14] and is derived from the evolving factor analysis (EFA) method proposed by Maeder and Zuberbühler [15] and Maeder [16]. To increase the discrimination of the study, chromatographic separations were performed with two mobile phases giving different k' values for all analytes. The results obtained were compared with those given by the usual spectral overlay technique.

3. Results and discussion

3.1. Chromatography

A preliminary study of the influence of the mobile phase pH on the capacity factor (k') values had shown that they were lowered for all compounds when the pH of the buffer was decreased from 6.0 to 3.0. The influence of phosphate concentration on k', the resolution and the asymmetry of the peaks are thus investigated at pH 3.

Fig. 1 shows that k' values decreased linearly when the phosphate concentration was increased

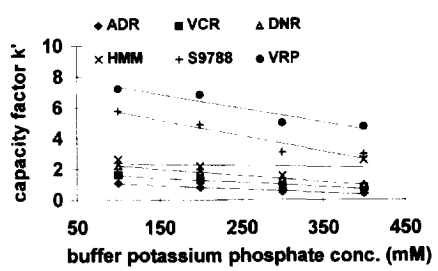

Fig. 1. Effect of phosphate buffer concentration in the mobile phase on the capacity factor k' of compounds.

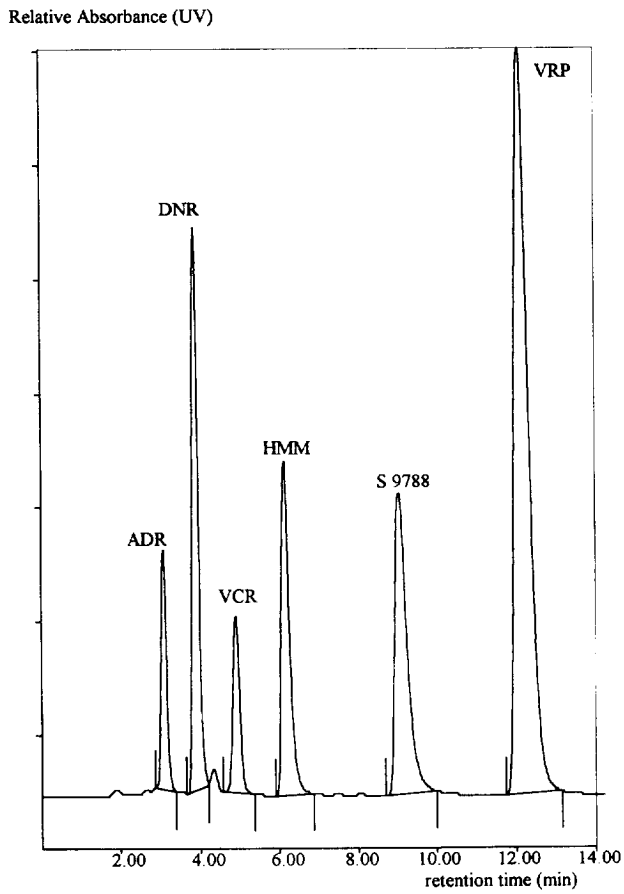

Fig. 2. Representative chromatogram of drugs extracted from cells. Amounts injected: ADR (0.2 nmol), DNR (0.2 nmol), VCR (1.0 nmol), HMM (0.4 nmol), S 9788 (4.0 nmol), VRP (2.0 nmol).

Table 1
Linear regression parameters and intra-day ($n=6$) and inter-day ($n=3$) mean coefficients of variation for the HPLC assay

Compound	Concentration range (nmol/10^6 cells)	Slope (mean±S.D.)	Intercept (mean±S.D.)	Correlation coefficient (mean±S.D.)	R.S.D.[a] (mean) (%)	
					Between-day	Within-day
ADR	0.1–1.0	0.0157±0.0005	−0.2±0.2	0.9987±0.0006	3.3	2.5
VCR	0.5–5.0	0.0074±0.001	−0.03±0.08	0.9991±0.0004	3.7	2.6
S 9788	2.0–20.0	0.00153±0.00006	−0.17±0.02	0.9990±0.0002	2.6	1.4
VRP	1.6–16.0	0.013±0.003	−0.6±0.4	0.998±0.002	4.3	1.2

[a] Mean coefficients of variation are expressed with regard to the internal standard.
Intra-day mean R.S.D. are calculated from six different concentrations. For each concentration, six samples were analysed.
Inter-day mean R.S.D. are calculated from six different concentrations. For each concentration, four samples were analysed on three different days.

Table 2
Detection and quantification limits of compounds

Compound	LOD (pmol injected)	LOQ (pmol injected)
ADR	2	5
VCR	4	13
DNR	2	7
HMM	2	8
S 9788	29	98
VRP	8	27

from 100 to 400 m*M*, except for HMM. However, 200 m*M* was found to be an optimum buffer concentration to obtain both a good resolution of all peaks and a minimum time analysis. Most of the peaks had shown an improved symmetry at higher phosphate concentrations of the buffer (400 m*M*). At 200 m*M*, the addition of triethylamine as tailing suppressor was necessary to have an acceptable symmetry of the DNR, S 9788 and VRP peaks. Fig. 2 illustrates the separation of the six drugs dissolved in the mobile phase.

3.2. Linearity and precision

The linearity of the method was estimated by measuring the peak area ratios of the drug to an I.S., both extracted from cells at different concentrations. DNR was used as I.S. to determine ADR and VCR, and HMM was the I.S. for S 9788 and VRP quantification. The linearity of the method was statistically demonstrated for all drugs ($P<0.05$) and intercepts were shown to be practically zero ($P<0.05$). Results collected in Table 1 show that the within- and between-day precisions (R.S.D.) were always less than 5% and the mean values were 2.0 and 3.5%, respectively.

3.3. Limits of detection and quantification

The limits of detection and quantification for the six drugs extracted from cells were at the pmol level (Table 2) and the ranges were respectively 2–29 for the LOD and 5–98 for LOQ.

Table 3
Influence of amount extracted on the mean recoveries and standard deviations of drugs extracted from KB-3-1 human cancer cells

Compound	Spiked amount (pmol)	I.S.	Absolute recovery (mean±S.D.) (%)	Relative recovery (mean±S.D.) (%)
ADR	25	DNR	89±3	97±3
	50		92±4	100±4
	100		92±4	100±4
	150		93±2	101±2
	200		94±2	101±3
	250		96±3	101±3
VCR	125	DNR	92±9	101±9
	250		92±4	100±4
	500		93±3	101±3
	750		92±2	100±2
	1000		92±2	100±2
	1250		92±1	100±2
S 9788	500	HMM	91±1	100±2
	1000		91±2	98±2
	2000		93±4	98±4
	3000		93±3	99±3
	4000		93±2	100±2
	5000		93±2	100±2
VRP	400	HMM	92±2	100±2
	800		93±8	101±8
	1600		93±8	101±8
	2400		93±2	101±2
	3200		93±5	101±5
	4000		95±1	103±1

3.4. Extraction

In the method described, the addition of $AgNO_3$ was essential to break strong anthracycline interactions with cells, but had no effect on the extraction recoveries of the other analytes. Ultrasonication and CH_3CN were respectively used to complete the destruction of the cell structure and to precipitate proteins. After centrifugation, the supernatant was diluted with 200 μl of phosphate buffer at pH 3.0 to reconstitute the mobile phase composition before injection.

3.5. Recoveries

The absolute recoveries of drugs, calculated by comparison of extracts from spiked samples with the same concentration prepared in the mobile phase, ranged from 89 to 95% and were concentration independent. The relative values are still better and very close to 100%. A very good precision for between-day recoveries was recorded (Table 3).

3.6. Specificity

Results obtained by the evoluting factor analysis with two mobile phases, as well as those obtained by the spectral overlay technique, demonstrated the peak purity for the six analytes extracted from cells. Moreover, no interference peak was found in the chromatogram of blank cell samples. The specificity was checked for two cell lines (KB-3-1 and KB-A-1). Figs. 2 and 3 show chromatograms of a drug free sample and of the six drugs extracted from KB cells, respectively.

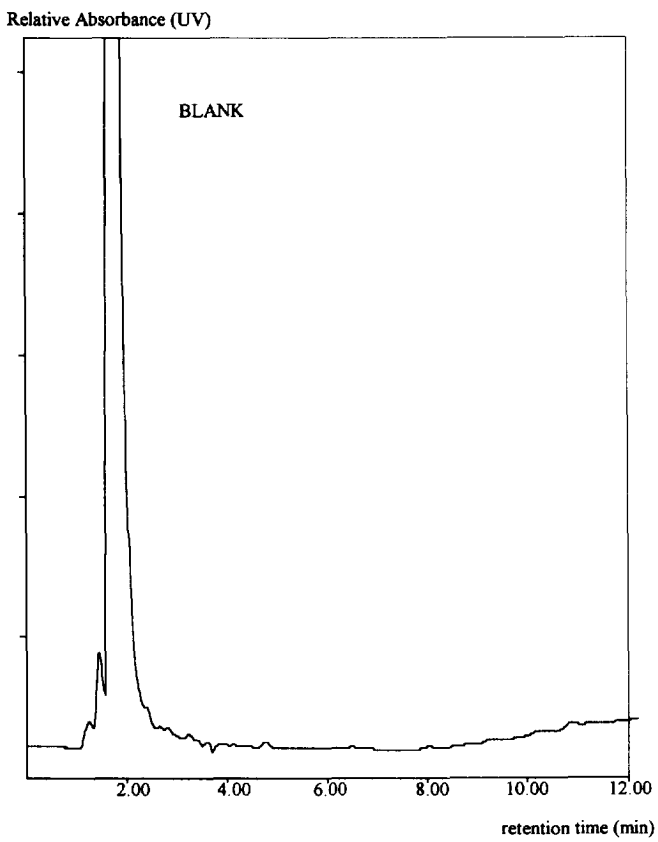

Fig. 3. HPLC chromatogram of blank sample extracted ($2 \cdot 10^6$ drug-sensitive KB-3-1 cells).

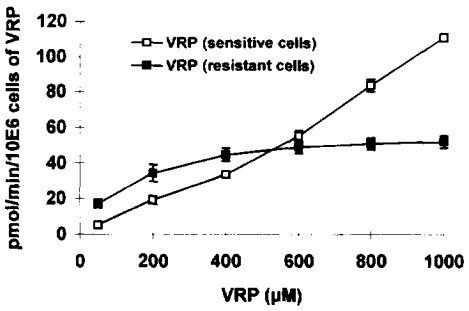

Fig. 4. Kinetics of uptake of VRP by drug-sensitive KB-3-1 cells and by drug-resistant KB-A1 cells. The initial rates of uptake (pmol min^{-1} 10^6 $cells^{-1}$ of intra-cellular VRP) were plotted as a function of VRP extra-cellular concentration incubated for 5 min. The error bars represent the S.E.M. obtained in three experiments (shown when larger than symbol size).

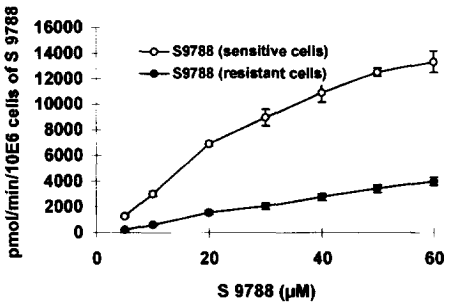

Fig. 5. Kinetics of uptake of S 9788 by drug-sensitive KB-3-1 cells and by drug-resistant KB-A1 cells. The initial rates of uptake (pmol min^{-1} 10^6 $cells^{-1}$ of intra-cellular S 9788) were plotted as a function of S 9788 extra-cellular concentration incubated for 5 min. The error bars represent the S.E.M. obtained in three experiments (shown when larger than symbol size).

3.7. Uptake and accumulation studies

The initial rate of uptake vs. concentration curves of VRP and S 9788, incubated with sensitive and resistant cells, are represented by Figs. 4 and 5. Fig. 4 shows that the uptake rate of VRP has a limiting value in resistant cells, while the relationship between the rate and the concentration is almost linear in sensitive cells. At low incubation concentrations, uptake rates were higher in resistant cells than in sensitive cells and became lower at saturation when the concentrations were increasing. Contrary to VRP uptake (Fig. 5), S 9788 uptake is limited at high concentration in sensitive cells and increases in direct proportion to the incubated concentration in resistant cells. However, the uptake rate is well above in sensitive cells for any incubation concentration. When comparing the behaviour of VRP and S 9788, S 9788 has the higher accumulation capacity. Further investigations are needed to understand the link between the transport mechanism and the modulation activity of these drugs. Accumulation of ADR and S 9788 in sensitive and resistant cells was studied either by incubation of one drug with cells or by co-incubation of the cytotoxic and the modulator drugs ADR and S 9788, respectively (Figs. 6 and 7). In sensitive cells, the kinetic behaviour of ADR is not influenced by S 9788, also, the kinetics of this drug are not modified by ADR. In resistant cells, ADR concentrations are increased by addition of S 9788 in the medium, but the kinetic curve of this drug is only moderately changed by

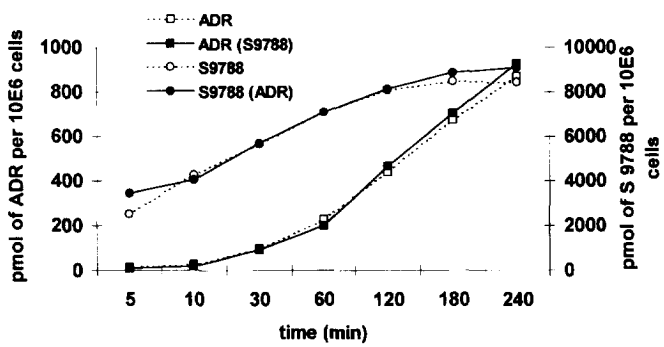

Fig. 6. Uptake of ADR and S 9788 which were incubated either alone or co-incubated with sensitive cells (KB-3-1). When co-incubated, the uptake of both drugs was simultaneously determined. The intracellular ADR amount has been determined in the absence (□) and in the presence (■) of 5 μ*M* S 9788.The intracellular S 9788 amount has been determined in the absence (○) and in the presence (●) of 5 μ*M* ADR. Average of results from three experiments done on different days.

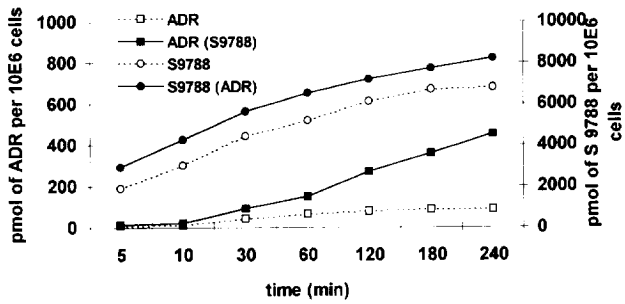

Fig. 7. Uptake of ADR and S 9788 which were incubated either alone or co-incubated with resistant cells (KB-A1). When co-incubated, the uptake of both drugs was simultaneously determined curves. The intra-cellular ADR amount have been determined in the absence (□) and in the presence (■) of 5 μ*M* S 9788.The intra-cellular S 9788 amount has been determined in the absence (○) and in the presence (●) of 5 μ*M* ADR. Average of results from three experiments done on different days.

ADR. From these results, a competition mechanism between S 9788 and ADR, to enter sensitive or resistant cells, should be excluded.

4. Conclusions

The HPLC method described is a useful tool to improve the understanding of transport mechanisms of cytotoxins and modulators of resistance in cells maintained in vitro. It should be applicable for toxicokinetic studies with few modifications. The main advantages of this assay are its specificity and its versatility for multiple applications in the particular field of multidrug resistance. Recoveries and precision were excellent for all drugs, despite the differences in their chemical origin.

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