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Assay for determination of daunorubicin in cancer cells with multidrug resistance phenotype

Petra Krumpochova[a,1], Andrea Kocurova[b,1], Petr Dolezel[a], Petr Mlejnek[a,*]

[a] Department of Biology, Faculty of Medicine and Dentistry, Palacky University Olomouc, Hnevotinska 3, Olomouc 77515, Czech Republic
[b] Department of Biochemistry, Faculty of Science, Palacky University Olomouc, Slechtitelu 11, Olomouc 77515, Czech Republic

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ABSTRACT

A sensitive assay for direct determination of intracellular level of daunorubicin (DRN) in resistant leukemia cells with overexpressed P-glycoprotein has been developed. This assay is based on a rapid separation of cells from media and fast cut-off of DRN transportation by centrifugation of cells through a layer of silicone oil. Cell pellets were extracted using 1% (v/v) formic acid in 50% (v/v) ethanol in water. The cell extracts were subsequently analysed by liquid chromatography (HPLC) coupled a low-energy collision tandem mass spectrometer equipped with an electrospray ionization source (ESI–CID–MS/MS) operated in the multiple-reaction monitoring (MRM) mode. Calibration curve was linear from 0.4 to 250 nM with correlation coefficient (r^2) better than 0.998. The limit of quantitation (LOQ) was 0.4 nM. The assay has been successfully applied to a determination of intracellular content of daunorubicin in sensitive K562 and resistant K562/Dox and K562/HHT300 cells.

1. Introduction

Daunorubicin (daunomycin, cerubidine, DRN) is an anthracycline antibiotic originally isolated from *Streptomyces peucetius*. DRN is an important antitumor agent used in the treatment of solid tumors, lymphomas, and acute lymphoblastic as well as myelocytic leukemias. The broader clinical application of DRN is, however, limited by tissue specific toxicity, and the selection of multiple mechanisms of drug resistance, including P-glycoprotein (P-gp, *MDR1*, *ABCB1*) mediated resistance [1,2].

P-gp, a product of the *ABCB1* (*MDR1*) gene, is a member of the ABC family of membrane transporters (ATP-binding cassette) that translocate wide variety of endogenous and exogenous substrates across cytoplasmatic membrane. The transport is driven by ATP hydrolysis [3]. P-gp was originally described as the first membrane transporter participating in the development of the multidrug resistance (MDR) phenotype [4]. Since then a large number of reports documented positive correlation between reduced intracellular accumulation of cytotoxic drug and increased expression of P-gp [5,6]. It is therefore assumed that the P-gp mediated efflux decreases the drug concentration in cancer cells which results in the failure of chemotherapy at least in *in vitro* models. However, the findings regarding to the significance of the P-gp expression for development of clinical drug resistance to chemotherapy of many cancers are rather controversial. Nevertheless, the overexpression of P-gp in clinical trials correlates with poor outcome of chemotherapy [7–9].

Possible involvement of P-gp is obviously measured indirectly using fluorescent probes such as rhodamine 123 or DRN, which serve as generalised substrates for P-gp [10,11]. This measurement demonstrates ability of P-gp to transport its substrates across the cytoplasmatic membrane. No information about actual intracellular drug level is provided. However, if we want to prove that resistance to a particular drug is due to the overexpression of P-gp, a clear quantitative relationship among intracellular drug level, P-gp expression and cell sensitivity/resistance must be established. To do that, we need an assay for the precise quantitative determination of intracellular level of a particular anticancer drug in sensitive as well as in resistant cancer cells.

DRN, which belongs to the well characterised substrates of P-gp [12,13], is a low molecular weight hydrophobic fluorescent molecule, which offers several analytical strategies for detection. Probably the easiest way for assay of intracellular level of DRN seems to be the measurement of fluorescence of intact cells using fluorimetry [14,15] or flow cytometry [16]. However, we do not think that this strategy might be used for precise measurement of DRN content in both sensitive and resistant cells, which overexpress drug transporters. Indeed, more than 90% of intracellular DRN is bound to nuclear DNA [14,15], which quenches DRN fluorescence [17]. In addition, DNA content in sensitive and resistant cells might not be the same, as malignancy is obviously accompa-

* Corresponding author. Tel.: +420 585632158; fax: +420 585632966.
E-mail address: mlejnek_petr@volny.cz (P. Mlejnek).
[1] These authors equally contributed to this work.

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nied with extensive aneuploidisation of cancer cells [18]. Therefore, accuracy of the fluorescence spectroscopy based assays for the measurement of DRN contents in sensitive and resistant cells might be compromised.

Here, we describe a straightforward assay for precise determination of the intracellular level of DRN in leukemia cells with MDR phenotype. The simultaneous separation of cells from media and fast cut-off of DRN transportation was accomplished by centrifugation of cells through a layer of silicone oil. Quantitation of DRN in high-performance liquid chromatography separated acidic ethanolic extracts was completed using electrospray ionization tandem mass spectrometry (positive ion mode) operating in the multiple-reaction monitoring (MRM) mode.

2. Experimental

2.1. Chemicals

Daunorubicin hydrochloride, IUPAC name (8S,10S)-8-acetyl-10-[(2S,4S,5S,6S)-4-amino-5-hydroxy-6-methyl-oxan-2-yl]oxy-6,8,11-trihydroxy-1-methoxy-9,10-dihydro-7H-tetracene-5,12-dione (DRN; purity ≥ 95%, HPLC grade) was obtained from Sigma (Sigma, Saint Louis, MO, USA). Formic acid (FA, ≥98% puriss p.a., ACS), ammonium acetate (puriss p.a.), and dimethylsulfoxid (DMSO; 99.9% for molecular biology) were purchased from Sigma–Aldrich (Germany). Methanol (MetOH; LC–MS grade) and ethanol (EtOH; HPLC grade) were obtained from Biosolve (The Netherlands). MiliQ® water (18.2 MΩ cm) was purified by MiliQ water purification system (Millipore, Bedford, MA, USA).

2.2. Preparation of standards for calibration

Stock solutions of DRN were prepared by dissolving the accurately weighed amount of DRN in 1% (v/v) FA in 50% (v/v) MetOH/water and stored at −20 °C. These solutions were found to be stable for at least 6 months and for at least 48 h when stored at −20 °C and at 4 °C, respectively. In addition, DRN proved to be stable over repetitive (5 times) freeze–thaw cycles. LC–ESI–MS/MS analysis was used to test the freeze–thaw and storage stability. Serial dilutions of the calibration standards (STDs) of DRN ranging from 0.4 to 250 nM were prepared by adding a determined volume of stock solutions to 0.1% (v/v) FA in 5% (v/v) EtOH/water. Quality controls (QCs), in nominal concentration of 10, 50, and 200 nM were also prepared by adding a determined volume of stock solutions to 0.1% (v/v) FA in 5% (v/v) EtOH/water, freshly on each experiment day.

2.3. Quantitation of daunorubicin by LC–ESI–MS/MS analysis

The HPLC system consisted of UltiMate 3000 RS pump, degasser, autosampler and column compartment (Dionex, Germering, Germany). Separations were performed at ambient temperature on a Polaris $C_{18\text{-A}}$ 250 × 2.0 mm (i.d.), 5 μm particle size column (Varian Inc., Lake Forest, CA, USA) connected with a guard C_{18} 4.0 × 2.0 mm (i.d.) precolumn (Phenomenex, Torrance, CA, USA). Solvents used for separation were A (0.25% FA (v/v) in 90% MetOH/water (v/v)) and B (0.25% FA/water (v/v)). The flow rate was 300 μl/min with linear gradient elution from 0 to 6 min (45–90% of solvent A), from 6 to 7 min (90–45% of solvent A) and from 7 to 10 min (45% of solvent A). Sample injection volume was set at 5 μl. The effluent was introduced into the API 3200 triple quadrupole mass spectrometer (MDS SCIEX, Ontario, Canada) and electrospray ionization in positive ion mode was used for detection. The mass spectrometer was operated in the multiple-reaction monitoring (MRM) mode. Daunorubicin was monitored by MRM transition 528 > 321 (dwell-time = 300 ms). Ion spray probe parameters were set to the following values: needle voltage 5500 V, declustering potential 26 V, temperature 400 °C, curtain gas (nitrogen) 1.38 bar, nebulizer gas (zero air quality) 3.45 bar, turbo V-gas (zero air quality) 3.45 bar. The nitrogen pressure in the second quadrupole was measured at 3.7×10^{-8} bar, the collision energy and entrance potential was set at 31.0 eV and 4.0 V, respectively. The instrument was operated in unit resolution.

2.4. Cell culture

Human chronic myelogenous leukemia K562 cells, obtained from ECACC, were cultured in RPMI-1640 medium supplemented with a 10% calf foetal serum and antibiotics in 5% CO2 atmosphere at 37 °C. K562/Dox and K562/HHT300 cells, which both overexpress P-gp (*ABCB1*, *MDR1*), were kindly provided by prof J.P. Marie (University of Paris 6, France). K562/Dox and K562/HHT300 cells were cultured under the same conditions. More detailed characterisation of K562/Dox and K562/HHT cell lines is given elsewhere [19]. The cell density and viability was measured using automatic analyzer Vi-CELL (Beckman Coulter, USA). Vi-CELL determines cell viability utilizing the trypan blue dye exclusion method [20].

2.5. Extraction of daunorubicin

We adopted a protocol originally developed by Harris and van Dam [21] with a modification by Andreasen and Schaumberg [22]. Briefly, cells at the density of $0.2–0.8 \times 10^6$/ml (usually 0.4×10^6/ml) were incubated in growth medium with daunorubicin for 4 h at 37 °C and 5% CO_2. After incubation time cells were layered on silicone oil (density: 1.035 g/ml; Sigma–Aldrich, Germany) in centrifugation tube and sedimented by centrifugation (6000 rpm/5 min/22 °C). Medium and oil layer were carefully removed. Cell pellet was extracted using defined volume (200–1200 μl; usually 800 μl) of ice cold solution of either (a) 1% (v/v) FA + 50% (v/v) MetOH in water, (b) 1% (v/v) FA + 50% (v/v) EtOH in water, (c) 0.1 M NaOH, which was neutralised by an equal volume of 3% (v/v) FA in MetOH, or (d) 10% sodium dodecyl sulphate (SDS; at ambient temperature). Cell extracts were clarified by centrifugation (18,000 rpm/10 min/4 °C). Clarified cell extracts were 10 times diluted with water and then analysed using LC–ESI–MS/MS analysis. Resulting matrix solution was 0.1% (v/v) FA in 5% (v/v) EtOH/water.

2.6. Flow cytometric analysis of cellular DNA content

Cells were washed in phosphate buffered saline (PBS) and then fixed in ice-cold 70% EtOH in PBS. Fixed cells were washed in PBS and then stained for at least 30 min in PBS containing propidium iodide (PI; 10 μg/ml) and RNase A (100 μg/ml) prior to flow-cytometric analysis on a Cytomics FC 500 System (Beckman Coulter, USA). The principle is as follows. The fluorescent dye PI binds stoichiometrically to the DNA. The stained cells are then measured in the flow cytometer and the emitted fluorescent signal is considered to be a measurement of the cellular DNA content [23].

3. Results and discussion

3.1. Overview

The general rationale of this manuscript was to determine the actual contribution of P-gp to the MDR phenotype in cancer cells. Therefore, it was essential to accurately assay the intracellular levels of DRN in the resistant as well as in the sensitive cells. For this reason, we avoided the direct measurement of DRN content, within the intact cells using fluorescence spectroscopy, as the DNA

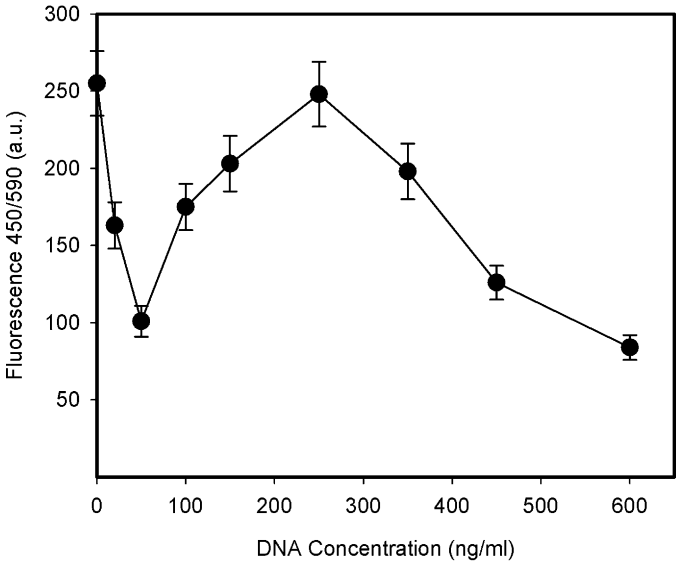

Fig. 1. Effect of DNA concentration on DRN fluorescence. DRN (100 nM – final concentration) was dissolved in 25 mM sodium HEPES (pH = 7.0) containing defined amount of human DNA, as indicated. Sample fluorescence was monitored at 450/590 nm. The experimental points represent mean values from three replicate experiments with standard deviations.

interacts with DRN [24] and substantially will influence its fluorescence. Obviously, DNA quenches DRN fluorescence [17]. However, our results revealed that the effect of DNA on DRN fluorescence was more complex and that the DNA besides quenching the DRN fluorescence, could also increased it tremendously (Fig. 1). Importantly, both effects depend on the ratio between DRN and DNA (Fig. 1). These facts suggest that the accuracy of this simple assay for the measurement of DRN content in cancer cells, which are usually aneuploid [18], could be compromised. Indeed, we observed that K562 cells and their resistant variants K562/HHT300 and K562/Dox cells differed substantially in amount of genomic DNA (Fig. 2). Therefore, we decided to determine intracellular DRN content upon extraction.

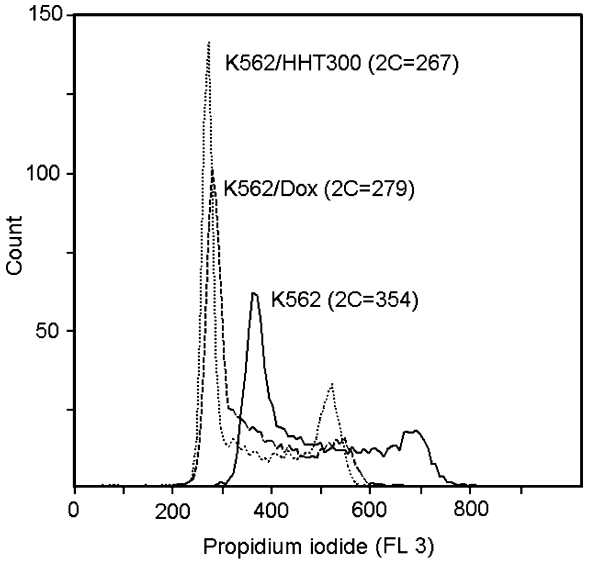

Fig. 2. Estimation of cellular DNA content in leukemia cells. K562 cells (solid line); K562/Dox cells (dashed line); K562/HHT300 (dotted line). 2C = DNA content of cells (in G1 phase of cell cycle), which corresponds to a diploid genome (2*n*). Histograms represent typical results.

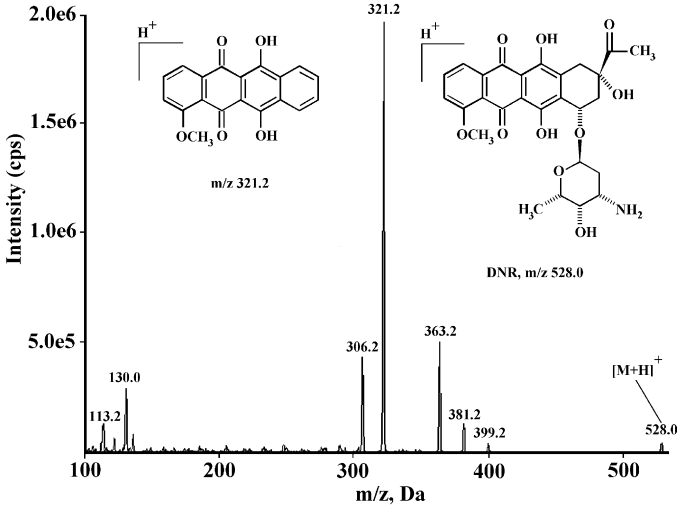

Fig. 3. MS/MS spectrum of DRN and its dissociation pattern. Precursor ion *m*/*z* 528; the most abundant product ion *m*/*z* 321.

3.2. Assay procedure

DRN was separated on a C_{18} silica reverse-phase column. DRN had a retention time 6.9 ± 0.06 min, and analysed on-line by a low-energy collision dissociation tandem mass spectrometry CID–MS/MS analysis using the MRM mode. The precursor protonated molecule $[M+H]^+$ of DRN at *m*/*z* 528 was produced in the positive ESI mode and was utilized to acquire product ion spectrum (Fig. 3). The loss of the aminoglycoside part and the next loss of one water molecule generated a product ion of *m*/*z* 363. Afterwards, the loss of a $CO{=}CH_2$ group resulted in the formation of the most abundant product ion at *m*/*z* 321 [25] and (Fig. 3). The transition from *m*/*z* 528 to 321 was used in our MRM analysis for detection and precise quantification of DRN.

The selectivity and specificity of the assay were investigated by analysis of three blank cell extracts at the analytical conditions described in Section 2. For specified MRM transition, no significant interferences from endogenous compounds near the retention time of measured drug were observed (Fig. 4).

3.3. Linearity, limit of detection (LOD), and limit of quantification (LOQ)

The calibration curve ranged from 0.4 to 250 nM and the linearity, with correlation coefficient $r^2 > 0.998$, was achieved in the appropriate investigated ranges (Fig. 5). The back-calculated concentrations of standards were compared with their nominal values and were within 100 ± 11.7% value of the nominal concentration of DRN.

Limit of detection (LOD), defined as signal-to-noise ratios of 3:1, were determined for 0.13 nM. The limit of quantification (LOQ), defined as signal-to-noise ratios of 10:1, was determined for 0.4 nM with an inter-assay accuracy of 115.0% and precision of 16.2% (*n* = 18), that agree with the requirements of FDA document [26].

3.4. Precision and accuracy

The intra- and inter-assay precision and accuracy for DRN was tested by analysis of three different QC sample concentration (10, 50, and 200 nM) in six replicates on 3 separate days (*n* = 18). The precision was expressed as the relative standard deviation (RSD). Accuracy was assigned as the ratio between the back-calculated concentration and the nominal value of QC samples concentrations, and expressed as a percentage. The intra- and inter-day

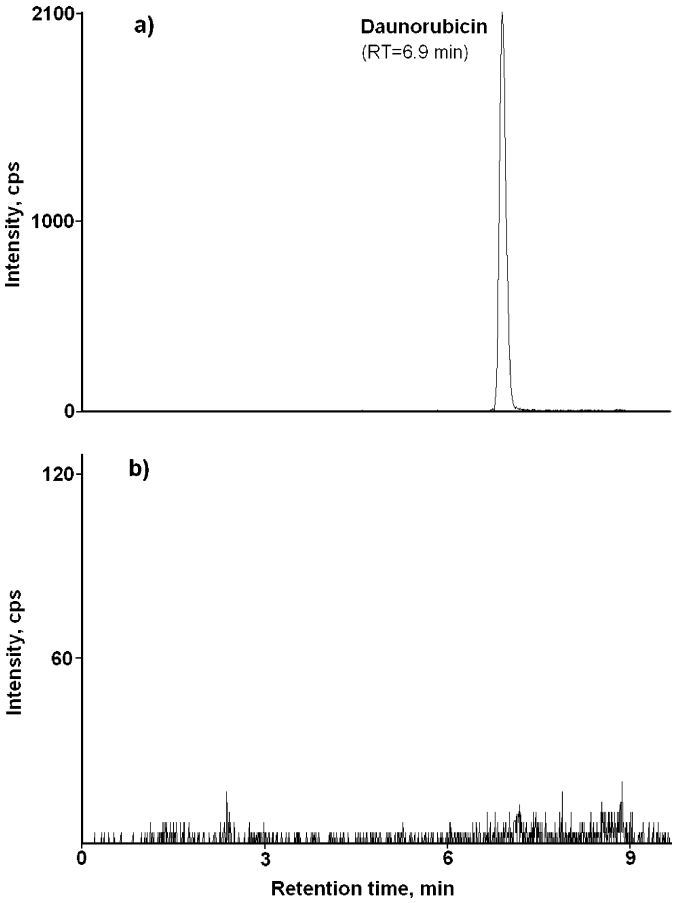

Fig. 4. Representative HPLC–MS–MS ion-chromatograms of cell extracts for DRN in MRM mode. Panel (A) Extract from K562 cells incubated in a standard growth medium supplemented with 25 nM DRN for 4 h. Panel (B) Extract from K562 cells incubated in a standard growth medium for 4 h.

precision (RSD) values ranged from 2.8–5.9% to 3.7–7.5%, respectively. The intra- and inter-day accuracy was between 92.4–105.0% and 95.2–106.1%, respectively. These results are in a good agreement with the requirements of FDA document [26].

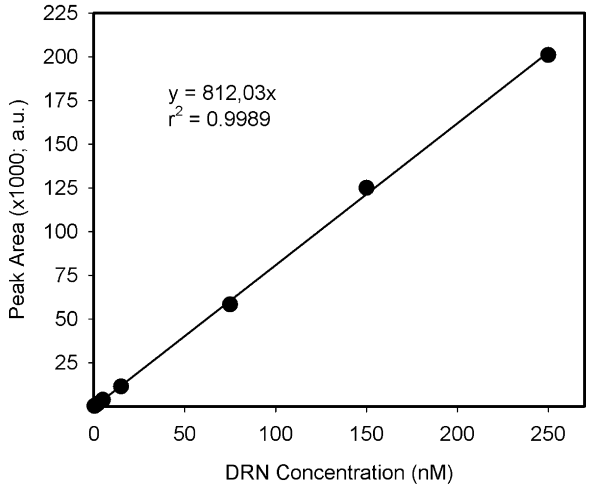

Fig. 5. Calibration curve for DRN assay using LC–ESI–MS/MS. Each point represents a mean value from triplicate measurements. Parameters of calibration curve: $y = 812.03x$; $r^2 = 0.9989$.

Table 1
Matrix effect [ME = (B/A) × 100%] for DRN standard solutions and real sample extracts ($n = 5$).

DRN (nM)	ME% (mean ± SD)[a]		
	B = K562 cell extract	B = K562/Dox cell extract	B = K562/HHT300 cell extract
10	92.0 ± 4.2	104.7 ± 3.3	94.1 ± 3.8
50	96.5 ± 3.6	93.1 ± 4.1	101.8 ± 3.1
200	92.9 ± 4.7	95.9 ± 2.9	93.7 ± 4.0

[a] A = DRN standards in 0.1% (v/v) FA + 5% (v/v) EtOH (blank matrice is absent).

3.5. Evaluation of the matrix effect

Matrix effect (ME) defined as a change in the ionization process of an analyte due to the co-eluting compounds which may result either in its suppression or enhancement. The evaluation of ME is based on the procedure described by Matuszewski et al. [27]. ME is calculated using the formula: ME = (B/A) × 100%, where A and B represent two different sets of samples. Set A represents the chromatographic peak areas for DRN standards prepared in 0.1% (v/v) FA + 5% (v/v) EtOH. Set B represents the chromatographic peak areas for DRN standard solutions mixed with extracts prepared from untreated cells using 1% (v/v) FA + 50% (v/v) EtOH and then 10 times diluted with water to achieve the same final concentrations of DRN as in the set A. The final nominal concentrations of DRN standards were 10, 50, and 200 nM, i.e., the same concentrations, which were used for QCs. As shown in Table 1, matrix exhibited insignificant effect on quantitative determination of DRN.

3.6. Effect of composition of extraction solution on extraction efficiency of DRN

DRN is a weak hydrophobic base [28], which is tightly associated with nuclear DNA [24] and therefore we extracted K562 cells with 1% (v/v) FA in 50% MetOH/water (v/v). However, we found that after cell extraction sedimented nuclei still exhibited intensive red fluorescence when volume of extraction solution was up to 400 μl. This indicated that a significant amount of DRN still remained bound to DNA. Therefore, we used a larger volume of extraction solution to achieve higher extraction efficiency (Fig. 6). In addition, we tested also other extraction solutions, including (a) 1% (v/v) FA in 50% EtOH/water (v/v); (b) 10% w/v SDS in water, according to [15]; and (c) 0.1 M NaOH to disrupt the DRN binding to the DNA. As demonstrated in Fig. 6, the most effective extraction solutions were 10% (w/v) SDS and 1% (v/v) FA in 50% EtOH/water (v/v), less effective was 1% (v/v) FA in 50% MetOH/water (v/v) and the least effective was 0.1 M NaOH.

However, the extraction solution could affect the output signal intensity of the particular assay for quantified compound and thus the appropriate values of LOQs. To determine the effect of extraction solution on the assay sensitivity, stock solutions of DRN were diluted in the tested extraction solutions, the resulting samples were further 10 times diluted with distilled water, and the signal intensity of DRN was recorded by LC–ESI–MS/MS. We observed that the lowest LOQs values were achieved for 1% (v/v) FA in 50% (v/v) MetOH (or EtOH)/water (Table 2). Significantly higher LOQ

Table 2
Effect of composition of the extraction solution on LOQ values for LC–ESI–MS/MS assay of DRN.

Extraction solution	LC–ESI–MS/MS assay (nM)
1% FA (v/v) in 50% MetOH/water (v/v)	0.5
1% FA (v/v) in 50% EtOH/water (v/v)	0.4
10% SDS	21.5
0.1 M NaOH (neutralized FA; pH = 3.5)	0.6

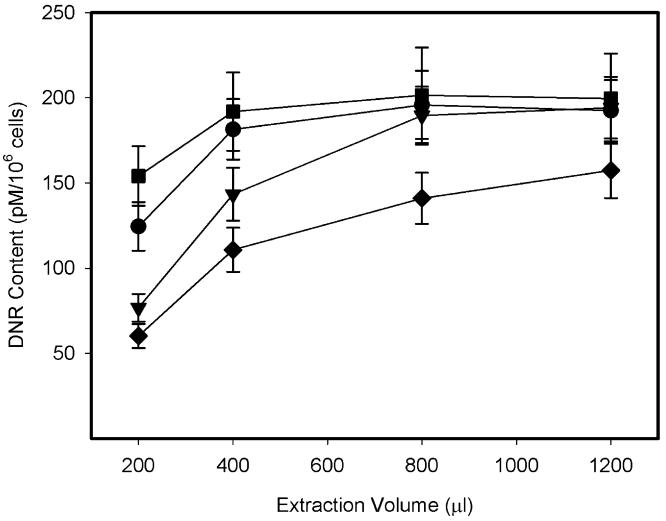

Fig. 6. Effect of composition of the extraction solution on the extraction efficiency of DRN. Cells were incubated in growth medium containing 1.0 μmol/l DRN at 37 °C for 4 h. Cells were afterwards separated from growth medium and cell pellet was extracted with various volumes of extraction solution as indicated. 1% (v/v) FA + 50% (v/v) MetOH in water (triangles); 1% (v/v) FA + 50% (v/v) EtOH in water (circles); 0.1 M NaOH, which was neutralised by an equal volume of 3% (v/v) FA in MetOH (diamonds); or 10% SDS (at ambient temperature; squares). The experimental points represent mean values from three replicate experiments, with standard deviations.

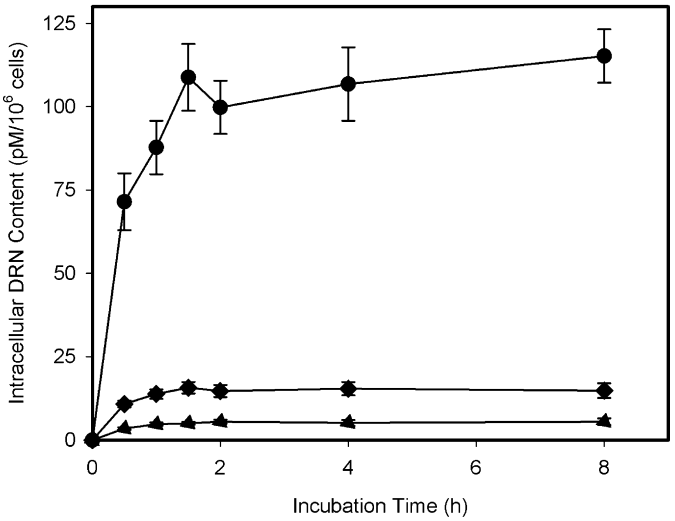

Fig. 7. Time course of intracellular level of DRN. Cells were incubated in the growth medium containing of 0.3 μmol/l DRN at 37 °C. At defined time intervals cells were extracted using 800 μl of 1% (v/v) FA in 50% EtOH/water (v/v), as described in Eonds; K562/HHT300 (triangles). The experimental points represent mean values from three replicate experiments, with standard deviations.

provided 0.1% NaOH neutralized with FA (pH = 3.7) (Table 2). Importantly, 10% SDS dramatically diminished MS signal of DRN and dramatically increased the LOQ value for LC–ESI–MS/MS (Table 2).

Therefore, we chose 1% (v/v) FA in 50% (v/v) EtOH/water as extraction solution, which provided best result for our assay (Fig. 6 and Table 2).

We tested also different diluted acids, including trichloroacetic acid (1% w/v) or HCl (0.3 M) in combination with 50% (v/v) EtOH (or MetOH)/water [17], which gave comparable results found for 1% (v/v) FA in 50% (v/v) EtOH/water, however, their LOQs values were somewhat higher (not shown).

3.7. Time course of intracellular level of DRN

We determined the steady-state level of DRN, when its influx and efflux, and/or metabolism are equilibrated. Cells were incubated with DRN for a defined incubation time interval before the cell extraction. We observed that DRN reached the steady state level after 1–1.5 h and remained without significant changes from 1.5 to 8 h (Fig. 7).

3.8. Quantification of intracellular DRN content in sensitive and resistant cells

We applied our method for determination of DRN content in sensitive K562 and resistant K562/Dox and K562/HHT300 cells. As shown in Fig. 8 we observed a linear relationship between extracellular and intracellular DRN content within the concentration range used in all cell lines. However, the DRN content in resistant cells was significantly lower than that in sensitive ones, as expected. We observed 9.5 and 26.8 fold decreases in the intracellular content of DRN in K562/Dox and K562/HHT300 cells, respectively, as compared with sensitive K562 cells (Fig. 8).

Intracellular level of DRN might be determined in intact cells using fluorimetry [14,15] or flow cytometry [16]. Although this simple approach failed to provide absolute values of intracellular DRN content in cells, it enables to compare relative values for intracellular DRN content provided that the cell fluorescence is proportional to the DRN amount. We determined the relative intracellular levels of DRN using flow cytometry (not shown) which provided 16.5 and 22.7 fold decreases in the intracellular content of DRN in K562/Dox and K562/HHT300 cells, respectively, as compared with sensitive K562 cells. This ratio, however, substantially differs from that obtained by our method (see above). Considering the facts that (i) DRN fluorescence is strongly affected by DNA content, which modulates DRN fluorescence in a rather complex way (Fig. 1), and that (ii) DNA content varies among studied cell lines (Fig. 2), the relative values for intracellular DRN levels measured

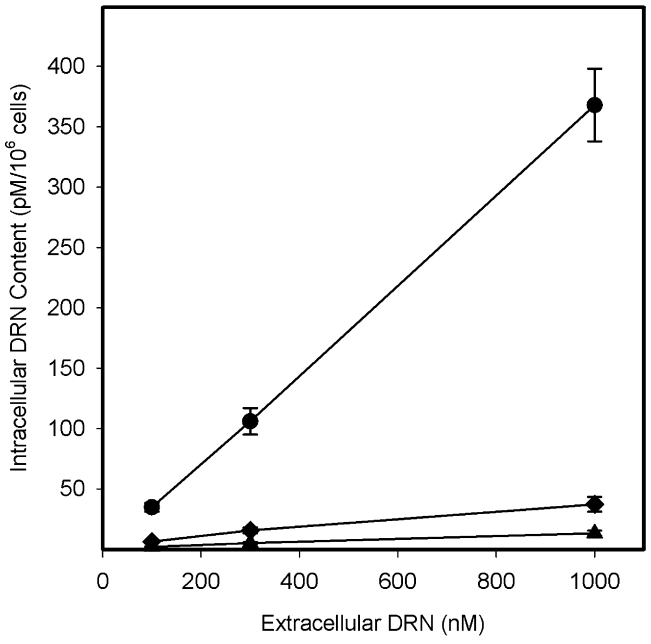

Fig. 8. DRN contents in sensitive and resistant cells. K562 cells (circles); K562/Dox cells (diamond) cells. K562 cells and their resistant variants K562/Dox and K562/HHT300 were incubated in the presence of various concentrations of DRN. After 4 h incubation cells were subjected to extraction and DRN content was determined in all cell lines. K562 cells (circles); K562/Dox cells (diamonds); K562/HHT300 (triangles). The experimental points represent mean values from three replicate experiments, with standard deviations.

using flow cytometry can be hardly taken as more reliable than that obtained using LC–ESI–MS/MS assay of DRN upon extraction.

The precision presented here is shown in the calibration curve, which is mostly based on the LC–ESI–MS/MS method reliability (see Sections 3.3 and 3.4). However, the whole assay is limited by the very nature of the complex sample preparation, not in any analytical method. Precision of the whole assay with real samples was somewhat lower but the RSDs do not exceed 15%. Also the real value of LOQ for DRN in growth medium, which was possible to determine inside cells, was somewhat higher. In sensitive K562 cells the LOQ was 5 nmol/l for extracellular DRN when cell concentration was 4×10^5 cells per ml. This real LOQ value is suitable for monitoring of DRN effects on cells as IC_{50} value for DRN in K562 cells is approximately 20 nmol/l (not shown). Under the same conditions, the LOQs were 51 and 138 nmol/l for K562/Dox and K562/HHT300 cells, respectively. Although, these values seemed to be high, they are suitable for monitoring of drug effect on resistant cells as the IC_{50} values for DRN in resistant K562/Dox and K562/HHT300 cells are within micromolar range (not shown).

The assay presented here is comparable to that recently published by Sakai-Kato et al. for doxorubicine [29]. However, they used ultra-high-performance liquid chromatography which provided approximately 40 fold lower sensitivity than LC–ESI–MS/MS application (our report). In addition, we used simple single-step extraction procedure, which is less laborious and cheaper than enzyme based cell lysis with subsequent deproteination [29].

To prevent leakage of the drug from resistant cells during extraction procedure a rapid separation of cells from medium was accomplished by centrifugation through a silicone oil. Similar approach was successfully applied in the assay for precise determination of intracellular levels of imatinib and its main metabolite in Bcr-Abl positive cells [30].

4. Conclusions

Our results indicated that presented approach is suitable for the precise determination of intracellular DRN levels in both sensitive and resistant cells *in vitro*. High sensitivity of our assay enables to determine intracellular drug levels at sub-cytotoxic extracellular DRN concentrations. In addition, this assay can be used as a tool to study quantitative relationship between expression of drug transporters and cell resistance to DRN.

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