Talanta 83 (2011) 1466–1471

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A non-radioactive assay for precise determination of intracellular levels of imatinib and its main metabolite in Bcr-Abl positive cells

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ARTICLE INFO

Article history:
Received 17 May 2010
Received in revised form 3 November 2010
Accepted 14 November 2010
Available online 19 November 2010

Keywords:
K562 cells
P-glycoprotein
Multidrug resistance
N-desmethyl imatinib
CGP 74588
Centrifugation through silicone oil

ABSTRACT

Multidrug resistance (MDR) is often associated with overexpression of the P-glycoprotein (P-gp, *ABCB1*). It was demonstrated that the P-gp mediated efflux decreases the drug concentration in cancer cells which results in the failure of chemotherapy. However, the MDR phenotype in cancer cells obviously involves various mechanisms. Therefore, if we want to estimate a contribution of the P-gp expression to the MDR phenotype, a clear quantitative relationship between the intracellular drug level and cell sensitivity must be established. To achieve this goal, a sensitive and non-radioactive assay for precise determination of intracellular levels of imatinib and its main metabolite N-desmethyl imatinib (CGP 74588) has been developed. The assay is based on an optimised extraction of cells with 4% formic acid after their separation from the growth medium by centrifugation through a layer of silicone oil. Cell extracts are subsequently analyzed by LC/MS/MS. Calibration curves were linear from 1 to 500 nmol/l for imatinib and from 2 to 500 nmol/l for CGP 74588, with correlation coefficients (r^2) better than 0.998 and 0.996, respectively. The limit of quantitation (LOQ) was 1 nmol/l for imatinib and 2 nmol/l for CGP 74588. Our method has been successfully applied to the determination of intracellular levels of imatinib in sensitive K562 and their resistant variant, K562/Dox cells.

1. Introduction

Imatinib (Gleevec, formerly referred to as STI571; Novartis, Basel, Switzerland) is a first rationally developed tyrosin kinase inhibitor (TKI) which becomes an effective drug used for therapy of Philadelphia chromosome-positive (Ph+) chronic myelogenous leukemia (CML) patients in all stages with few side effects [1,2]. Philadelphia chromosome is characterized by the translocation t(9;22)(q34;q11), and production of fusion Bcr-Abl tyrosine kinase with oncogenic activity that plays the central role in maintaining the malignant phenotype in CML [3,4]. Despite the fact that the introduction of imatinib lead to a substantial revolution in the therapy of Ph+ CML, it fails in approximately 25% of patients due to the development of resistance [5]. In the majority of patients, resistance is related to the reactivation of the Bcr-Abl tyrosine kinase activity. This can either result from gene amplification or, more frequently, point mutations that disrupt the binding of imatinib to Bcr-Abl itself [6]. In addition to these "classical mechanisms of resistance", there has been suggested a different one that coincides with the decreased intracellular imatinib level due to the P-glycoprotein (P-gp) overexpression [7].

P-gp, encoded by the *ABCB1* (*MDR1*) gene, functions as an ATP-dependent efflux pump for a wide variety of endogenous and exogenous substrates including chemotherapeutic agents. Experiments *in vitro* suggested that many of newly developed TKI molecules may serve as substrates for the human drug transporters, including P-gp, and thus might be involved in the multidrug resistance (MDR) phenotype of cancer cells [8]. Recently, the P-gp overexpression was confirmed as a clinically relevant mechanism of resistance to imatinib treatment [9,10]. However, there is an increased body of evidence that more than one factor might contribute to the MDR phenotype in cancer cells [11].

To estimate contribution of P-gp expression to the overall resistance, a clear quantitative relationship between the intracellular drug level and the cell sensitivity must be established. Because the precise quantitative determination of intracellular concentration of an anticancer drug in general is not a trivial experimental task, several alternative approaches have been adopted. Contribution of efflux transporters is obviously measured indirectly using fluorescent probes such as rhodamine 123 or daunorubicine, which serve as generalised substrates for P-gp [12,13]. This measurement demonstrates the ability of P-gp to transport its substrates across the cytoplasmatic membrane. No information about the actual intracellular drug level is provided. Only few researchers tried to measure the intracellular level of imatinib directly [10,14,15].

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doi:10.1016/j.talanta.2010.11.028

Unfortunately, these methods are mostly focused on a precise instrumental management of samples, lesser attention is paid to the sample preparation.

Here, we describe a straightforward non-radioactive assay for precise determination of the intracellular levels of imatinib and its main metabolite CGP 74588. Rapid separation of cells from medium was accomplished by centrifugation through silicone oil and the contents of imatinib and CGP 74588 in acidic extracts were quantified using LC coupled with MS/MS.

2. Experimental

2.1. Chemicals

Formic acid (FA, 98% for LC–MS), trichloroacetic acid (TCA; 99%, Sigma ultra), ammonium acetate (puriss. p.a.), and dimethylsulfoxid (DMSO; 99,9% for molecular biology) were purchased from Sigma–Aldrich (Germany). Methanol (LC–MS grade) was obtained from Biosolve (The Netherlands). MiliQ® water (18.2 MΩ cm) was purified by MiliQ water purification system (Millipore, Bedford, MA, USA).

Imatinib mesylate (a salt form with active moiety imatinib; purity ≥ 98%) and its main metabolite N-desmethyl imatinib (CGP 74588) mesylate (a salt form with active moiety N-desmethyl imatinib; purity ≥ 98%) were kindly provided by Novartis (Basel, Switzerland).

2.2. Preparation of standards for calibration

Stock solutions were prepared by dissolving accurately weighted amounts of imatinib mesylate and CGP 74588 mesylate in DMSO and stored at −20 °C. These solutions were found to be stable for at least 6 months when stored at −20 °C. In addition, imatinib and CGP 74588 proved to be stable over repetitive freeze–thaw cycles [16]. LC/MS/MS analysis was used to test the freeze–thaw and storage stability. Serial dilutions of the calibration standards (STDs) of imatinib mesylate and CGP 74588 mesylate (ranging from 1 to 500 nmol/l and from 2 to 500 nmol/l, respectively) in 0.4% FA (w/v) were done. Three quality controls (QCs) in nominal concentration of 50, 150, and 400 nmol/l were prepared freshly on each experiment day by adding a determined volume of stock solutions to FA diluted with water (0.4% (w/v)).

2.3. Instrumentation

2.3.1. Chromatography

The LC 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). The mobile phase was composed of methanol–water (65:35, v/v) containing 7 mmol/l ammonium acetate. The HPLC flow rate was 300 μl min^{-1} and sample injection volume was set at 10 μl.

2.3.2. Mass spectrometry

An API 3200 triple quadrupole mass spectrometer (MDS SCIEX, Ontario, Canada) with electrospray ionization in positive ion mode was used for detection. The mass spectrometer was operated in the multiple-reaction monitoring (MRM) mode. Imatinib (active moiety) and CGP 74588 (active moiety) were monitored by MRM transitions 494.2 > 394.0 and 480.2 > 394.0 (dwell-time = 300 ms), respectively.

Ion spray probe parameters were set to the following values: needle voltage 5500 V, declustering potential 71 V for imatinib and 66 V for CGP 74588, 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 set at 3.7×10^{-8} bar, the collision energy and entrance potential was set at 33.0 eV and 5.0 V, respectively. The instrument was operated in unit resolution.

2.4. Data processing

A PC workstation with Analyst 1.5 software was used for data acquisition and processing. A 3-point smoothing algorithm was applied to all ion-chromatograms. The calibration curves were generated daily using peak areas of each concentration level by linear least-squares regression analysis (with weighting by $1/x$) according to the equation $y = a + bx$, where y was the peak area, x was the concentration of analyte, a was the intercept, and b was the slope of the regression line.

2.5. Cell culture

A suspension of human chronic myelogenous leukaemia K562 cells, obtained from ECACC, was cultured in RPMI-1640 medium supplemented with a 10% calf fetal serum and antibiotics in 5% CO_2 atmosphere at 37 °C. K562/Dox cells, which represent a MDR variant of K562 cells with overexpressed P-glycoprotein (*ABCB1*, *MDR1*), were kindly provided by Prof. J.P. Marie (University of Paris 6, France). K562/Dox cells were cultured under the same conditions.

For experiments with cells, imatinib mesylate and CGP 74588 mesylate were dissolved in DMSO. The final concentration of DMSO in culture medium was approximately 0.1%.

2.6. Cell extraction

Cells at the density of 0.4×10^6/ml were incubated in the growth medium with imatinib or CGP 74588 for defined time period in 5% CO_2 atmosphere at 37 °C. A rapid separation of cells from medium was accomplished by centrifugation through silicone oil (density: 1.035 g/ml; Sigma–Aldrich, Germany) as described elsewhere [17,18] with slight modifications. Briefly, cells in growth medium (10 ml per sample) were layered on silicone oil in a centrifugation tube and sedimented by centrifugation (6000 rpm/5 min/22 °C). Peletted cells propelled through silicone oil layer were extracted using 400 μl of ice cold solution of either (a) 4% (w/v) trichloroacetic acid; (b) 4% (w/v) formic acid; (c) 100% methanol; (d) 50% (v/v) methanol in water; (e) 1% (w/v) trichloroacetic acid + 50% (v/v) methanol in water; or (f) 1% (w/v) formic acid + 50% (v/v) methanol in water. Cell extracts were clarified by centrifugation (14,000 rpm/10 min/4 °C). Clarified cell extracts were 10 times diluted with water and then analyzed using LC coupled with tandem MS detection. Alternatively, cell pellets were re-extracted in the same solutions and resulting samples were analyzed as described above.

3. Results and discussion

3.1. Overview

Combination of liquid chromatography and tandem mass spectrometry represents a powerful analytical technique which has very high sensitivity and specificity. Although this approach requires expensive equipment, it has become a standard method for monitoring of anticancer drugs, including imatinib and CGP 74588 in body fluids as well as in cell extracts in recent years [19–21]. However, monitoring of drug levels in cells requires also

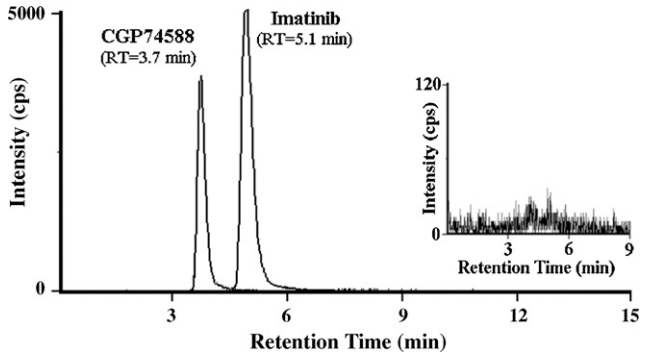

Fig. 1. Representative LC/MS/MS ion-chromatograms of cell extracts for imatinib and CGP 74588 in MRM mode. Extract from K562 cells incubated in a standard growth medium supplemented with 1 μmol/l imatinib + 0.6 μmol/l CGP 74588 for 4 h. Inserted window: extract from K562 cells incubated in a standard growth medium for 4 h. *Y*-axis unit: cps = counts per second. Picture represents a typical example of analysis.

an efficient separation of cells from media and a fast cut-off of drug transportation especially in cells which express drug transporters. Some authors believe that this could be accomplished by cell sedimentation using standard centrifugation with subsequent washing with an ice cold buffer. However, our experiments with resistant cell lines revealed that this approach was not sufficient enough and that there was a significant leakage of the drug probably due to the drug transporter function. This effect can be abrogated when cells are centrifuged through a layer of silicone oil using a method originally developed by Harris and van Dam for beef heart mitochondria [17] and later applied to thymocytes [18].

3.2. Assay procedure

Imatinib and CGP 74588 were separated on the C_{18} silica reverse-phase column with retention times 5.1 ± 0.05 and 3.7 ± 0.04 min, respectively, and on-line analyzed by selected MRM transitions of 494.2 > 394.0 for imatinib and 480.2 > 394.0 for CGP 74588, respectively (Fig. 1). The multiple-reaction monitoring transitions from $[M+H]^+$ ion to appropriate most abundant product ions allowed precise quantification of the analyzed compounds. The selectivity and specificity of the assay were investigated by analysis of three blank cell extracts at the analytical conditions described in Section 2. No significant interferences from endogenous compounds near the retention times of measured drugs were observed for any specified MRM transition (Fig. 1).

3.3. Linearity, limit of detection, and limit of quantification

The calibration curves (for standards dissolved in 0.4% FA) constructed from 5 point were linear from 1 to 500 nmol/l for imatinib ($y = 2.1383x$; $r^2 = 0.9984$), and from 2 to 500 nmol/l for CGP 74588 ($y = 1.4097x$; $r^2 = 0.9966$). The back-calculated concentrations of standards were compared with their nominal values and were within 100 ± 15% value of the nominal concentration of imatinib, and 100 ± 17% value of the nominal concentration of CGP 74588.

Limits of detection (LOD), defined as signal-to-noise ratios of 3:1, were 0.4 nmol/l and 0.6 nmol/l for imatinib and CGP 74588, respectively. The limit of quantification (LOQ), defined as signal-to-noise ratios of 10:1, were 1 nmol/l and 2 nmol/l for imatinib and CGP 74588, respectively.

The concentration range of imatinib and CGP74588 in cell extracts was inside the extent of QC samples concentration (50–400 nmol/l). This range was in the linear region of the calibration curve of imaninib and CGP74588, respectively.

Table 1
Precision and accuracy data for the LC/MS/MS analysis of imatinib and CGP 74588 in human leukemia cells.

Analyte	QC (nmol/l)	Intra-assay precision ($n = 5$)		Inter-assay precision ($n = 15$)	
		RSD (%)	Bias (%)	RSD (%)	Bias (%)
Imatinib	50	6.3	1.5	10.4	−5.0
	150	5.1	−2.2	8.1	3.7
	400	4.8	−3.3	11.6	3.5
CGP 74588	50	5.0	−2.9	9.1	6.1
	150	3.2	−4.3	7.8	−5.8
	400	4.4	3.8	6.5	−4.2

3.4. Precision and accuracy

The intra- and inter-assay precision and accuracy for each analyte were tested by analysis of QC samples prepared for three different concentrations in replicates of at least five on 3 separate days (Table 1). The precision was expressed as the relative standard deviation (RSD). Intra-assay precision (repeatability) was evaluated by replicate ($n = 5$) analysis of the QC samples; calculated averaged RSDs were 5.4 ± 0.8% for imatinib and 4.2 ± 0.9% for CGP74588. Inter-assay precision was evaluated by replicate analysis of the QC samples on 3 different days ($n = 15$); calculated averaged RSDs were 10.0 ± 1.8% for imatinib and 7.8 ± 1.3% for CGP74588. Accuracy was described as percentage bias of nominal versus measured concentrations. All data are summarised in Table 1.

3.5. Matrix effect and quantitation

In order to evaluate effects of the sample matrix on the whole method (extraction and quantification procedures), spiked samples were analyzed. For this purpose, K562 and K562/Dox cells were cultured in the complete growth medium without or with imatinib at 0.5 or 1.0 μM concentration for 4 h and than extracted with 4% FA. Essentially the same procedure was applied for cells incubated with CGP 74588. Before the LC/MS/MS analysis, cell extracts were 10-times diluted with water and spiked with standard solutions at three different concentrations of imatinib (25, 100, and 400 nmol/l) or CGP 74588 (25, 100, and 400 nmol/l). Quantitation was done by integration of the area under the unique MRM chromatograms and the obtained data were plotted against concentrations of standard solutions. Each data set was fitted by a linear curve (weighting by $1/x$) using Analyst 1.5 software and resulting values of slopes for each curve (mean for $n = 3$) are shown in Table 2. Values of slopes for lines constructed from analysis of both standards were compared with those obtained for spiked samples. As demonstrated in Table 2, there was a good agreement of calculated values of slopes of curves for standard solutions and slopes of curves for spiked samples. The differences between calculated values of slopes of curves for standard solutions and slopes of curves for spiked samples were ≤4.9% and ≤4.2% for imatinib and CGP74588, respectively, when expressed as RSDs. It indicated that the influence of matrix effect on quantitative determination of both analytes was not significant.

3.6. Effect of extraction solution on limits of quantification

Selection of extraction solution might affect the signal intensity of the analyzed compounds and thus the appropriate values of LOQs. Imatinib and its N-desmethylated metabolite, CGP 74588, are both weak bases, more hydrophobic than hydrophilic and therefore diluted acids (4% TCA or 4% FA), methanol (100% or 50% MetOH in water (v/v)), or combinations of either acid with methanol (1% TCA in 50% MetOH, or 1% FA in 50%MetOH), could be used as extraction solutions. In addition, all tested extraction solutions effectively precipitate proteins (not shown). To evaluate the effect of

Table 2
Slope values of calibration curves for STDs and real sample extracts with various concentrations of imatinib and CGP 74588 in cultivated media.

	STDs (0.4% FA)		Sample of human leukemia cells (4% FA in matrix)									
			K562 + 0 μmol/l		K562 + 0.5 μmol/l		K562 + 1 μmol/l		Dox + 0.5 μmol/l		Dox + 1 μmol/l	
	Imatinib	CGP 74588	Imatinib	CGP 74588	Imatinib	CGP 74588	Imatinib	CGP 74588	Imatinib	CGP 74588	Imatinib	CGP 74588
Slope ($n = 3$)	2.16	1.43	2.29	1.44	2.32	1.41	2.23	1.50	2.05	1.40	2.10	1.56

Note: Dox stands for K562/Dox cells.

extraction solution composition on the assay sensitivity, stock solutions of imatinib and CGP 74588 were diluted in the tested extraction solutions and the resulting samples were further 10 times diluted with distilled water and the LOQ parameters were determined for both compounds. As demonstrated in Table 3, 0.4% FA (w/v) provided the best results. On the other hand, 4% TCA exhibited a strong suppressive effect on assay sensitivity of both compounds. Nevertheless, this suppression diminished upon subsequent dilution with distilled water (Table 3). The signal intensity of imatinib and CGP 74588 dissolved in methanol or methanol–water was somewhat lower than in acidified methanol (Table 3).

3.7. Effect of extraction solution on extraction efficiency

Our results indicated that among chosen extraction solutions the most efficient one was 4% FA for both imatinib and CGP 74588 (Fig. 2). The other extraction solutions were significantly less efficient (Fig. 2a). Accordingly, second extraction revealed that the lowest residual drug content within once extracted cells was found for 4% FA (Fig. 2b). Extraction of the cell pellet with 100% methanol provided unsatisfactory results as it dissolved traces of residual silicone oil and made the extract "milky", which hampered quantitation of both drugs. Traces of silicone oil could be removed by centrifugation. Our results indicated that extraction efficiency of 100% methanol was similar to that found for 50% MetOH in water (v/v; not shown). We further found that relatively large volumes of extraction solution (400 μl) must be used to achieve high extraction efficiency. Indeed, when extraction volumes decreased below 400 μl, extraction efficiency declined dramatically for all extraction solutions except for 4%FA, which decreased only slightly (not shown). It is necessary to note that one million of K562 (or K562/Dox) cells represent a volume of approximately 4 μl (not shown). These results indicated that imatinib and also its N-desmethylated metabolite might be tightly associated with some cellular compartments such as nuclei (DNA) or lysosomes.

3.8. Time course of intracellular levels of imatinib and CGP 74588

Each drug in the growth medium requires some time to reach its constant level within cells (i.e., steady-state level) when its influx and efflux, and/or metabolism are equilibrated. To determine the steady-state level of tested drugs, cells were incubated with either imatinib or CGP 74588 for defined incubation time interval before cell extraction. We observed that imatinib and CGP 74588 achieved the steady state levels within cells very rapidly. After 1.5–12 h in the culture of K562 or K562/Dox cells, intracellular levels of imatinib and CGP74588 were without significant changes (Fig. 3). Our data also revealed that imatinib was metabolised within K562 cells

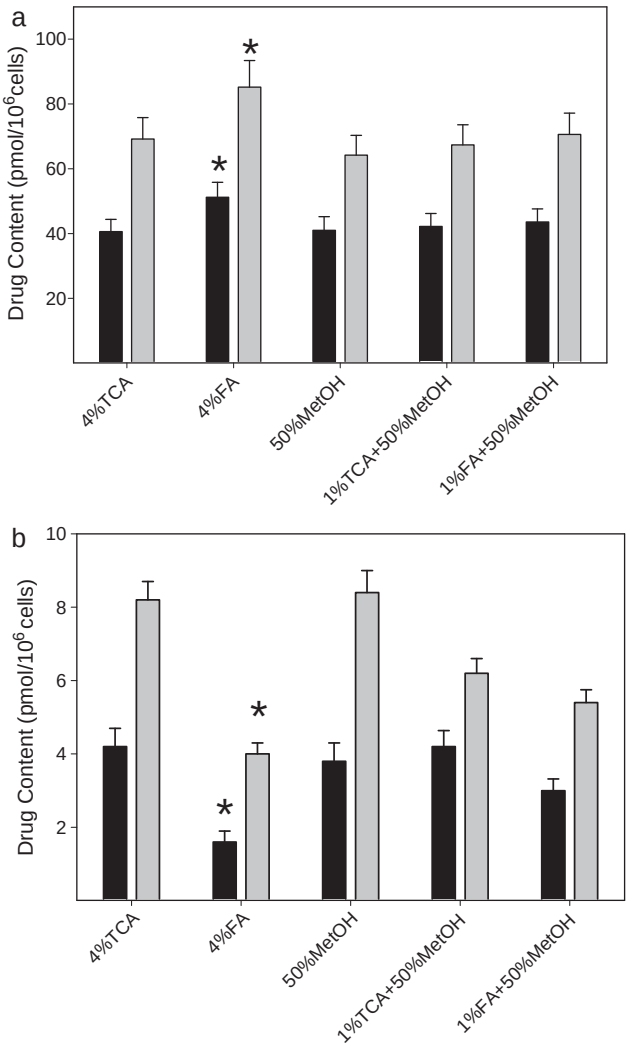

Fig. 2. Effect of composition of the extraction solution on extraction efficiency of imatinib and CGP 74588. Cells were incubated in growth medium in the presence of 1 μmol/l imatinib and 1 μmol/l CGP74588 at 37 °C for 4 h. Cells were afterwards separated from growth medium and cell pellet was repeatedly extracted as described in Section 2. Panel (a) first extraction; panel (b) second extraction. Imatinib (black columns); CGP74588 (gray columns). *Denotes significant change in intracellular content of imatinib and CGP 74588 ($P < 0.05$) in K562 cells extracted using 4% FA compared to K562 cells extracted using other extraction solutions. The experimental points represent mean values from three replicate experiments, with standard deviations.

Table 3
LOQ [nmol/l] for imatinib and CGP 74588 in various sample matrix.

Sample matrix	Imatinib	CGP 74588
0.4% TCA	1.4	2.8
4% TCA	2.2	4.4
0.4% FA	**1.0**	**2.0**
4% FA	1.5	2.1
10% MetOH	1.4	2.8
100% MetOH	1.3	2.3
5% MetOH	1.7	3.6
50% MetOH	1.4	2. 5
5% MetOH + 0.1% TCA	1.2	2.7
50% MetOH + 1% TCA	1.9	3.4
5% MetOH + 0.1% FA	1.1	2.3
50% MetOH + 1% FA	1.4	2.2

The lowest values of LOQ are in bold.

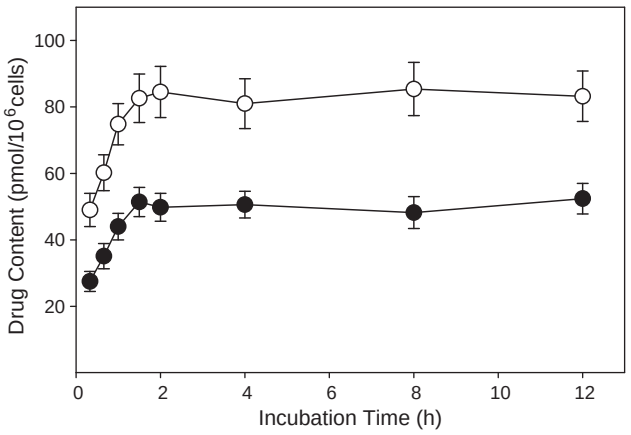

Fig. 3. Time course of intracellular level of imatinib and CGP 74588. Cells were incubated in the growth medium in the presence of either 1 μmol/l imatinib or 1 μmol/l CGP 74588 at 37 °C. At defined time intervals cells were extracted using 4% FA, as described in Section 2, and the imatinib or CGP 74588 contents were determined. Imatinib (filled circles); CGP74588 (open circles). The experimental points represent mean values from three replicate experiments, with standard deviations.

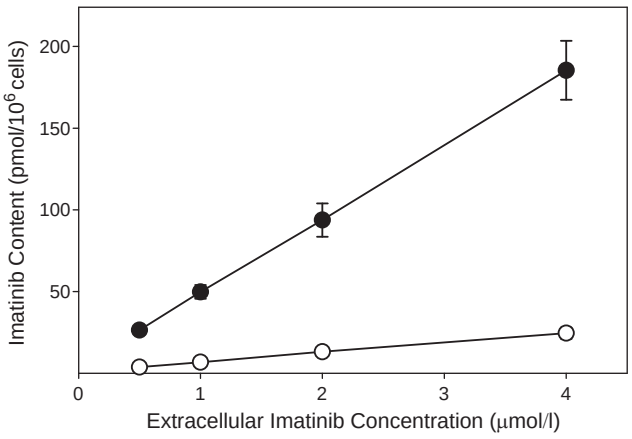

Fig. 5. Imatinib contents in sensitive and resistant cells. K562 cells and their resistant variant K562/Dox were incubated in the presence of various concentrations of imatinib. After 4 h incubation cells were subjected to extraction and imatinib content was determined in both cell lines. K562 cells (filled circles); K562/Dox cells (open circles). The experimental points represent mean values from three replicate experiments, with standard deviations.

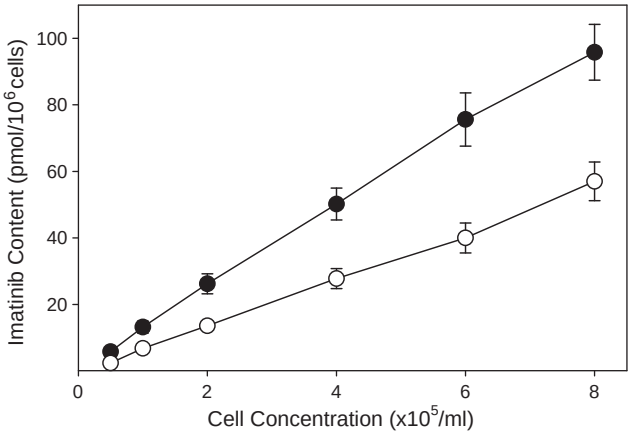

Fig. 4. Relationship between intracellular imatinib content and cell concentration. K562 cells at various concentrations were incubated in the growth medium in the presence of 0.5 μmol/l (open circles) or 1 μmol/l (filled circles) imatinib at 37 °C for 4 h. Cells were extracted using 4% FA and imatinib content was determined, as described in Section 2. The experimental points represent mean values from three replicate experiments, with standard deviations.

very slowly as approximately 1% of imatinib was converted into CGP 74588 (not shown). Conversion of imatinib into CGP 74588 within K562/Dox cells was under detection limits (not shown). Our results are in a good agreement with other authors. Indeed, imatinib is mainly metabolised by CYP3A4 [22], the most abundant cytochrome P-450 in human liver [23].

Our results also revealed that CGP74588 accumulated in sensitive K562 cells in significantly higher amount than imatinib (Figs. 2 and 3). This finding was rather surprising as there is only a little structural difference between imatinib and its N-desmethyl metabolite.

3.9. Cell concentration

An important factor for assessment of intracellular drug level is the number of cells. Cells cultured *in vitro* are usually maintained in the interval of 10^4–10^6 cells per ml. Therefore, we tested precision of the assay within this interval. We found a linear relationship between intracellular drug content and cell concentration within the tested interval (from 5×10^4 to 8×10^5 cells per ml; Fig. 4). However, detailed analysis of results revealed that precision of the assay declined when the cell concentration was below 10^5 cells per ml. While determination of the drug content in cells with the concentration maintained between 1×10^5 and 8×10^5 cells per ml provided results with RSD up to 15%, determination of the drug content in cells with a lower concentration provided results with RSD which exceeded 20% (not shown).

Similarly to other authors, we presented here the precision shown in the calibration curves, which is mostly based on the LC/MS/MS method reliability. 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 and as mentioned above, the RSDs do not exceed 15%, when cell concentrations were maintained from 10^5 to 8×10^5 cells per ml. Also value of LOQ for imatinib in growth medium was somewhat higher. In sensitive K562 cells the LOQ was 22 nmol/l for extracellular imatinib when cell concentration was 4×10^5 cells per ml. Although this value might seem to be high, it is suitable for monitoring of cytotoxic effects of imatinib as its EC_{50} value in K562 cells is approximately 170 nmol/l (not shown). Both sample preparation including the better control of the biological uncertainty and the more precise analytical determination method are essential criteria for accuracy of the assay. Anyway, the presented approach seems to be suitable for the intracellular imatinib and CGP 74588 determination.

3.10. Imatinib content in sensitive and resistant cells

Intracellular level of an anticancer drug is an important factor that influences the cell sensitivity especially in cells with the MDR phenotype caused by overexpression of drug efflux transporters such as P-gp. Therefore, we tested our assay to determine imatinib contents in sensitive K562 and resistant K562/Dox cells, which overexpress P-gp (not shown). As shown in Fig. 5, we observed a linear relationship between extracellular and intracellular contents of imatinib in both cell lines. As expected, the imatinib content was approximately 7–8-times lower in the resistant cells than that in the sensitive ones (Fig. 5). Similarly, resistant K562/Dox cell exhibited significantly decreased intracellular level of CGP 74588 in comparison to that found in sensitive K562 cells (not shown).

We further compared our approach for quantification of intracellular level of imatinib, which relies on cell centrifugation through a layer of silicone oil, with the standard sedimentation of cells with

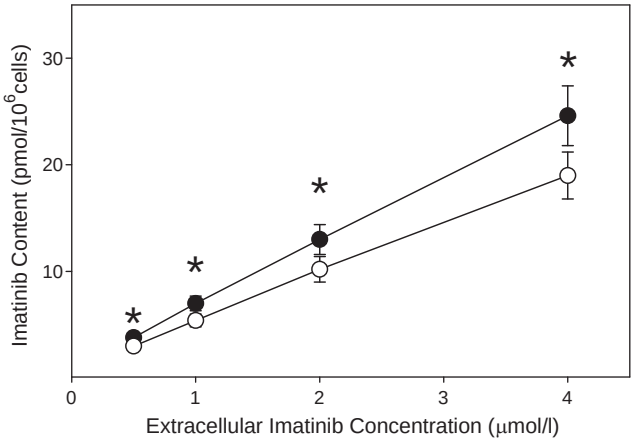

Fig. 6. Effect of sample preparation on the determination intracellular level of imatinib in cells. K562/Dox cells were incubated in growth medium containing imatinib together with 3 μmol/l CsA. After 4 h incubation cells were subjected to extraction and imatinib content was determined in cells. Cells centrifugated through a layer of silicone oil (filled circles); cells subjected to a standard centrifugation with subsequent washing in ice cold PBS (open circles). *Denotes significant change in intracellular content of imatinib ($P < 0.05$) in K562/Dox cells centrifugated through a layer of silicone oil compared to K562/Dox cells subjected to a standard centrifugation with subsequent washing in ice cold PBS. The experimental points represent mean values from three replicate experiments, with standard deviations.

subsequent washing in ice cold phosphate buffered saline (PBS). We found a significant difference between both approaches in resistant K562/Dox cells cultured in a growth medium containing imatinib in combination with cyclosporine A (CsA), an efficient inhibitor of P-gp (Fig. 6). Indeed, intracellular level of imatinib was significantly higher when drug transportation was quickly interrupted using cell separation from the growth medium by centrifugation through a layer of silicone oil, in comparison to standard sedimentation of cells with subsequent washing in ice cold PBS (Fig. 6). These results indicated that this significant difference was likely due to the leakage of the drug mediated by P-gp function since the same difference was not apparent in sensitive K562 cells, which did not express P-gp (not shown). Similar results were obtained for CGP 74588 (not shown). In addition, the above mentioned difference proportionally increased when the temperature of washing PBS was gradually elevated (not shown). Silicone oil centrifugation in contrast to standard centrifugation enable to determine the intracellular level of drugs very precisely as the trapped extracellular volume is small, less than 2% of the cell pellet volume [24, unpublished results].

Our results clearly demonstrate that the standard centrifugation method with washing step is sufficient for precise determination of intracellular level of imatinib or CGP 74588, however, it fails to determine intracellular drug levels in resistant cells expressing P-gp. Indeed, it underestimates intracellular drug levels in resistant cells probably due to residual activity of P-gp. In contrast, centrifugation of cells through silicone oil eliminates leakage of drugs. We believe that approach developed approximately 40 years ago [17,18] is still advantageous and would provide reliable results for precise determination of intracellular levels of anticancer drugs especially in cells expressing not only P-gp but also other drug transporters.

Acknowledgements

This work was supported by grant NR/9482-3 (Ministry of Health) and partly by grants MSM 6198959216 (Ministry of Education, Youth and Sports) and LF_2010_013 (Internal grant agency of Palacky University).

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