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Short communication

Simultaneous determination of AMN107 and Imatinib (Gleevec®, Glivec®, STI571) in cultured tumour cells using an isocratic high-performance liquid chromatography procedure with UV detection

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Abstract

A reversed phase high-performance liquid chromatographic (HPLC) method with UV detection was developed for the simultaneous determination of imatinib (Gleevec®, Glivec®, STI571) and AMN107 in cultured tumour cells, using clozapine as an internal standard. The compounds of interest were extracted by liquid–liquid extraction using TOXI-TUBES® A extraction tubes. Chromatographic separation was performed on a Phenomenex Gemini C18 reversed phase column (150 mm × 2.0 mm, 5 μm particle size), using a mixture of 65% CH_3OH (methanol) and 35% NH_4Ac (Ammonium acetate) buffer (20 mM, pH 10). Separation was achieved under isocratic conditions at a flow rate of 0.5 ml/min. Imatinib, clozapine and AMN107 are detected by UV detection at 260 nm. Calibration curves were linear from 50 to 7500 ng/ml with correlation coefficients (r^2) better than 0.998. The limit of quantitation (LOD) was 50 ng/ml. The method has been successfully applied to a cellular kinetics study.

Keywords: Imatinib; AMN107; HPLC; Cell culture

1. Introduction

Gastrointestinal stromal tumours (GIST) are the most common mesenchymal tumours of the gastrointestinal tract, representing approximately 20–30% of all soft tissue sarcomas [1]. These tumours are characterized by the ubiquitous expression of the stem cell factor (CSF) receptor [2]. The development of the KIT tyrosine kinase inhibitor imatinib (Gleevec®, Glivec®, STI571) (Fig. 1A) has revolutionized the treatment of GIST [3,4]. The drug is taken orally on a daily basis in order to suppress the growth of the tumour. Although most GIST patients show a good response to imatinib, some patients may develop resistance after a long period of treatment, and this has led to the development of novel, more effective, inhibitors.

AMN107 (Novartis Pharma AG) (Fig. 1B), a novel and highly potent inhibitor of the tyrosine kinase activity of the Bcr–Abl oncoprotein, is undergoing phase II clinical trials in chronic myelogenous leukaemia patients [5]. Furthermore, it is currently being evaluated in a phase I–II trial in GIST, either as a single agent or as a combination treatment with imatinib.

Several studies have reported on the role of P-glycoprotein (PGP) as a transporter of imatinib in transformed cell lines, indicating that MDR1 expression confers resistance [6–8]. High levels of PGP in tumour cells may efficiently reduce intracellular drug levels. Preliminary data in MDR1 over-expressing CCRF cells indicate favourable cellular uptake of AMN107 when compared to imatinib [9].

Our study assessed the cellular uptake of imatinib in comparison with AMN107 in two human GIST patient-derived cell lines (GIST882 and GIST GDG1), which express constitutively activated KIT [10]. In order to investigate the cellular uptake of the tyrosine kinase inhibitors imatinib and AMN107, alone and in combination, in gastrointestinal stromal tumour patient-derived cell lines (GIST882 and GIST GDG1), a new

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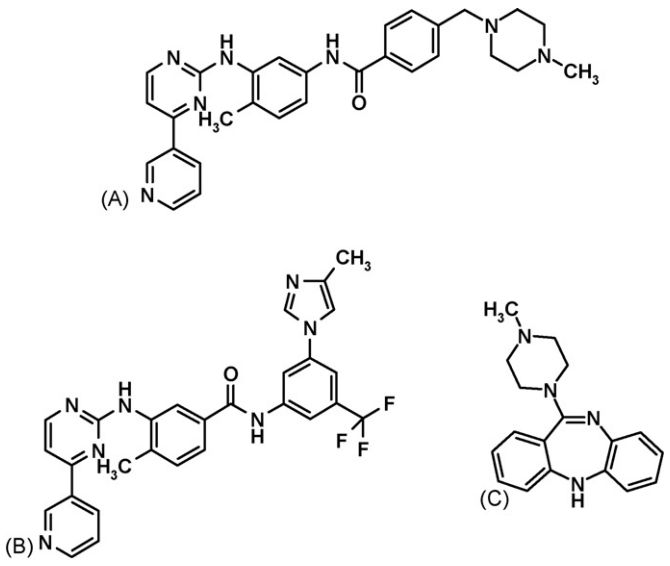

Fig. 1. Chemical structures of IMATINIB (A), AMN107 (B) and clozapine (I.S.) (C).

and simple analytical HPLC method for the simultaneous analysis of AMN107 and imatinib had to be developed.

Bakhtiar et al. [11] were the first to report assays capable of fast, sensitive and robust analyses of imatinib mesylate and its metabolite (CGP 74588) in blood plasma, using monkey plasma and a semi-automated solid phase extraction procedure with liquid chromatography–tandem mass spectrometry. They used LC–MS/MS and the sample pre-treatment procedure applied included either a solid phase extraction (SPE) [11] or a protein precipitation step [12]. The coupling of MEasurement of SEDiment (MESED) technology with LC–MS/MS resulted in assays for imatinib mesylate in the red blood cells (RBC) of patients treated with the signal transduction inhibitor [6]. Other LC based assays using detection methods which are not based on mass spectrometry have been published recently [13–15].

A sensitive HPLC method has been developed by Widmer et al. [13] for the assay of imatinib in human plasma, employing off-line solid phase extraction followed by HPLC coupled with UV-diode array detection. Imatinib is analyzed using a Nucleosil C18 column and a gradient elution program with a solvent mixture comprising methanol and water, both containing 0.05% ammonium acetate.

An isocratic online-enrichment HPLC-assay was developed by Schleyer et al. [14], permitting the analysis of imatinib and its main metabolite N-desmethyl-STI (N-DesM-STI) in plasma, urine, cerebrospinal fluid (CSF), culture media and cell preparations, in various concentrations, using UV detection at 260 nm. The analytical procedure consists of an online concentration of imatinib and N-DesM-STI in the HPLC system followed by elution on a ZirChrom-PBD analytical column.

Velpandian et al. [15] developed a HPLC method with UV detection for the estimation of imatinib in the plasma of patients. Samples were prepared by precipitating the plasma proteins with methanol, and a 50 μl aliquot of supernatant was then subjected to analysis. The assay was conducted using a C8 column (250 mm × 4.6 mm, 5 micron particle size), under isocratic elution with 0.02 M potassium dihydrogen phosphate–acetonitrile (7:3, v/v), at a flow rate of 1 ml/min, with detection using a photodiode array at 265 nm.

Thus far, the determination of AMN107 by HPLC and UV detection has not been described. For the purpose of cellular studies, we have developed a HPLC method for the determination of imatinib and AMN107 in one run by photo diode array (PDA) detection, using clozapine (Fig. 1C) as an internal standard.

2. Experimental

2.1. Materials

Methanol and water (HPLC grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate was purchased from Sigma–Aldrich (Bornem, Belgium). TOXI-TUBES® A extraction tubes (Varian, Inc., 25200 Commercentre Dr. Lake Forest, CA 92630, USA) were used for liquid–liquid extraction. AMN107 and imatinib were a kind gift from Novartis Pharma AG (Basel, Switzerland), and the internal standard, clozapine was supplied to us by Sigma–Aldrich (Bornem, Belgium). The purity of the standards used was over 99.9%.

2.2. Instrumentation

The analytical system consisted of a Waters 2690 HPLC and a 996 photodiode array detector (Waters, Milford, MA, USA). The compounds were separated on a Phenomenex Gemini C18 reversed phase column (150 mm × 2.0 mm, 5 μm). Millenium software (Waters, Milford, MA, USA) was used for recording the data and performing calculations, which were made in the internal standard mode using peak area ratios.

2.3. Preparation of calibration and quality control standards

Stock solutions of AMN107 and imatinib (0.2 mg/ml) were produced by dissolving 20 mg in 100 ml methanol in a volumetric flask. The internal standard was prepared by dissolving 100 mg in 100 ml methanol in a volumetric flask. All solutions were stored at −20 °C.

Seven calibration standards ranging from 50 to 7500 ng/ml (50, 150, 450, 2000, 3000, 4500 and 7500 ng/ml) were prepared by diluting the stock solution in 200 μl drug free cell suspensions, and stored in Eppendorf tubes at −20 °C. Quality controls were prepared in nominal concentrations of 250, 2500, and 5000 ng/ml, and also stored in aliquots at −20 °C.

2.4. Cultured tumour cells

The GIST882 cell line was a kind gift from Dr. Jonathan Fletcher (MIT Cancer Center and Department of Biology, Cambridge, MS). These cells express an activating KIT mutation (K642E) in the first part of the cytoplasmic split tyrosine kinase domain (exon 13) [17]. They were grown in culture flasks in DMEM/F12 medium supplemented with 10% foetal bovine

serum (FBS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin, at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. GIST GDG1 is an imatinib resistant GIST cell line, derived from a patient with progressive GIST, and was grown in the same medium as GIST882.

2.5. Sample preparation and HPLC analysis

Approximately 10^6 GIST882 or GIST GDG1 cells were seeded in 60 mm Petri-dishes and treated for 2 h with single agent imatinib or AMN107, or with the combination. Drug uptake was determined at three concentrations; 0.01, 1 and 10 μM. After incubation, the medium was removed; cells were washed twice with ice-cold PBS, and lysed in a cold buffer containing 0.5% Triton X-100, 50 mM Tris–HCl (pH 7.5), 150 mM NaCl and a protease inhibitor cocktail. Lysates were rocked for 30 min at +4 °C and then centrifuged at 14,000 rpm for 30 min at +4 °C. Supernatants were transferred to a fresh tube for intracellular drug concentration measurements.

After cell lysis, 300 μl internal standard solution (500 ng/ml) and 3 ml water were added to 200 μl cell lysate. After vortexing, the solution was transferred to a toxi-tube and mixed gently for 5 min. After centrifugation, the organic phase was transferred to a glass reaction tube and evaporated until dry. The residue was redissolved in 100 μl of a NH_4Ac-buffer (20 mM, pH 10) for HPLC injection (50 μl). The toxi-tube appeared to be excellent for the biological matrix of interest. This finding is relatively new, since toxi-tubes are generally used for urine, another biological matrix commonly used in toxicological research.

Chromatographic separation was performed on a Phenomenex Gemini C18 reversed phase column (150 mm × 2.0 mm, 5 μm), maintained at 30 °C in a column heater. The mobile phase consisted of a mixture of 65% CH_3OH and 35% NH_4Ac buffer (20 mM, pH 10). Separation was achieved under isocratic conditions at a flow rate of 0.5 ml/min. Imatinib, clozapine and AMN107 were detected by UV detection at 260 nm. The chromatographic run was completed in 20 min.

3. Results and discussion

3.1. Chromatography

Fig. 2 shows representative chromatograms of blank cells and cells treated with 1 μM imatinib and 1 μM AMN107, corresponding to 493 ng/ml and 533 ng/ml, respectively. Clozapine was added as an internal standard to the lysate, resulting in a final concentration of 750 ng/ml lysate. The three peaks of interest are symmetrical and baseline resolved. Imatinib, clozapine and AMN107 are well separated from co-extracted material under the described chromatographic conditions, at retention times of 4.0, 9.4 and 17.5 min, respectively. The peaks were of good shape and no interference with matrix constituents was observed.

3.2. Linearity, limit of quantification (LOQ), precision, accuracy and recovery

The calibration curve ranged from 50 to 7500 ng/ml (50, 150, 450, 2000, 3000, 4500 and 7500 ng/ml). Standard response

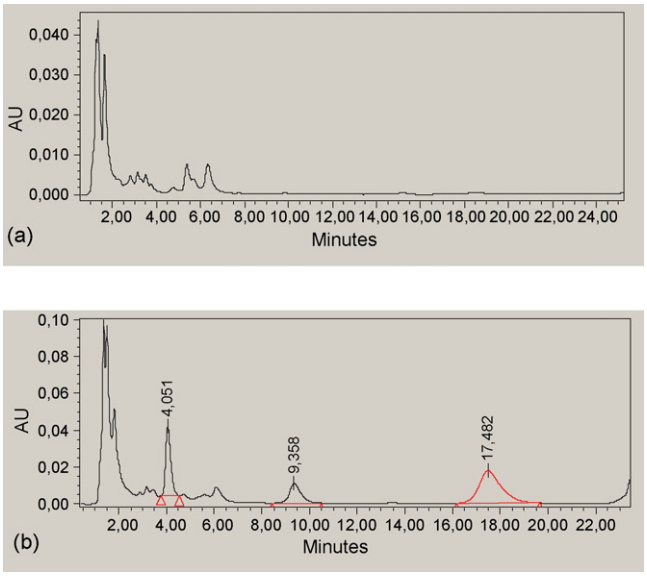

Fig. 2. (a) Representative chromatogram of an extract from blank cells. (b) Representative chromatogram of an extract from cells treated for 2 h with imatinib (Rt 4.05 min) and AMN107 (Rt 17.48 min) (1 μM). The concentration of clozapine (Rt 9.36 min) was 750 ng/ml cell lysate.

curves were generated daily using a weighted ($1/x$) least-squares linear regression model.

Linearity, with correlation coefficients $r^2 > 0.998$, was achieved in the range investigated. The back-calculated concentrations of all calibrators were compared with their respective nominal values, and were within 100 ± 15% of the nominal value. The LOQ, defined as the concentration of the lowest calibrator calculated to be within ±20% of the nominal value and with a percentage relative standard deviation (R.S.D.) of less than 20%, was 50 ng/ml. The intra-assay precision (repeatability) and inter-assay precision (reproducibility) are shown in Table 1.

Intra-assay precision was evaluated by replicate ($n = 5$) analysis of the QC samples.

Inter-assay precision was evaluated by replicate analysis of the QC samples in several experiments performed on ten different days by two operators. A comparison of the calcu-

Table 1
Precision and accuracy data for the HPLC analysis of imatinib and AMN107 in cells

Imatinib	Intra-assay precision ($n = 5$)		Inter-assay precision ($n = 10$)	
QC (ng/ml)	R.S.D. (%)	Bias (%)	R.S.D. (%)	Bias (%)
250	5.2	−0.9	13.5	−3.0
2500	4.5	−2.3	8.4	−3.9
5000	5.0	3.0	10.2	5.2
AMN107	Intra-assay precision ($n = 5$)		Inter-assay precision ($n = 10$)	
QC (ng/ml)	R.S.D. (%)	Bias (%)	R.S.D. (%)	Bias (%)
250	3.8	−2.1	8.1	4.2
2500	2.9	6.4	6.5	6.2
5000	3.6	4.4	7.8	5.9

lated concentrations of the QC samples with their respective nominal values was used to assess the accuracy (bias) of the method. Recovery was estimated by comparing the response of a 750 ng/ml calibrator when AMN107 and imatinib were added before the extraction step ($n = 3$) with the response obtained when both compounds were added after sample preparation ($n = 3$). Clozapine was added before the extraction step in both conditions. The recovery of the method was $75.2 \pm 5.5\%$ for AMN107 and 79.3 ± 7.2 for imatinib.

The intra-assay precision (repeatability) and the inter-assay precision (reproducibility) were highly satisfactory, with all relative standard deviations less than 15% (Table 1).

In cases of special interest, the assay can be used to measure both agents in the fluid used to wash the cells. The MESED technology should then be applied as described in a series of papers by us, e.g. Prenen et al. [6]. The technology allows the measurement of AMN107 and imatinib in the washings with an accuracy of $\leq$4.3% of the total concentration in the medium and cells. The data are not presented here because they are considered to be beyond the scope of this report.

3.3. Results of the intracellular uptake of imatinib and/or AMN107 in the two GIST cell lines

In both GIST cell lines, the uptake of AMN107 was significantly higher than that of imatinib: in GIST882 cells uptake was 7-fold higher (50,611 ng AMN107/mg protein *versus* 7002 ng imatinib/mg protein at an exposure of 10 μM), while in GIST GDG1 cells uptake was 10-fold higher (62,150 ng AMN107/mg protein *versus* 5970 ng imatinib/mg protein at an exposure of 10 μM) [16]. The protein content was determined using a protein assay reagent (Bradford, BioRad).

Preliminary data have demonstrated that the expression of the transporter proteins ABCG2/BCRP and SLC221A1/OCT1 in the GIST cell line 882 are relatively low compared to ABCB1/MDR1. The expression of the latter protein is between that of the cell lines tested before by us: 2780^{AD} *versus* A2780. However, expression alone is not sufficient to provide an insight into drug uptake and release by active pumping. Testing the expression and activity of proteins of interest concomitantly requires assays using flow cytometry and doubling labelling techniques, as published by us earlier [18].

3.4. Applicability of the method

We report a new HPLC method for the simultaneous determination of imatinib and AMN107 in a single chromatographic run. It requires 200 μl of cell suspension, takes 30 min to prepare a batch of 20 samples, and 20 min for a chromatographic run. The method can be adopted easily in many laboratories using commonly available HPLC equipment.

The selectivity of the assay described will be exploited optimally when monitoring both substances in body fluids and/or tissues during animal and clinical pharmacokinetic studies of concomitant imatinib and AMN107 administration. A sensitivity of 50 ng/ml and an assay speed of 50 min are already promising. However, the particular biological matrix investigated may well demand some fine adjustment of the sample pre-treatment and chromatographic conditions. Although the method has not been validated for plasma analysis, we performed the assay on spiked plasma. Fig. 3 shows the chromatogram of plasma spiked with STI-571, clozapine and AMN-107. The concentration of all 3 compounds was 200 ng/ml plasma. Furthermore, possible interference by products formed in vivo, such as imatinib metabolites, needs to be excluded.

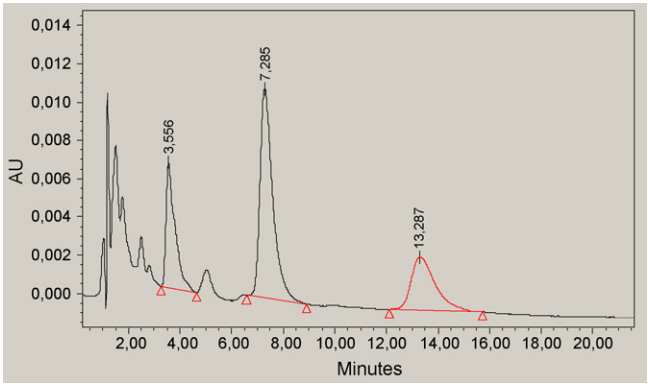

Fig. 3. Representative chromatogram of an extract from plasma spiked with 200 ng/ml imatinib (Rt 3.56 min), clozapine (Rt 7.29 min) and AMN107 (13.29 min).

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