Journal of Chromatography B, 1038 (2016) 88–94

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Development and validation of LC–MS/MS assay for the simultaneous determination of methotrexate, 6-mercaptopurine and its active metabolite 6-thioguanine in plasma of children with acute lymphoblastic leukemia: Correlation with genetic polymorphism

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ARTICLE INFO

Article history:
Received 15 September 2016
Received in revised form 15 October 2016
Accepted 26 October 2016
Available online 28 October 2016

Keywords:
6-Mercaptopurine
Acute lymphoblastic leukemia
Individualized therapy
LC–MS/MS
Leukemia
Methotrexate

ABSTRACT

Individualized therapy is a recent approach aiming to specify dosage regimen for each patient according to its genetic state. Cancer chemotherapy requires continuous monitoring of the plasma concentration levels of active forms of cytotoxic drugs and subsequent dose adjustment. In order to attain optimum therapeutic efficacy, correlation to pharmacogenetics data is crucial. In this study, a specific, accurate and sensitive liquid chromatography tandem mass spectrometry (LC–MS/MS) has been developed for determination of methotrexate (MTX), 6-mercaptopurine (MP) and its metabolite 6-thioguanine nucleotide (TG) in human plasma. Based on the basic character of the studied compounds, solid phase extraction using a strong cation exchanger was found the optimum approach to achieve good extraction recovery. Chromatographic separation was carried out using RP-HPLC and isocratic elution by acetonitrile: 0.1% aqueous formic acid (85:15 v/v) with a flow rate of 0.8 mL/min at 40 °C. The detection was performed by tandem mass spectrometry in MRM mode via electrospray ionization source in positive ionization mode. Analysis was carried out within 1.0 min over a concentration range of 6.25–200.00 ng/mL for the studied analytes. Validation was carried out according to FDA guidelines for bioanalytical method validation and satisfactory results were obtained. The applicability of the assay for the monitoring of the MTX, MP and TG and subsequent application to personalized therapy was demonstrated in a clinical study on children with acute lymphoblastic leukemia (ALL). Results confirmed the need for implementation of reliable analysis tools for therapeutic dose adjustment.

1. Introduction

It is a well-recognized fact that individuals respond differently to therapeutics. Trial-and-error and decisions based mainly on the clinical picture are the main procedures used by medical practitioners to optimize a therapeutic dosage for each individual patient. As a result, adverse drug reactions or lack of efficacy are commonly observed in some patients [1]. The interindividual differences in therapeutic response could be attributed to differences in: (i) drug absorption, (ii) distribution, (iii) metabolism, and (iv) elimination [2]. Genetic factors were found responsible for approximately 15–30% of interindividual variability; up to 95% with some drugs [3]. Patients of the same weight or body surface area recieving the same treatment regimen may show differences in drug levels in plasma up to 600-fold [3]. In such cases, modern medication management strategies based on theraputic drug monitoring (TDM) in biological fluids should be implemented [4]. Pharmacogenetics has

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http://dx.doi.org/10.1016/j.jchromb.2016.10.035
1570-0232/

been outlined in 1960s as "... *the study of heredity and the response to drugs*" [5]. Investigation of genetic polymorphisms in key metabolizing enzymes and correlation with differences in therapeutic outcomes has been recommended since then. Consideration of such variability in efficacy and toxicity, at equivalent doses should provide safe and effective medicine.

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children. The chemotherapeutic agent 6-Mercaptopurine (6-MP) is used in most of the treatment protocols of ALL [6]. Inactivation of MP by xanthine oxidase (XO), suggested the concurrent administration of XO inhibitors such as methotrexate (MTX) in order to increase bioavailabity and thus efficacy of MP [7,8]. Administration of MP (oral, 75 mg/m^2/day) with weekly doses of MTX (intravenous, 40 mg/m^2/week) represents the core of maintenance therapy for ALL [6,9]. However, resistance to chemotherapy and toxicity have been a concern in approximately 80% of patients [10].

MP (3,7-Dihydropurine-6-thione hydrate) is a pro-drug metabolized by hypoxanthine phosphoribosyl transferase into the active cytotoxic metabolite 6-thioguanine nucleotide (TG), Fig. 1 [11]. MP is also metabolized by thiopurine methyltransferase (TPMT) and XO into inactive 6-methylmercaptopurine (6-MMP) and thiouric acid, respectively [8,12,13]. High TG levels have been correlated with good therapeutic efficacy, whereas high 6-MMP levels were associated with liver toxicity [9,14,15]. The TDM of MP and key metabolites in plasma has been suggested in order to predict efficacy and toxicity [16–18] especially when the teatment protocol is not achieving the desired therapeutic outcomes at theoretically adequate doses [19,20].

MTX is N-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino] benzoyl] -L-glutamic acid (Fig. 1) [11]. Occurance of side effects has been highly variable amoung patients receiving MTX [21] that was attributed to genetic polymorphism in folate metabolizing enzymes [21,22]. The elimination of MTX is controlled by the adenosine triphosphate–binding cassette class transporter (ABCC2) and it has been reported that polymorphisms in ABCC2 gene is linked to the variability of pharmacokinetics of MTX at high doses [23]. As per ALL treatment protocols, all patients receiving MTX routinely should receive also calcium folinate [24] to protect from MTX side effects [25]. Determination of the MTX plasma concentration was thus required after high dose MTX therapy to predict and prevent toxicity through modification of the calcium folinate administration schedule [26,27].

In previous work by our research group, polymorphisms in genes encoding MTX and 6-MP drug metabolizing enzymes when combined with HCV infection was investigated in Egyptian children with ALL [4]. Correlation of obtained results with plasma concentration levels of MTX, MP and TG requires reliable assay protocols. Moreover, determination of plasma concentration levels using sensitive, accurate and precise bioanalytical techniques should provide an early and affordable alarming signal in such cases. In literature, several methods were developed for the analysis of either MTX [28–32] or MP and its metabolites [33–36] in human plasma. To the best of our knowledge, there is no reported assays for the simultaneous determination of MTX, MP and their metabolites. Here, LC–MS/MS assay for the simultaneous determination of MTX, MP and its active metabolite TG in human plasma was developed and validated. The application of cation exchange chromatography along with LC–MS/MS for selective extraction and determination of the studied compounds helped to overcome limitations in plasma sample volume obtained from young children. The assay was successfully applied in a clinical study intended to correlate plasma concentration levels of the studied drugs with genetic polymorphism in ALL children and therapeutic outcomes.

2. Experimental

2.1. Materials

MTX, 6MP, TG and TU (internal standard, IS) were purchased from Sigma-Aldrich (USA). Methotrexate vials (25 mg/mL) and 6-mercaptopurine tablets (50 mg/tablet) were obtained from Orion pharma, Finland and GSK, USA, respectively. Human blank plasma was obtained from the Holding Company for Biological Products and Vaccines (VACSERA), Egypt. All other chemicals and solvents were of HPLC grade and were obtained from Sigma Aldrich (USA). Ultra-pure water was obtained using a MilliQ UF-Plus system (Millipore, Germany); resistivity > 18 MΩ cm^{-1} at 25 °C and TOC < 5 ppb.

2.2. Instruments

A vacuum manifold and manually packed strong cation exchanger sorbent (Bondesil SCX, 40 μm, 100 mg) from Agilent Technologies (USA) were used for solid phase extraction (SPE) of the studied drugs. Analysis was achieved using Waters Aquity UPLC connected to Waters Aquity Ultra Performance LC autosampler. Mass spectrometric detection was performed using Waters Aquity TM TQD (triple quadrupole detector) in multiple reaction monitoring (MRM) mode. An electrospray ionization (ESI) interface in positive ionization mode was used. Hardware control and data acquisition and treatment were carried out using MassLynx 4.1 SCN805 software.

2.3. Liquid chromatographic and mass spectrometric conditions

Separations were carried out using Acquity UPLC BEH shield RP 130 Å, 1.7 μm, 2.1 × 150 mm column (Waters, USA) and a mobile phase of acetonitrile: 0.1% aqueous formic acid (85:15 V/V) with a flow rate of 0.8 mL/min at 40 °C. Each component of the mobile phase was degassed before use in an ultrasonic bath for 10 min. The transitions of molecular ions were: MTX (m/z 455.34 > 308.22), MP (m/z 152.89 > 119.00), TG (m/z 168.01 > 151.08) and IS (m/z 128.94 > 111.90). The main working parameters of the mass spectrometer were summarized in Table S1.

2.4. Standard solutions and calibration curves

Stock solution of MTX, MP and TG were prepared in 25 mL volumetric flask using methanol containing 50 μL ammonia at a concentration of 100 μg/mL. Aliquots were taken from the stock solutions and diluted with methanol to prepare working solution of 8.00 μg/mL. Stock solution of IS (TU) was prepared in methanol at a concentration of 100 μg/mL. Stock and working standard solutions were stored at 4–8 °C away from direct light. Quality control samples were prepared at three concentration levels; low (QCL), medium (QCM) and high (QCH) with concentrations of 8.00 ng/mL, 100.00 ng/mL and 190.00 ng/mL, respectively. Aliquots were taken from the working standard solutions, diluted into 500 μL plasma followed by 500 μL 2% formic acid solution and loaded to the SPE cartridges after vortex mixing for 1 min.

2.5. Sample preparation

All sample preparation steps were carried out away from direct light in amber glass vials or glass ware covered with aluminum foil. Preconditioning of SPE cartridges was carried out using 3 mL methanol followed by 3 mL 2% (v/v) aqueous formic acid. Aliquots of 1 mL of each of the prepared samples were loaded slowly at mild vacuum. Washing of the cartridges was performed using 2 mL of 2% (v/v) aqueous formic acid followed by 1 mL 2% (v/v) methanolic