Microchemical Journal xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Microchemical Journal

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

Robust and sensitive LC/MS-MS method for simultaneous detection of acetylsalicylic acid and salicylic acid in human plasma

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ARTICLE INFO

Article history:
Received 13 July 2016
Received in revised form 6 November 2016
Accepted 6 November 2016
Available online xxxx

Keywords:
Aspirin
Salicylic acid
LC/MS-MS
Validation
Human plasma

ABSTRACT

A liquid chromatography-tandem mass spectrometry method was developed for the simultaneous determination of acetylsalicylic acid (ASP) and its major metabolite, salicylic acid (SAL) in human plasma. The method was validated. ASP and SAL were extracted by single-step liquid-liquid extraction using *tert*-butyl methyl ether. The ions were detected in multiple reaction monitoring mode at *m/z* 179.0/137.0 transition for ASP and *m/z* 136.9/93.0 for SAL. The lower limit of quantification for ASP and SAL was 1 and 80 ng/ml, respectively. The method was successfully applied for the characterization of the plasma concentration levels of ASP and SAL after oral administration of Aspirin Protect 100 to healthy volunteers. The present method can contribute to the improvement of ASP and SAL determination in patients under antithrombotic therapy, and to the reduction of the risk for ASP resistance associated with bioavailability/exposure issues (non-compliance, misdosing, poor absorption).

1. Introduction

Aspirin (ASP) is a well-known salicylate drug (Fig. 1), which is one of the least expensive and most widely used anti-inflammatory, analgesic and antipyretic agent [1]. In addition, ASP at low doses (<100 mg/day) is applied for thrombosis prophylaxis, because it selectively inhibits cyclo-oxygenase (COX)-dependent platelet aggregation [2]. The mechanism of action and the effects of ASP depend on the dose taken [3]. At low doses (30–100 mg/day), ASP irreversibly acetylates COX-1, which is responsible for the synthesis of prostanoids and for the generation of thromboxane A2. The antithrombotic effect of ASP is attributed to the selective inhibition of COX-1 dependent production of thromboxane A2 in platelets [4,5]. High-dose ASP (2–4 g/day) inhibits both COX-1 and COX-2, blocks prostaglandin production, and displays anti-inflammatory, antipyretic and analgesic effects. At extremely high doses (6–8 g/day), ASP has been reported to be as effective as cortisone in treatment of rheumatic disorders [6]. ASP has a short half-life, approximately 15–20 min in plasma, because it is rapidly hydrolyzed by esterases to salicylic acid (SAL) (Fig. 1) [7,8]. SAL is metabolized to gentisic acid or biotransformed by conjugation with glycin or glucuronic acid [7]. SAL also displays some anti-inflammatory, antipyretic and analgesic activity; however, the pharmacological activity of SAL is far from that of the parent compound [9]. The efficacy and safety aspects of ASP therapy require the establishment of the optimal dose for the particular indication. Therefore, optimization of ASP dosing is essential for the avoidance of both adverse drug reactions and ASP resistance. Misdosing of patients appears to be rather frequent; 10–20% of patients with cardiovascular disease are resistant to ASP [10]. ASP resistance is the inability of ASP to protect patients from thrombotic complications, to cause a prolongation of the bleeding time, or to inhibit thromboxane biosynthesis [11,12]. One of the possible reasons of ASP failure is the reduced bioavailability of ASP which is induced by inadequate intake of ASP, inadequate ASP dosing, and reduced absorption or increased metabolism of ASP [12].

The bioavailability of ASP can be monitored by the simultaneous determination of ASP and SAL plasma levels in patients, and the optimal ASP dosing can be tailored individually. Hereby the risk of the clinical atherothrombotic vascular events in patients can be reduced. In addition, the guideline of the European Medicines Agency on the investigation of bioequivalence requires the determination of plasma levels of both ASP and SAL for a new ASP formulation since 2010 [13]. For the measurement of salicylates, several techniques are available, such as colorimetric, fluorometric, enzymatic, gas or liquid chromatographic assays [14]. Bioanalytical HPLC-UV methods have also been reported for

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http://dx.doi.org/10.1016/j.microc.2016.11.005
0026-265X/

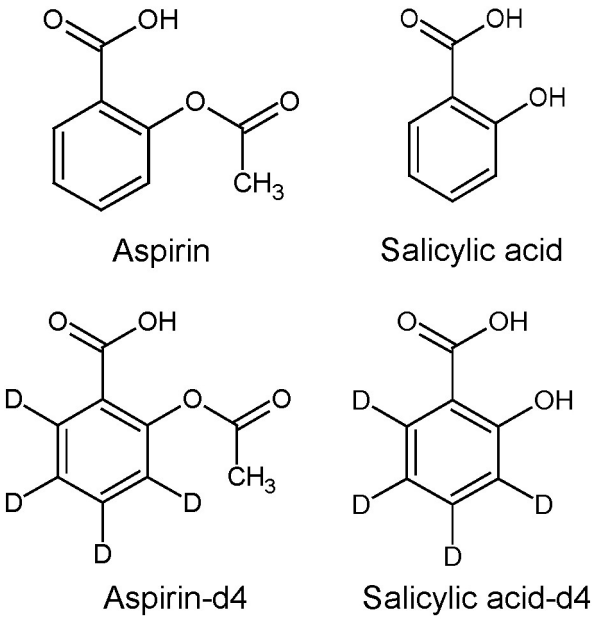

Fig. 1. Chemical structure of aspirin, salicylic acid and their deuterated analogues (aspirin-d4 and salicylic acid-d_4).

the quantification of ASP and its metabolites [15–21]. However, the liquid chromatography-tandem mass spectrometry (LC-MS/MS) offers the most sensitive and selective methods for measuring salicylates simultaneously from biological matrices. The number of the published LC-MS/MS methods that are suitable for the simultaneous determination of ASP and SAL from plasma is still limited. Bae et al. [22] applied sample preparation methods separately for the parent compound and its deacetylated metabolite, whereas others reported multi-step sample preparation (protein precipitation followed by a liquid-liquid extraction) [23], one-step solid-phase extraction [24], or even direct injection of the supernatant of precipitated plasma into the LC-MS/MS system [25]. To the best of our knowledge, no published LC-MS/MS method with a one-step liquid-liquid extraction is available for the simultaneous extraction and quantification of ASP and SAL in human plasma. The main advantages of the present method over the previously published methods are the single-step liquid-liquid extraction, the better retention on the HPLC column to lower the risk of matrix effect, the more symmetric peak shapes and the inexpensive materials necessary. The aim of the current work has been the development of a sensitive, selective and economical LC/MS-MS method for assaying ASP resistance in patients.

2. Experimental

2.1. Chemicals and reagents

ASP and SAL (analytical standards) were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA), while ASP-d4 and SAL-d4 (2-hydroxybenzoic-3,4,5,6-d4 acid) (internal standards) were obtained from Toronto Research Chemicals Inc. (Toronto, Canada) and C/D/N Isotopes Inc. (Pointe-Claire, Canada), respectively. *Tert*-butyl methyl ether (TBME) (LiChrosolv) and acetic acid (glacial, 100% Suprapur) were procured from Merck Inc. (Darmstadt, Germany). Formic acid (puriss p.a., 98–100%) and potassium fluoride (ACS reagent, ≥99.0%) were purchased from Sigma-Aldrich Inc. Acetonitrile (ultra gradient HPLC grade) was from J.T. Baker (Deventer, The Netherlands). Blank human plasma was purchased from Sera Laboratories International Ltd. (Haywards Heath, United Kingdom).

2.2. Chromatographic conditions

The Agilent 1100 series HPLC system consisted of a G1312A binary pump, a G1322A degasser, a G1329A autosampler and a G1330A autosampler thermostat. The HPLC column was an InertSustain C18 (2.1 × 75 mm, 3 μm) (GL Sciences Inc., Nishi Shinjuku, Japan), kept at ambient temperature (23 ± 3 °C). Other HPLC columns tested for separation were Inertsil ODS-4 (2.1 × 75 mm, 3 μm) (GL Sciences Inc.) and ACE-3 C18 (2.1 × 75 mm, 3 μm) (Advanced Chromatography Technologies Ltd., Aberdeen, UK). The HPLC mobile phases were Milli-Q water containing 0.2% formic acid (eluent A) and acetonitrile containing 0.2% formic acid (eluent B). The analytes were eluted by a gradient program. After sample injection (0 min), a mixture of 80% eluent A and 20% of eluent B was linearly changed to a mixture of 35% eluent A and 65% of eluent B until 4.0 min. Then eluent B was linearly reversed back to 20% at 4.1 min. Finally, the initial combination of the eluents was kept up to 6.5 min for equilibration of the column. The flow rate was set at 0.3 ml/min, and the injected sample volume was 10 μl. A diverter valve was used to discard the LC effluent during the first 2.5 min and the last 1.0 min of each chromatographic run.

2.3. Mass spectrometric conditions

The mass spectrometer was an API2000 triple quadrupole instrument equipped with an electrospray ionization (ESI) interface (Applied Biosystems, Toronto, Canada). Data were acquired and processed by the Analyst software (version 1.4.2). The source parameters, curtain gas, collision gas, ion spray voltage, source temperature, nebulizer gas and auxiliary gas were set at 30 psi, 3 psi, −500 V, 400 °C, 60 psi and 60 psi, respectively. The parameters of the mass spectrometer, declustering potential, entrance potential, collision energy, collision entrance potential and collision exit potential for ASP and ASP-d4 were −15 V, −10 V, −10 V, −10 V and −4 V, respectively. These parameters for SAL and SAL-d4 were −60 V, −15 V, −42 V, −15 V and −4 V, respectively. Detection of the ions was performed in multiple reaction monitoring (MRM) mode, monitoring the transition of the *m/z* 179.0 parent ion to the *m/z* 137.0 product ion for ASP, the *m/z* 136.9 parent ion to the *m/z* 93.0 product ion for SAL, the *m/z* 183.0 parent ion to the *m/z* 141.0 product ion for ASP-d4 and the *m/z* 140.9 parent ion to the *m/z* 97.0 product ion for SAL-d4. The dwell time was 400 ms for all components.

2.4. Sample preparation

The primary stock solutions of ASP and SAL for the preparation of calibrator (STC) and quality control (STQ) samples were prepared by separate weighing (MX5 balance, Mettler-Toledo International Inc., Switzerland). The primary stock solutions of ASP, SAL and internal standards (STC-ASP 100 μg, STQ-ASP 200 μg, STC-SAL 1 mg, STQ-SAL 2 mg, ASP-d4 50 μg and SAL-d4 100 μg) were prepared in acetonitrile containing 0.2% acetic acid. The working solutions used for spiking the calibrator and quality control (QC) samples were diluted separately by spiking an appropriate volume of the stock solutions to achieve the final concentrations for ASP, SAL and the internal standards. The dilutions were performed in acetonitrile containing 0.2% acetic acid. Stock and working solutions were stored at −20 ± 5 °C.

A simple liquid-liquid extraction method was followed for the extraction of ASP and SAL from human plasma. Because of the instability of ASP in plasma, potassium oxalate and sodium fluoride were used as anticoagulant, and the sample preparation was performed in ice-water bath. The enzymes in human plasma were inhibited by 12% formic acid and 450 mg/ml potassium fluoride solution. The plasma and the 12% formic acid solution were mixed in 10:1 volume ratio. An aliquot of 275 μl acidified human plasma, 10 μl of 450 mg/ml potassium fluoride solution, 10 μl of the corresponding standard working solution and 10 μl of IST solution were mixed. 2 ml of TBME was added to the

Please cite this article as: D. Sirok, et al., Robust and sensitive LC/MS-MS method for simultaneous detection of acetylsalicylic acid and salicylic acid in human plasma, Microchem. J. (2016), http://dx.doi.org/10.1016/j.microc.2016.11.005