Determination of acetylsalicylic acid and its major metabolite, salicylic acid, in human plasma using liquid chromatography–tandem mass spectrometry: application to pharmacokinetic study of Astrix® in Korean healthy volunteers

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ABSTRACT: The first liquid chromatography–tandem mass spectrometry (LC/MS/MS) method for determination of acetylsalicylic acid (aspirin, ASA) and one of its major metabolites, salicylic acid (SA), in human plasma using simvastatin as an internal standard has been developed and validated. For ASA analysis, a plasma sample containing potassium fluoride was extracted using a mixture of ethyl acetate and diethyl ether in the presence of 0.5% formic acid. SA, a major metabolite of ASA, was extracted from plasma using protein precipitation with acetonitrile. The compounds were separated on a reversed-phase column with an isocratic mobile phase consisting of acetonitrile and water containing 0.1% formic acid (8:2, v/v). The ion transitions recorded in multiple reaction monitoring mode were m/z 179 → 137, 137 → 93 and 435 → 319 for ASA, SA and IS, respectively. The coefficient of variation of the assay precision was less than 9.3%, and the accuracy exceeded 86.5%. The lower limits of quantification for ASA and SA were 5 and 50 ng/mL, respectively. The developed assay method was successfully applied for the evaluation of pharmacokinetics of ASA and SA after single oral administration of Astrix® (entero-coated pellet, 100 mg of aspirin) to 10 Korean healthy male volunteers. Copyright © 2008 John Wiley & Sons, Ltd.

KEYWORDS: aspirin; salicylic acid; LC/MS/MS; pharmacokinetics; Astrix®

INTRODUCTION

Aspirin (ASA) is the most widely used as an analgesic, anti-inflammatory and antipyretic drug. In addition, low-dose ASA is employed as an antithrombotic agent to inhibit cyclooxygenase-dependent platelet aggregation (Hennekens et al., 1989). After administration of ASA, ASA is rapidly hydrolyzed in the body to produce salicylic acid (SA), which is the compound that is primarily responsible for the pharmacological activity of ASA. SA is further metabolized to gentisic acid, salicyluric acid and other conjugates (Mays et al., 1984). A number of methods have been reported for the analysis of ASA and its metabolites in plasma samples, including HPLC (Peng et al., 1978; Buskin et al., 1982; Bakar and Niazi, 1983; Mays et al., 1984; O’Kruk et al., 1984; Shen et al., 1990; Kees et al., 1996; McMahon and Kelly, 1998; Pirola et al., 1998 and references therein), GC (Walter et al., 1974; Rance et al., 1975) and capillary electrophoresis (Hansen et al., 1998). The method using on-line solid-phase extraction (SPE) by a methylimidazole-immobilized-strong anion-exchanger (MC-SAX) in a column-switching HPLC system achieved the highest sensitivity among the assay methods previously reported. The lower limit of quantification of ASA and SA was 60 ng/mL in 100 μL of plasma (Yamamoto et al., 2007 and references therein).

Astrix®, containing 100 mg of aspirin in the form of enteric-coated, controlled-release pellets, is low-dose aspirin. To date, almost no pharmacokinetic data on ASA and SA after oral administration of Astrix® (entero-coated pellet, 100 mg of aspirin) has been reported in the literature due to assay limitations (stability in biological fluids and sensitivity). Limited information...
on the pharmacokinetic data of low-dose ASA is available (Bochner et al., 1991; Benedek et al., 1995; Bukhari et al., 2005). A more sensitive assay had to be developed in order to characterize the pharmacokinetics of low-dose ASA. The objective of this study was to develop and validate a reliable LC/MS/MS method for measurement of ASA and SA in human plasma after low-dose aspirin oral administration.

EXPERIMENTAL

Chemicals

Astrix® capsules were supplied by Hanmi Pharmaceutical Company (Seoul, Korea). Aspirin (ASA), SA, simvastatin (internal standard of ASA and SA, IS), potassium fluoride and formic acid were purchased from Sigma–Aldrich Chemical Company (St Louis, MO, USA). Other chemicals were of reagent grade or HPLC grade, and therefore were used without further purification.

Analytical system

The plasma ASA and SA concentrations were quantified using liquid chromatography–mass spectrometry with an API3000 LC/MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization interface used to generate negative ions [M – H]-. The compounds were separated on a reversed-phase column (Luna C18, 50 x 2.0 mm i.d.; 3 µm particle size; Phenomenex, Torrance, CA, USA) with an isotropic mobile phase consisting of acetonitrile and water containing 0.1% formic acid (8:2, v/v). The mobile phase was eluted using an Agilent 1100 series HPLC system (Agilent, Wilmington, DE, USA) at 0.2 mL/min.

The mass spectrometer was operated in the negative ion mode at ~3800 eV and 400°C. The operating conditions were optimized by flow injection of a mixture of all analytes and were determined as follows: nebulizing gas flow, 9 L/min; curtain gas flow, 12 L/min; collision gas (nitrogen) pressure, 3.58 x 10^{-5} Torr; and collision energy, ~20 eV. Quantification was performed by multiple reaction monitoring (MRM) of the deprotonated precursor ion and the related product ion for ASA or SA using internal standard method with peak area ratios and a weighting factor. The mass transitions used for ASA, SA and internal standard were m/z 179 → 137, 137 → 93 and 435 → 319, respectively.

Preparation of plasma standards and quality controls

Stock solutions of ASA, SA and IS were dissolved in acetonitrile at a concentration of 1 mg/mL. Standard solutions of ASA and SA in human plasma were prepared by spiking with an appropriate volume of the variously diluted stock solutions, giving final concentrations of 5, 20, 50, 100, 200 and 500 ng/mL for ASA, and 50, 200, 500, 1000, 2000 and 5000 ng/mL for SA. The calibration curves were prepared and assayed along with quality control (QC) samples and each batch of clinical plasma samples. The QC samples were prepared at three different concentration levels of 20, 100 and 500 ng/mL for ASA, and 200, 1000 and 5000 ng/mL for SA in blank plasma. All prepared plasma samples and stock solutions were stored at ~80°C (Revco ULT 1490 D-N-S; Western Mednics, Asheville, NC, USA).

Sample preparation

For ASA analysis, to glass tubes containing a 1 mL aliquot of human plasma, 20 µL of 50% potassium fluoride, 10 µL of internal standard (20 µg/mL) and 1 mL of 0.5% formic acid were added: 0.5% formic acid was used to acidify the plasma samples. After vortex-mixing, 5 mL of organic solvent (ethylacetate:ether = 4:1, v/v) was added and vortexed and then the mixture was centrifuged at 3000g for 10 min at 4°C. The organic layer was separated and evaporated to dryness at ambient temperature in a Speed-Vac (Savant, Holbrook, NY, USA). The residue was reconstituted with a 100 µL aliquot of the mobile phase, and a 5 µL aliquot was injected directly onto the LC/MS/MS system. All procedures were conducted at 4°C in an ice bath and within 20 min.

For SA analysis, a 400 µL aliquot of acetonitrile (including IS at a concentration of 10 µg/mL) was added to a 100 µL aliquot of human plasma. After vortex-mixing and centrifugation at 16,000g for 10 min, the supernatant was transferred into a clean Eppendorf tube. A 5 µL aliquot of the supernatant was injected directly onto the LC/MS/MS system. All prepared samples were kept in an autosampler at 4°C until injection.

Method validation

Specificity. Specificity of the method was established by measuring seven independent sources of blank human plasma or plasma samples spiked with ASA and IS, and SA and IS.

Linearity and lower limits of quantification. Linearity was assessed by preparing and analyzing ASA and SA, standard samples over 5–500 ng/mL for ASA and 50–5000 ng/mL for SA with six concentration points in human plasma. Calibration curves were analyzed by weighted linear regression (1/x^2 for ASA and 1/x for SA) of the peak area of analytes (ASA or SA) over those of IS.

The lower limits of quantification (LLOQ) for ASA and SA were determined based on at least 10 times of signal-to-noise ratio and sufficient precision (within 20%) and accuracy (80–120%).

Accuracy and precision. Intra- and inter-day accuracy and precision for this method were determined at three different concentration levels on three consecutive days, and on each day six replicates were analyzed with an independently prepared calibration curve. The accuracy was expressed by (mean observed concentration)/(nominal concentration) x 100% and the precision by relative standard deviation (RSD, %).

Recovery. The recovery of ASA and SA from the sample preparation procedure was determined by a comparison of the peak area of ASA and SA in spiked plasma samples (three replicates each of QC levels) with that of ASA and SA.
in samples prepared by spiking extracted drug-free plasma samples with the same amounts of ASA and SA.

**Stability.** The stability of stock solutions of ASA, SA, and IS at a concentration of 100 μg/mL was evaluated at 4°C for 7 days by comparison with freshly prepared solutions at corresponding concentrations. The stability experiments of ASA and SA in plasma samples were assessed by analyzing three replicates of stability samples at concentrations of 20 and 100 ng/mL for ASA, and 200 and 1000 ng/mL for SA under three conditions: after short-term storage (for 30 min in an ice-bath for ASA and for 24 h at room temperature for SA), after three freeze–thaw cycles, and after sample preparation (for 12 h at 4°C, autosampler stability).

**Clinical application**

Ten healthy male subjects who gave written informed consent took part in this study. Health problems, drug or alcohol abuse and abnormalities in laboratory screening values were exclusion criteria. The protocol of this study was approved by the Institutional Review Board of Busan Paik Hospital (Busan, Korea) and this study was performed according to the rules of Good Clinical Practice.

After an overnight fast, all the subjects were given a Astrix® capsule. Approximately 9 mL blood samples were collected via the cannula at the following times: predose, 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 8, 10 and 12 h after the administration. Blood samples were collected into chilled EDTA tubes containing a 20 μL aliquot of 50% potassium fluoride (to minimize the hydrolysis of ASA to SA in human blood). The chilled blood samples were centrifuged immediately at 2000 g for 10 min at 4°C and the plasma samples were frozen at −80°C until LC/MS/MS analysis.

The total area under the plasma concentration–time curve from time zero to time infinity or the last measured time (AUC ∞: 6 h for ASA and 12 h for SA) in plasma was calculated using the trapezoidal rule–extrapolation method; this method uses the logarithmic trapezoidal rule for the calculation of the area during the declining plasma-level phase (Chiou, 1978) and the linear trapezoidal rule for the rising plasma-level phase. The area from the last datum point to time infinity (for the calculation of AUC) was calculated by dividing the last measured plasma concentration by the terminal-phase rate constant. Standard methods (Gibaldi and Perrier, 1982) were used to calculate the following pharmacokinetic parameters using the noncompartmental analysis (WinNonlin®; Pharsight Corporation, Mountain View, CA, USA).

The peak plasma concentration (C max) and time to reach a C max (T max) were read directly from the experimental data.

**RESULTS AND DISCUSSION**

ASA is rapidly hydrolyzed by esterases in the gut wall, liver, plasma and red blood cells to form SA, with a half-life of only 15–30 min (Needs and Brooks, 1985). It was reported (Harris and Riegelman, 1967; Sørensen, 1983) that the half-life of ASA (SA formation through hydrolysis), when incubated in plasma at 37°C, was about 1 h. Therefore, we added enzyme inhibitors (50% potassium fluoride) to all plasma and/or blood samples in order to prevent enzymatic hydrolysis of ASA into SA and all sample preparation procedures were conducted at 4°C in an ice bath and within 20 min. Owing to differences in plasma concentration ranges of ASA and SA after oral administration of ASA, ASA and SA were separately analyzed.

**Method validation**

The ions of [M – H]+, m/z 179 → 137 for ASA, m/z 137 → 93 for SA, and m/z 435 → 319 for IS were selected for the MRM (–) due to their high intensity and stability, and no significant solvent adduct ions and fragments ions were observed.

**Specificity.** No interference peaks were detected for the ASA and IS, and SA and IS from seven different sources of human plasma. The typical chromatograms of blank plasma, plasma spiked with 5 ng/mL of ASA (200 ng/mL of SA for SA analysis) and IS, and a volunteer plasma at 3.5 h after single oral administration of Astrix® are shown in Fig. 1. The retention times of ASA and IS were approximately 1.06 and 1.67 min for ASA analysis. The corresponding values were 1.0 and 1.72 min for SA analysis. The total chromatographic run time was 3.0 min.

**Linearity and lower limits of quantification.** The plasma concentration ranges were 5–500 ng/mL for ASA and 50–5000 ng/mL for SA. The calibration model was selected on the basis of the analysis of the data by linear regression with/without intercepts and weighting factors (1/x2 and 1/x). The best linear fit and least-squares residuals for the calibration curve were achieved with 1/x2 and 1/x weighing factor for ASA and SA. The mean correlation coefficient (r) of the respective weighted calibration curves generated during the validation were 0.998 (0.997–0.999) for ASA and 0.999 (0.998–0.999) for SA. The lower limits of quantification (LLOQ) for ASA and SA were 5 and 50 ng/mL.

**Accuracy and precision.** The intra- and inter-day accuracy and precision for ASA and SA are listed in Table 1. The mean intra-day RSD (%) for ASA and SA were 2.67% (range 1.50–4.30%) and 3.90% (range 1.50–7.90%). The corresponding values for inter-day were 7.37% (range 6.40–9.30%) and 7.13% (range 6.0–7.80%). The intra-day accuracies of ASA and SA were 7.37% (range 6.0–7.90%). The corresponding values for inter-day were 104–108% and 91.2–98.5%.

**Recovery.** The absolute recoveries for ASA at three concentrations of 20, 100 and 500 ng/mL were 64.4 ± 4.96, 61.1 ± 4.47 and 63.5 ± 5.94%. The corresponding
Determination of ASA and SA in human plasma by LC/MS/MS method

**Original Research**

Figure 1. Chromatograms of acetylsalicylic acid (ASA) (I) and simvastatin (internal standard, II): (a) blank plasma, (b) blank plasma spiked with 5 ng/mL of ASA and simvastatin (20 μg/mL), and (c) volunteer plasma at 3.5 h after single oral administration of Astrix® (ASA: 75 ng/mL); and chromatograms of SA (III) and simvastatin (internal standard, IV): (d) blank plasma, (e) blank plasma spiked with 200 ng/mL of salicylic acid (SA) and simvastatin (10 μg/mL) and (f) volunteer plasma at 3.5 h after single oral administration of Astrix® (SA: 2075 ng/mL).

values for SA at three concentrations of 200, 1000 and 5000 ng/mL were 31.8, 27.6 and 29.3%.

**Stability.** Stock solutions of ASA, SA, and IS in acetonitrile were stable for 7 days at 4°C; more than 95.0% of the spiked amounts were recovered. The stability data of ASA and SA in human plasma under three conditions are listed in Table 2. As shown in Table 2, no significant degradation of ASA and SA in human plasma was observed under all conditions studied.

Clinical application

After single oral administration of Astrix® to 10 Korean healthy male volunteers, the mean plasma concentration–time profiles of ASA and SA are shown in Fig. 2(A) and (B), and some relevant pharmacokinetic parameters are listed in Table 3. After reaching the $C_{max}$ of ASA, the plasma concentrations of ASA declined in a mono-exponential fashion [Fig. 2(A)]. The $T_{max}$ values of ASA were long, 3.25 h (range 2–4.5 h; Table 3), and this
Table 1. Intra- and inter-day precision and accuracy data for assays of acetylsalicylic acid (ASA) and salicylic acid (SA) in human plasma (n = 6)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Nominal concentration (ng/mL)</th>
<th>Precision</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean a</td>
<td>RSD (%)</td>
<td>Accuracy (%)</td>
</tr>
<tr>
<td>Intra-day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASA</td>
<td>20</td>
<td>17.1 ± 0.100</td>
<td>1.50</td>
<td>86.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>89.4 ± 3.82</td>
<td>4.30</td>
<td>89.4</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>430 ± 9.20</td>
<td>2.20</td>
<td>87.6</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>192 ± 15.1</td>
<td>7.90</td>
<td>95.9</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1030 ± 15.2</td>
<td>1.50</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>5120 ± 120</td>
<td>2.30</td>
<td>102</td>
</tr>
<tr>
<td>SA</td>
<td>200</td>
<td>20.7 ± 1.92</td>
<td>9.30</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>105 ± 6.73</td>
<td>6.40</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>538 ± 34.5</td>
<td>6.40</td>
<td>108</td>
</tr>
</tbody>
</table>

Inter-day

ASA

|         |         |         |         |         |
|         |         |         |         |         |
| SA      | 200     | 197 ± 11.9 | 6.00   | 98.5 |
|         | 1000    | 979 ± 74.2 | 7.60   | 97.9 |
|         | 5000    | 4560 ± 357 | 7.80   | 91.2 |

a Values are mean ± standard deviation.
ASA, acetylsalicylic acid; SA, salicylic acid; RSD, relative standard deviation.

Table 2. Stability of acetylsalicylic acid (ASA) and salicylic acid (SA) in human plasma a

<table>
<thead>
<tr>
<th>Conditions</th>
<th>ASA</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 ng/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 ng/mL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) ASA

Short-term stability (30 min) | 91.3 ± 5.53 | 95.1 ± 7.80 |
Three freeze–thaw cycles | 94.9 ± 3.96 | 105 ± 3.99 |
Autosampler stability (12 h) | 91.9 ± 8.04 | 92.4 ± 9.48 |

(b) SA

200 ng/mL | 92.5 ± 2.06 | 100 ± 7.66 |
1000 ng/mL | 103 ± 3.55 | 107 ± 2.55 |
Autosampler stability (12 h) | 99.2 ± 7.84 | 100 ± 3.59 |

a Values are means ± standard deviation.

Table 3. Pharmacokinetic parameters of acetylsalicylic acid (ASA) and salicylic acid (SA) after single oral administration of Astrix® (enter-coated pellet, 100 mg of aspirin) to 10 Korean healthy male volunteers

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ASA</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC∞ (ng h/mL)</td>
<td>297 ± 87.9</td>
<td>18200 ± 5130</td>
</tr>
<tr>
<td>AUClast b (ng h/mL)</td>
<td>279 ± 83.3</td>
<td>17100 ± 4640</td>
</tr>
<tr>
<td>Terminal half-life (h)</td>
<td>0.649 ± 0.300</td>
<td>2.04 ± 0.200</td>
</tr>
<tr>
<td>Cmax (ng/mL)</td>
<td>170 ± 96.7</td>
<td>3780 ± 865</td>
</tr>
<tr>
<td>Tmax c (h)</td>
<td>3.25 (2–4.5)</td>
<td>4.75 (3–6)</td>
</tr>
</tbody>
</table>

a Values are means ± standard deviation.
b 6 h for ASA and 12 h for SA.
c Median (ranges).

could be due to continuous absorption of ASA from the intestine. The conversion of ASA to SA after single oral administration of Astrix® was rapid and considerable; SA was detected in plasma from the first blood sampling time (1 h) and the AUCSA/AUCASA ratio was 61.3.

Figure 2. Plasma concentration–time profiles of (a) acetylsalicylic acid (ASA; ●) and (b) salicylic acid (SA; ○) after single oral administration of Astrix® (enter-coated pellet, 100 mg as aspirin) to 10 Korean healthy male volunteers. Vertical bars represent standard deviation.
CONCLUSION

In conclusion, this validated method had acceptable accuracy, precision and sensitivity and was successfully applied for the evaluation of pharmacokinetics of ASA and SA after a single oral administration of Astrix® (entero-coated pellet, 100 mg of aspirin) to 10 Korean healthy male volunteers. This is the first reported study of plasma concentration of ASA and SA using LC/MS/MS; it allows a suitable sensitivity of ASA (LLOQ, 5 ng/mL) and can be carried out in a short time (within 3 min for ASA and SA).

Acknowledgments

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