(54) Title: METHOD FOR ANALYZING ASPIRIN IN PLASMA WITH LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Fig. 1

MB2 (179.45) C1 (42): 26 MCA score from Sample 1 (TrainSampleName) of Aspirin_InHepatic_Hepa_m.wff

(57) Abstract: The present invention relates to a method for analyzing aspirin in plasma with liquid chromatography-mass spectrometry, which can stably and easily analyze ASA and SA without loss of their amounts even after sampling.
DESCRIPTION

METHOD FOR ANALYZING ASPIRIN IN PLASMA WITH LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

FIELD OF THE INVENTION

The present invention relates to a method for analyzing aspirin in plasma with liquid chromatography-mass spectrometry.

BACKGROUND OF THE INVENTION

Acetylsalicylic acid (ASA, aspirin) has been widely used as an analgesic, anti-inflammatory, antipyretic and antithrombotic drug. ASA is rapidly hydrolyzed *in vivo* to produce salicylic acid (SA) responsible for the pharmacological activity. Therefore, in order to analyze the amounts of ASA, it is required to determine SA level in blood with liquid chromatography-mass spectrometry (LC-MS).

However, two problems are associated with the high performance liquid chromatography (HPLC) analysis of ASA and SA in biological fluids. Firstly, ASA hydrolyzes to SA in protic solvents such as water or methanol, and also in plasma. ASA in water has the best stability when pH is 2-3, and at pH 7 about 20% of ASA is degraded to SA within a day. Also, since the half-life of ASA is about one hour in plasma at 37°C, the partial degradation of ASA may occur after sampling and before injecting it into the HPLC system. Secondly, a part of SA may be lost by sublimation during sample treatment comprising evaporation step. Therefore, there was a difficulty in determining the accurate amount of ASA by employing HPLC.

Accordingly, a number of methods have been reported for the analysis of ASA to solve these problems.

Frieder Kees *et al* disclose a method for analyzing ASA by way of...
acidifying a sample to adjust its pH in the range of 2 to 3, cooling and storing at -70°C before use, in order to prevent the degradation of ASA in plasma (Frieder Kees et al., Journal of Chromatography B, 677, 172-177 (1996)).

Also, Bouche et al disclose a method for analyzing ASA with ESI(-)-LC-MS/MS (M.P. Bouche et al., Combining selectivity from chromatography and fast ion separation by MS in bioanalysis). However, the method has a long analyzing time, a tailing phenomenon and poor stability due to high pH. Also, the method was not suitable for quantification of human plasma, since the limit of quantification (LOQ) of ASA and SA represents 80 ng/ml and 60 ng/ml, respectively, which means low sensitivity.

Further, Soo Kyung Bae et al and Xiangrong et al disclose methods for analyzing ASA by extraction using a mixture of acetonitrile and aqueous 0.1% formic acid in a ratio of 80:20 (v/v (°)) and 63:37 (v/v (°)), respectively, as a mobile phase. However, both methods fail to completely isolate ASA (Soo Kyung Bae et al, Biomed. chromatogram., 22: 590-595 (2008)); and (Xiangrong et al., Biomed. chromatogram., 23: 973-979 (2009)).

Therefore, the present inventors have endeavored to determine the accurate levels of ASA and SA in plasma, and found a stable and easy method for analyzing ASA and SA in plasma with liquid chromatography-mass spectrometry.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a method for analyzing aspirin (ASA) in plasma with liquid chromatography-mass spectrometry.

In accordance with one aspect of the present invention, there is provided a method for analyzing aspirin in plasma with liquid chromatography-mass spectrometry which comprises the steps of:

1) adding an internal standard material and extracting solvent to a plasma sample, centrifuging the sample, and collecting a supernatant
therefrom;
2) concentrating and dissolving the supernatant in acetonitrile; and
3) subjecting the resulting mixture obtained in step 2) to liquid chromatography-mass spectrometry for a quantitative analysis of aspirin in plasma.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings, which respectively show:

Figs. 1 to 3: MS/MS spectrum results of aspirin, salicylic acid and ibuprofen, respectively; and

Fig. 4: liquid chromatography results for 250 ng/ml of aspirin, 78.125 ng/ml of salicylic acid, and 500 ng/ml of ibuprofen (ISTD).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for analyzing aspirin in plasma with liquid chromatography-mass spectrometry which comprises the steps of: 1) adding an internal standard material and extracting solvent to a plasma sample, centrifuging the sample, and collecting a supernatant therefrom; 2) concentrating and dissolving the supernatant in acetonitrile; and 3) subjecting the resulting mixture obtained in step 2) to liquid chromatography-mass spectrometry for a quantitative analysis of aspirin in plasma.

In step 1) of the present invention, an internal standard material and extracting solvent is added to a plasma sample, and the sample is centrifuged to collect a supernatant. In one embodiment of the present invention, the internal standard material may be ibuprofen, and the extracting solvent may be methyl tert-butyl ether (MTBE) containing 0.5% acetic acid. ASA in plasma has the best stability when pH is 2 to 3, and therefore, MTBE containing 0.5% acetic
acid used as the extracting solvent is useful in maintaining a certain pH level, thereby achieving the stability of ASA.

In step 2) of the present invention, the supernatant may be dried and concentrated. The dried concentrates may be dissolved in acetonitrile. In one embodiment of the present invention, the dried concentrates may be dissolved in 50% acetonitrile.

In step 3) of the present invention, the resulting mixture obtained in step 2) may be subjected to liquid chromatography-mass spectrometry for a quantitative analysis of aspirin in plasma. In one embodiment of the present invention, the plasma sample obtained in step 2) may be introduced to the liquid chromatography (LC) column, and mass spectrophotometer (MS). The plasma sample applied to MS may be ionized and quantified for analyzing the amount of ASA in plasma.

In one embodiment of the present invention, a mobile phase used in LC may be a mixture of acetonitrile and formic acid, preferably, a mixture of acetonitrile and 0.01% formic acid in a ratio ranging from 65 : 35 to 75 : 25 (v/v (%)), preferably, 70 : 30 (v/v (%)).

In MS, the plasma sample may be ionized in a negative ionization mode, and quantified in a multiple reaction monitoring (MRM) mode.

MS/MS spectrum results of ASA, SA and ibuprofen quantified in the MRM mode are shown in Figs. 1 to 3, respectively.

As shown in Fig. 1, MS/MS spectrum of ASA quantified in MRM mode represents that Q1 is about 179 (m/z), and Q3 is about 137 and 93 (m/z).

As shown in Fig. 2, MS/MS spectrum of SA quantified in MRM mode shows that Q1 is about 137 (m/z), and Q3 is about 93 and 65 (m/z).

As shown in Fig. 3, MS/MS spectrum of ibuprofen quantified in MRM mode demonstrates that Q1 is about 205 (m/z), and Q3 is about 161 (m/z).

The following Examples are intended to further illustrate the present invention without limiting its scope.
Example 1: Construction of calibration curves

Standard samples of ASA and SA were prepared by sequentially diluting standard solutions of 100 µg/mL of ASA and 1 mg/mL of SA with a dog's blank plasma, respectively, giving final concentrations of 2 to 1,000 ng/mL for ASA and 20 to 10,000 ng/mL for SA. 20 µL of the standard samples of different concentration levels were taken to each test tube with a cap. 50 µL of internal standard material (ibuprofen 500 ng/mL) was added to the test tube followed by adding 1 mL of methyl tert-butyl ether containing 0.5% acetic acid thereto. The mixture was centrifuged at 1,400 rpm for 10 min. A supernatant was collected, and then dried and concentrated at 35°C using a vacuum dryer. The dried concentrates were dissolved in 500 µL of 50% acetonitrile (ACN) and subjected to the liquid chromatography-mass spectrometry (LC-MS) as follow.

The sample thus obtained was applied to LC with the following conditions.

<LC conditions>

System          HP1 100 series (Agilent Co., USA)
Column         xterra MS C18 (diameter 2.1 mm x length 50 mm, particle size 3.5 µm, Waters)
Column temperature    30°C
Mobile phase     ACN/0.01% formic acid= 70/30 (v/v (%))
Flow rate       200 nL/min
Injection volume 5 µL
Analysis time    5 min.

Then, the sample which passed through the LC column was applied to MS with the following conditions, and ionized in the negative ionization mode and then quantified in MRM mode as shown in Table 1.

<MS conditions>

System          API 4000 Qtrap (Applied Biosystems/MDS
Ionization mode: Turbo ion spray ionization mode (negative)

Curtain gas (CUR): 15 psi

Collision gas (CAD): Medium

Ion voltage: -4500V

GS 1: 50 psi

GS 2: 40 psi

Turbo gas temperature: 450°C

CUR, CAD, GS (1, 2): Nitrogen.

### Table 1

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Q1/Q3 (m/z)</th>
<th>DP (V)</th>
<th>EP (V)</th>
<th>CE (V)</th>
<th>CXP (V)</th>
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<tbody>
<tr>
<td>ASA (for quantification)</td>
<td>179.0/93.0</td>
<td>-25</td>
<td>-10</td>
<td>-30</td>
<td>-15</td>
</tr>
<tr>
<td>ASA (for identification)</td>
<td>179.0/137.0</td>
<td>-25</td>
<td>-10</td>
<td>-23</td>
<td>-10</td>
</tr>
<tr>
<td>SA (for quantification)</td>
<td>137.0/92.7</td>
<td>-55</td>
<td>-10</td>
<td>-28</td>
<td>-9</td>
</tr>
<tr>
<td>SA (for identification)</td>
<td>137.0/64.9</td>
<td>-55</td>
<td>-10</td>
<td>-40</td>
<td>-9</td>
</tr>
<tr>
<td>Ibuprofen (ISTD)</td>
<td>205.0/161.0</td>
<td>-50</td>
<td>-10</td>
<td>-10</td>
<td>-10</td>
</tr>
</tbody>
</table>


Calibration curves were constructed based on the ratio of peak areas for ASA and SA to those for the internal standard material, and calculated by a weighted \((1/x)\) least squares regression. LC chromatograms for 250 ng/ml of ASA, and 78.125 ng/ml of SA are shown in Fig. 4.

**Example 2:** Sample analysis

A blood sample was collected from a dog administered with aspirin, and stored at -70°C. The sample was thawed at room temperature, well mixed in a desktop mixer for 0.5 min. 20 µL of the resulting sample was transferred into a
test tube with a cap. 50 µl of the internal standard material (ibuprofen 500 ng/mL) was added to the test tube followed by adding 1 mL of methyl tert-butyl ether containing 0.5% acetic acid thereto. The mixture was centrifuged at 1,400 rpm for 10 min. A supernatant was collected, and then dried and concentrated at 35 °C using a vacuum dryer. The dried concentrates were dissolved in 500 µL of 50% acetonitrile and subjected to the LC-MS according to the procedure described in Example 1. Quantification of ASA and SA in plasma was performed using the calibration curves constructed in Example 1.

As shown in the above, the method for analyzing aspirin in plasma with LC-MS according to the present invention can stably and easily analyze ASA and SA without loss of their amounts even after sampling.

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes may be made to the invention by those skilled in the art which also fall within the scope of the invention as defined by the appended claims.
WHAT IS CLAIMED IS:

1. A method for analyzing aspirin (ASA) in plasma with a liquid chromatography-mass spectrometry (LC-MS) which comprises the steps of:
   1) adding an internal standard material and extracting solvent to a plasma sample, centrifuging the sample, and collecting a supernatant therefrom;
   2) concentrating and dissolving the supernatant in acetonitrile; and
   3) subjecting the resulting mixture obtained in step 2) to liquid chromatography-mass spectrometry for a quantitative analysis of aspirin in plasma.

2. The method of claim 1, wherein the internal standard material is ibuprofen.

3. The method of claim 1, wherein the extracting solvent is methyl tert-butyl ether (MTBE) containing 0.5% acetic acid.

4. The method of claim 1, wherein the liquid chromatography is performed with a mobile phase which is a mixture of acetonitrile : 0.01% formic acid in a ratio ranging from 65 : 35 to 75 : 25 (v/v(%)).

5. The method of claim 4, wherein the liquid chromatography is performed with a mobile phase which is a mixture of acetonitrile : 0.01% formic acid in a ratio of 70 : 30 (v/v(%)).
Fig. 2
Fig. 3

[Diagram of a chemical structure and a graph with m/z and intensity values]
Fig. 4

XIC of NRM (6 pairs): 179.0/93.0 amu from Sample 12 (250) of calibration-ASA, wiff (Turbo Spray)

Max. 1.0e4 cps.

ISTD

Salicylic acid

Aspirin

Time, min

Intensity, cps

9.9e-4 9.0e-4 8.0e-4 7.0e-4 6.0e-4 5.0e-4 4.0e-4 3.0e-4 2.0e-4 1.0e-4