Human serum albumin binding of 3, 5, 6, 7, 8, 3’, 4’-heptamethoxyflavone, a citrus flavonoid possessing a neuroprotective effect

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Abstract

3, 5, 6, 7, 8, 3’, 4’-heptamethoxyflavone (HMF), which is present in citrus fruits, has been reported to induce brain-derived neurotropic factor (BDNF) production, and have an anti-inflammatory effect. However, its pharmacokinetics is obscure. Therefore, as the first study of HMF pharmacokinetics, the reversible binding of HMF to human serum albumin (HSA) has been examined. For the binding examination and further pharmacokinetic study of HMF, a simple HPLC assay method was established first. The HPLC system equipped with a UV detector (HPLC-UV) and an isocratic mobile phase were used. The accuracy of intra-assay validation at each concentration from 1 to 100 µM was from 97.2 to 101.6%, and the precision of intra-assay validation was less than 1.60%. For inter-assay validation, the accuracy was from 97.1 to 104.5%, and the precision was less than 2.24% from 1 to 100 µM of HMF. The reversible binding of HMF to HSA was performed by the equilibrium dialysis method. The bound fraction of HMF to 4.6% HSA decreased from around 70% to 55% as the total concentration of HMF increased. This concentration dependency of the reversible binding suggests that HMF may have a specific binding site on the HSA molecule. The HPLC method established in this study is now being used for further investigation of HMF pharmacokinetics, such as intestinal absorption.

Key Words: heptamethoxyflavone, human serum albumin, protein binding, citrus fruits, HPLC, assay validation

Area of Interest: Information and computing approach for drug design and ADMET study
1. Introduction

Natural products have several functions, which are not only pharmacological activities, but also fascinating characteristics of ADME (absorption, distribution, metabolism, and excretion). For example, phloridzin and phloretin, which are contained in an apple, inhibit not only Na\(^+\)/glucose cotransporter 1 (SGLT1) but also the intestinal glucuronidation metabolism to increase the intestinal absorption of phenolic compounds [1]. Polyphenols contained in green teas also inhibit the intestinal glucuronidation metabolism to increase the intestinal absorption of phenolic compounds [2].

3, 5, 6, 7, 8, 3', 4'-heptamethoxyflavone (HMF) (Figure 1) is contained in Kawachi Bankan, a special citrus fruit (a local product of Ehime prefecture in Japan), has been suggested to have a neuroprotective effect after brain ischemia by inducing neurotrophic factor (BDNF) production and anti-inflammatory effects in mice [3-5]. Although HMF has been shown such activities, its pharmacokinetics remains unclear. Therefore, as the first study of HMF pharmacokinetics, the reversible binding of HMF to human serum albumin (HSA) has been examined. For the binding examination and further pharmacokinetic study of HMF, a simple HPLC assay method was established as well.

![Figure 1. Structure of 3, 5, 6, 7, 8, 3’, 4’-heptamethoxyflavone (HMF)](image_url)

2. Materials and Methods

2.1 Chemicals and reagents

Flavone, acetonitrile (HPLC grade), and albumin from human serum (HSA, 019-10503) were purchased from Wako Pure Chemical Industries (Osaka, Japan). HMF was kindly supplied by USHIO Chemix, Co Ltd. (Shizuoka, Japan). Other chemicals were of reagent grade.

2.2 HPLC assay

The HPLC system (Jasco, Tokyo, Japan) consisted of a pump (PU-2089), a UV detector (UV-2075) with an integrator (807-IT), an automated sampler (AS-2057), and a column (Mightysil RP-18, 6.0 mm i.d., 150 mm length, Kanto Chemicals, Tokyo). The mobile phase consisted of 50% acetonitrile and 0.05% phosphoric acid in water, and the flow rate was 1.5 ml/min. A UV spectrum of HMF, dissolved in the mobile phase, was obtained with a UV-visible spectrophotometer (V-630, Jasco, Tokyo, Japan).
2.3 Reversible binding experiment

Reversible binding of HMF to HSA was performed with the equilibrium dialysis method using a semi-permeable membrane (Spectra/Pore 2, 12000-14000 Da of molecular weight cut-off, Spectrum Laboratories Inc, California, USA). One milliliter of HMF solution, dissolved in a modified Krebs-Ringer bicarbonate phosphate buffer (pH 7.4) [6], was added to a chamber on one side. One milliliter of 4.6% HSA (albumin concentration in vivo) was added to a chamber on the other side. The equilibrium dialysis apparatus was incubated for 20 h at 37 ºC with 100 oscillations/min in an incubator (BR-40LM, TAIITEC, Koshigaya, Japan). One hundred microliters of solution was sampled from each chamber, and mixed with 10 μL of 60% perchloric acid in a siliconized microtube. Then, 40 μL of 2.5 mM flavone (internal standard) solution, dissolved in 25% acetonitrile and 5.6% DMSO in purified water, and 50 μL of acetonitrile, was added into the microtube. After centrifugation of the mixture at 10,000 rpm (desktop microfuge MC-150, TOMY, Tokyo, Japan) for 5 min, the resultant supernatant was used for the HPLC assay.

2.4 Data analysis

A weighted linear regression analysis of the HPLC assay was performed, and the accuracy and precision (coefficient of variance) were calculated using the following equations:

\[
\text{Accuracy} (%) = \left( \frac{\text{Observed value}}{\text{Theoretical value}} \right) \times 100
\]

(1)

\[
\text{Precision} (%) = \left( \frac{\text{Standard deviation}}{\text{Mean}} \right) \times 100
\]

(2)

Bound fraction \((f_b)\) was calculated according to equation 3:

\[
f_b (%) = \left( \frac{C_b}{C_t} \right) \times 100
\]

(3)

where Cb and Ct represent the concentration of HMF bound to HSA, and the total concentration of HMF, respectively.

3. Results and Discussion

3.1 HPLC assay condition

A UV spectrum of HMF, dissolved in the mobile phase, showed a local maximum wavelength around 342 nm (Figure 2a). An HPLC chromatogram of HMF and flavone (internal standard) detected at 342 nm showed that the retention times of HMF and flavone were at approximately 7.5 and 9 min (Figure 2b), respectively.

3.2 Validity of the HPLC assay

This assay condition was validated. A typical calibration curve of HMF is shown in Fig. 1c and was analyzed with three kinds of weight conditions (a unit, an inverse of the concentration, and an inverse square of the concentration). A calibration curve of HMF was analyzed with three kinds of weight conditions (a unit, an inverse of the concentration, and an inverse square of the concentration). Table 1 shows the precision (coefficient of variance, CV) at each concentration,
which was calculated under the various weight conditions. Among these conditions, the mean of precision was smallest when calculated with the inverse square of the concentration. In addition, the accuracy under the respective weight conditions, a unit, an inverse of the concentration, and an inverse square of the concentration, were from 78.1 to 102.2%, from 94.1 to 103.4%, and 97.1 to 104.5%, respectively. This indicates that the accuracy under the weight condition of an inverse square of the concentration is closest to 100%. Therefore, the calibration curve was analyzed with the weight of the inverse square of the concentration in the HPLC assay.

The accuracy and precision of intra-assay and inter-assay validation are also summarized in Table 2. The accuracy of intra-assay validation at each concentration from 1 to 100 μM was from 97.2 to 101.6%, and the precision of intra-assay validation was less than 1.60 (1.595)%%. For inter-assay validation, the accuracy was from 97.1 to 104.5%, and the precision was less than 2.24%. These values clearly indicate that the present method of HPLC assay is applicable to HMF determination.

Figure 2a-c. UV spectrum (a), HPLC chromatogram (b), and calibration curve of the HPLC assay (c) of HMF
Assay conditions: column, Mightysil RP-18 (6.0 mm i.d., 150 mm length); mobile phase, 50% acetonitrile and 0.05% phosphoric acid in water; flow rate of mobile phase, 1.5 L/min; UV detector, 342 nm; IS, internal standard: flavone.
Table 1. Influence of weight on precision (%) in HPLC assay (n=5)

<table>
<thead>
<tr>
<th>Concentration μM</th>
<th>Weight 1</th>
<th>Weight 1/C</th>
<th>Weight 1/C²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.89</td>
<td>1.40</td>
<td>0.752</td>
</tr>
<tr>
<td>2</td>
<td>2.70</td>
<td>1.40</td>
<td>1.60</td>
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<tr>
<td>5</td>
<td>1.04</td>
<td>1.08</td>
<td>0.902</td>
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<tr>
<td>10</td>
<td>0.454</td>
<td>0.494</td>
<td>0.785</td>
</tr>
<tr>
<td>20</td>
<td>0.873</td>
<td>0.954</td>
<td>0.593</td>
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<tr>
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<td>0.805</td>
<td>0.762</td>
<td>1.15</td>
</tr>
<tr>
<td>100</td>
<td>0.180</td>
<td>0.523</td>
<td>0.577</td>
</tr>
<tr>
<td>Mean</td>
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<td>0.946</td>
<td>0.908</td>
</tr>
<tr>
<td>SD</td>
<td>1.36</td>
<td>0.377</td>
<td>0.360</td>
</tr>
</tbody>
</table>

Table 2. HPLC assay validation of HMF

<table>
<thead>
<tr>
<th>Concentration μM</th>
<th>Intra-assay (n=5)</th>
<th>Inter-assay (3 days)</th>
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<tbody>
<tr>
<td></td>
<td>Mean μM</td>
<td>Accuracy %</td>
</tr>
<tr>
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<td>99.4</td>
</tr>
<tr>
<td>2</td>
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<td>101.3</td>
</tr>
<tr>
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<td>99.5</td>
</tr>
<tr>
<td>100</td>
<td>97.2</td>
<td>97.2</td>
</tr>
</tbody>
</table>

3.3 Reversible binding of HMF to HSA

The reversible binding of HMF to 4.6% HSA is shown in Figure 3. The bound fraction was HMF concentration dependent, and decreased from around 70% to 55% as the HMF total concentration increased. This result suggests that the binding of HMF to HSA consists of specific (saturable) binding and non-specific (non-saturable) binding.

Plasma protein binding of a drug is one of the determinants of pharmacokinetics and pharmacodynamics because an unbound drug in the blood can permeate plasma membranes to bind to a constituent triggering a pharmacological effect [7]. The result obtained in this study indicates that the reversible binding of HMF to serum albumin is not so high as to strongly prevent the distribution and pharmacological action of HMF in humans.
4. Conclusion

A simple HPLC method for the determination of HMF was established to be applicable for pharmacokinetic study. The accuracy and precision of intra- and inter-assay validation were satisfactory. The mobile phase was isocratic and the running time per assay was within 12 min. The HPLC assay of HMF was used to study the human serum albumin binding of HMF, and showed that the bound fraction of HMF to 4.6% HSA was around 55-70%. The concentration dependency of the reversible binding suggests that HMF may have a specific binding site on the HSA molecule. Further study of HMF pharmacokinetics, such as intestinal absorption, which requires quantitative analysis, is now ongoing using the present HPLC method.

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References


