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**HPLC Plasma Assay of a Novel Anti-MRSA Compound, Kaempferol-3-O-Alpha-L-(2",3"-di-p-coumaroyl)hhamnoside, from Sycamore Leaves**

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious pathogen that is resistant to current antibiotic therapy. Thus, there is an urgent need for novel antimicrobial agents that can effectively combat these new strains of drug-resistant “superbugs”. Recently, fractionation of an extract from *Platanus occidentalis* (American sycamore) leaves produced an active kaempferol molecule, 3-O-alpha-L-(2",3"-di-p-coumaroyl)hhamnoside (KCR), in four isomeric forms; all four isomers exhibit potent anti-MRSA activity. In order to further the preclinical development of KCR as a new antibiotic class, we developed and validated a simple analytical method for assaying KCR plasma concentration. Because KCR will be developed as a new drug, although comprising four stereoisomers, the analytical method was devised to assay the total amount of all four isomers. In the present work, both a plasma processing procedure and an HPLC method have been developed and validated. Mouse plasma containing KCR was first treated with ethanol and then centrifuged. The supernatant was dried, suspended in ethanol, centrifuged, and the supernatant was injected into an HPLC system comprising a Waters C18, a mobile phase composed of methanol, acetonitrile, and trifluoroacetic acid and monitored at 313 nm. The method was validated by parameters including a good linear correlation, a limit of quantification of 0.27 µg/mL, and high accuracy. In summary, this method allows a rapid analysis of KCR in the plasma samples for pharmacokinetics studies.

**Keywords:** Methicillin-resistant *Staphylococcus aureus* (MRSA), American sycamore extract, HPLC method validation.

Hemophilic-resistant *Staphylococcus aureus* (MRSA) is a serious pathogen that can resist conventional antibiotic therapy. MRSA causes patient mortality and extends hospital stays, resulting in higher healthcare costs; MRSA alone is estimated to kill around 19,000 people every year in the U.S. [1, 2]. Further, MRSA has recently emerged as a community-associated pathogen (CA-MRSA), infecting individuals who have not been hospitalized or undergone a medical procedure within the last year [3]. MRSA is also known to be a common skin colonizer and causes the same spectrum of infections as methicillin-susceptible *S. aureus* (MSSA) infections. Over 50% of *Staphylococcus* cases around the world are known to be caused by MRSA [4].

Currently, vancomycin is widely used as the drug of choice for MRSA infections even though it is only successful in 35-57% of the infections [5]. Vancomycin has to be administered by i.v. injection/infusion, which may require prolonged hospitalization. Oral antibiotic choices for MRSA include trimethoprim-sulfamethoxazole (TMP-SMX), clindamycin, doxycycline, minocycline, linezolid, and rifampin. TMP-SMX is a sulfonamide antibiotic used as the initial antibiotic for CA-MRSA infection, but it is associated with several potentially serious adverse reactions and is not suitable for general use [6]. Clindamycin is another antibiotic frequently used as an initial therapeutic option, but resistance to clindamycin can develop rapidly [7,8]. Doxycycline and minocycline also have been reported to be effective antibiotics for MRSA/CA-MRSA [9, 10]. Linezolid has been used in central nervous system (CNS) MRSA infections and has similar efficacy as vancomycin. Its oral bioavailability allows oral administration thus decreasing hospital stays and therapy costs [11]. Rifampin has been used in combination with other antibiotics, such as TMP-SMX, clindamycin, or doxycycline/minocycline since resistance may develop rapidly if it is used as monotherapy [12]. However, the most effective anti-MRSA drug used currently is daptomycin, a cyclic lipopeptide derived from *Streptomyces roseosporus*. Daptomycin is useful in treatments of short duration and persistent MRSA un-affected by other drug treatments [13]. Its mechanism of action involves binding to the bacterial cell membrane, which causes depolarization of the membrane potential leading to inhibition of bacterial growth. Unfortunately, the FDA has reported serious side effects for daptomycin, including an increase in blood creatine phosphokinase, rhabdomyolysis, skin exfoliation and skin ulcers. Thus, there are great and urgent needs for novel antimicrobial agents that can effectively combat these new strains of drug-resistant “superbugs” [14]. One of the most promising sources of anti-MRSA agents is from natural products.

Currently, 60-75% of pharmaceutical sales for infectious diseases and cancer are from products derived from natural sources and approximately 60% of those compounds commercially available or in the late stages of clinical trials for the treatment of infectious diseases or cancer are of natural product origin [15]. Rapid emergence of multi drug-resistant microbial pathogens is a serious problem to US human health.

*Platanaceae* is a family of flowering plants, which has a long history of use in traditional folk medicines as antimicrobial and antiseptic herbal remedies. *Platanus occidentalis*, also known as American sycamore, is one of the species of *Platanus* native to North America [16]. Sycamore material has been widely used in production of antimicrobial agents.
folk medicine [17]. Native Americans used sycamore for various medicinal purposes, including cold and cough remedies, as well as dermatological, respiratory and gastrointestinal aids. Sycamore bark is currently taken as a dietary aid to gain weight and as an analgesic for internal pains. Sycamore extracts have also been used in cosmetic preparations and as an antidiarrheal for dysentery [18]. The extensive human contact and use of this plant has led to the classification of sycamore material as generally regarded as safe (GRAS) by the US FDA.

American sycamore is a native US species already in culture for timber and pulp production. As a result, leaf material for drug production will be highly scalable. In a recent study, Ibrahim et al. isolated a group of glycosides from the leaf extract of American sycamore [5]. Fractionation of the extract produced an active kaempferol molecule, 3-O-alpha-L-(2",3"-di-p-coumaroyl)-rhamnoside (KCR), in four stereoisomeric forms (EE, EZ, ZE, and ZZ) (Figure 1). All four isomers exhibit potent anti-MRSA activity, both in cultures and in animal models [5,19]. Active metabolites of these anti-MRSA extracts are likely to possess a novel mechanism of action as they do not show cross-resistance or structural homology with established antibiotics. This represents the first plant-derived antibiotics to prevent growth of MRSA both on surfaces and systemically.

![Figure 1: Structures of the four stereoisomers of KCR](image)

In order to further the preclinical development of KCR as an antibiotic for MRSA, we have developed and validated an analytical method suitable for assaying plasma concentration of KCR. Because KCR will be developed as a new antibiotic in that all four isomers are active, the analytical method was developed to assay the total amount of all four isomers.

**Experimental**

**Instruments:** A Waters Alliance HPLC system [including a Waters 2690 Separations Module, a Waters 2996 Photodiode Array Detector, a Waters C18 column, and the Waters Empower software] (Milford, Massachusetts, USA) was used in this study for the determination of KCR. A Beckman Coulter Allegra 21R Centrifuge (Fullerton, California, USA) was employed to prepare the plasma and plasma samples for HPLC injection, and an Eppendorf Vacufuge™ to evaporate the solvent during the sample prep.

**Reagents:** KCR (HPLC peak purity >99%) was extracted and purified as previously described [5]. Individual stereoisomers (KCR in EE, EZ, ZE, and ZZ forms) were provided by Dr Jim McChesney (Ironstone Separations, Inc., MS). All solvents were HPLC grade and obtained from Burdick & Jackson (Muskegon, Michigan, USA).

**Preparation of control plasma samples:** Control mouse blood samples were collected by cardiac puncture using a 1 mL syringe (with a 25-gauge needle) containing heparin as anticoagulant. Once the maximum amount of blood was collected, it was transferred to a 1.5 mL conical centrifuge tube and centrifuged at 14,000 rpm (equivalent to 19,280 g) at 4°C for 15 min to separate plasma and red blood cells (RBC). Plasma was transferred and stored in a freezer at -20°C. To prepare individual spiked standards, 50 µL of KCR stock solutions in ethanol were added individually to 200 µL of plasma samples, and 800 µL of ethanol was then added to each vial. Each aliquot was vortexed for 10 sec and centrifuged at 14,000 rpm for 15 min at room temperature. Each supernatant was removed and collected in a 1.5 mL conical Eppendorf® tube and evaporated to dryness employing an Eppendorf Vacufuge® at 45°C. The residue was reconstituted in 200 µL of ethanol, centrifuged, and the supernatant was injected (10 µL injection) into the HPLC system. To prepare a plasma control, 4x of ethanol (v/v) was added to each plasma sample, and processed in the same way as the spiked plasma standards before HPLC analysis.

**HPLC procedure:** KCR in the eluted fraction was determined using a Waters Nova-Pak® C18 reversed-phase column (3.9 x 300 mm) with a 4 µm particle size. The injection volume was 10 µL; the column oven was at room temperature; the mobile phase was methanol/acetonitrile/aqueous formic acid (0.3%), 60/3/37, v/v, at a flow rate of 0.75 mL/min. Detection was made at 313 nm. The results of the analysis were expressed in µg of KCR per mL of plasma.

**Calibration curve:** The calibration curve was obtained with mouse plasma samples spiked with known amounts of KCR stock solution in ethanol, and each analyte was extracted according to the protocol described above. The calibration curve was obtained by plotting the peak area against its concentration in 5 spiked plasma standards (18.75, 6.25, 2.5, 1.25, 0.25 µg/mL) and performing a linear regression analysis.

During the method development, we tried methanol and acetonitrile for sample preparation, but the recovery percentages were low; ethanol yielded better results in extracting KCR from plasma samples.

The data gathered allow detection of KCR over a 100-fold range of concentrations. The method developed here was validated according to the U.S. Pharmacopoeia [20] and the analytical performance parameters considered were specificity (selectivity), linearity, accuracy, precision, sensitivity, stability and ruggedness; the analytical details are shown below:

1. **Specificity (selectivity):** The absorption spectrum of KCR showed a maximum absorption at 313 nm and was chosen for quantitative analysis; there was no interfering peak from plasma. A typical chromatogram of KCR at 313 nm (Figure 2) consisted of a group of four peaks, which were identified as EZ, ZZ, EE, and ZE, respectively by comparing with individual stereoisomeric standards provided by Dr McChesney. The use of a UV photodiode array detector allowed the confirmation of the identity for KCR both by their retention times (Figure 2) and UV spectra.

2. **Linearity:** A calibration curve of KCR was prepared to determine the linearity of the method over the range of 0.25 – 18.75 µg/mL. This range was chosen as previous in vitro studies have indicated a mean inhibitory concentration (MIC) in the order of 1 – 4 µg/mL for KCR. Integrated peak areas
A simple method to determine KCR in plasma

References


Table 1: Ruggedness of the method and sample stability.

<table>
<thead>
<tr>
<th>Flow Rate (mL/min)</th>
<th>Day 0</th>
<th>Day 0, night (intraday)</th>
<th>Day 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75</td>
<td>Control</td>
<td>100.3</td>
<td>99.2</td>
</tr>
<tr>
<td>0.65</td>
<td></td>
<td>100.3</td>
<td>98.2</td>
</tr>
<tr>
<td>0.80</td>
<td></td>
<td>100.3</td>
<td>99.7</td>
</tr>
</tbody>
</table>

Ruggedness and stability are shown in % recovery.

In summary, we have successfully developed a simple method to determine KCR in plasma. We have shown that each of the parameters discussed above provides a good basis for further biological evaluation of KCR and the method described herein is suitable for analyzing plasma samples of KCR. The method is useful for future studies to evaluate both the pharmacokinetics and therapeutic efficacy of KCR in infected mice.

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