Determination of Rottlerin in Pancreatic Cancer Cells and Mouse Xenografts by RP-HPLC Method

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Abstract

Rottlerin is a natural polyphenolic ketone isolated from the pericarps of Mallotus philippinensis. In previous studies we showed that parenteral administration of rottlerin reduced tumor growth in murine xenograft models of pancreatic cancer. The aim of this study was to develop a simple and validated method for the quantitative determination of rottlerin in plasma and tumor tissues of mice fed a rottlerin diet. A xenograft model of pancreatic cancer was prepared by injection of 2×10⁶ HPAF-II cells subcutaneously into nude mice. One week before tumor implantation, mice were randomly allocated to standard diet (AIN76A) and standard diet supplement with 0.012% rottlerin (n=6 per group). Mice were sacrificed after 6 weeks on diets. Rottlerin was extracted from the plasma and tissues using protein precipitation-extraction and analyzed by reverse-phase HPLC-DAD method. The same HPLC method was also applied to determine rottlerin levels in conditioned culture media and in cell lysates from HPAF-II cells exposed to 25 μM concentration of rottlerin. A substantial amount of rottlerin was detected in tumor (2.11 ± 0.25 nmol/g tissue) and plasma (2.88 ± 0.41 μM) in mice fed rottlerin diet. In addition, significant levels of rottlerin (57.4 ± 5.4 nmol/mg protein) were detected in cell lysates from rottlerin-treated HPF-A-II cells. These data indicate that rottlerin is efficiently absorbed in cells and tissues both in vivo and in vitro and suggest a strong potential for rottlerin as a preventive or adjuvant supplement for pancreatic cancer.

Keywords: Rottlerin; Tissue distribution; HPLC; Pancreatic cancer; In vivo; Cell uptake

Introduction

Rottlerin (1-[6-[(3-acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2H-1-benzopyran-8-yl]-3-phenyl-2-propen-1-one, also known as mallotoxin) is a natural polyphenolic ketone (Figure 1) isolated from the pericarps of Mallotus philippinensis (common names Monkey-puzzle, Monkey Face Tree, Kamala Tree). It is a traditional Indian medicine that is used against tapeworm, scabies, and herpetic ringworm. Recent scientific research has demonstrated that rottlerin has a range of molecular targets and anti-tumor activities, such as cell growth suppression [1], apoptosis [2], anti-angiogenesis [3] and inhibition of reactive oxygen species formation [4]. Rottlerin is most well-known as an inhibitor of protein kinase C (PKC) with selectivity for PKC δ [5]. It is also a mitochondrial uncoupler that depolarizes the mitochondria membrane potential, reduces cellular ATP levels and activates 5'-AMP activated protein kinase (AMPK) and affects the mitochondrial production of reactive oxygen species [6,7]. Moreover, rottlerin can target many key regulatory kinases including p38 regulated / activated kinase, cAMP-dependent protein kinase, casein kinase II, glycogen synthase, kinase 3-beta, AKT/PKB, and calmodulin-dependent kinases [8].

Our research team demonstrated recently that rottlerin at concentration range of 2.5-10 μM has potent proapoptotic and antitumor activities in pancreatic cancer in vitro, which is mediated by disrupting the interaction between prosurvival Bcl-2 proteins and proapoptotic BH3-only proteins [2]. Importantly, we showed rottlerin significantly reduced tumor growth in both pancreatic cancer subcutaneous and orthotopic xenograft murine models [2]. Thus rottlerin may represent a promising novel agent for the treatment of pancreatic and other cancers. However, the bioavailability of this natural compound in experimental animals has not been reported. The aims of this study were to develop a simple and validated HPLC-UV method for the quantitative determination of rottlerin in plasma, liver, pancreas and tumor tissues of mice fed with a rottlerin diet, and for the examination of rottlerin uptake by cultured pancreatic cancer cells after cells were exposed to rottlerin.

Materials and Methods

Reagents and chemicals

Rottlerin (96% purity), butyldihydroxytoluene (BHT), ascorbic...
acids and β-glucuronidase/sulfatase (type H-5 from Helix Pomatia) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Internal standard 3', 4', 6'-trihydroxyflavone (97% purity) was purchased from Indotine (Hillsborough, NJ, USA). All solvents used were HPLC grade from Fisher Scientific (Pittsburg, PA, USA).

**Sample preparation**

For the analysis of rottlerin in plasma, 10 μL of 3', 4', 6'-trihydroxyflavone (IS, 100 μM), and 200 μL acetone containing 1% of BHT were added to 100 μL of plasma. The resulting mixture was thoroughly vortex-mixed for 2 min and then centrifuged at 1,300×g for 5 min. The precipitate was extracted one more time with acetone.

**Stability of rottlerin in the diet**

Rottlerin (n=6 per group) was diluted with ethanol to obtain calibration standards at 0.5, 1, 3, 6 and 24 h during cell incubation with rottlerin. Aliquots were subjected to HPLC analysis. The precipitate was dried, weighed and dissolved in protein lysis buffer for protein concentration measurement using 2D Quant kit (Amersham, Piscataway, NJ, USA).

**Method validation**

The specificity was evaluated by analyzing the chromatograms of blank plasma samples from six mice for possible interferences at the retention time of rottlerin and the IS. The analytical response was expressed as rottlerin to IS peak area ratios versus concentrations. The limit of detection (LOD) in plasma and tissues was defined as the lowest concentration resulting in a signal-to-noise ratio of 3:1. The intra-day and inter-day precision and accuracy were determined by replicative analysis of three QC samples at concentrations of 200, 2000 and 8000 ng/mL for rottlerin on the same day and on three consecutive validation days. The stability was analyzed by comparing the chromatograms of control plasma to make rottlerin at concentrations of 200, 2000 and 8000 ng/mL. All solutions were stored at -20°C.

**Animals and diets**

Animal studies were approved by the Chancellor’s Animal Research Committee of the University of California, Los Angeles, in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Six-week-old male athymic nude mice (Charles River Laboratories, San Diego, CA, USA) were used for the subcutaneous xenograft model of pancreatic cancer as described earlier [9]. Briefly, one week before tumor implantation, mice were randomly assigned to a control group fed with standard diet (AIN-76A, Dyets, Bethlehem, PA, USA) and to a rottlerin group fed with standard diet supplement with 0.012% w/w of rottlerin (n=6 per group). Stability of rottlerin in the diet was analyzed at day 0 (as control), 1, 2 and 3 by HPLC following vigorous extraction with ethyl acetate containing BHT as an antioxidant. Diet was replaced every 2 or 3 days based on the stability analysis. After one week on diets, each mouse was inoculated in the right flank region by subcutaneous injection with the human pancreatic tumor cell line HPAF-II (American Type Culture Collection, Manassas, VA, USA, # CRL-1997; 2×10^5 HPAF-II cells suspended in 0.2 mL of culture medium). After incubation, cells and conditioned media were collected and analyzed as indicated in the following sections.
days, respectively. The intra-and inter-day precisions were expressed by the relative standard deviation (% RSD), while the relative error was used to evaluate the accuracy. The extraction recovery was determined by comparing the ratio of the analyte peak areas of the extracted QC samples with the standard solutions of the same concentration.

**Statistical analysis**

Descriptive statistics, such as mean and SD, were used to summarize the results. Data were analyzed by paired student t-test. Statistical significance was defined by p-value of 0.05.

**Results and Discussion**

Understanding the absorption, distribution, metabolism of a bioactive compound is important for its application as a potential chemopreventive or therapeutic agent. Extensive experimental evidence has demonstrated the correlations between tumor size and levels of the bioactive compounds found in tumor in various animal models [10,11]. To the best of our knowledge, pharmacokinetics and tissue bioavailability studies that relate efficacy and toxicity have not been carried out for rottlerin. In this paper, we describe the development of an analytical methodology which would allow the quantitative analysis of rottlerin in the mouse plasma, tissues and in pancreatic cancer cells.

Plant phenolic compounds are often found in the plasma and tissues of animals as the conjugates of glucuronide and sulfate of the parent compound. Particularly in plasma, the conjugates may be the predominant form [9,12,13]. Therefore, we first treated plasma and tissue samples by adding β-glucuronidase/sulfatase to hydrolyze glucuronide and sulfate conjugates. After the incubation and liquid-liquid extraction, we found the samples with added β-glucuronidase/sulfatase did not produce a higher peak area by HPLC analysis in comparison to the samples without enzyme treatment, suggesting that rottlerin may not form conjugates as other phenolic compounds. Therefore, we used protein precipitation-extraction with acetone and determined the levels of rottlerin in plasma and tissues.

Polyphenolics are known to be more stable in acidic condition. We tested the short-term (0.5, 1, 1.5 and 24 h) stability of rottlerin at pH 5.0, 7.4 and 9.4 under room temperature or 37°C. Results from the HPLC analysis showed that rottlerin, similar to the other polyphenolics, is more stable at lower pH and temperature conditions (data not shown). Water with 0.1% ortho-phosphoric acid and acetonitrile were used as mobile phase. Figure 2 illustrates a typical chromatogram of tumor tissue of control diet-fed mice (A) in comparison to those of rottlerin-fed mice (B). The HPLC profile of mouse plasma and other tissues shows a peak with same retention time at 21.7 min as standard reference rottlerin. On-line UV-VIS maxima of the diode-array detector responses facilitated the confirmation of the compound. Under the selected chromatographic condition, separation of rottlerin was achieved without the interference of endogenous peaks within the time frame of a single analysis. For the calibration curves, there were linear relationship between peak area and concentration in the range. The equation for rottlerin was y = 59.614x – 0.0625 with R^2 = 0.9996. The lower limit of quantitation for rottlerin was 135 ng/mL.

To determine the in vivo effect of rottlerin on pancreatic tumor growth we used a xenograft mouse model as described in the Material and Methods section. Mice were fed standard diets supplemented with rottlerin (0.012% or 120 mg/kg diet) for 6 weeks. Based in daily food consumption, we estimated that the mice received an average of 0.5 mg/mouse/day rottlerin. Rottlerin in the diet was stable at room temperature for 3 days as determined by HPLC analysis.

The sample preparation method described above was applied to the measurement of rottlerin in plasma, selected tissues, and tumors obtained from mice fed control and rottlerin diet for 6 weeks. While we determined the distribution of rottlerin in pancreas and tumor as target tissues, we also investigated liver uptake as liver is a site of metastases for pancreatic cancer. Table 1 shows the average concentration of rottlerin in plasma, tumor, pancreas and liver. Levels of rottlerin were highest in liver (6.56 ± 0.83 nmol/g), followed by pancreas (2.46 ± 0.29 nmol/g) and tumor (2.11 ± 0.25 nmol/g) tissues. Average plasma level is 2.88 ± 0.41 μM. The extraction efficiency is 87.2% for plasma and 85.2-104.5% for tissues and tumors. Of note, mice fed rottlerin diet grew smaller tumors than those fed control diet (unpublished data). No rottlerin was detected in samples collected from mice fed control diet.

To examine the stability of rottlerin during the extraction procedure, we spiked rottlerin to liver tissue homogenates in pH 7.0 buffer and to plasma of control mice. The resulting samples were then incubated at 37°C for 2 h under dark condition and aliquots of each sample were taken at 40, 80, and 120 min. Table 2 lists the rottlerin concentration in each time point in comparison to the baseline. The data show that rottlerin is stable in both plasma and liver tissue homogenate under the conditions indicated.

We next examined the accumulation of rottlerin in HPAF-II cells treated with 25 μM rottlerin, and the fate of rottlerin in the incubation media. Cells were collected at 3 and 24 h after rottlerin stimulation and conditioned media were taken at 0.5, 1.0, 3.0, 6.0 and 24 h (n=4 for each time point). Samples were prepared immediately for HPLC analysis. The equation for rottlerin was y = 59.614x – 0.0625 with R^2 = 0.9996. The lower limit of quantitation for rottlerin was 135 ng/mL.

![Representative HPLC chromatograms of mouse tumor monitored at 286 nm. (A) Tumor from a mouse treated with control diet (top) and (B) from a rottlerin-treated mouse (bottom). The peak at 15.3 min represents BHT added as antioxidant, IS, internal standard.](image-url)

**Table 1:** Rottlerin concentration in plasma and tissues of mice fed 0.012% rottlerin diet for 6 weeks (n=6).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Plasma (μM)</th>
<th>Liver tissue (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.19 ± 0.06</td>
<td>6.01 ± 0.38</td>
</tr>
<tr>
<td>40</td>
<td>6.29 ± 0.29</td>
<td>5.48 ± 0.11</td>
</tr>
<tr>
<td>80</td>
<td>6.62 ± 0.15</td>
<td>5.86 ± 0.56</td>
</tr>
<tr>
<td>120</td>
<td>6.29 ± 0.21</td>
<td>5.56 ± 0.64</td>
</tr>
</tbody>
</table>

**Table 2:** Stability of rottlerin in plasma and liver tissue homogenates with time (n=3).

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UV absorption

![UV absorption graph](image-url)
medium with time. Our data can be used to provide important information on evaluating the potential chemo-preventive or therapeutic efficacy of rottlerin.

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**References**


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**Table 3**: Summary of precision and accuracy from quality control (QC) samples of mouse plasma extracts of rottlerin (n=3 days, 3 replicates per day).

<table>
<thead>
<tr>
<th>Spiked (μg/ml)</th>
<th>Measured (μg/ml)</th>
<th>Accuracy (%)</th>
<th>Precision (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.208</td>
<td>104.1</td>
<td>9.7</td>
</tr>
<tr>
<td>2</td>
<td>2.02</td>
<td>100.9</td>
<td>0.9</td>
</tr>
<tr>
<td>8</td>
<td>8.01</td>
<td>100.1</td>
<td>1.8</td>
</tr>
</tbody>
</table>

**Figure 3**: Accumulation of rottlerin in pancreatic cancer HPAF-II cells. A. Rottlerin uptake in HPAF-II cells after stimulation with 25 μM rottlerin for 3 and 24 h; and B. Change of rottlerin level in cultured media. *P<0.05, **P<0.01.