EFFECT OF 70% ETHANOL EXTRACT OF ORTHOSIPHONIS STAMINEUS BENTH LEAVES ON THE PHARMACOKINETIC PARAMETERS OF FUROSEMIDE IN MALE WHITE RATS

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ABSTRACT

Objective: This study aimed to observe the effect of the 70% ethanol extract of Orthosiphon stamineus Benth leaves on the pharmacokinetic parameters of furosemide in white male rats.

Methods: 18 Sprague-Dawley male rats were divided into three groups: The normal control group was given only 1% carboxymethyl cellulose, the furosemide group was given 7.2 mg/200 g body weight (BW) suspension of furosemide, and the combination group was given 700 mg/kg BW suspension of the 70% ethanolic extract of O. stamineus Benth leaves for 4 days followed by a 7.2 mg/200 g BW suspension of furosemide. On the 4th day of treatment, we performed orbital sinus blood sampling on the eyes of the rats and analyzed the levels of furosemide in plasma using high-performance liquid chromatography.

Results: Therefore, the results showed that the administration of the 70% ethanol extract of O. stamineus Benth leaves improves the pharmacokinetic parameters of furosemide on C$_{max}$ and the area under the curve (p<0.05).

Conclusion: This study concludes that the 70% ethanol extract of O. stamineus Benth leaves improves the pharmacokinetic parameters of furosemide in white male rats.

Keywords: Cat’s whiskers leaves, Furosemide, Orthosiphon stamineus Benth, Pharmacokinetic, 70% ethanolic extract.

INTRODUCTION

Furosemide is an anthranilic acid derivative drug included in powerful diuretics. Based on the research conducted by Bragatto et al. [1], a single dose of 40 mg of furosemide given orally produced significant results in increasing the volume of urine and had a pharmacokinetics ratio interval of C$_{max}$ (93.63-121.92%), area under the curve (AUC$_{0-\infty}$) (96.80-115.72%), and AUC$_{0-t}$ (98.45-117.43%). Another study mentions that the administration of furosemide with a dose of 8 mg/200 g body weight (BW) produced AUC$_{0-\infty}$ 13.52±5.229 µg/mL hr with C$_{max}$ 22.34±4.39 µg/mL [2]. Indonesian herbal medicine has been used for hundreds of years, long before the advent of modern medicine that involves a diagnostic laboratory and chemicals [3]. The results of basic medical research in 2010 showed that about 59.12% of Indonesia’s population consumes herbs [4]. Cat’s whiskers is a medicinal plant that has been empirically shown to act as a diuretic. Tensigard® is a phytopharmacological preparation containing extracts of cat’s whiskers leaves combined with celery extract and is used as an antihypertensive due to its diuretic properties.

The use of cat’s whiskers leaf extract and furosemide at the same time can cause a pharmacokinetic interaction that can increase or decrease furosemide levels in plasma. As a result, drug toxicity and drug effectiveness may also increase or decrease [5]. The diuretic effect of cat’s whiskers leaf extract is demonstrated by the inhibition of adenosine receptor A$_1$, which will inhibit sodium reabsorption in the proximal tubules [6], and the diuretic mechanism of furosemide diuretic inhibits reabsorption of electrolytes Na$^+$K$^+$/2Cl$^-$ in the ascending limb of the loop of Henle in the thickest epithelium section. The diuretic effect of cat’s whiskers leaf extract and furosemide was similarly with inhibiting sodium reabsorption in the nephron [7]. The changes in the levels of the combined drugs will affect the pharmacokinetic parameters of furosemide, and this study observes the influence of cat’s whiskers leaf extract on the pharmacokinetic parameters of furosemide in white male rats using blood plasma samples.

MATERIALS AND METHODS

Materials
The material sample used in this study was the 70% ethanol extract of cat’s whiskers leaf (Orthosiphon stamineus Benth.) obtained from the Research Center for Spices and Medicinal Plants. Determination of cat’s whiskers leaf was conducted by the Indonesian Institute of Sciences, Bogor Botanical Gardens. Animals used in this study were 30 Sprague–Dawley rats: 12 samples for the optimization of timing and 18 for the actual test. The rats were male, approximately 3 months old and approximately 200 g in weight, and were obtained from the Institut Pertanian Bogor. The chemicals used include furosemide (Kimia Farma), furosemide standard (BPFI), propanolol HCl (BPFI), carboxymethyl cellulose (CMC) (Brauaco Chemical), methanol (high-performance liquid chromatography (HPLC) grade, Merck), phosphoric acid (85%) (Merck), ethyl acetate (Merck), human blood plasma from Palang Merah Indonesia, Aqua Bistid distilled water, food standards for test animals, anhydride acetic acid, Mayer’s reagent, Dragendorff’s reagent, Bouchard’s reagent, Molisch’s reagent, iron (III) chloride reactant solution, hydrochloric acid, zinc powder, and magnesium powder.

Instruments
The instruments used in this study were stomach probes, syringes (Terumo), animal scales, an analytical balance (Ohaus), microhematocrit capillary tubes, a set of HPLC LC-10AD VP tools (Shimadzu) consisting of a pump, an injector, C$_18$ column of RP-18e (Waters, Sunfire TM 5 μm; 250 × 4.6 mm), detector SPD-20A ultraviolet-visible (UV-Vis), DGU-12A degasser, SCL-10A VP controller, and data processing on a computer, UV-1601 (Shimadzu), pH meter (Eutech pH 700), vortex (Maxi Mix II), microcentrifugator (Spectrafuge 16 M), refrigerator (LabTech), centrifugator (Digisystem), evaporator (Caliper), microtube, centrifuge tubes, 0.45 µm membrane filter, micropipette and pipette (blue tip/ yellow tip), reaction tube, test tube rack, vaporizer cup, beaker (Pyrex), spatula, stir bar, measuring glass (Pyrex), and flask (Pyrex).
Test material preparation

Preparation of the test material included producing CMC solution of 1%, producing the furosemide suspension, producing the 70% ethanol extract suspension of cat’s whiskers leaves extract, producing a mobile phase of methanol-water (50:50) that adjusted to phosphoric acid (85%) at pH of 3.0, producing furosemide solution for optimization, producing propranolol HCl solution for internal standard solution, and producing furosemide solutions for calibration curve.

Table 1: Group division and animal testing

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Provision of CMC 1%</td>
</tr>
<tr>
<td>Furosemide</td>
<td>Provision of furosemide suspension 7.2 mg/200 g BW</td>
</tr>
<tr>
<td>Combination</td>
<td>Provision of 70% cat’s whiskers ethanol extract 700 mg/kg BW + furosemide suspension 7.2 mg/200 g BW</td>
</tr>
</tbody>
</table>

CMC: Carboxymethyl cellulose; BW: Body weight

The solution from the extraction (20 mL) was injected to HPLC by chromatographic conditions chosen using C18 column (250 × 4.6 mm, 5 µm), UV-Vis detector [11]. Measurements were taken at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 8, and 10 hrs after administration of the furosemide.

Optimization of condition analysis

Wavelength analysis involved the use of a UV-Vis spectrophotometer at a concentration of 10 µg/mL. Optimization of the mobile phase ratio included three comparisons: Methanol–water (50:50 v/v) pH 3.0, methanol–water (55:45 v/v) pH 3.0, and methanol–water (60:40 v/v) pH 3.0, using solutions containing 10 mg/mL of furosemide and 100 mg/mL of propranolol HCl. The pH modification of the mobile phase was at pH 2.8, pH 3.0, and pH 3.2. Further modification of the flow rate was 0.8, 1.0, and 1.2 mL/min. Each optimization was compared to the retention time (tR, the peak area (A), tailing factor (T), the number of theoretical plates (N), HETP, and the resolution (R). The coefficient of variation (CV) was obtained after 6 repetitions of the test.

Optimization of extraction method

250 µL of plasma containing 10 µg/mL of furosemide in a centrifugation tube added 50 µL containing 50 µg/mL propranolol HCl, and then, vortex a 10 seconds. The mixture was added ethyl acetate as much as 10 times of the mixture, and then, vortex for 1 minute. The mixture was centrifuged for 20 minutes at 3000 rpm. The supernatant was taken and evaporated with nitrogen gas for 15 minutes at 60°C. The residue was dissolved in 200 mL of the mobile phase, and as much as, 20 mL of the solution was injected into the HPLC instrument on the condition of the selected analysis. Optimization time of vortex was 30 seconds, 1 minute, and 1.5 minutes. Furthermore, optimization time of centrifugation was 10 minutes and 20 minutes. Each optimization was performed by observing the maximum peak of the analysis.

Validation method

Producing calibration curve and linearity test

A calibration curve was created with plasma comprising one blank sample (plasma without raw), a zero sample (plasma raw), and 7 concentration gradients of calibration curve: 1, 5, 10, 15, 20, 30, and 40 µg/mL. Each concentration for the calibration curve was added raw in as much as 50 µL from 50 ppm and extracted as in the sample. Linearity was determined by calculating the correlation coefficient of the linear regression of the calibration curve. In addition, the calculated value % difference for each produced calibration curve with the requirements should not be more than ±15%, except for the lowest quantitation limit determination (LLOQ) solution, which should not be more than ±20%.

LLOQ

We prepared a standard furosemide solution in plasma at LLOQ concentration (based on the calculation of the calibration curve series) and then added internal standard and prepared as in the sample. The injection was repeated 5 times. From the measurement data, we then calculated the value % difference and CV with the requirements of not more than 20%. If the solution at the tested concentration met the requirements, then it was retested by half of the initial concentration. This process was performed until the lowest concentration was obtained that still met the requirements, otherwise known as LLOQ.
Accuracy and precision
We prepared a plasma containing the solution at LLOQ concentrations and at low (QC-L), moderate (QC-M), and high (QC-H) concentrations. The results were as follows:
- QC-L: 3 µg/mL
- QC-M: 5 µg/mL
- QC-H: 10 µg/mL

Each concentration received the standard preparation of 100 µL. Tests were repeated 5 times for each concentration in a day (within-run) and interday (between-run) for at least 2 different days with a minimum of three test iterations. We then calculated the % difference as the parameter of accuracy and % CV as a precision parameter. The % difference and CV value should not exceed ±20% of the sample LLOQ and should not exceed ±15% for all quality control samples.

Pharmacokinetic parameters of furosemide in blood
Pharmacokinetic parameters were calculated as the absorption rate constant (Ka), the peak time (tmax), the peak plasma concentration (Cmax), the elimination rate constant (Ke), the half-life (t1/2), the volume of distribution (Vd), the clearance (Cl), and the AUC. Each of these pharmacokinetic parameters was analyzed using SPSS to determine the normality as well as the significance of the data.

RESULTS AND DISCUSSIONS
Phytochemical screening of the 70% ethanol extract of cat’s whiskers leaves
Organoleptic evaluation is useful for knowing the characteristics of cat’s whiskers leaf extract. Observations showed that the 70% ethanol extract of cat’s whiskers leaf was in the form of a slightly thick liquid, had a distinctive smell, and was a blackish-green color. The yield produced in the simplex process of the cat’s whiskers plant was 7.43%.

Furthermore, a phytochemical screening process was conducted to determine the class of bioactive compounds contained in the extract. Phytochemical screening conducted at the Research Institute for Spices and Medicinal Plants showed positive results for alkaloids, phenols, flavonoids, and terpenes. Phytochemical screening was also performed at the Phytochemistry Laboratory of the Faculty of Pharmacy, UI, and the results are shown in Table 2.

Time optimization of the 70% ethanol extract of cat’s whiskers leaves
Based on the average, we can see that the volume mean of urine was the most in the group given the 70% ethanol extract of cat’s whiskers leaves for 4 days followed by the furosemide dose on day 4. The first urine volume measurement was 11.166 mL; at the 2nd hr, the volume rose to 15.833 mL; at the 3rd hr, it measured at 17.50 mL; and the amount remained stable from 4 to 6 hrs (at a volume of 18.50 mL). Time for 4 days of the extract was used as the actual test. Optimization results for the average volume of rat urine for 6 hrs are shown in Table 3.

Optimization of condition analysis
The optimum wavelength selected for furosemide analysis was 236 nm. This wavelength was chosen because it is the greatest absorption of furosemide. In mobile phase composition, furosemide and propranolol HCl, respectively, have a retention time of 5.472 and 2.990 minutes; theoretical plates (N) of 1851.523 and 1258.122; HETP of 0.0315 and 0.0199; tailing factor (T) of 1.641 and 0.880; and a resolution of 6.154. Then, that mobile phase is modified to methanol–water (55:45 v/v) pH 3.0 and methanol–water (50:50 v/v) pH 3.0. The composition of methanol–water (55:45 v/v), furosemide and propranolol HCl, respectively, had a retention time of 7.465 and 3.043 minutes; theoretical plates (N) of 2514.007 and 1515.980; HETP of 0.0099 and 0.0165; tailing factor (T) of 1.695 and 1.750; and resolution of 9.658. The composition of methanol–water (50:50 v/v), furosemide and propranolol HCl, respectively, had a retention time of 10.677 and 3.340 minutes; theoretical plates (N) of 402.193 and 2926.168; HETP of 0.0062 and 0.0085; tailing factors (T) of 1.689 and 1.874; and resolution of 15.914.

The pH optimization of mobile phase, methanol–water (50:50 v/v) pH 2.5 showed that retention time of furosemide and propranolol HCl was 9.291 and 4.040 minutes, respectively, whereas the composition of methanol–water (50:50 v/v) pH 3.5 retention time of furosemide and propranolol HCl was 13.257 and 3.451 minutes, respectively. The flow rate was used as an initial condition, and it was 1.0 mL/minute. The flow rate of 1.0 mL/minute obtained retention time of furosemide and propranolol HCl, respectively, was 10.577 and 3.335 minutes. The flow rate of 1.2 mL/minute retention time in furosemide and propranolol HCl, respectively, was 13.449 and 4.713 minutes and resolution of 14.508 (≥1.5); T=1.527 (≤2); and N=3726.252 (≥2.000). This study showed that % CV was <2.0%. The results obtained by each system suitability test performed yielded a CV value of <2.0%. Time analysis of each injection was 15 minutes.

Optimization of extraction method
Data optimization with a vortex agitaion time of 30 seconds resulted in a furosemide area of 304.907; vortex agitaion for 1 minute resulted in a furosemide area of 318.954; and vortex agitaion for 1.5 minutes resulted in a furosemide area of 285.781. Playback of time optimization data by centrifugation, namely, the screening of 10 minutes, showed a

<table>
<thead>
<tr>
<th>Identification</th>
<th>Results</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Orange precipitate (Dragendorff’s reagent), yellow (Mayer’s reagent), and redish-brown (Bouchard’s reagent)</td>
<td>Positive, contains alkaloids</td>
</tr>
<tr>
<td>Phenol</td>
<td>Bluish-black color</td>
<td>Positive, contains phenol</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Color change occurred</td>
<td>Positive, contains flavonoids</td>
</tr>
<tr>
<td>Terpene</td>
<td></td>
<td>Positive, contains terpenes</td>
</tr>
</tbody>
</table>

Table 2: Results of phytochemical screening of the 70% ethanol extract of cat’s whiskers leaves

<table>
<thead>
<tr>
<th>P Treatment group</th>
<th>The average volume of urine at hours of mL±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>2.2±1.0</td>
</tr>
<tr>
<td>Furosemide</td>
<td>8.0±1.0</td>
</tr>
<tr>
<td>Combination for 4 days</td>
<td>11.2±3.2</td>
</tr>
<tr>
<td>Combination for 7 days</td>
<td>9.8±1.4</td>
</tr>
</tbody>
</table>

Information: Control=With CMC 1%; Furosemide=Furosemide of 7.2 mg/200 g BW, combination=Extract of Cat’s whiskers leaf of 700 mg/kg+furosemide 7.2 mg/200 g BW. CMC: Carboxymethyl cellulose, BW: Body weight, SD: Standard deviation

Table 3: Average volume of rat urine for 6 hrs in a preliminary test

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The rat plasma obtained was analyzed using HPLC with previously validated analysis conditions. The plasma furosemide concentration was obtained in the administration of the single and combined furosemide with 70% ethanol extract of cat’s whiskers leaves calculated by pharmacokinetic parameters. The mean of pharmacokinetic parameter data obtained can be seen in Table 4.

### Description

The time optimization of a comparative analysis of selected mobile phases is a mobile phase composition of methanol–water mixture (50:50 v/v) that adjusted by phosphoric acid to a pH of 3.0. The selection was based on the retention time and resolution between the analyte and internal standard. In this mobile phase composition, separation and retention time of internal standard was a little longer than the mobile phase of methanol–water (55:45) and methanol–water (60:40).

In the mobile phase of methanol–water (50:50 v/v) pH 3.0, the peak of the analyte on the chromatogram was more pointed and had a better resolution than pH 2.5 and pH 3.5. The flow rate used for analysis was 0.8 mL/minute with resolution was 14.508 (≥1.5); T<sub>90</sub> = 1.527 (≤2); and V<sub>d</sub> = 537.90±81.71* (397.06±17.22%).

**Accuracy and precision**

Accuracy and precision were tested by intra- and inter-days. The intraday accuracy measurement values obtained for LLOQ concentration % difference ranged from −13.25% to −19.27%, and for the QC concentration, it ranged from −1.50% to −19.27%. For the interday precision measurements, we obtained a CV value of <4.75% for LLOQ and QC concentrations. For the interday measurement accuracy for LLOQ concentration, we obtained a range of % difference from −11.66% to +19.27%, and for the QC concentration, the range % difference was −0.68% to +14.96%. The measurement of the interday precision was a CV value of <5.79%. The value % difference and CV for both intraday and interday met accuracy and precision standards.

### Pharmacokinetic analysis of furosemide in rat blood plasma

The rat plasma obtained was analyzed using HPLC with previously validated analysis conditions. The plasma furosemide concentration was obtained in the administration of the single and combined furosemide with 70% ethanol extract of cat’s whiskers leaves and furosemide did not result in a significant decrease (p>0.05) in the rate of absorption but also did not significantly increase the rate of elimination. An increase in the rate of elimination means more drugs were being released from the body. This corresponds to the diuretic mechanism of increasing urine volume. The time of plasma concentration peak did not change, while the half-life decreased. Rises in value elimination rate cause a decline in how long the drug is present in the body [12].

Decreased clearance did not differ significantly. Clearance value is directly proportional to the volume of distribution and the elimination rate [12]. The decline in value is due to the volume of distribution clearance, which also decreased significantly while the elimination rate increased. The volume of distribution shows the amount of drug distribution in the body. The volume of distribution is also related to the amount of drug in the plasma. If the volume of distribution increases, the drug concentration in the plasma will be small and otherwise [12]. The peak plasma concentration increased significantly (p<0.05), as well as the AUC. This increase indicates an increase in the plasma concentration of therapeutic furosemide. Increasing concentrations of furosemide are still relatively safe because it does not reach a toxic level. Under the U.S. Food and Drug Administration and the World Health Organization guidelines on bioequivalence, furosemide plasma concentrations reach toxicity in the interval of 25-30 mg/mL [13].

**Table 4: The mean of pharmacokinetic parameters in the treatment group (±SD values)**

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Unit</th>
<th>Furosemide</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ka</td>
<td>1 hr</td>
<td>0.96±0.08</td>
<td>0.88±0.07</td>
</tr>
<tr>
<td>t&lt;sub&gt;max&lt;/sub&gt;</td>
<td>hr</td>
<td>2.5±0.5</td>
<td>2.5±0.6</td>
</tr>
<tr>
<td>Q&lt;sub&gt;p&lt;/sub&gt;</td>
<td>ug/mL·1&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>13.63±2.01*</td>
<td>18.16±0.81*</td>
</tr>
<tr>
<td>T&lt;sub&gt;0&lt;/sub&gt;</td>
<td>1 hr</td>
<td>0.70±0.22</td>
<td>0.78±0.04</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>hr</td>
<td>3.0±0.80</td>
<td>2.5±0.28</td>
</tr>
<tr>
<td>V&lt;sub&gt;d&lt;/sub&gt;</td>
<td>mL</td>
<td>357.90±81.71*</td>
<td>397.06±17.22*</td>
</tr>
<tr>
<td>Cl</td>
<td>hrs mL·1&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>364.64±100.28</td>
<td>308.51±13.23</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0→t&lt;/sub&gt;</td>
<td>ug mL·1&lt;sup&gt;−1&lt;/sup&gt;·hr</td>
<td>46.92±4.91*</td>
<td>62.86±3.99*</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0→∞&lt;/sub&gt;</td>
<td>ug mL·1&lt;sup&gt;−1&lt;/sup&gt;·hr</td>
<td>124.2±1.22</td>
<td>143.7±2.65</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;inf&lt;/sub&gt;</td>
<td>ug mL·1&lt;sup&gt;−1&lt;/sup&gt; hr</td>
<td>59.34±4.27*</td>
<td>77.23±6.43*</td>
</tr>
</tbody>
</table>

*Indicates significance value <0.05, that means on the two groups have significantly different values. AUC: Area under the curve, SD: Standard deviation

Based on the results of the phytochemical screening, cat’s whiskers leaves contain flavonoids, alkaloids, terpenes, and phenols. The increase in pharmacokinetic parameters in male rats caused by the presence of methoxy flavonoids that can induce diuretic through the activity of the adenosine A<sub>1</sub> receptor antagonist that is involved in the regulation of the flow rate of urine and the excretion of sodium [14,15]. Inhibition
of these receptors would inhibit sodium and water reabsorption in the proximal tubules [6].

Administration together between furosemide, which is a potent diuretic, with 70% ethanol extract of cat’s whiskers leaves can cause interactions that lead to the inhibition of sodium reabsorption in the nephron. Furosemide and flavonoids found in cat’s whiskers leaves have similar diuretic properties. Furosemide is a diuretic that inhibits the reabsorption of electrolytes Na+/K+/2Cl− in the ascending limb of the loop of Henle in the thick epithelial part, and flavonoids found in the cat’s whiskers plant can inhibit the reabsorption of sodium and water in the proximal tubules through inhibition of adenosine receptor A1. The diuretic effect of cat’s whiskers leaves also suspected to be due to the methyltriparichromene compound A (MRC-A). MRC-A diuretic effect is shown by an increase in the volume of urine and excretion of Na+, K+, and Cl−.

CONCLUSION

Based on these research results, this study concludes that the joint administration of 70% ethanol extract of cat’s whiskers leaves (O. stamineus Benth) and furosemide can improve the pharmacokinetic parameters of furosemide in white male rats, results that were indicated by significant increases (p<0.05) in peak plasma concentration (Cpmax) and AUC.

The authors also recommend performing the further studies on the toxicity of the concurrent use of 70% ethanol extract of the cat’s whiskers plant with furosemide.

REFERENCES