CHAPTER 4

MATERIALS AND METHODS

The laboratory session of this clinical study was conducted (between May 2008 and October 2008) at the Clinical Pharmacology Unit, Faculty of Medicine, Chiang Mai University, Thailand.

1. Drug formulations

Glucophage®, the reference drug, was supplied in the commercially available tablets containing 850 mg active substance (OLIC (Thailand) Limited, Thailand, (under the license of Merck Sante s.a.s., FRANCE), Lot No. 806001). The generic metformin HCl, the test drug, was supplied as tablets containing 850 mg active substance (The Government Pharmaceutical Organization (GPO), Bangkok, Thailand, Lot No. R51029).

2. Subjects

The number of subjects was determined based on the error variance (within-subject coefficient of variation (% CV)) of AUC and C_{max}. The significant level desired (α) of 5% and the expected mean deviation from the reference product compatible with bioequivalence of 0.95-1.00, while the power was >80%. The sample size of 24 deemed adequate with reference to previous study, however, the enrolled subjects were 26 to allow for possible drop-outs or withdrawals. Twenty six healthy nonsmoking Thai male volunteers aged between 18-50 years old with body mass index (BMI) within 18-25 kg/m^2 were enrolled into the study based on the following criteria:

Inclusion criteria

1. All subjects were healthy as confirmed by medical history, vital sign, physical, hematological examination including electrocardiogram (ECG), complete blood count (CBC), liver function test (LFT), blood glucose, blood

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urea nitrogen (BUN), serum creatinine (C_r). In addition, subject must have seronegative for hepatitis B surface antigen (HBs-Ag).

2. All subjects did not take any medications, substances or foods which interact with circulatory, gastrointestinal, liver and renal function 1 month prior to the study day and throughout the participation period.
3. All subjects did not drink caffeinated and alcoholic and did not consume caffeine-containing foods or any beverages for 7 days prior to the study and agreed to continue their refraining until the last collection time point of phase II.
4. All subjects agreed to comply with the study protocol after the aims and procedure for the study were explained and signed the written informed consent before entering the study.

Exclusion criteria

1. Subjects with known contraindication or hypersensitivity to metformin and its exicipients.
2. Subjects with known history of gastrointestinal disease, liver and/or kidney disease, allergic disease and other diseases that might alter the bioavailability of metformin.
3. Subjects with known history of recent cigarette smoking, alcoholism or drug abuse.
4. Subjects taking other drug within 1 month before enroll to the study.
5. Subjects previously enrolled in other drug study within 3 months.

Withdrawal criteria

1. Subjects who experienced adverse drug reaction during the study.
2. Subjects could not comply with the study protocol.
3. Subjects voluntarily withdrew from the study.
4. Subjects who required other medication (which interfere with the level of metformin) during the study period.

3. Ethics

The protocol was approved by the Human Research Ethic & Committee of the Faculty of Medicine, Chiang Mai University.

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4. Study design

The study design was a single dose, randomized, balanced two-period, two-sequence crossover with a one-week washout period between phase I and phase II dosing. The design was in accordance to the Thai Food and Drug Administration (FDA) guideline of bioequivalence testing (15). Equal numbers of subjects were randomly assigned to two dosing sequences (Test-Reference, Reference-Test) as shown in Table 1:

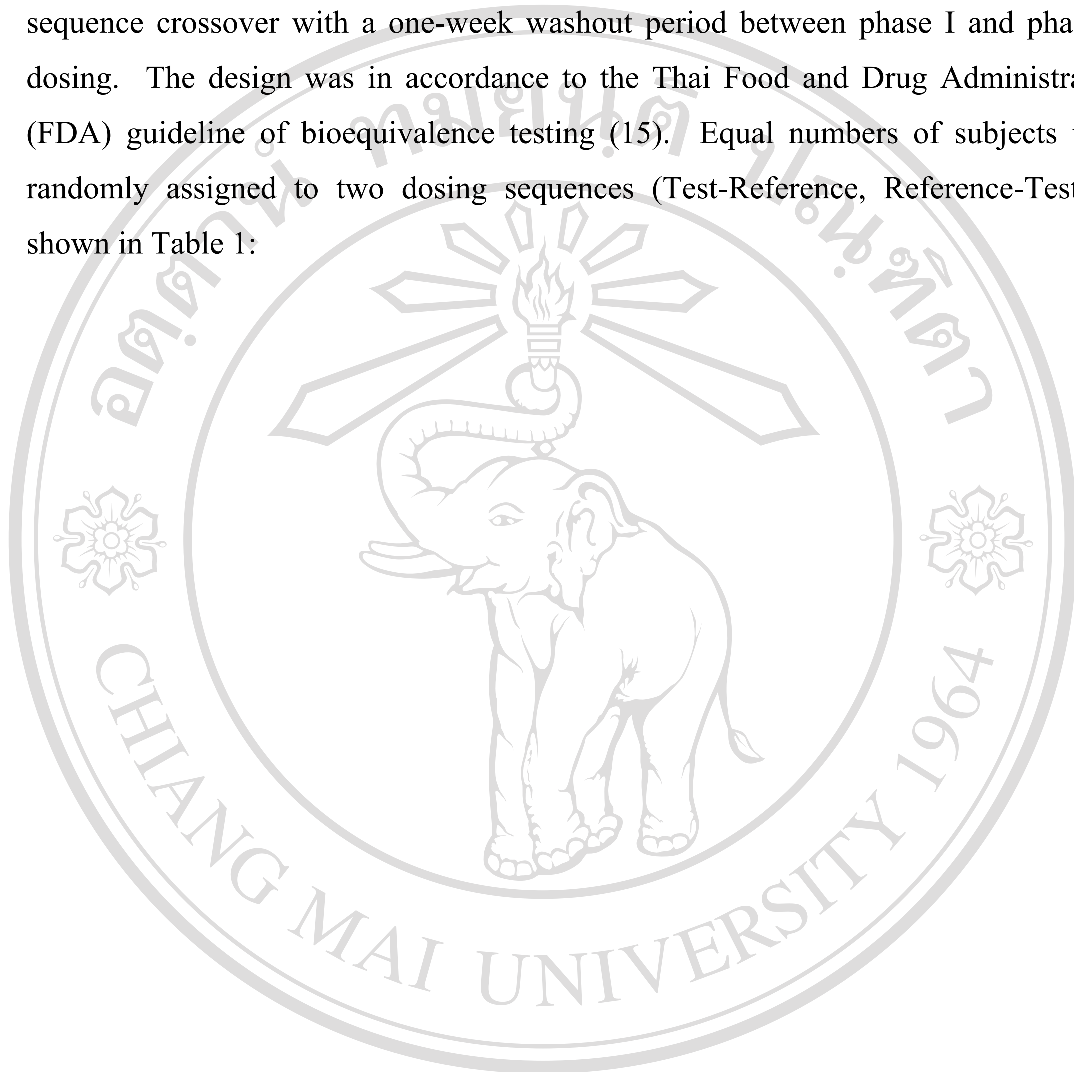

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Table 1 Schedule of visits and sequence of drug administrations

Subject No.	Pre-study visit (screening)	Dispense medication (period 1)	Dispense medication (period 2)	Post-study visit (adverse event report)
1	13 July 08	19 July 08 (T)	26 July 08 (R)	31 July 08
2	13 July 08	19 July 08 (T)	26 July 08 (R)	31 July 08
3	13 July 08	19 July 08 (T)	26 July 08 (R)	31 July 08
4	13 July 08	19 July 08 (R)	26 July 08 (T)	31 July 08
5	13 July 08	19 July 08 (T)	26 July 08 (R)	31 July 08
6	13 July 08	19 July 08 (R)	26 July 08 (T)	31 July 08
7	13 July 08	19 July 08 (R)	26 July 08 (T)	31 July 08
8	13 July 08	19 July 08 (T)	26 July 08 (R)	31 July 08
9	13 July 08	19 July 08 (R)	26 July 08 (T)	31 July 08
10	13 July 08	19 July 08 (R)	26 July 08 (T)	31 July 08
11	13 July 08	19 July 08 (T)	26 July 08 (R)	31 July 08
12	13 July 08	19 July 08 (T)	26 July 08 (R)	31 July 08
13	13 July 08	19 July 08 (T)	26 July 08 (R)	31 July 08
14	13 July 08	20 July 08 (R)	27 July 08 (T)	01 Aug 08
15	13 July 08	20 July 08 (R)	27 July 08 (T)	01 Aug 08
16	13 July 08	20 July 08 (R)	27 July 08 (T)	01 Aug 08
17	13 July 08	20 July 08 (T)	27 July 08 (R)	01 Aug 08
18	13 July 08	20 July 08 (R)	27 July 08 (T)	01 Aug 08
19	13 July 08	20 July 08 (T)	27 July 08 (R)	01 Aug 08
20	13 July 08	20 July 08 (R)	27 July 08 (T)	01 Aug 08
21	13 July 08	20 July 08 (T)	27 July 08 (R)	01 Aug 08
22	13 July 08	20 July 08 (R)	27 July 08 (T)	01 Aug 08
23	13 July 08	20 July 08 (T)	27 July 08 (R)	01 Aug 08
24	13 July 08	20 July 08 (R)	27 July 08 (T)	01 Aug 08
25	13 July 08	20 July 08 (T)	27 July 08 (R)	01 Aug 08
26	13 July 08	20 July 08 (R)	27 July 08 (T)	01 Aug 08

(T = Test product, R = Reference product)

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5. Drug administration

Twenty-six volunteers were admitted to the Clinical Pharmacology Unit of the Faculty of Medicine, Chiang Mai University in the evening before the study day and fasted overnight for at least 8 h. In the morning of the study day (day 1), the vital signs and fasting blood glucose level were determined before drug administration using the Accu-Chek Advantage meter (Roche Diagnostics) and the results of the glucose level were read within one minute. Volunteers were enrolled if their vital signs were normal, no symptom of hypoglycemia (sweating, nervousness, tremulousness, faintness, palpitation or hunger) and fasting glucose level > 40 mg/dL. Volunteers were given either one tablet of Glucophage®or one tablet of the 850-mg generic metformin with 240-mL glucose solution. Volunteers would remain upright for 4 h and were fasted 2 h after drug administration. Twenty percent of glucose solution and lunch were served at 2 h and 4 h after dosing, respectively. Since the plasma elimination $t_{1/2}$ of metformin following oral dosing is about 6.2 h, the washout period between each phase was set at least one week to ensure the total clearance of the drug. After a washout period of one week, volunteers were switched to receive the different brand of metformin in the same manner. Identical meal and fluid were served during the two study periods. Blood pressure, heart rate as well as clinical symptoms of hypoglycemia were monitored every hour during the first 4 h after dosing.

6. Plasma sample collections

An intravenous catheter connected to an injection plug was used for serial blood sample collections. Venous blood samples (10 mL each) were collected before (0 h) and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 8, 10, 14, 24 and 30 h after dose administration. The blood samples were centrifuged for 10 min at 3,000 rpm to separate the plasma. Thereafter, the plasma samples were immediately kept at -20 °C until assay.

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7. Volunteers safety monitoring

Blood glucose level, blood pressure and heart rate were determined before dosing to prevent the risk of hypoglycemia. All Volunteers were monitored for the symptoms and signs of hypoglycemia include sweating, flushing or pallor, numbness,

chilliness, hunger, trembling, headache, dizziness, increased pulse rate, palpitations, increased blood pressure and apprehensiveness. If hypoglycemia was suspected, blood glucose level was determined and volunteers were given a rapid intravenous administration of 20 mL of 50% glucose solution followed by continuous infusion of 10% glucose solution at a rate necessary to maintain blood glucose levels between 50-120 mg/dL. Thereafter, volunteers were monitored closely for at least 48 h. In addition, this study was conducted under close supervision of licensed study physicians/nurses and emergency kits were available in the Clinical Unit, if needed.

8. Follow up visit

Volunteers returned to the Clinical Unit for safety follow up at one week ($\pm$ 2 days) after the last dose. Physical examination, vital sign record, assessment for adverse events, blood sample for CBC, BUN, C_r, FBG, aspartate aminotranferase (AST), alanine aminotransferase (ALT), alkaline phoaphatase (ALP), total bilirubin and urinalysis were done in the morning of the follow up day.

9. Determination of plasma metformin concentrations

Metformin plasma concentrations were determined by high performance liquid chromatography (HPLC) with UV detection. The method of metformin determination was modified from the assay validation method employed in previous study (37, 38).

9.1 Instruments

Pump	LC-10ADvp, Shimadzu Co., Kyoto, Japan
De-gasser	DGU-14A, Shimadzu Co., Kyoto, Japan
UV detector	SPD-M10Avp, Shimadzu Co., Kyoto, Japan
System controller	SCL-10Avp, Shimadzu Co., Kyoto, Japan
Column oven	CTO-10ASvp, Shimadzu Co., Kyoto, Japan
Auto-injector	SIL-10ADvp, Shimadzu Co., Kyoto, Japan
Sample concentrator	Eppendorf Concentrator 5301, Germany
Analytical balance	Satorius BP 61, Germany
Microtube centrifuge	Eppendorf 5410, Germany
Serofuge	Hermle Z22A, Germany
Vacuum box	J&W Scientific, USA

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Micropipettes	Gilson, USA
Microliter syringe	Hamilton, Switzerland
Vortex mixer	Vortex-2 Genie Scientific Inc, USA

9.2 Chemicals reagents

Methanol	AR grade J.T. Baker, USA
Acetonitrile	AR grade J.T. Baker, USA
Sodium dihydrogen phosphate (NaH_2PO_4)	AR grade Merck, Germany
Sodium dodecyl sulphate	AR grade Fisher, England

9.3 Test materials

Standard	Metformin hydrochloride [1, 1-Dimethyl biguanide hydrochloride], Batch No. MT-08740607, Manufauring date June-2007
Internal Standard (IS)	Phenytoin [5, 5-Diphenylhydantoin sodium], Sigma-Aldrich

9.4 HPLC assay conditions

Mobile phase	10 mM NaH_2PO_4 and 10 mM sodium dodecyl sulphate (pH 5.1)/acetonitrile (500/250, v/v).
Analytical column	Inersil® C8, 150 x 4.6 mm 5 µm, GL Sciences Inc., Tokyo, Japan
Guard column	Inersil® C8, 10 x 4.0 mm 5 um, GL Sciences Inc., Tokyo, Japan
Flow rate	1 mL/min
Temperature	25 ^{o}C
UV-detector wavelength	234 nm
Injection volume	20 µL

9.5 Preparation of reagents

9.5.1 Standard solution

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1. Stock solution for metformin 100,000 ng/mL was prepared by dissolving 0.0100 g of metformin HCl into 100.0 mL of methanol in a volumetric flask.
2. Standard working solutions of metformin of 100,000, 50,000, 25,000, 12,500, 6,250, 3125, 625 and 250 ng/mL were prepared by serial dilution of stock solution with appropriate volume of methanol.
3. Stock internal standard solution was prepared by dissolving 0.0500 g of phenytoin into 100.0 mL of methanol in a volumetric flask to give the concentration of 500,000 ng/mL
4. Stock standard solutions were aliquot and stored at -20 ^{o}C until required.

9.5.2 Standard plasma

1. Preparation of calibration standards plasma samples

Each of the nine aliquots (250 μL) of drug free plasma were spiked with 10 μL of methanol for blank sample, 10 μL of working IS and metformin working standard solutions (250, 625, 3,125, 6,250, 12,500, 25,000, 50,000 and 100,000 ng/mL) to give spiked concentrations of 0, 25, 125, 250, 500, 1000, 2000 and 4000 ng/mL, respectively.

2. Quality control (QC) plasma samples

They were prepared by adding the following volumes of the metformin stock solution 15 μL (50,000 ng/mL), 190 μL and 380 μL (100,000 ng/mL) into each 10 mL volumetric flask and then adjusted to volume with blank plasma to give spiked concentrations of 75, 1,900, and 3,800 ng/mL, respectively. Stock QC samples were aliquoted and stored at -20 ^{o}C until required.

9.5.3 Mobile phase

1. Accurately weighed 0.78 g of NaH_2PO_4 and 1.44 g of sodium dodecyl sulphate.
2. Transfered the solid into a 1 L plastic bottle added 500 mL of deionized water using a volumetric flask.
3. Mixed well until complete dissolution of the solid.
4. Added 250 mL of acetonitrile and 20 mL of methanol using a 250-mL volumetric flask and a 50-mL graduated cylinder, respectively.
5. Mixed well.

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6. Filtered the solution on a 0.45 μm membrane filter using a solvent filtration apparatus and a vacuum pump.
7. The solution was stored at room temperature for a maximum of 2 days.

9.6 Plasma extraction

The calibration standard samples, quality control samples and unknown plasma samples were treated identically as follow:

9.6.1 The plasma sample (250 μL) were spiked with 10 μL of IS (except a blank plasma sample which was spiked with 10 μL of methanol), mixed well by vortex mixer.

9.6.2 Added 380 μL of acetonitrile, then mixed well by vortex mixer immediately.

9.6.3 Centrifuged at 14,000 rpm for 5 min.

9.6.4 Transferred 100 μL of the supernatant to injection vial, and 20 μL was injected onto the HPLC system.

10. Validation of HPLC method

Validation of HPLC method was performed as proposed by the Thai FDA. The parameters in validation procedure included 1) specificity, 2) calibration curves, linearity and lower limit of quantification (LLQ), 3) precision, accuracy and recovery, 4) stability of metformin and internal standard in standard solution and spiked plasma samples.

10.1 Specificity

A mixture of metformin and IS in mobile phase was injected for making a typical chromatogram of each run. Blank plasma and metformin and IS spiked samples were injected onto the same HPLC condition after preparation by liquid-liquid extraction procedure for testing the plasma interference.

The retention times from this injection were used as references for the reminder of the samples. However, fluctuations in retention times were liable to occur due to change in temperature and column performance.

10.2 Calibration curve

Calibration curves established in plasma containing 25.0 to 4000.0 ng/mL of metformin with IS were used to calculate metformin concentrations for assay validation and unknown samples in clinical assay. Linear regression of

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concentrations versus peak height ratios of metformin/IS gave coefficients of determination (r^2) of 0.9900 or better.

LLQ was assessed by determining 5 aliquots of 25.0 ng/mL metformin in plasma with a set of calibration curves

10.3 Precision, accuracy and recovery

For the intra-day assay validation, a set of 5 control samples from each of 3 different concentrations (75, 1,900, 3,800 ng/mL) of QC samples were evaluated with a single calibration curve.

For the inter-day assay validation, the 3 sets of 5 control samples from each of 3 different concentrations (75, 1,900, 3,800 ng/mL) of QC samples were studied on 3 independent days with concurrent 3 standard calibration curves.

The accuracy of the mean value must be within $\pm$15% of the theoretical value (between 85-115%), except at the LLQ, where it must not deviate by more than $\pm$20%. The precision represented by the CV must be less than $\pm$15%, except for the LLQ, where it must not exceed $\pm$20% of the CV.

Assay recovery was determined by comparing the peak height of metformin standard sample dissolved in mobile phase with the peak height of metformin in plasma from a set of 3 different concentrations of QC samples.

10.4 Stability test

10.4.1 Freeze-thaw stability test was determined by comparing the metformin concentrations in 3 aliquots of each low and high levels QC sample after freeze-thaw of 3 cycles, with the concentrations of metformin after immediately spiking QC samples.

10.4.2 Short-term stability test was determined by comparing the metformin concentrations in 3 aliquots of each of the low and high levels QC sample kept at room temperature for 8 h with QC samples which freshly prepared standard solution.

10.4.3 Long-term stability test was determined by comparing the metformin concentrations in 3 aliquots of each low and high levels QC sample at the time of the beginning of the study with other QC samples which stored at -20 ^{o}C until the end of the study.

10.4.4 Post-preparative stability test was to determine the time effect to the drug and internal standard after plasma preparation and standing in the auto-sampler for

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injection onto the analytical column. The post-preparative stability time was calculated from number of samples in a batch size sample of one subject (2 visits) x sample run time (34 samples x 11 min, approximated 7 h).

10.4.5 Stock solution stability was tested by storage of metformin and the IS for 2 months at -20 ^{o}C. Then, the stability was evaluated by comparing the instrument response of the 3 aliquots of each low and high levels QC sample prepared by the stock standard solution with the freshly prepared standard solution.

The deviation for the stability test from the fresh reference values must not exceed the acceptance criteria of $\pm$15%.

10.5 Study phase validation

To evaluate study phase validation, the concentrations of QC samples were determined in the same manner and the same run time period of unknown samples. In each analytical run of 2 subjects' samples, three aliquots of each low, medium and high concentration were determined. The concentrations of QC samples of all analytical run were analyzed for the accuracy and precision. At least 67% (4 out of 6) of QC samples should be within 15% deviation of their respective nominal value.

11. Statistical methods and data analysis

11.1 Pharmacokinetic analysis

C_{max} and T_{max} were obtained directly by visual inspection of each subject's plasma concentration-time profile. $AUC_{0-\infty}$ and $t_{1/2}$ were determined by non-compartmental analysis. The slope of the terminal log-linear portion of the concentration-time profile was determined by least-squares regression analysis and used as the elimination rate constant (K_e). The elimination $t_{1/2}$ was calculated as $0.693/K_e$. The AUC_{0-t} from time zero to the last quantifiable point (Ct) was calculated using the trapezoidal rule. Extrapolated AUC from Ct to infinity ($AUC_{t-\infty}$) was determined as Ct/Ke. Total $AUC_{0-\infty}$ was the sum of AUC_{0-t} + $AUC_{t-\infty}$. The calculation was performed by using the TopFit , a pharmacokinetic data analysis program for PC.

11.2 Statistical analysis

The individual pharmacokinetic parameters (T_{max}, C_{max}, and AUC) which represented the rate and extent of drug absorption were presented as mean (median

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for T_{max}), standard deviation (SD), % CV, minimum and maximum values. An analysis of variance (ANOVA) was used to determine the statistical differences of these pharmacokinetic parameters such as variability between subjects, treatment groups, study periods and formulations. Statistical analysis of AUC and C_{max} was performed on logarithmically (ln) transformed data using ANOVA appropriate for the design. Thereafter, using the variance estimate (S^2) obtained from the ANOVA, the 90% confidence intervals (CI) for the ratio $\frac{\text{Test}}{\text{Reference}}$ of AUC as well as C_{max} values were calculated using the following formula:

$$90\%\ \text{CI}\ (\mu_T - \mu_R) = (X_T - \bar{X}_R) \pm t^v_{0.1}\sqrt{\frac{2S^2}{n}}$$

where

- X_T, $\bar{X}_R$ were the observed means of the (ln) transformed parameters (either C_{max} or AUC) for the test (T) and the reference (R) product
- S^2 was the variance obtained from the ANOVA
- n was the number of subjects
- $t^v_{0.1}$ was the tabulated two-tail *t* value for 90% CI
- v was the number of degree of freedom of the error mean square

The antilogarithm of the CI (μ_T- μ_R) would express the bioequivalence as a ratio of the test product and the reference product [$\frac{\text{Test}}{\text{Reference}}$]. Regarding the analysis of T_{max}, the limits for the bioequivalence range was expressed as untransformed data (absolute differences) and the 90% CI was calculated.

11.3 Bioequivalence acceptance criteria

The bioequivalence interval of 0.8-1.25 for the ratio [$\frac{\text{Test}}{\text{Reference}}$] of the average $AUC_{0-\infty}$ and C_{max} was accepted by the Thai FDA (15). Regarding analysis of T_{max}, the limit for the bioequivalence range was expressed as untransformed data (absolute differences) and the stipulated bioequivalence range of T_{max} difference [Test-Reference] was ± 20% of the T_{max} of the reference formulation (14).

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