The Effect of GLM 002, an Oriental Medicine, on Blood Pressure and Plasma Lipids in Spontaneously Hypertensive Rats

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Inhibition of angiotensin converting enzyme (ACE) activity is one of the common antihypertensive methods functioned by drugs such as captopril, lisinopril and enalapril to serve as inhibitors of ACE. This study was designed to compare the effects of enalapril, an angiotensin-converting enzyme inhibitor and GLM002, an oriental medicine, on tail systolic pressure, aorta and plasma properties in spontaneously hypertensive rats (SHR) after 4 weeks of treatment. During the treatment, blood pressure was depressed to normal in GLM002 and enalapril groups. The treatments of enalapril and GLM002 were discontinued in 4 weeks. One week after the treatment stop, systolic blood pressure was smoothly increased in both groups; the increment of blood pressure was slightly greater in GLM002-SHR, but the increment of plasma ACE activity was proportionately similar in each group. In the aspects of the triglyceride, HDL and total cholesterol level, those levels were slightly different among each group. We also conducted clinical dosage of GLM002 to the patients who have mild and severe hypertension for approximately 7 weeks. Clinical treatments also showed remarkable efficiencies on blood pressure (systolic blood pressure, diastolic blood pressure), complete blood count (CBC) routine, differ count (NEUTRO, LYM, MONO, EOS and BASO) and R-chemistry. We conclude that GLM002, like already proven enalapril, plays a role as an angiotensin-converting enzyme inhibitor, and can be suggested as a drug candidate for curing hypertension.

Key words : Angiotensin converting enzyme, spontaneously hypertensive rats (SHRs), hypertension, Enalapril

Introduction

A number of mechanisms have been suggested in the manifestation of hypertension. The renin-angiotensin system (RAS) among the many mechanisms plays an important role in the regulation of systemic blood pressure.[1,2] The renin-angiotensin cascade starts with the cleavage of angiotensinogen by renin to form angiotensin I (Ang I) and culminates in the conversion of the inactive decapeptide Ang I to the vasoconstrictor hormone angiotensin II (Ang II). Ang II influences vascular tone by several mechanisms.[3]

Traditionally, the RAS has been viewed as an endocrine system, however, a number of studies have shown multiple pathways of Ang II production in peripheral tissues, and suggested that Ang II generated locally may contribute to a sustained elevation in blood pressure.[4] These systems are regulated by the models of angiotensin converting enzyme and inhibitors.

Angiotensin converting enzyme (ACE) inhibitors and Ang II type1 (AT1) receptor antagonists both block the RAS.[5] Although these two types of drugs have very similar overall effects, the manifestation of their actions may be different. Since ACE is also involved in the cleavage of brandykinin, neurotensin, met-enkephalin and substance P as well as Ang I [6], the use of ACE inhibitors could lead to a potentiation of the effects of these peptides. It is reported that some of the therapeutic features of ACE inhibitors and some of their side effects may be attributed to angiotensin independent mechanisms.[7,8]

In contrast, AT1receptor antagonists specifically block the RAS. Such higher specificity may reduce the incidence of side effects. Several clinical studies have been conducted to compare the efficacy of Ang II antagonists with that of ACE inhibitors. The antihypertensive efficacy of GLM002 could be comparable tothat of ACE inhibitors such as captopril, enalapril or lisinopril. [9,10] GLM002 shows the large decrement on levels of

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lipid concentrations while other antihypertensive drugs show their own therapeutic features.[11]

This report indicates GLM002 showed the usefulness of lipid resolvent in rat with hypertension. GLM002 may work as a lipid resolvent which specifically decrease in rat aorta and plasma lipid. In several rat models of hypertension such as spontaneously hypertensive rats, DOCA - salt induced model, 2 kidney 1 clip (2K1C), as a renal hypertension model, and so on were introduced.[12,13,14] SHR is adopted as a rat model of this study in order to approach the most common hypertension mechanism. The present study was conducted to compare the hypertensive effects of GLM002 and enalapril, the active metabolite of enalapril and GLM002 were also evaluated using biochemical test and the measurement of ACE activity.

Materials and methods

1. Animal

Muce used were 10 weeks old, weighed 150~200 g, and assigned as SHR-Saline treated group, SHR-Enalapriltreated group and SHR-GLM002 treated group. Mice were stabilized for 2 weeks before treated, administered orally with 30 mg of GLM002 for GLM002 group, and with 30 mg of enalapril for SHR-EN group. A treatment was conducted twice a day for 4 weeks and withdrawal of dosage for 2 weeks. Rats were housed under identical conditions with free access to food (Sam Taco: Kimchun, Korea) and water until termination. SHR-NT group is treated with 3 ㎖ of saline as a placebo.

2. Determination of ACE inhibition in vitro

We evaluated the efficiency of angiotensin converting enzyme inhibition with GLM002 in vitro and compared with that of enalapril as a positive control. One gram of GLM002 was dissolved in 3 ㎖ of 0.05 % triton-X 100 and 0.1 g NaOH at 80 ℃, and diluted with 10 mg/㎖, 5 mg/㎖, 2 mg/㎖ and 1 mg/㎖ of water. And enalapril was also diluted using H_2O with same fraction of concentration as described above.

ACE activity was determined by a modified method of Friedland et al. as described in detail by Santos et al. Five units of angiotensin converting enzyme (ACE) (Sigma, A-6778) was incubated with 5 μM hippuryl-histidyl-leucine (Hip-His-Leu), an artificial substrate (Aldrich 85,905-20) in 500 ㎕ of 0.4 M sodium borate buffer pH 8.3 containing 0.9 M sodium chloride.

Ten ㎕ of enalaprildiluted with different concentrations as described above was added to each reaction mixture. The enzyme reaction was stopped by the addition of 1.2 ㎖ of 0.34 M NaOH. Blank samples were prepared by reversing the order of addition of enzyme and NaOH. One hundred ㎕ of o-phthaldialdehyde dissolved in methanol (20 mg/㎖) were added to each tube, and 10 min later the reaction was terminated by addition of 200 ㎕ of 3 N HCl. The reaction mixture was then centrifuged at 800 × g for 5 min at room temperature, and the product histidyl-leucine (His-Leu) was measured fluorometrically (365 nm excitation and 495 nm emission wavelengths). Standard curves for His-Leu (1-60 nmol) were prepared under the same conditions. All measurements were made in duplicate. ACE activity was expressed in nanomoles His-Leu per min per ㎖ plasma.

3. Systolic BP Measurement

Mice were removed from the animal room and taken to the facility, allowed free access to water, and kept in a quiet before measuring blood pressure (BP). Two weeks before the initiation of treatment with GLM002 or enalapril for each group, SHRs were trained for the measurement of BP by the tail-cuff method. Each time, rats were placed in a room at 37°C for 1 hour and systolic BPwas measured in unrestrained animals.

Once the rats were considered to be trained and not susceptible to stress from the tail-cuff procedure, systolic BP measurements were performed using a programmed physiography (model - 4CH, MK3SO297M) with photoelectric transducer. Basally and at week 10, systolic BP was measured at the same time (11 AM) for 2 consecutive days. Eight systolic BP measurements were carried out on each of these days in each animal, with the maximum and the minimum being rejected. The mean of 8 consecutive readings was used as the measurement of systolic BP of each rat, and systolic BP was determined twice a week during the control (1 week) and experimental (4 weeks) periods. The mean value of direct systolic BP (125 ± 4 mmHg) compared with that of indirect measurements (130 ± 5 mmHg) showed 96% correlation.

4. Plasma ACE Activity.

Blood samples were collected through the aorta with heparinized syringes while the animals were anesthetized with xylazine hydrochloride and ketamine. The blood samples were centrifuged at 800 × g for 15min at 4 ℃, and the plasma was stored at -20 ℃ until the assay. Plasma ACE activity was determined by the modified method of Friedland et al.[15] as described in detail by Santos et al.[16,17] Mouse plasma (10 ㎕) was incubated with 5 μM hippuryl-histidyl-leucine (Hip-His-Leu) an artificial substrate (Aldrich 85,905-20) in 500 ㎕ of 0.4 M sodium borate buffer, pH 8.3, containing 0.9 M sodium chloride. The enzyme reaction was stopped by the

addition of 1.2 ㎖ of 0.34 M NaOH. Blank samples were prepared by reversing the order of adding enzyme and NaOH.

One hundred ㎕ of methanol containing o -phthaldialdehyde by the concentration of 20 mg/㎖ were added to each tube, and 10 min later the reaction was terminated by addition of 200 ㎕ of 3 N HCl. The reaction mixture was then centrifuged at 800 × g for 5 min at room temperature. The product histidyl-leucine (His-Leu) was measured fluorometrically (365 nm excitation and 495 nm emission wavelengths). Standard curves for His-Leu(1-60 nmol) were prepared under the same conditions. All measurements were made in duplicates. ACE activity was expressed in nanomoles His-Leu per min per ㎖ plasma.

5. Tissue ACE activity.

Isolation of ACE from tissue in the rat aorta. Mouse aorta tissue was washed with saline at 4 ℃, chopped into small pieces, and homogenized with 10-fold volume of 50 mMsodium borate buffer, pH 7.4, by using a homogenizer. The homogenates were centrifuged at 12,000 × g for 10min at 4 ℃, and the supernatant collected for the measurement of ACE activity. *Measurement of aorta ACE activity.* Instead of plasma 50 ㎕ of tissue sample is assayed according to the protocol above. *Calculation of ACE activity.* We prepared standard curve (2,500 μM, 1,000 μM, 500 μM, 250 μM, 100 μM, 50 μM, 25 μM of His-Leu) by diluting the final reaction product, His-Lue. The formula calculating ACE activity by using the standard curve is as following;

Plasma = (Sample-Blank)/15min [nmol His-Leu/min/ml]
Tissue = (Sample-Blank)/15min/ tissue$_{weight}$ [nmolHis-Leu/min/ g $_{tissue}$]
Tissue $_{조직량}$ (g) = concentration $_{protein}$ (mg/ml) 50 l

6. Biochemical measurement in the plasma.

Blood samples were taken at random order between 08.00 and 10.00 h after 16 h fasting period. Blood was collected under anesthesia from the heart. Plasma of the animals were cooled and kept at 4°C until ultracentrifugation. Plasma total cholesterol concentration, triglyceride and HDL were determined using enzymatic test kits (Embiel kit). For each group, aliquots of plasma from all animals were pooled at -25 ℃ until the assay carried out.

7. Drugs.

GLM002 was prepared and provided by Green life Co., Ltd., seoul, Korea. Enalapril and Hip-His-Lue were purchased from SigmaChemical Co. Main components of GLM002 are Glycyrrhiza uralensis, Rehmannia glutinosa, Uncaria rhynchophylla, Scutellaria baicalensis, Coptis chinensis, Anemarrhena asphodeloides, Gardenia jasminoides, Garthamus tinctorius, Kalopanax pictum, Gastrodia elata, and Angelica gigas.

8. Statistical Analysis.

Statistical analyses were performed with analysis of student t-test, and expressed as the means ± S.E.M. Differences between groups were considered to be significant at $P<0.05$.

9. Clinical treatment.

We also conducted a clinical dosage to the patients who have mild or severe hypertension for approximately 7 weeks. The result of clinical treatment was showed by measuring blood pressure (systolic and diastolic blood pressure), complete blood count (CBC) routine (WBC; white blood cell count, RBC; red blood cell count, HB; hemoglobin, HT; Hematocrit, MCV; mean corpuscular volume, MCH; mean corpuscular hemoglobin, MCHC; mean corpuscular hemoglobin concentration and PLT; psittacosis-lymphogranulomavenereumtrachoma), differ count (NEUTRO, LYM, MONO, EOS and BASO), R-chemistry (GOT; glutamic oxaloacetic transaminase, GPT; glutamic pyruvic transaminase, GGT; gamma-glutamyl transferase, ALP; alkaline phosphatase, GLU; glucose, T-P; total protein , ALB; albumin, T-B; total bilirubin and D-B; direct bilirubin) , lipid (CHOLE; cholesterol, TG; triglyceride and HDL; high density lipid).

Result

1. Determination of Inhibitory activity of GLM002 to ACE in vitro.

We have compared the inhibitory activities to angiotensin converting enzyme between GLM002 as a natural substance and enalapril as a positive control in vitro. The values of IC_{50} of GLM002 and enalapril were analyzed. The results are shown as Table 1. Enalapril inhibited 50% of activities of angiotensin converting enzyme at the concentration of 0.68 ± 0.09 mg/㎖, whereas GLM002 at 2.34 ± 0.78 mg/㎖.

Table 1. Comparison for inhibition of ACE activity (IC_{50}) in vitro

ACE inhibitor	IC_{50} (mg/ml)
Enalapril	0.68 ± 0.09
GLM002	2.34 ± 0.78

The data show mean ± standard deviations. Enalapril inhibited 50% of activities of angiotensin converting enzyme at the concentration of 0.68 ± 0.09 mg/㎖, whereas GLM002 at 2.34 ± 0.78 mg/㎖.

2. Body weight changes of mice and heart and body ratio.

Ratios of heart and body weights in all groups are similar, and body weights of rats are consistently increased without fluctuation during the study (Table 2, Fig. 1).

Table 2. Weights of liver, heart and kidney collected from untreated, enalapril treated, and GLM002 treated SHRs.

Group	Liver weight (g)	Heart weight (g)	Kidney weight (g)	Ratio (heart/body weight)	N
SHR-NT	11.53 ± 0.79	1.28 ± 0.11	2.58 ± 0.19	0.032	10
SHR-PC	10.62 ± 0.56	1.11 ± 0.25	2.39 ± 0.04	0.031	10
SHR-GLM002	11.26 ± 0.33	1.15 ± 0.09	2.62 ± 0.20	0.037	10

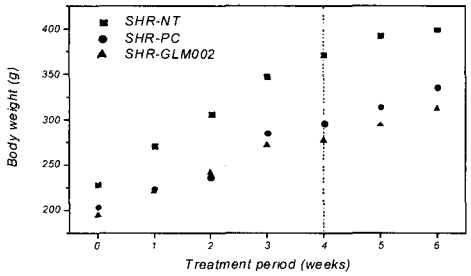

Fig. 1. Graphs of weekly changes in body weight. All groups are consistently increased without fluctuation during the study.

3. Changes of systolic blood pressure.

The weekly changes of blood pressure in the each group of mice are shown in Fig. 2. SHR-NT group showed consistent increment (4.8 ± 0.6 mmHgper week) of blood pressure. SHR-PC group treated with enalapril rapidly decreased blood pressure 2 weeks later while decreased at a low rate in 2 weeks. The withdrawal of treatment was conducted in both treated group after 4 weeks later. Blood pressure of SHR-PC group significantly increased after withdrawal of enalapril. The blood pressure of SHR-GLM002 treated group has showed less increment in contrast to SHR-PC group, and even remained stable after treatment is discontinued (Fig. 2).

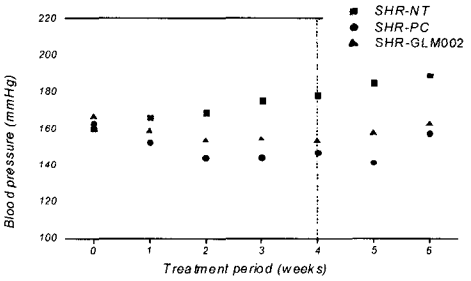

Fig. 2. A graph of weekly changes of blood pressure for each group. SHR-NT group showed consistent increment (4.8 ± 0.6 mmHg per week) of blood pressure while SHR-PC group treated with enalapril decreased rapidly 2 weeks later meanwhile slightly decreased 2 weeks later. However, Blood pressure of SHR-PC group increased after withdrawal of enalapril. The blood pressure of SHR-GLM002 treated group has showed less increment in contrast to SHR-PC group and even maintained the blood pressure as it was after treatment is discontinued (dotted line).

4. HDL, Total cholesterol and Triglyceride concentrations in Mouse Plasma.

The results of evaluation for HDL, total cholesterol and triglyceride concentration in the rat plasma are shown in Fig. 3. The concentrations of HDL in the SHR-NT (52.71 mg/dl ± 10.53 mg/dl) and SHR-PC (53.14 mg/dl ± 18.57mg/dl) groups were slightly lower than SHR-GLM002 (57.49 mg/dl ± 4.02 mg/dl) group. The concentrations of total cholesterol in both of SHR-PC and SHR-GLM002 groups were significantly lower by 20.7% and 24.6% individually comparing with SHR-NT group. Also the concentration of triglyceride in the SHR-PC group was higher (8.1%) than in the SHR - NT group that was higher than the concentration of triglyceride in the SHR-GLM002 group (9.8%).

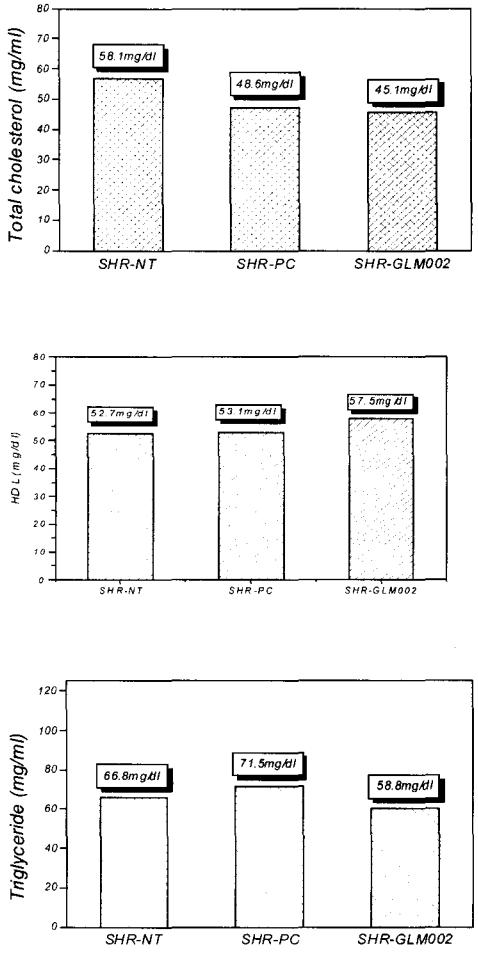

Fig. 3. The concentration of HDL, total cholesterol and triglyceride in rat plasma. The evaluation results for HDL, total cholesterol and triglyceride concentration in the rat plasma are shown in Fig 3. The concentrations of HDL in the SHR-NT (52.71 mg/dl ±10.53 mg/dl) and SHR-PC (53.14 mg/dl ±18.57 mg/dl) groups were slightly lower than SHR-GLM002 (57.49 mg/dl ± 4.02 mg/dl) group. The concentrations of total cholesterol in both of SHR-PC and SHR-GLM002 groups were significantly lower by 20.7% and 24.6% individually comparing with SHR-NT group. Also the concentration of triglyceride in the SHR-PC group was higher (8.1 %) than in the SHR-NT group that was higher than that of triglyceride in the SHR-GLM002 group (9.8%).

5. ACE activity in the tissue and plasma.

ACE activities in rat plasma and aorta are shown in Table 3. The ACE plasma activity in SHR-PC group ($p < 0.0001$) and SHR - GLM002 group ($p < 0.05$) decreased significantly than in the SHR - NT group. And the ACE tissue activities in SHR-PC and SHR - GLM002 groups ($p < 0.0001$) decreased significantly than in the SHR - NT group.

Table 3. ACE activity in the tissue and plasma for each group (nmol His-Lue/min·ml).

Group	Plasma	Aorta tissue	N
SHR-NT	4.83 ± 0.64	5.41 ± 0.43	10
SHR-PC	1.36 ± 0.41**	1.89 ± 0.41**	10
SHR-GLM002	4.15 ± 0.93*	3.86 ± 1.12**	10

The data show mean ± standard deviations, *p<0.05, **p<0.0001: significantly different from the control group. N: Number of rats used in the group.

6. Clinical treatment.

Hypertension patients, age from 40 to 50, daily took 0.3 g of GLM002 daily for 7 weeks and their values of CBC routine, DIFF count, R-chemistry and lipid showed dramatic antihypertensive efficiency (Figs 5, 6, 7, and 8). For GLM002 a significant decrement in BP was showed after the treatment and also steadiness of BP after withdrawal of the treatment (Fig. 4). In CBC routine, all measurements were in the normal range. DIFF count and R-chemistry also showed level in the normal range. Moreover, changes of lipid in the patients were significantly decreased, and also showed stable efficiency after withdrawal of treatment.

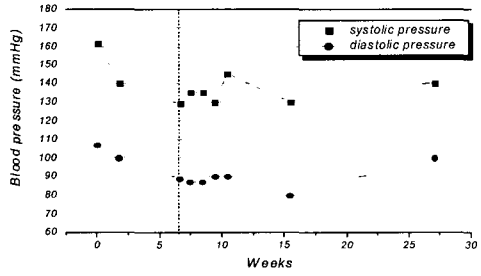

Fig. 4. Changes of BP(systolic and diastolic pressures) in the patients. The significant decrement in the BP was showed after the treatment, and also steadiness of BP after withdrawal of GLM002 over 15 weeks. Discontinued treatment was marked as a dotted line after 4 weeks of experiment.

Discussion

Many antihypertensive drugs, which are exclusively focused on depressing the blood pressure, have been used for curing a hypertensive even though damaged kidney, heart and brain because of their side effects.[18,19] It is the most main appearance in the most of antihypertensive medicines that withdrawal of treatment bring out increment of blood pressure again. That's one of the reasons combined dosage were requisite in the demand of need, such as diuretic, angiectasis, α,β- blocker, ACE inhibitor, antagonist and so on.[20] Considering that a desirable drug is physioactive substance extracted from galenicals protects heart, kidney and other organs from side effect as well as stabilizes the blood pressure after the withdrawal of treatment.

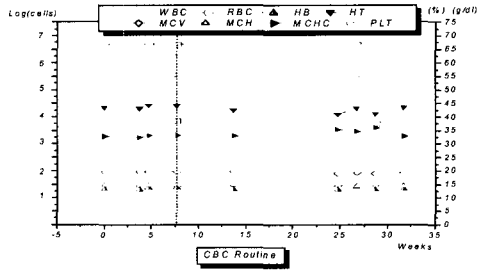

Fig. 5. Change of CBC routine in the patients. All results were in normal ranges (WBC - $4 \times 10^3 \sim 1.0 \times 10^4$ cells, RBC - $1.0 \times 10^6 \sim 5.9 \times 10^6$ cells, HB - 12 ~ 17 g/dl, HT - 39 ~ 50%, MCV - 80 ~ 100, MCH - 25 ~ 35 cells, MCHC - 30 ~37%, PLT - $1.5 \times 10^5 \sim 4.5 \times 10^5$ cells). Unit of Blank symbols are belonged in cells counts, half shade symbols is g/dl, and shaded symbols' are % unit. Discontinued treatment was marked as a dotted line after 4 weeks of experiment.

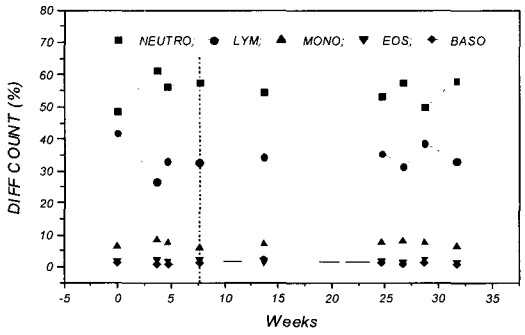

Fig. 6. Changes of DIFFcount in the patients. All results were in a normal range (NEUTRO - 40~75 %, LYM - 22~40 %, MONO - 4~8 %, EOS - 1~5 %, BASO - 0~2 %). Discontinued treatment was marked as a dotted line after 4 weeks of experiment.

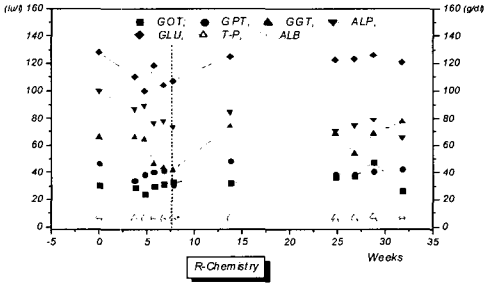

Fig. 7. Changes of R-chemistry in the patients. All ranges were in a normal range (GOT - 0~40 (Iu/ℓ), GPT - 0~45 (Iu/ℓ), GGT - 0~70 (Iu/ℓ), ALP - 50~250 (Iu/ℓ), GLU - 70~130 (Iu/ℓ), T-P - 6.5~8.0 (g/dl), ALB - 3.7~5.2 (g/dl)). Discontinued treatment was marked as a dotted line after 4 weeks of experiment. Unit of blank symbols are belonged in g/dl, and shaded symbols' are Iu/dl.

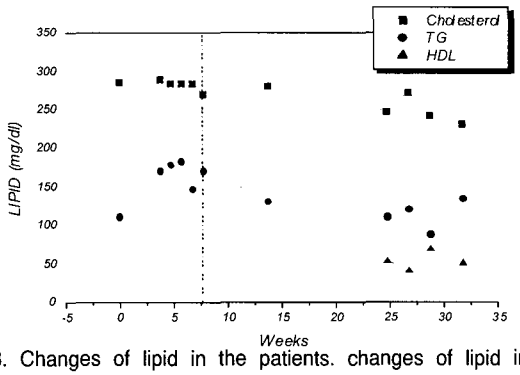

Fig. 8. Changes of lipid in the patients. changes of lipid in the patients were significantly decreased, and also showed steadiness of efficiency after withdrawal of treatment. (Normal range - Total cholesterol 120~270 mg/dl, HDL - 35~65 mg/dl, TG - 30~160 mg/dl). Discontinued treatment was marked as a dotted line after 4 weeks of experiment.

This study demonstrated that a variety of galenical materials regarded to have an antihypertensive effect were checked for the efficiency on various concentrations in vitro. After that the most suitable galenical selected among many was orally treated to the SHR, a genetically primary hypertension model, for 4 weeks. And withdrawal of the drug was also conducted to check its tonicity. In results, there was a significant decrement on plasma and tissue ACE activities, biochemical measurement, total cholesterol, HDL, and triglyceride concentration. The comparison between GLM002 and enalapril, a widely used ACE inhibitor, showed that IC_{50} value of enalapril (0.68 mg/㎖ ± 0.09 mg/㎖) was much lower than that of GLM002 (Table 1) in vitro. There was a significant and continuous decrement of BPfor both of GLM002 treated GLM002 group and enalapril treated SHR-PC group, compared with BP of the 3ml saline treated SHR-NT group. After withdrawal of each drug slightincrements were finally observed.

However, the BP of GLM002 group treated with GLM002 showed less decrement than SHR-PC group treated with enalapril, and GLM002 group kept BP in low levels compared against that of SHR-NT group (Fig. 2). In the view of the results, GLM002, a galenical extract, can inhibits ACE activity so that RAS, produces angiotensin Ⅱ, angiotonic substance, is blocked and accelerates brandykinn, vasodilator substance results in the decrement of blood pressure. In the results of comparisonin ACE activity in mouse plasma and aorta tissue of each group SHR-PC group showed dramatical decrement and SHR-GLM002 group showed outstanding inhibition efficiency in the aorta tissue whereas showed slight inhibition efficiency in the plasma to the ACE activity (Table 3). When we compared with biochemical results the concentration of HDL both plasma and aorta in the SHR-GLM002 group was higher than other groups and the concentration of total cholesterol and triglyceride was significantly lower than others. (Fig. 3).

In additions, hypertension patients, age from 40 to 50, daily took 0.3 g of GLM002 for 7 weeks and their values of CBC routine, differ count, R-chemistry and Lipid showed great antihypertensive efficiency (Figs 5, 6, 7, and 8). The significant decrement in BP was showed after the treatment and also stability of BP after withdrawal of GLM002 over 15 weeks (Fig 4). The value of CBC routine (WBC, RBC, HB, HT, MCV, MCH, MCHC and PLT), differ count (NEUTRO, LYM, MONO, EOS and BASO), R-chemistry (GOT, GPT, GGT, ALP, GLU, T-P, ALB, T-B and D-B), lipid (CHOLE, TG and HDL) were similar with the value of normotensive person with no toxicity.

The fact demonstrates that GLM002, a galenical extract, can specifically cure a hypertension by deceasing inside blood vessel the concentration of lipid, lipoprotein and cholesterol which is a main factor of obesity, arteriosclerosis, cerebral thrombosis and hypertension. GLM002 shows the its own therapeutic features on lipid level in rat and human plasma. A number of antihypertensive drugs, such as ACE inhibitor, diuretic, channel blocker, angiectasis, and so on, have been presently used, but use of a single drug is unable to show prevailing efficacies. This is the reason why each of antihypertensive drug only works through a few pathways of therapeutic features out of numerous hypertension mechanisms. But dual or triplex treatments can produce much better efficiency comparing a single treatment. Although GLM002 wasn't able to demonstrate a superior efficacy, but merely comparable with other remedies according to this study.

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