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# High Performance Gel-Chromatography as a Parameter for the Progress of Hepatic Failure in Dogs*)

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## ABSTRACT

Hepatic failure dogs induced by hepatic ischemia were prepared from mongrel dogs by occluding their hepatic and gastroduodenal arteries without anesthesia 48 hours after operation of their end to side port-caval anastomosis. During the process, they were divided into two groups; the 60-minute temporary hepatic ischemia group and the continuous hepatic ischemia group.

The plasma was sampled with the lapse of time from the above dogs after their hepatic ischemia and was analyzed using a high performance gel-chromatography (TSK G2000SW). The progress of hepatic failure caused distinct increase in the 12 abnormal Peaks a-l, of which Peak a and b fell in a region of middle molecular weights on the chromatography. Especially, Peak b increased greatly showing a good correlation with the condition of progressing hepatic failure.

Therefore, it is considered that the measurement of variation of such peaks, especially of Peak b, by the high performance gel-chromatography will prove to be a parameter indicating the degree of hepatic failure in progress.

## INTRODUCTION

Fulminant hepatic failure is a total metabolic disorder that leads to a sudden deterioration in the patient's condition. The purpose of its treatment is how to improve metabolic disorder, how to regenerate liver and how to maintain the patient's life until he reaches a sufficient level of hepatic regeneration. So, it is important to determine the degree of hepatic injury and the course of regeneration, and to predict the prognosis.

At present, the products of metabolic disorder in the condition of hepatic failure have not been clarified. Therefore, an attempt is being made to determine a parameter for evaluating the degree of hepatic failure by means of a high performance liquified chromatography (HPLC)[3,7,8,11]. Special attention is being given to the middle molecular weight substances of 500-5,000.

In this study, the authors performed an acute hepatic ischemia on mongrel dogs to induce hepatic failure condition to them and reviewed their applicability as hepatic failure models. Further, they analyzed the plasma taken from the hepatic failure dogs by means of HPLC and reviewed the result to prove if it can be used as a parameter to indicate the degree of hepatic failure in progress.

*) 川西秀樹，西亀正之，土谷太郎，江崎治夫：肝不全の進行に対する指標としての高速ゲル・クロマトグラフィーの有用性

## MATERIALS AND METHOD

### 1. Preparation of Hepatic Failure Dogs

Using mongrel dogs (BW 10–20 kg), acute hepatic ischemia models were prepared in accordance with the Abouna method[1]. After subjected to a fast of 24 hours, they were anesthetized by intravenously injecting 25 mg/kg of pentobarbital and placed under controlled respiration in the room temperature using a respirater through and intratracheal intubated tube. Immediately before the operation, canulation was performed in the external jugular vein for fluid infusion and blood samplin During the operation, they were maintained with 10–15 ml/kg/hr of saline. Fifty mg/kg of antibiotics-ABPC was injected intramuscularly for 2 days.

The operation method used was that, after laparotomy by the upper median incision and completely cutting apart the lesser omentum and phrenic artery, the end to side port-caval anastomosis of about 2.0 cm diameter was performed. The hepatic and gastroduodenal arteries were separated and a nylon thread of 0.5 mm diameter was tied around them. The thread was led through inside a vinyl tube of 0.5 cm diameter to outside the body and fixed in the subcutaneous of the wall. The dogs were given water freely for 2 days after the operation. Forty-eight hours after the operation, a hepatic ischemia was performed by pulling the buried nylon thread to prepare two groups of models of 60-minute temporary hepatic ischemia and continuous hepatic ischemia. After the ischemia procedure, the dogs were given 2–4 ml/kg/hr of glucose fluid (saline containing 5% of glucose) until their death or consciousness improved to being able to drink water had been reached. Fifty mg/kg of antibioticus-ABPC was intramuscularly injected at every 12 hours.

The degrees of coma were classified in accordance with the Smith method[14], as follows:

Grade 0: animal normal
Grade I: hypersalivation, ataxia, walk into objects; sleeps slightly more than normal but easily arousable.
Grade II: marked staxia, hypersalivation becomes marked; walks "through" objects, sleeps most of the time.
Grade III: frank hepatic coma but responsive to pain, awakes with difficulty, animal cannot stand unaided.
Grade IV: animal deeply comatose, unresponsive to pain; death usually follows shortly.

The dogs that fell into a coma and died within one week after ischemia were classified as the hepatic failure death group. Each of such cases were subjected to autopsy immediately after death and those which were considered to have been clearly caused by improper operation (bleeding in abdominal cavity, peritonitis) were excluded. The dogs that survived the operation for 4 weeks were classified as the survival group and sacrificed.

### 2. Analysis of Hepatic Failure Plasma by High Performance Liquid Chromatography (HPLC)

A gel-chromatography by the HPLC method was used to separate hepatic failure plasma.

A HPLC unit used was Toyo Soda HLC-803D and UV-8 Model II for detection at UV 220 nm. The column used was TSK-GEL G2000SW (Toyo Soda Co., Ltd., Tokyo Japan) of silica gel, 0.75×60 cm. Elution was achieved with a phosphate buffered saline (0.2 M, pH 6.9) at a flow rate of 0.6 ml/min. at 25°C. Prior to analysis, plasma samples were deproteininzed by ultrafiltration through Amicon Centriflo GF50A (Amicon Co., Ltd., Massachusetts, U. S. A.). The 10 $\mu$l of deproteinized sample was injected into the column. The peaks eluted were named alphabetically according to the elution time. The concentration level of each peak was measured and indicated in PHU/ml (peak height unit/ml of sample, where 1 PHU is equivalent to 0.01 absorbance unit of UV). All the peaks emerged in 60 minutes.

## RESULTS

### 1. Survival Time

Of the 22 dogs of the 60-minute temporary hepatic ischemia, 18 fell into coma and died of hepatic failure within one week. Their mean survival time was 55.0±37 hours. The 18 dead dogs were widely divided into two groups—one is the short-survived group of 11 that died within 40 hours marking the mean survival time of 27.6±7.2 hours, and the other, the long-survived group of the remaining 7 that died between the 40th hour and the 7th day marking the survival time of 98.1±21.7 hours.

**Table 1.** Survival of fulminant hepatic failure induced by hepatic ischemia in dogs

| Survival time | No of dogs (%) | Mean survival time |
|---|---|---|
| temporary ischemia group | 22 | |
| ~40 hrs | 11(50) | 27.6±7.2 hrs |
| 40 hrs~7 days | 7(32) | 98.1±21.7 hrs |
| 7days~4 weeks | 2(9) | 7.5 days, 24.5 days |
| survivors | 2(9) | 4 weeks |
| continuous ischemia group | 5 | 19.3±4.1 hrs |

(Mean±SD)

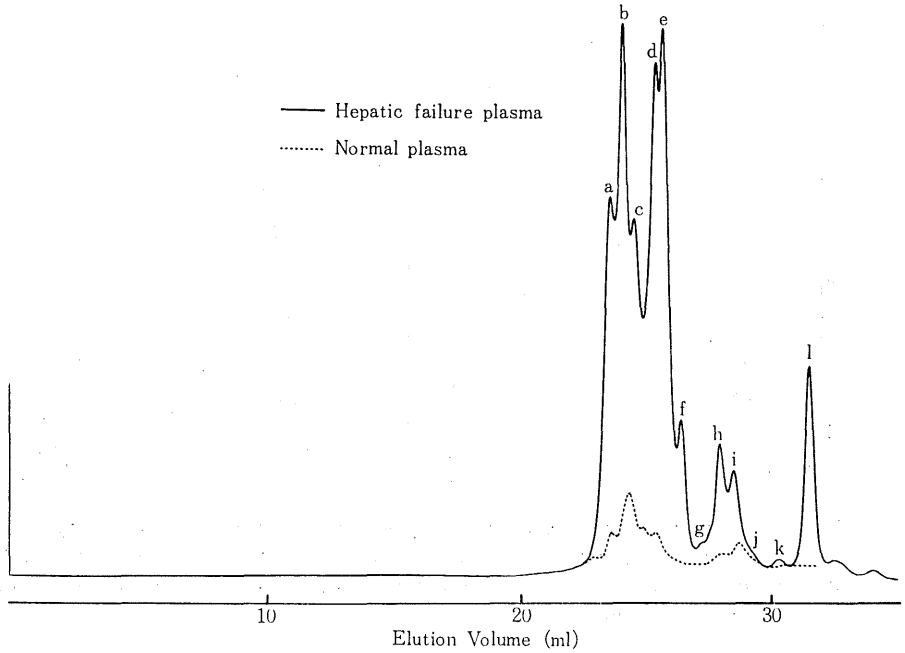


**Fig. 1.** Typical patterns of high performance gel-chromatography taken from a dog in normal and hepatic failure conditions.

Four dogs survived for more than 7 days, of which 2 survived for 4 weeks and were sacrificed (Table 1).

The condition of progress to a coma was observed as follows: The dogs in the short-survived group fell into a coma from about 6 hours after ischemia and more than half of them, into Coma III or IV about 12 hours after ischemia. The state of coma rapidly took a sharp turn for the worse thereafter, causing all the cases to die at Coma IV. On the other hand, those in the long-survived group which fell into a coma from about 6 hours after ischemia in the same manner as for the former group, showed gradual progress of coma thereafter. Three of them died at Coma III and 4, at Coma IV. Those in the survived group also fell into a coma from about 6 hours after ischemia but up to Coma III, showing an almost improved condition to eat and drink in the 1st week.

On the contrary, 5 dogs in the continuous hepatic ischemia group falling into a coma from about 6 hours after ischemia rapidly took a

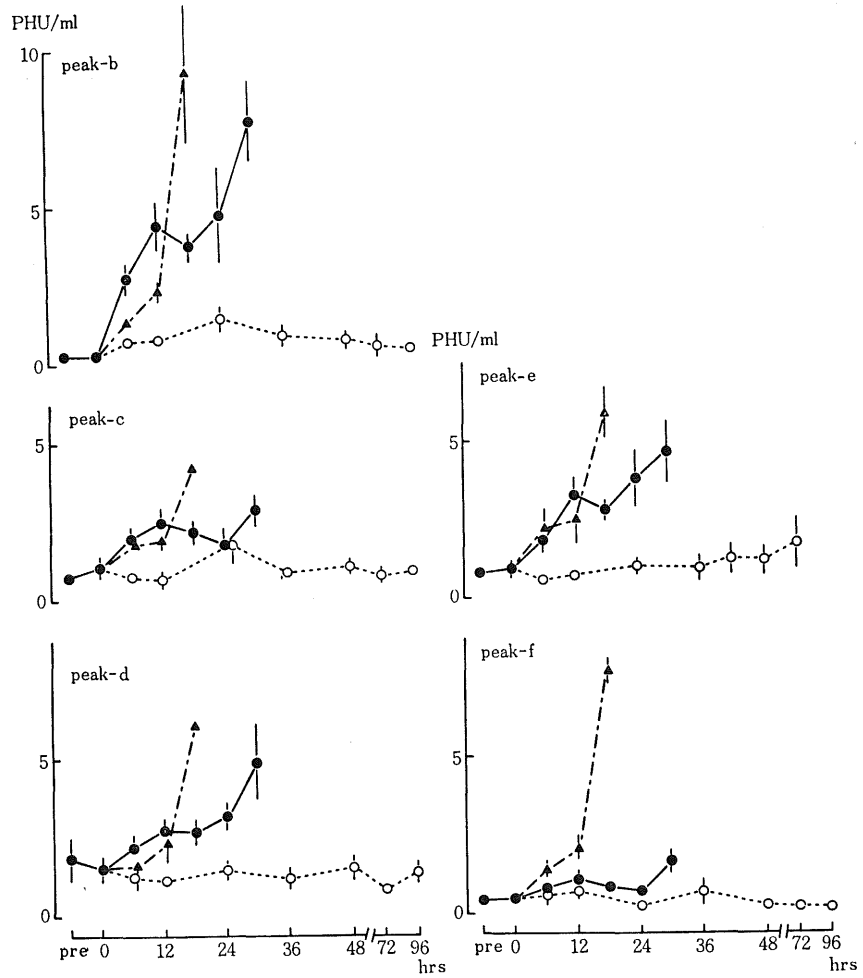


**Fig. 2.** Changes in the chromatographic peak level in the progress of hepatic failure in dogs. Temporary hepatic ischemia: short survived group (—●—, n=6), long survived group (···○···, n=4). Continuous hepatic ischemia (-·-▲-·-, n=5). PHU=Peak height units. Mean±SE.

bad turn and all died at Coma IV between 15.5 and 27 hours marking the mean survival time of 19.3±4.1 hours (Table 1).

## 2. Variation in Gal-Chromatography Pattern

Fig. 1 shows typical chromatograms of plasma of normal and hepatic failure dogs (Coma IV). The 12 peaks of a-l were observed. Variation was particularly distinct at Peaks b, c, d, e and f. Peaks a and b are cut off by the 500-dalton cut-off membrane (Ultra-filter UHO5, Toyo Roshi Co., Ltd., Tokyo, Japan) which are considered to contain middle molecular weight substances.

In ovservation of variations at Peaks b, c, d, e and f of 6 dogs in the short-survived group, 4 in the long-survived group of the temporary hepatic ischemia group and 5 of the continuous hepatic ischemia group (Fig. 2), those of the short-survived group showed an increase in variation as hepatic failure progressed, with a distinct variation at Peak b to 17.4 times and 28.5 times the normal value after 24 hours and 30 hours of ischemia, respectively. On the other hand, those of the long survived group showed only variations as little as 5.3 times at Peak b and 2.5 times at Peak c, respectively. Those of the continuous hepatic ischemia group showed nearly linear increases at all peaks.

Table 2 shows the values at each peak of

**Table 2.** Changes in the chromatographic peak level at normal and grades III and IV coma

| Peak | Mean peak levels (PHU/ml) at | | |
|---|---|---|---|
| | Normal | Grade III coma | Grade IV coma |
| b | 0.22±0.10 | 4.08±0.79 | 5.49±2.45 |
| c | 0.63±0.24 | 2.34±0.90 | 2.67±0.59 |
| d | 0.83±1.33 | 2.50±0.86 | 4.63±2.28 |
| e | 0.83±1.33 | 2.92±1.35 | 4.98±2.52 |
| f | 0.50±0.13 | 0.97±0.18 | 1.28±0.30 |

PHU=Peak height units. Mean±SD (n=6)

Coma III and IV into which the dogs in the short-survived group fell. Peak b shows distinct increases reaching 18.5 times and 25 times the normal value at Coma III and IV, respectively. Peak b rises abruptly when Coma III has been reached. When it progresses to Coma IV, the peak b does not increase so much, but Peaks d and e increase rapidly reaching nearly the same level as Peaks b, d and e. From these facts, Peak b is considered most sensitive of all peaks to indicate the dogs' fall into a coma.

As described above, it may be said that the pattern of variation in the gel-chromatography, especially Peak b, would clearly show the degree of coma in hepatic failure.

## DISCUSSION

To prepare the experimental models of fulminant hepatic failure, various methods have been used, including total hepatectomy[9,15], hepatic ischemia (temporary[1,5] and continuous[10]), application of chemicals[2], etc. When considering any one of these methods which simulated the clinical hepatic failure and yet achieves clinically applicable effects of treatment that has been given to the models, it must be, at least, of reversibility and reproducibility and provide death from hepatic failure, as described by Terblanche et al.[14]. From this point of view, the authors selected mongrel dogs as models that were subjected to non-laparotomical hepatic ischemia 48 hours after the end to side port-caval anastomosis was performed.

The models that were subjected to continuous ischemia showed an abrupt change immediately after ischemia and all died within 15.5–27 hours.

On the other hand, of the 22 dogs of the 60-minute temporary hepatic ischemia models, 2 were found survived meeting the requirement of reversibility. However, there was a considerably wide difference from 17.5 to 132 hours in the survival time of the hepatic failure death group. In a detailed observation, this group was divided into two groups at the boundary of 40 hours. Moreover, a histopathological observation showed that all cases in the short-survived group indicated panlobular massive hepatic necrosis with little survived hepatocyte. While, in the long-survived group, panlobular necrosis and confluent necrosis existed together even with about 20% of survived hepatocyte, showing a clear difference with those in the short-survived group. However, there was little difference observed between the two groups in the general liver function test including coagulative function.

A distinct difference between the two groups were observed in variation in a gel-chromatography. This high performance liquid chromatography, especially the gel-chromatography has been frequently used in the analysis of hepatic failure plasma.

Hughes et al.[6] have observed an abnormal peak in substances with molecular weights below 10,000 by analyzing the plasma of a hepatic failure patient using the Sephadex G25 chromatography. They assume that these substances would cause activation disturbance of Na·K-ATPase in brain cell microsome leading to brain edema.

Also, Chang et al.[3] and Leber et al.[8] have found an abnormal peak in molecular weights of 500–5,000 by analyzing plasma of galacto-

samine hepatic failure rats and hepatic failure patients using the Sephadex G15 chromatography. Inoue et al.[7] used the polyvinyl alcohol copolymer gel-chromatography in an analysis of the plasma of a hepatic failure patient and reported that the variation of the peak could be used in judgement of the effect of plasmapheresis treatment.

All the researches conducting various studies, as above, point out the importance of the so-called middle molecular (MM) fraction.

The authors used TSK G2000SW of silica gel at UV 220 nm to indicate the variations in Peaks b, c, d, e and f as hepatic failure progressed. Peak b, in particular, showed the best correlation with the degrees of progress and coma.

The cause that clearly divided into two groups the hepatic failure models induced by temporary hepatic ischemia prepared by the authors was considered due to the difference in the degree of hepatocyte necrosis. That is, in the short-survived group, the rapidly increased amount of substances escaped from a large part of the hepatocyte that had fallen into a necrosis caused encephlopathy that might lead the models to death. In the long-survived group, on the other hand, although the degree of hepatocyte necrosis was a little less and the increase in the amount of escaped substances was not so abrupt that its models were brought to death, the insufficient amount of the remaining hepatocyte absolutely necessary for life maintenance might lead them to death of hepatic failure, thereafter. As described above, it is considered that the 60-minute temporary hepatic ischemia models are clinically similar to those of fulminant hepatitis.

This test has not been able to identify what the substances emerging during gel-chromatography are and how they function. Moreover, several substances are considered being overwrapped at these peaks with those of middle molecular weights mainly contained at Peak b, leading to an anticipation of existence of considerable amount of substances of small molecular weights at this peak at the same time. Thus, it will be impossible to discuss the substances causing hepatic failure only by the gel-chromatography. However, it is considered useful for predicting the degree of progress and after care because of the good correlation between its peak variation and the degree of hepatic failure.

As to MM, especially, so far considered to be peptidelike substance[8], its molecular weight is only known as lying in the renge of 500–5,000. However, in both clinical cases and animal test, it is certain that MM increases as the coma condition progresses. In addition, its increase in the brain passing through the blood brain barrier. Shi and Chang[13] identified MM in the brain by HPLC and observed a reduction in it by ACAC-hemoperfusion. Also, Denis et al.[4] have observed a reduction in the fluorescent MM in the brain by calculating the concentration of such MM from the difference between the tyrosin levels by the fluorimetric method and the resin chromatographic method.

As above, the measurement of MM is significant for use as a parameter for the advance of hepatic failure.

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