BLOOD COAGULATION TESTS IN TOXICOLOGICAL STUDIES
- REVIEW OF METHODS AND THEIR SIGNIFICANCE
FOR DRUG SAFETY ASSESSMENT -

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ABSTRACT — In general toxicological studies, prothrombin time and activated partial thromboplastin time are routinely measured to assess blood coagulation. Special (problem-driven) tests for blood coagulation are of significance to detect abnormalities and investigate the mechanism of toxicity in detail. In this review, we compiled widely scattered information on blood coagulation testing from different fields in the biological area, and reviewed the methods available and their significance in toxicological studies. The relevant literature cited here reports large species differences in platelet aggregation, coagulation factors or fibrinolysis, and technical limitations. However, the following tests are basically applicable to laboratory animals; (1) assays for individual coagulation factors and protein induced by vitamin K absence or antagonists (PIVKA) to investigate coagulation factor abnormalities; (2) platelet aggregation-, platelet adhesion-, platelet release-tests and von Willebrand factor assay to screen and/or investigate platelet dysfunction; (3) fibrin/fibrinogen degradation products (FDP), D-dimer and thromboelastogram to detect fibrinolytic abnormalities, and assays for plasminogen, plasmin and their activator/inhibitor to investigate fibrinolysis in detail; and (4) bleeding-time to grossly evaluate blood coagulation capability in vivo. An appropriate battery of these tests provides significant information for risk assessment of drugs.

KEY WORDS: Blood coagulation, Platelets, Fibrinolysis, Toxicological study, Laboratory animals

INTRODUCTION

According to the guidelines (e.g., guideline from MHLW* Japan) based on ICH**, prothrombin time (PT) and activated partial thromboplastin time (APTT) are required as routine parameters to evaluate the effects of drugs on blood coagulation in general toxicological studies. These data, together with other findings such as platelet counts and histopathological findings, allow a gross interpretation of the status of blood coagulability. However, further examination will be necessary if we want to identify the target of drugs on blood coagulation or investigate their mechanism. Bloom (1993) described platelet aggregation and coagulation factor (VII, VIII, etc.) assays as “the special (problem-driven) tests” to be performed in the toxicological studies. A recent textbook listed platelet aggregation, plasma fibrinogen concentration, clotting factor assays, thrombin time and bleeding time as the “problem-driven tests” necessary to characterize the effects of drugs (Bloom and Brandt, 2001). The blood coagulation system constitutes numerous complex reactions of platelets, coagulation factors and fibrinolysis factors. We should consider different toxicological mechanisms in each drug when any abnormality is found in the blood coagulation system. It is of importance to choose “the special (problem-driven) tests” in accordance with the characteristics of the drug or toxicological events.

On the other hand, there exist large species differences regarding platelet aggregation (Macmillan and Sim, 1970; Dodds, 1978; Kurata et al., 1995), coagulation factors and fibrinolysis (Karges et al., 1994; Ravanat et al., 1995; Dodds, 1997). In addition, methodological limitations in each species are an essential issue that complicates the examinations done in laboratory animals. Almost all kits for clinical pathological
tests done in humans employ an immunological method. These often lack or have decreased cross-reactivity with other species. For a toxicological study, clinical pathologists may have to choose the most suitable method or modify a method for use in laboratory animals.

Currently, useful information regarding the methods that can be used to examine blood coagulation in laboratory animals is scattered in the literatures of toxicology, pharmacology, laboratory animal science, comparative science and veterinary medicine. We reviewed the literature in an attempt to compile this widely scattered information in one place, so that future investigators may grasp the nature and significance of the method used in each blood coagulation test.

* : Ministry of Health, Labour and Welfare
** : ICH: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

COAGULATION FACTOR TESTS

As shown in Fig. 1, the cascade of reactions leading to blood coagulation consists of two main pathways (Mischke and Nolte, 2000; Roberts et al., 2001). One is "the extrinsic pathway" initiated by the reaction of tissue thromboplastin, and followed by factors III (tissue thromboplastin), VII and X (Stuart-Power factor). The other is "the intrinsic pathway", which is initiated by factor XII (Hageman factor) activation followed by factors XI, IX and VIII. The extrinsic (i.e., activated factor VII; abbreviated, factor VIIa) or intrinsic (i.e., factor IXa) pathway activates factor X, in the presence of phospholipid, Ca²⁺ and factor V (proaccelerin). Factor Xa catalyzes the reaction from prothrombin (factor II) to thrombin (factor IIa) which is involved in the transformation of fibrinogen (factor I) into fibrin.

Many of these factors are synthesized in the liver. Therefore, any agent that impairs liver function may cause a decrease in the production of coagulation factors (Bloom and Brandt, 2001). The importance of

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Fig. 1. Cascade of blood coagulation factors. The blood coagulation cascade is divided into the intrinsic and extrinsic pathways. "a" (e.g., XIIa) means activated form (e.g., activated XII). Please note factor XIII is described in this figure. Factor XIII is not usually involved in the intrinsic or extrinsic pathway. This figure was reproduced with permission from the Japanese Society of Laboratory Animals, Japan, from a textbook concerning blood examinations used to assess drug safety (blood coagulation section described by Kurata M.).
coagulation factors tests is the possibility to detect the toxicity of drugs based on their effects on coagulation factor activity or on vitamin K-related coagulation [see the section of protein induced by vitamin K absence or antagonists (PIVKA)].

In this section, we briefly describe sample preparation, storage and artifactual errors, and reviewed screening tests, individual coagulation factors and representative intrinsic inhibitors.

Sample preparation, sample storage and artifactual errors

Citrated plasma is separated from citrated blood (a ratio of 9 volume of blood and 1 volume of a 3.2 or 3.8% sodium citrate solution) by centrifugation (e.g., ≥2000 × g, ≥10 min, at 4°C), and used for PT, APTT, fibrinogen and coagulation factor assays.

The plasma is usually kept cold in ice. Tabata et al. (1995) reported that the PT and APTT of various laboratory animal species (mice, rats, cynomolgus monkeys, rabbits and dogs) were stable up to 24 hr at 4°C, except for the APTT of rats and cynomolgus monkeys.

On the other hand, Iazbik et al. (2001) reported that storage up to 30 days at −30°C did not significantly affect PT and APTT in dogs. We confirmed the stability of canine PT and APTT during storage at −80°C for up to 4 months. However, rat APTT was prolonged after one-week at −80°C. A similar result was reported in a human sample of frozen plasma (Rao et al., 2000). The effect of storage or freezing differs according to the species, and rat APTT is especially unstable.

As mentioned above, blood collected for coagulation tests is mixed at a ratio of 9 volumes of blood per 1 volume of citrate solution. An inadequate blood collection procedure (e.g., a relatively small volume of blood) or an increased hematocrit (e.g., due to dehydration) resulted in an increase of the ratio of citrate to plasma. The excess of citrate is known to induce prolongation of PT and thromboplastin time (PTT) in human plasma (Koecke et al., 1975; Ingram and Hills, 1976). Similar effects have been observed on these PT and APTT in plasma from rats and dogs (O'Brien et al., 1995; Kurata et al., 1998).

Prothrombin time

Quick et al. (1935) introduced prothrombin time (PT) as a screening test for factors VII, X, V and II of the extrinsic pathway. The value of PT is highly significant, because it allows an accurate evaluation of the capacity of the extrinsic pathway. PT is one of the standard parameters recommended in the guidelines for toxicological studies (e.g., MHLW guideline).

Briefly, the method consists in measuring the time up to clot formation after adding the “PT reagent” (a combination of tissue thromboplastin and Ca²⁺). Although the original method described by Quick et al. (1935) was a manual operation under observation, at present automatic analyzers are usually employed in PT. Mechanical principles to determine plasma coagulation are divided into two categories: (1) monitoring turbidity of plasma during plasma coagulation [e.g., ACL series, Beckman, Lexington, MA (currently, the same apparatus is obtained from Instrumentation Laboratory, Lexington, MA); CA series, Sysmex, Kobe, Japan], and (2) monitoring plasma viscosity during coagulation (e.g., STA series, Roche Diagnostica Stago, Asnieres, France; KC series, Amelung, Lebrinksweg, Germany). It is known that PT varies depending on the PT reagents employed (Kase, 1978). Therefore, it is necessary to establish a reference range in each laboratory, and under each experimental condition.

Table 1 summarizes the species differences and the reagents used to measure PT. The shortest value is found in dogs. Rats, especially males, are known to show a prolonged PT under fasting condition (Tabata et al., 1995). This may be related to a deficiency in vitamin K and estrogen (Jolly et al., 1977; Uchida et al., 1986).

Activated partial thromboplastin time

Proctor and Rapaport (1961) introduced activated partial thromboplastin time (APTT) as a screening test for the intrinsic pathway (factors XII, XI, IX, VIII, X, V and II). A prolonged APTT is caused by deficiency of factors XII, XI, IX, VIII, or by inhibitors (heparin, etc.) (Seligsohn and Coller, 2001). APTT, same as PT, is of high significance in the gross evaluation of intrinsic pathway activity. APTT is a standard parameter recommended by the guidelines for toxicological studies (e.g., MHLW guideline).

To determine APTT, a commercially available APTT reagent (Table 2) is added to the citrated plasma and the mixture is placed in a test tube at 37°C. An APTT reagent consists of a surface activator and a phospholipid that activate the intrinsic pathway. Therefore, Ca²⁺ is added to start clot formation, and the time until elapsed coagulation is recorded. The same automatic analyzers used to determine PT can be usually employed.

Blood coagulation tests.
Table 2 shows the APTT in different species and the reagents used to measure APTT. Similar to PT, the shortest value of APTT was found in dogs. In rats, prolongation of APTT under fasting conditions was the same as described for PT. It is apparent from our data that APTT is affected by the reagents employed in the test. This again emphasizes the importance of establishing a reference range in each laboratory.

**Fibrinogen**

Fibrinogen is a glycoprotein synthesized in the liver, and found in plasma and the α-granules of platelets (Roberts et al., 2001). Fibrinogen plays a central role in hemostasis by forming a cross-linking meshwork of fibrin. Fibrinogen value, taken together with those of PT and APTT, allows a rough evaluation of drug-target in the blood coagulation cascade in toxicological studies.

Fibrinogen is also known as one of the acute

<p>| Table 1. Prothrombin time in different species as determined using various PT reagents. |
|----------------------------------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Species</th>
<th>PT (sec)</th>
<th>PT Reagents</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>1) 12.1</td>
<td>1) Thromborel, Dade, Behring,</td>
<td>1) Karges et al., 1994</td>
</tr>
<tr>
<td></td>
<td>2) 16.7 (M), 17.0 (F), 20.1 (M)<em>, 17.7 (F)</em></td>
<td>Marburg, Germany</td>
<td>2) Tabata et al., 1995</td>
</tr>
<tr>
<td></td>
<td>3) approx. 17</td>
<td>2) Ortho Brain Thromboplastin,</td>
<td>3) Our data</td>
</tr>
<tr>
<td></td>
<td>4) approx. 14</td>
<td>Ortho Diagnostic Inc. Raritan, NJ</td>
<td>4) Our data</td>
</tr>
<tr>
<td>Mice</td>
<td>1) approx. 10</td>
<td>3) SRA Neoplatin Plus, Roche</td>
<td>3) Our data</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diagnostics GmbH, Mannheim Germany</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4) Thrombocheck PT,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>International Reagent, Kobe, Japan</td>
<td></td>
</tr>
<tr>
<td>Guinea pigs</td>
<td>1) 44.7</td>
<td>1) Ortho Brain Thromboplastin,</td>
<td>1) Karges et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ortho Diagnostic Inc. Raritan, NJ</td>
<td></td>
</tr>
<tr>
<td>Dogs</td>
<td>1) 10.8</td>
<td>1) General Diagnostics, Morris Plains, NJ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2) 7.4</td>
<td>2) Thromborel, Dade, Behring,</td>
<td>1) Feingold et al., 1986</td>
</tr>
<tr>
<td></td>
<td>3) 7.1 (M), 6.7 (F)</td>
<td>Marburg, Germany</td>
<td>2) Karges et al., 1994</td>
</tr>
<tr>
<td></td>
<td>4) approx. 7.5</td>
<td>3) Ortho Brain thromboplastin,</td>
<td>3) Tabata et al., 1995</td>
</tr>
<tr>
<td></td>
<td>5) approx. 8</td>
<td>Ortho Diagnostic Inc. Raritan, NJ</td>
<td>4) Our data</td>
</tr>
<tr>
<td>Cynomolgus monkeys</td>
<td>1) 12</td>
<td>1) SRA Neoplatin Plus, Roche</td>
<td>5) Our data</td>
</tr>
<tr>
<td></td>
<td>2) approx. 9</td>
<td>Diagnostics GmbH, Mannheim Germany</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4) Thrombocheck PT,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>International Reagent, Kobe, Japan</td>
<td></td>
</tr>
<tr>
<td>Pigs</td>
<td>1) 11.4</td>
<td>1) Thromborel, Dade, Behring,</td>
<td>1) Karges et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Marburg, Germany</td>
<td>2) Tabata et al., 1995</td>
</tr>
<tr>
<td>Rabbits</td>
<td>1) 9.9</td>
<td>1) Thromborel, Dade, Behring,</td>
<td>1) Karges et al., 1994</td>
</tr>
<tr>
<td></td>
<td>2) 8.1 (M), 8.5 (F)</td>
<td>Marburg, Germany</td>
<td>2) Tabata et al., 1995</td>
</tr>
<tr>
<td>Humans</td>
<td>1) 12.1</td>
<td>1) Ortho Brain thromboplastin,</td>
<td>1) Tabata et al., 1995</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ortho Diagnostic Inc. Raritan, NJ</td>
<td></td>
</tr>
</tbody>
</table>

M: Male animals, F: Female animals.

* Fasting condition.

b: Ortho Diagnostic Inc. was thereafter renamed to Ortho Clinical Diagnostic Inc. Currently, the same reagent can be obtained from Instrumentation Laboratory, Orangeburg, SC.
Blood coagulation tests.

Phase proteins released in an inflammatory reaction (Mischke and Nolte, 2000). An increase in plasma fibrinogen is an index of inflammation in vivo. Lewis et al. (1989) reported an increase in plasma fibrinogen from 1.0-1.7 mg/mL (normal) to 4.3-5.2 mg/mL in a rat model of adjuvant-induced inflammation. A two-fold increment in plasma fibrinogen was reported in rats intraperitoneally injected with lipopolysaccharide (Silveira and Limoas, 1990).

In general, fibrinogen is measured by the "thrombin time" method (Clauss, 1957). A commercially available reagent containing thrombin (e.g., Dade Thrombin Reagent, Dade Behring, Marburg, Germany; STA Fibrinogen, Roche Diagnostics, GmbH, Mannheim, Germany) is added to the citrated plasma. As the time required for fibrin clot formation is propor-

| Table 2. Activated partial thromboplastin time in different species as determined using various reagents. |
|------------------------|------------------------|------------------------|------------------------|
| Species                | APTT (sec)             | APTT Reagents           | References             |
| Rats                   |                        | 1) Pathromtin, Dade Behring, Marburg, Germany |
|                        | 1) Pathromtin, Dade Behring, Marburg, Germany |
|                        | 2) Ortho Brain Thromboplastin, Ortho Diagnostic Inc. Raritan, NJ |
|                        | 3) SRA APTT, Roche Diagnostics GmbH, Mannheim Germany |
|                        | 4) Dade Actin, Dade Behring, Marburg, Germany |
| Mice                   | 1) approx. 23          | 1) Ortho Brain Thromboplastin, Ortho Diagnostic Inc., Raritan, NJ |
| Guinea pigs            | 1) 52.8                | 1) Pathromtin, Dade Behring, Marburg, Germany |
| Dogs                   | 1) 18.3                | 1) General Diagnostics, Morris Plains, NJ |
|                        | 2) 17.7                | 2) Pathromtin, Dade Behring, Marburg, Germany |
|                        | 3) approx. 12          | 3) Ortho Brain Thromboplastin, Ortho Diagnostic Inc., Raritan, NJ |
|                        | 4) approx. 18          | 4) SRA APTT, Roche Diagnostics GmbH, Mannheim, Germany |
|                        | 5) approx. 14          | 5) Dade Actin, Dade Behring, Marburg, Germany |
| Cynomolgus monkeys     | 1) 36.8                | 1) Pathromtin, Dade Behring, Marburg, Germany |
|                        | 2) 21.9 (M), 20.6 (F)  | 2) Ortho Brain Thromboplastin, Ortho Diagnostic Inc., Raritan, NJ |
| Pigs                   | 1) 16.6                | 1) Pathromtin, Dade Behring, Marburg, Germany |
| Rabbits                | 1) 21.4                | 1) Pathromtin, Dade Behring, Marburg, Germany |
|                        | 2) approx. 25          | 2) Ortho Brain thromboplastin, Ortho Diagnostic Inc., Raritan, NJ |
| Humans                 | 1) 27.3                | 1) Ortho Brain thromboplastin, Ortho Diagnostic Inc., Raritan, NJ |

M: Male animals, F: Female animals.

*: Fasting condition.

b: Ortho Diagnostic Inc. was thereafter renamed to Ortho Clinical Diagnostic Inc. Currently, the same reagent can be obtained from Instrumentation Laboratory, Orangeburg, SC.
tional to fibrinogen concentration in this assay system, the fibrinogen concentration is obtained based on the time elapsed up to clot formation, using commercial human standard. Although it may be a rare case, one must bear in mind that the presence of thrombin inhibitor (e.g., heparin) produces an artifactual decrease in fibrinogen when using the thrombin time method.

To avoid the above problem, an alternative method, the heat precipitation method, can be used. The method (Millar et al., 1971) is, briefly, as follows. Whole blood (with EDTA or heparin) is withdrawn into a microhematocrit capillary, and centrifuged at 12000 rpm for 5 min to separate blood cells from plasma. The capillary is then heated at 56°C for 3 min, and again centrifuged for 3 min. The fibrinogen concentration is calculated from the ratio of fibrinogen and plasma in the centrifuged capillary (Fig. 2). Using a similar principle, Lewis et al. (1989) employed "cold" precipitation (4°C, 18 hr) with ammonium sulphate and protein measurement to measure rat fibrinogen.

In these methods, fibrinogen levels ranged from 100 to 300 mg/dL, which was comparable among laboratory animals (Table 3).

**Table 3.** Fibrinogen concentration as determined using various methods in different species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Fibrinogen concentration</th>
<th>Methods</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1) 200-300 mg/dL</td>
<td>1) Thrombin time</td>
<td>1) Our data</td>
</tr>
<tr>
<td></td>
<td>2) 250-300 mg/dL</td>
<td>2) Heat precipitation</td>
<td>2) Our data</td>
</tr>
<tr>
<td></td>
<td>3) 100-170 mg/dL</td>
<td>3) Cold precipitation</td>
<td>3) Lewis et al., 1989</td>
</tr>
<tr>
<td>Dogs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1) 321.5 mg/dL</td>
<td>1) Thrombin time</td>
<td>1) Feingold et al., 1986</td>
</tr>
<tr>
<td></td>
<td>2) 200-300 mg/dL</td>
<td>2) Thrombin time</td>
<td>2) Our data</td>
</tr>
<tr>
<td></td>
<td>3) 196 mg/dL</td>
<td>3) Heat precipitation</td>
<td>3) Blaisdell and Dodds, 1977</td>
</tr>
<tr>
<td>Rabbits</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1) 235 mg/dL</td>
<td>1) Heat precipitation</td>
<td>1) Blaisdell and Dodds, 1977</td>
</tr>
<tr>
<td>Humans</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1) 204.2 mg/dL</td>
<td>1) Thrombin time</td>
<td>1) Feingold et al., 1986</td>
</tr>
</tbody>
</table>

**Fig. 2.** Measurement of fibrinogen level in the heat precipitation method. The length of the fibrinogen layer (A) is measured using a microscope equipped with vernier (0.01 mm unit). The length of plasma layer (B) is measured using appropriate ruler (0.01 mm unit). The calculation is as follows: Fibrinogen concentration (mL/100 mL) = (length of fibrinogen layer (mm) (A) / length of the plasma layer (mm) (B)) × 100 (Millar et al., 1971); or fibrinogen concentration (mg/100 mL) = (length of the fibrinogen layer (mm) (A) / length of the plasma layer (mm) (B)) × 10000 (Blaisdell and Dodds, 1977).
Chromogenix, Molndal, Sweden) in rats, dogs and cynomolgus monkeys (Hayashi et al., 2001).

**Protein induced by vitamin K absence or antagonists (PIVKA)**

The “protein induced by vitamin K absence or antagonists” (PIVKA) is an abnormal coagulation factor produced under vitamin-K deficiency.

Factors II, VII, IX, X and protein C are vitamin-K dependent and are synthesized in the liver (Nelsestuen et al., 2000). All of them possess γ-carboxylglutamate (Gla) at the N-terminal. In case of vitamin K deficiency, abnormal proteins having a non-carboxylated glutamate (Glu) at the N-terminal (i.e., PIVKA) are released. Some drugs affect the synthesis of vitamin K-dependent coagulation factors; e.g., warfarin, some rodenticides, antibiotics, α-tocopherol (Bloom and Brandt, 2001). It is important to determine the level of PIVKA to investigate any such toxicological mechanism.

Harauchi et al. (1986) and Ishii et al. (1997) described a method to determine the level of PIVKA in rats. Briefly, PIVKA is separated from prothrombin using a barium salt, and converted to a thrombin-like substance by *Echis carinatus* venom. The activity of the thrombin-like substance is measured spectrophotometrically by adding a chromogenic substance (S-2238, Kabi Diagnostics, Piscataway, NJ). Mount et al. (2003) measured PIVKA using reagents from a clinical kit for humans (Thrombotest, Accurate Chemical and Scientific Corp, Westbury, NY) in dogs, and stated its significance in the detection of anticoagulant poisoning.

**Intrinsic coagulation inhibitors**

There are several intrinsic coagulation inhibitors. These include antithrombin III, protein C, protein S, heparin, heparin cofactor II, tissue factor pathway inhibitors (TFPI) and annexin V (Johnstone, 2000; Roberts et al., 2001). Among these factors, antithrombin III is introduced in this review, as this is a physiologically potent inhibitor and is well investigated in experimental animals.

1. **Antithrombin III**

Antithrombin III (ATIII) is a serine-protease inhibitor produced in the liver and endothelial cells, and accounts for approximately 80% of the thrombin-inhibitory capacity (Johnstone, 2000). It is known that heparin, an anticoagulant, activates ATIII. Owens et al. (1986) reported ATIII assays in rats by an immunologic assay and its activity using a synthetic chromogenic substrate (S-2237, Kabi Diagnostics, Piscataway, NJ). Nobutaka et al. (2000) measured ATIII activity in rats using a commercially available kit for human clinical pathology that employs a synthetic substrate (Testzym S, ATIII, Daiichi Pure Chemicals, Tokyo, Japan). Karges et al. (1994) showed that the level of ATIII was comparable in level among dogs, pigs, monkeys and humans.

Another possibility is to measure the thrombin-

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**Table 4. Coagulation factors and types of plasma used to measure individual coagulation factors in different species.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Coagulation factors</th>
<th>Types of plasma*</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>II, VII</td>
<td>II: Rat deficient plasma</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>VIII: Deficient plasma</td>
<td>Owens et al., 1986</td>
</tr>
<tr>
<td>Rats</td>
<td>II, V, VII, VIII, IX, X, XI, XII</td>
<td>Deficient plasma&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Nobutaka et al., 2000</td>
</tr>
<tr>
<td>Rats</td>
<td>V, VII, VIII, IX, XI, XII</td>
<td>Deficient plasma&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Laudes et al., 2002</td>
</tr>
<tr>
<td>Dogs</td>
<td>VIII</td>
<td>Deficient plasma&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Feingold et al., 1986</td>
</tr>
<tr>
<td>Dogs</td>
<td>II, V, VII, X, XI, XII</td>
<td>Deficient plasma&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Mischke, 2001</td>
</tr>
<tr>
<td>Dogs</td>
<td>V, VIII</td>
<td>Deficient plasma&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Curry et al., 2002</td>
</tr>
<tr>
<td>Rats, Dogs Rabbits, Pigs, Guinea pigs, Cynomolgus monkeys</td>
<td>II, V, VII, VIII, IX, X, XII</td>
<td>Deficient plasma</td>
<td>Karges et al., 1994</td>
</tr>
</tbody>
</table>

<sup>a</sup>: George King, Inc. (George King Biomedical, Inc., Overland Park, KS).
<sup>b</sup>: International Reagents, Kobe, Japan.
<sup>c</sup>: Factor VIII-deficient human plasma.
<sup>d</sup>: Diagnostica Stago, Roche Diagnostics (Roche Diagnostics GmbH, Mannheim, Germany), except for VII-deficient plasma which was from Dade, Behring, Marburg, Germany.
<sup>e</sup>: Diamed, Brazil.
<sup>*</sup>: The methods are based on the clotting time in combination with deficient plasma.
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ATIII complex (TAT). TAT complexes have been determined in mice, rats, rabbits, dogs, pigs and baboons employing an ELISA kit for use in humans (Enzygnost TAT, Behring, Germany) (Ravanat et al., 1995). Its use in rats was confirmed in other reports (Nobutaka et al., 2000; Elg et al., 2001).

PLATELET FUNCTION TESTS

Many drugs are known to affect platelet function; for example, nonsteroid anti-inflammatory agents, β-lactam-containing antibiotics, cardiovascular drugs, particularly β-blockers, psychotropic drugs, antihistaminics, and some chemotherapeutic agents (Bloom and Brandt, 2001). Currently, the guidelines for toxicological studies do not include routine parameters to assess platelet function.

Platelets aggregate mainly via a platelet specific glycoprotein (GP) IIb/IIIa receptor (platelet aggregation), adhere to blood vessels via the GPIa receptor and von Willebrand factor (vWF) (platelet adhesion), and release granules, dense bodies and lysosomes containing many active substances (release reaction) (Parise et al., 2001). The following section will introduce platelet aggregation, platelet adhesion and release reaction as platelet function tests. Thromboelastogram, a screening test, can also detect platelet function abnormality (see in Fibrinolysis tests).

Platelet aggregation
1. Sample preparation
Platelet-rich plasma (PRP) is separated from citrated blood by centrifugation at 200 g for 10 min or 1000 g for 2 min at room temperature. The remaining citrated blood is further centrifuged at ≥1500 g for ≥10 min to obtain platelet-poor plasma (PPP). PRP is diluted with PPP, and the platelet counts are adjusted to 2-5 × 10^11 cell/L, ideally to 3 × 10^11 cell/L.

2. Turbidimetric method
Changes in light transmittance in the PRP sample are considered to indicate platelet aggregation, which is called the turbidimetric method (Born, 1962). This is usually carried out in the presence of appropriate platelet aggregating agents (see the section on “platelet aggregation agents”) using a commercially available apparatus. An apparatus permitting a small sample (e.g., 0.1 mL for each sample) is useful for experimental animals. Fig. 3 depicts the analysis of a platelet aggregation pattern.

3. Impedance method
Cardinal and Flower (1980) introduced the impedance method to determine platelet aggregation. In this method, platelets aggregate on the electrode, and impedance changes during the aggregation process are recorded as an index of platelet aggregation. This method, in contraposition to light transmittance, makes

![Fig. 3. Indexes used to analyze platelet aggregation. For data evaluation, the following indexes are generally used: maximum aggregation (% from baseline to maximum aggregation as 100% transmittance of PPP), threshold (mM or µg/mL; the lowest concentration of the agonist inducing aggregation), lag time (sec; duration from the addition of the agonist to aggregation in the case of collagen- or arachidonic acid-aggregation). This figure was reproduced with permission of the Japanese Society of Laboratory Animals, Japan, from a textbook concerning blood examinations used to assess drug safety (blood coagulation section described by Kurata M.).](image-url)
it possible to test platelet aggregation in whole blood. With the whole-blood platelet aggregation method, (1) sample preparation is easy, and (2) the detection of platelet inhibition via adenosine-uptake inhibition in erythrocytes, such as a group of dipyridamole, is possible (Dawicki et al., 1985; Kurata et al., 1997). However, one of the disadvantages is its low sensitivity to adenosine 5’-diphosphate (ADP)- or platelet activating factor (PAF)-induced platelet aggregation in rats, dogs and rabbits (Kurata et al., 1995).

4. Platelet aggregating agents

Platelet aggregation is tested in the presence of appropriate platelet aggregating agents such as collagen, ADP, arachidonic acid (AA) and PAF. Among these agents, collagen and/or ADP are primarily used in human clinical pathology, since they are “physiological activators”. For safety studies employing laboratory animals, the uses of these two agents are also recommended. The explanation and use of collagen, ADP, AA and PAF are described below in detail. Although other substances (epinephrine, serotonin and thrombin) are also known as aggregating agents, these are not described in this review, because of less potent agonistic action (epinephrine and serotonin), or due to its complicated preparation for platelet washing procedure (thrombin). Regarding ristocetin-aggregation, it is described in the section of platelet adherence in this review, since it is considered as agglutination, and used for evaluating platelet adherence.

1) Collagen

Collagen in the subendothelium promotes platelet activation via multiple receptors (e.g., GPIIa/IIIa (α2β1) integrin, GPVI and GPV), followed by the release reaction and the intrinsic arachidonic cascade, resulting in tyrosine phosphorylation and activation of PLCγ2 (Emms and Lewis, 1986; Parise et al., 2001).

Commercially available collagen (e.g., Collagen Reagent Horm, Hormon-Chemie, Munich, Germany) is suspended in an appropriate isotonic buffer before use. Since collagen-induced aggregation is easily lost during storage of PRP for several hours, the measurement should be completed within approximately 3 hr after blood collection.

2) Adenosine 5’-diphosphate (ADP)

ADP is also a physiological agonist, as it is released from platelet granules when platelets are activated. ADP binds to at least three receptors, P2Y1, P2TAC and P2X1 in humans, leading to phospholipase β and subsequent phosphoinositide hydrolysis and Ca2+ influx (Parise et al., 2001). Ticlopidine, an anti-platelet drugs, is known to block the ADP receptor (Majerus and Tollefsen, 2001). ADP, as a reagent, is usually dissolved in saline or an adequate isotonic buffer in platelet aggregation tests.

3) Arachidonic acid (AA)

In the plasma membrane, many other agonists stimulated the release of AA from phosphatidylethanolamine (PC) and phosphatidylethanolamine (PE) (Parise et al., 2001). The metabolism of AA to thromboxane A2 (TXA2), mainly via cyclooxygenase-1 (COX-1), is the fundamental pathway contributing to platelet aggregation. TXA2 is a potent platelet agonist that stimulates its TXA2 receptor. Nonsteroidal anti-inflammatory agents inhibit the phospholipase A2/cyclooxygenase pathway and synthesis of thromboxane A2 (Bloom and Brandt, 2001). It is important to examine AA-induced platelet aggregation in a study of this type of substances. A commercially available reagent, AA with a hydrochloride base, is dissolved in saline or an adequate isotonic buffer. A base-free AA is also available as a suspension in PPP. To properly store the AA solution, the oxygen in the vial should be changed for an inactive gas (e.g., N2) to prevent oxidation.

4) Platelet activating factor (PAF)

PAF (mixture of 1-O-hexadecyl-2-acetyl-sn-glycerol-3-phosphocholine and 1-O-octadecyl-2-acetyl-sn-glycerol-3-phosphocholine) is a phospholipid ether produced by platelets, leukocytes and other cells. PAF induces G-protein-dependent inhibition of adenyl cyclase and activation of phospholipase (PLC), leading to protein kinase C (PKC) activation and Ca2+ influx (Parise et al., 2001). PAF is usually dissolved in an organic vehicle like ethanol, and then diluted with saline or an appropriate isotonic buffer. The final concentration of ethanol in PRP is recommended as low as possible (e.g., less than 0.01%), because ethanol at approximately 0.02% is known to inhibit platelet aggregation (Torres Duarte et al., 1995).

5. Species differences in platelet aggregation

There exist marked species differences in platelet aggregation (Macmillan and Sim, 1970; Addonizio et al., 1978; Dodds, 1978). In designing a study, it is of importance to know the characteristics of platelet aggregation in different laboratory animals. These characteristics are reviewed below.

1) Rat

Rat platelets show no response to AA when sodium citrate is employed as the anticoagulant. However, the response to AA was noted in platelets from a blood sample with heparin, probably because extracel-
lular Ca$^{2+}$ is an essential factor for AA-induced platelet aggregation in rats (Hwang, 1980). Dwyer and Meyers (1986) and Zhou et al. (2002) have reported inter-strain differences in aggregation of platelets from Sprague-Dawley rats obtained from different breeders. As for other characteristics, rat platelets do not respond to PAF because they lack the receptor for PAF (Inarrea et al., 1984; Kurata et al., 1995).

2) Dog

There exist large individual differences regarding canine platelet aggregation in response to collagen and ADP (Johnson et al., 1979; Clemmons and Meyers, 1984). This intra-species difference is due to a genetic difference in the response to TXA$_2$ (Johnson et al., 1991).

3) Cynomolgus monkey

PRP from cynomolgus monkeys obtained by separating platelets by centrifugation sometimes has an extremely low concentration of platelets. This is probably because in a population of this species the platelet gravity is close to other blood cells. Therefore, the centrifugation conditions (e.g., 200 g, 10 min or 1000 g, 2 min) must be changed for each individual monkey. The platelets aggregate in responses to collagen (Griffin et al., 1993) and ADP (Weiner et al., 1994).

4) Guinea pig

Guinea-pig platelets show excellent responses to collagen, ADP, AA and PAF. They also are relatively close to human platelets in terms of reactivity with platelet-aggregating agents (Kurata et al., 1995; Zhou et al., 2001). Guinea pigs would be useful to evaluate the platelet functional abnormalities for human risk assessment.

**Platelet adhesion**

In parallel with platelet aggregation, platelets adhere to the blood vessels via the receptor of GP$\mathrm{IIa}$ and the mediating protein of von Willebrand factor (vWF). Few methods are available to test platelet adhesion. Platelet retention and ristocetin-induced platelet aggregation are ex vivo methods to estimate gross adhesion activity of platelets. Alternatively, vWF, which reflects platelet adhesion, is measured in plasma. Recently, the measurement of vWF has become frequent.

1. **Platelet retention method**

A unique blood collection tube filled with small glass beads was developed to evaluate platelet adhesion (Salzman, 1963). The principle of this apparatus is based on the fact that platelets with higher adhesion activity show higher retention in this tube (Salzman, 1963; Yasunaga, 1976). Addonizio et al. (1978) reported that platelet retention is less in rhesus monkeys (75.5% of platelet retention) than in humans (92.5% of platelet retention). This method can be applicable to other species. However, it may be necessary to modify the method in rats and rabbits; e.g., to cut the glass beads tube to half its length, because these species show close to 100% of retention.

2. **Ristocetin-induced platelet aggregation**

Ristocetin-induced aggregation is also applicable for evaluating platelet adhesion. Ristocetin causes aggregation via reactions with the vWF and GPIb in platelets. Ristocetin-induced aggregation is considered to be agglutination, since ristocetin can aggregate formalin-fixed platelets. There is a great species-difference regarding ristocetin-induced platelet aggregation. Brinkhous et al. (1977) reported that ristocetin aggregates platelets from humans and cynomolgus monkeys, but not from rats, guinea pigs, rabbits and dogs. Leis et al. (1980), however, reported that in dogs ristocetin aggregates gel-filtrated platelets but not those in PRP. Some species-differences therefore might be due to the lack of methods optimized for a given species.

3. **von Willebrand factor (vWF)**

vWF is produced in endothelial cells and megakaryocytes, and plays an important role in platelet adhesion (Parise et al., 2001). vWF has been measured by enzyme-linked immunosorbent assay (ELISA) in rats (Ribau, et al., 1998) and dogs (Moser et al., 1998; Johnstone, 1999; Kageyama et al., 2002). Newsholme et al. (2000) reported cross-reactivity of the kit for use in humans with rat vWF (Asserachrom, Diagnostica Stago, American Bioproducts, Parsippany, NJ). In these studies, the plasma levels of vWF were expressed as a % change compared to canine or human normal control, except for a report by Moser et al. (1998), in which it was expressed in U/dL and the base line was between 15 and 20 U/dL.

**Release reactions**

Platelets release active substances from their dense bodies, $\alpha$-granules and lysosomes, in parallel with an increase in intracellular calcium concentration and consequent contraction of contractile proteins. The released substances include ADP, serotonin and ATP. ADP and serotonin induce or enhance platelet aggregation, while ATP serves as a partial antagonist of ADP-induced platelet aggregation (Parise et al., 2001). The test for release reaction would be useful to investigate...
the mechanism of platelet dysfunction in detail.

The ATP released from platelets is measurable ex vivo with a lumi-meter in the presence of luciferin and magnesium in human samples (Horie et al., 1997) and in canine samples (Soloviev et al., 1999). As an alternative method, a release of C-5HT into extracellular fluid can be detected by radioactivity, using platelets that incorporated C-5HT into dense body during incubation at 37°C (Inagaki et al., 1984).

**FIBRINOLYSIS TESTS**

Plasmin is derived from plasminogen, and plays a role of lysing fibrin and fibrinogen. This step is regulated by many factors that activate plasminogen (e.g., tissue plasminogen activator (t-PA), urokinase, kallikrein), or inhibit plasmin activity (e.g., α2-antiplasmin, α2-macroglobulin). t-PA is in turn inhibited by plasminogen activator inhibitor-1 (PAI-1) and PAI-2. As the endpoint of the fibrinolytic pathway, plasmin dissolves fibrin/fibrinogen to fibrin/fibrinogen degradation products (FDP) including D-dimer (Fig. 4).

There are drugs that inhibit the fibrinolytic pathway: e.g., tranexamic acid and aminocaproic (Bloom and Brandt, 2001). It is also of importance to know that the fibrinolytic pathway is located at the end of the blood coagulation system. The tests used to assess fibrinolysis are described below.

**Fibrin/fibrinogen degradation products (FDP) and D-dimer**

Plasmin degrades both fibrin and fibrinogen. During this reaction, fibrinogen degrades to fragment X (consisting of one domain E and two domain D), fragment Y (one domain E and one domain D), and eventually domain E and domain D. As domains D are cross-linked to each other during fibrin formation, degradation products from fibrin contain D-dimer. These degradation products are called FDP (fibrin/fibrinogen degradation products).

The significance of the measured FDP and D-dimer is to provide us with evidence of fibrinolysis events in vivo. D-Dimer, as a specific fibrin breakdown product, makes it possible to confirm the fibrin formation and its subsequent degradation in vivo.

There are reports showing the FDP level in dogs measured using a commercially available latex agglutination kit (FDP plasma, American Bioproducts, Parsippany, NJ) (Otto et al., 2000; Stokol et al., 2000; Scott-Moncrieff et al., 2001). In dogs, an FDP concentration of 10 µg/mL or more is considered as evidence of increased fibrinolytic activity (Roncales and Sancho, 2000). Regarding D-dimer measurement, an enzyme immunoassay and a semi-quantitative latex agglutination method are available for mice, rats and dogs. The methods and available kits for laboratory animals are summarized in Table 5.

![Fig. 4. Pathway of fibrinolysis. The main reaction of fibrinolysis is to degrade fibrin by plasmin derived from plasminogen. This figure was reproduced with permission from the Japanese Society of Laboratory Animals, Japan, from a textbook concerning blood examinations used to assess drug safety (blood coagulation section described by Kurata M.).](image-url)
Thromboelastogram

Thromboelastogram is a procedure that records the whole blood coagulation process using a special apparatus named “thromboelastography” (Hartert, 1951). During the blood coagulation process, the elasticity of the blood sample increases as the “gelatin-like” clot develops, and then it gradually decreases with clot degradation (i.e., fibrinolysis). The mechanical principle of thromboelastography is to convert this change in elasticity to electrical signals that are recorded as a spindle-shaped pattern (Fig. 5).

Briefly, native blood or immediately previously recalcified citrated blood is placed in a stainless steel cuvette heated at 37°C, which moves with a monitored slow turning motion. A hanging steel cylinder is dipped into the blood. The turning movement of the cylinder supplies the movement signal as changing elasticity of blood, leading to the spindle-shaped pattern (Mischke and Nolte, 2000). In the recalcified method, CaCl₂ solution is added to citrated blood to give a final concentration of 5-10 mM.

Thromboelastogram is an effective screening test for fibrin formation defects, which are more sensitive than in PT, APTT and thrombin time (Mischke and Nolte, 2000). It is equal or more sensitive than other tests, including D-dimer, and therefore more useful to evaluate fibrinolytic activity (Mallett and Cox, 1992).

From another aspect, thromboelastogram serves to detect hypercoagulation. Otto et al. (2000) reported a hypercoagulation pattern due to an increased concentration of fibrinogen level in dogs. Currently, there are only a few methods to evaluate hypercoagulation, although in toxicological studies the detection of hypercoagulation status is of great importance.

Fig. 5 shows the expected abnormal patterns. Excepting for “low-throughput” issue, thromboelastography is an effective tool to detect hyperfibrinolysis and hypercoagulation in toxicological studies. It is also significant to know that thromboelastogram detects platelet dysfunction and extreme hyperfibrinolysis as a thrombopenia pattern. Patterns of thromboelastograms in different laboratory animals are shown in Fig. 6.

Plasminogen and plasin

Plasminogen is a proenzyme synthesized primarily in the liver. Activation of plasminogen results from the cleavage of a single Arg-Val peptide bond, and yields the fibrinolytic serine protease, plasmin.

Measurements for plasminogen and plasmin activities are important in investigation of the effects of test substances on plasminogen activation. The values of both plasminogen and plasmin are highly relevant in the evaluation of the status of plasminogen activation (Aasen et al., 1978).

A synthesized peptide substrate containing chromogenic p-nitroanilide (H-D-Val-L-leucyl-L-lysyl-p-nitroanilide; S-2251) is used to measure plasmin activity, and streptokinase is usually added to activate plasminogen when measuring plasminogen in human plasma (Friberger et al., 1978). However, it is known that there are species differences regarding the sensitivity of plasminogen to streptokinase; e.g., low sensi-

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<td>2) Accuclot D-dimer, Sigma Diagnostics, St Louis, MO</td>
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*: Ravanat et al. (1995) reported cross-reactivity with samples from baboons, but negative cross-reactivity with samples from rats and dogs.

**: Current name is Mitsubishi Kagaku Iatron, Inc.

In our experience, it is possible to detect D-dimer of rhesus monkey using a latex-agglutination kit (Rapidia, D-dimer, Fuji Rebio, Inc. Tokyo, Japan).
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tivity in rabbits (Wolh et al., 1983). Karges et al. (1994) reported that the activity of plasminogen is 1/10 or less in rats, guinea pigs, rabbits, dogs and pigs compared to humans. Using urokinase as the activator, the plasminogen activity of rats or rabbits was found to be equal or 3 times higher than that of humans (Mussoni et al., 1979). Lanevschi et al. (1996) introduced a method using a kit (Coatest Plasminogen kit components, Kabi Pharmacia Hepar Inc, Franklin, OH; modification in incubation period, and substitution of urokinase for streptokinase) to determine plasminogen in dogs.

Endogenous inhibitor and activator of fibrinolysis

The measurement of plasmin inhibitors (e.g., \(\alpha_2\)-antiplasmin) and plasminogen activators (e.g., t-PA) is useful to confirm the contribution of intrinsic inhibitors and activators of fibrinolysis. Representative parame-

Fig. 5. Patterns of thromboelastograms in blood coagulation abnormalities due to different causes. The vertical and horizontal lines indicate changes in elasticity and time, respectively. The top pattern is the typical normal pattern in humans. The second corresponded to a case of thrombopenia; however, extreme platelet dysfunction and hyperfibrinolysis produces a similar pattern. The third pattern corresponds to a case of pronounced clot degradation due to hyperfibrinolysis. The last pattern corresponds to a state of hypercoagulation. This figure was reproduced with permission from the Japanese Society of Laboratory Animals, Japan, from a textbook concerning blood examination used to assess drug safety (blood coagulation section described by Kurata M.).
ters, $\alpha_2$-antiplasmin and t-PA, are introduced below.

$\alpha_2$-Antiplasmin is a glycoprotein synthesized in the liver and kidneys, and it is present in plasma and $\alpha$-granules of platelets. $\alpha_2$-Antiplasmin reacts very fast with plasmin, irreversibly inhibiting the enzyme by forming a stable 1:1 complex. Other plasmin inhibitors such as $\alpha_2$-macroglobulin may exert a limited role. Other plasma protease inhibitors such as $\alpha_1$-antitrypsin and $C_1$-inactivators have some antiplasmin activity but exert minimal physiologic effect (Darien, 2000).

Karges et al. (1994) reported relatively comparable values of $\alpha_2$-antiplasmin among rats, guinea pigs, dogs, pigs, cynomolgus monkeys and humans. In dogs, $\alpha_2$-antiplasmin is measurable using a kit for clinical use in humans (Coatest antiplasmin kit components, Kabi Pharmacia Hepar Inc., Franklin, OH) using substrate S-2251 (Lanevschi et al., 1996). They also introduced the utilization of another kit for clinical use in humans (Spectrolyte tPA/PAI kit components, American Diagnostica Inc., Greenwich, CT) with substrate

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Fig. 6. Patterns of thromboelastograms observed in different experimental animals. Miniature pigs, beagles, rats and guinea pigs show a “spindle-shaped” pattern. It is worthy to note that cynomolgus monkeys show a high rate of loss of elasticity, likely due to spontaneous hyperfibrinolysis. This figure was reproduced with permission of the Japanese Society of Laboratory Animals, Japan, from a textbook concerning blood examinations used to assess drug safety (blood coagulation section described by Kurata M.).
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S-2251 to determine t-PA in dogs (Lanevschi et al., 1996).

**BLEEDING TIME TEST**

The “bleeding time test” measures a period from giving small wound to hemostasis. This test is important to evaluate whether the test substance can affect hemostatic capability in vivo, especially to confirm the safety of a drug that causes some changes in the values of blood coagulation parameters. Since small wounds must be produced, satellite group or a separate study is necessary when applying this method in safety studies. The puncture site is blotted every 30 sec with filter paper. The duration of bleeding is recorded as the bleeding time. The bleeding time test is carried out without anesthesia in humans. In laboratory animal studies, appropriate anesthesia is recommended to be employed for an ethical reason, even though effects of anesthesia on hemostasis are suggested in humans (Faraday, 2002). Furthermore, it is recommended that operators confirm hemostasis at puncture sites after bleeding test, to avoid re-bleeding after the animals return to cages. The detailed procedures used in each species are described below.

**Rat**

Cohen et al. (1978) introduced a method called “tail template technique” according to which an incision 5 mm long and 1 mm deep is made on the tail to measure bleeding time. They reported a bleeding time of 80-150 sec using the “tail template technique”. Using the “tail template technique (10 mm long and 1.5 mm deep)”, Elg et al. (2001) found that melagatran, a thrombin inhibitor, prolonged bleeding time (15 min at control vs. 53 min at 2 μmol/kg, i.v.) in rats.

**Dog**

Because the skin of dogs is relatively thicker than that of humans or monkeys, the lancet usually employed for human skin is not appropriate. An adequate lancet and template should be used to induce a wound of the same size (2-3 mm width and 2-3 mm depth). If the puncture is done on a forelimb, the IVY method employed in human clinical pathology can be applied to avoid changes in blood pressure. In the IVY method, a manchette is attached to the upper forelimb and 40 mm Hg of pressure is applied. Using this procedure, the normal bleeding time in dogs was found to range from 2 to 3 min. A report by Nolte et al. (1997) showed that the bleeding time of dogs measured on the forelimbs was prolonged after an injection of acetylsalicylic acid (20 mg/kg) from 2.25 ± 0.76 min (normal) to 25 min. Kageyama et al. (2002) chose the inner lip of dogs as the bleeding test site, and reported that this was prolonged after an injection of antibody to vWF (< 5 min in normal vs. ≥ 30 min after injection).

**Cynomolgus / Rhesus monkey**

The procedure is essentially the same as that used in humans. A puncture is made in the forearm with a human lancet. Bleeding time is obtained by inflating a pediatric manchette placed on the upper extremity to 40 mm Hg and superficially piercing the forearm skin (1-5 mm wide by 1 mm deep) with the lancet employed to carry out the bleeding time test in humans. The bleeding time with the IVY method was found to be 1.5 ± 0.25 min in cynomolgus monkeys (Weiner et al., 1994), 6 ± 1 min in humans and 2.7 ± 1 min in rhesus monkeys (Addonizio et al., 1978). As an example of the usefulness of this method to detect drug effects, Weiner et al. (1994) reported that doxazosin, a selective α1-adrenergic inhibitor, prolonged bleeding time (33%) in cynomolgus monkeys. Prolongation of bleeding time accompanied with a decrease of platelet aggregation was also reported after the injection of antiplatelet glycoprotein (GP) IIb/IIIb monoclonal antibody in rhesus monkeys (Kaku et al., 1996).

**SUMMARY OF BLOOD COAGULATION TESTS AND THEIR SIGNIFICANCE**

Blood coagulation tests and their significance in toxicological studies are summarized according to their category.

**Coagulation factors tests**

Abnormalities of the extrinsic and intrinsic coagulation pathway can be screened by routine parameters PT and APTT. Together with the concentration of fibrinogen, these three parameters allow us a rough estimation of the impaired factor in the coagulation cascade. Assays for individual coagulation factors and PIVKA are significant to identify in detail the factor causing a dysfunction.

**Platelet function tests**

No routine parameters to assess platelet function are included in the current guidelines for toxicological studies. Platelet aggregation induced by collagen or ADP *ex vivo* is significant to reveal the effect of test substances on platelet function. For detection of drugs
affecting the AA cascade, AA-induced platelet aggregation is useful if added to evaluate the battery of parameters. Other platelet function tests, such as platelet adhesion test including vWF measurement and platelet release test, are also important to investigate the effects of test substances on platelet function.

**Fibrinolysis test**

No routine parameters to assess fibrinolysis are included in the current guidelines for toxicological studies. FDP, D-dimer and thromboelastography can be used to screen fibrinolysis abnormalities in laboratory animals. In addition to these screening tests, the values of plasminogen, plasmin and their activator / inhibitor are of significance to confirm plasmin activation or contribution of intrinsic activators and inhibitors.

**Bleeding time**

The bleeding time, which measures hemostatic time after provoking a small wound, might be of significance to grossly evaluate blood coagulation capability in vivo, especially to confirm the safety of drugs affecting blood coagulation parameters.

**FINALLY**

The risk of blood coagulation abnormalities is assessed on the basis of the clinical pathological findings and gross/histopathological findings (i.e., hemorrhage or thrombosis). An appropriate battery of blood coagulation tests provides significant information for risk assessment of drugs.

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