Monitoring hepatic cholesterol 7α-hydroxylase activity by assay of the stable bile acid intermediate 7α-hydroxy-4-cholesten-3-one in peripheral blood

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Abstract We describe an accurate method for monitoring the enzymatic activity of hepatic cholesterol 7α-hydroxylase (C7αOH; CYP7A1), the rate-limiting and major regulatory enzyme in the synthesis of bile acids. Assay of 7α-hydroxy-4-cholesten-3-one (C4), an intermediate in bile acid synthesis, revealed that the level of C4 in peripheral blood serum or plasma showed a strong correlation to the enzymatic activity of hepatic C7αOH, both at steady-state conditions (r=0.929) as well as during the rapid changes that occur during the diurnal phases. This assay should be of value in clarifying the regulation of bile acid synthesis in vivo in laboratory animals and humans since it allows for the monitoring of hepatic C7αOH activity using peripheral blood samples.—Gälman, C., I. Arvidsson, B. Angelin, and M. Rudling. Monitoring hepatic cholesterol 7α-hydroxylase activity by assay of the stable bile acid intermediate 7α-hydroxy-4-cholesten-3-one in peripheral blood. J. Lipid Res. 2003. 44: 859–865.

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The breakdown of cholesterol to bile acids in the liver is a major pathway for cholesterol elimination (1–3). Stimulation of this process results in adaptive responses that promote hepatic cholesterol synthesis and LDL receptor-mediated uptake. The ensuing lowering of plasma LDL cholesterol (LDL-C) has been shown to be beneficial in preventing coronary heart disease (4).

Bile acids are synthesized by two biochemical pathways (5): the classic (neutral) pathway, regulated by the enzyme cholesterol 7α-hydroxylase (C7αOH; CYP7A1) that directly converts cholesterol into 7α-hydroxycholesterol (6), and a second alternative (acidic) pathway where cholesterol is converted to oxysterols by sterol 27-hydroxylase prior to 7α-hydroxylation catalyzed by oxysterol 7α-hydroxylase (7). The importance of C7αOH as a regulator in whole-body cholesterol homeostasis is well established in both animals and humans (8–10). In addition to the classical ways of stimulating C7αOH activity by resin treatment or ileal resection (11, 12), new drugs are under development. Among those are inhibitors of the apical sodium-dependent bile acid transporter (SLC10A2) that blocks the active uptake of bile acids in the distal ileum (13–15) and antagonists for the hepatocellular bile acid receptor, farnesoid X receptor (16). Direct stimulation of C7αOH activity by, for example, hormonal treatment also holds some promise (17).

The increased interest in this field makes it important to have more reliable and rapid methods to assay changes in bile acid synthesis. Presently, bile acid synthesis can be estimated from analysis of fecal excretion (18) and from isotope dilution studies (19). Direct assay of the enzymatic activity of C7αOH or its mRNA requires liver biopsy. All these methods are laborious, costly, and do not allow for dynamic studies. It has previously been shown that an intermediary product in the synthesis of bile acids, 7α-hydroxy-4-cholesten-3-one (C4), is present in blood plasma, and that its plasma level appears to reflect bile acid synthesis (20). Later studies have also shown that the plasma level of C4 correlates with the enzymatic activity of C7αOH assayed in human hepatic microsomes (21) as well as with bile acid synthesis in humans (22). However, the originally described procedure is rather complex and, therefore, the study of larger series of samples has been difficult. Interestingly, to the best of our knowledge there are no available published data regarding the use of C4 to monitor bile acid synthesis in laboratory animals.

In the current paper, we describe a method for assay of C4 in serum or plasma that takes advantage of commer-
cally available columns for sample preparation. This allows the analysis of 50–75 samples per day compared with earlier described procedures where only 10–15 samples could be analyzed (20). By studying rats at different established steady-state conditions known to modulate bile acid synthesis in this species, we have tested the relevance of the data generated. We have found that the C4 level in rat serum strongly correlates with the enzymatic activity of hepatic C7αOH not only during steady-state conditions but also during the diurnal changes that occur in this species.

The described method should be of value for monitoring C7αOH activity and presumably bile acid synthesis, and will be of great use in further studies on the in vivo regulation of the dynamics of bile acid synthesis in both humans and laboratory animals.

MATERIALS AND METHODS

Instrumentation

The HPLC system used was the Hewlett Packard 1100 series (Hewlett Packard GmbH, Waldbronn, Germany). It consisted of an autosampler (G1313A), a column compartment (G1316A), a diode array detector (G1315A), a degasser (G1322A), and a pump (G1312A). The software used was HP Chemstation® for LC. The column was a Nova-Pak® C18 steel column, 3.9 × 300 mm, 4 μm particle size, (part. No. WAT011605, Waters Corp., Milford, MA).

Sample preparation columns were mounted in a Varian CEREX® SPE processor (Varian Inc. Palo Alto, CA), to which had been added a removable custom-made 5.5 × 21.4 × 8.4 cm stainless steel water-jacketed block heater with 24 positions, hole diameter 1.1 cm.

The column heater was connected to a Lauda E100 water bath (Lauda-Königshofen, Germany) set to 66°C in order to obtain 64°C column temperature.

The eluted samples were evaporated to dryness at 60°C under a stream of nitrogen using a Techne sample concentrator and a Technne Dry block, type DB3D (Technne, Cambridge, UK).

Chemicals

Acetonitril, methanol, chloroform, and hexane were HPLC grade (LiChrosolv®, Merck, Darmstadt, Germany). Pyridin and chlorotrimethylsilane were purchased from Merck and hexane (LiChrosolv, Germany). Pyridin and chlorotrimethylsilane were purchased from Merck and hexane (LiChrosolv, Merck, Darmstadt, Germany). Pyridin and chlorotrimethylsilane were purchased from Merck and hexane (LiChrosolv, Germany). Pyridin and chlorotrimethylsilane were purchased from Merck and hexane (LiChrosolv, Merck, Darmstadt, Germany). Pyridin and chlorotrimethylsilane were purchased from Merck and hexane (LiChrosolv, Merck, Darmstadt, Germany). Pyridin and chlorotrimethylsilane were purchased from Merck and hexane (LiChrosolv, Merck, Darmstadt, Germany).

Human blood samples

Human blood serum and plasma (Table 1) were obtained from the blood donor center and were aliquoted and stored at 4°C, −20°C, −85°C, and in liquid nitrogen. Human serum samples for the experiment shown in Fig. 5 were from five healthy males 26–33 years of age. The studies were approved by the Ethics Committee at Huddinge University Hospital.

Animals and treatments

Altogether, 67 male Sprague-Dawley rats (B and K Universal AB, Sollentuna, Sweden), 7 to 8 weeks old and weighing 250 g to 300 g, were used. All animals were kept under standardized conditions with free access to water and chow. Groups of rats received 2% cholestyramine (Questran, Bristol-Myers Squibb) for 7 days or 0.8% cholic acid (Sigma) for 15 days. Cholestyramine was mixed with ground rat chow, and the cholic acid diet was prepared by mixing ground rat chow with hot Mazola oil (9:1, v/v) (CPC Foods AB, Kristianstad, Sweden) into which cholic acid had been dissolved. The control group received ground rat chow. All rats were sacrificed the same day between 9 AM and 12 AM.

In the diurnal variation experiment, rats were sacrificed at 6 PM, 12 PM, 6 AM, and 12 AM. All animals were kept under standardized conditions with free access to water and chow. The light-cycle hours were between 6 AM and 6 PM. Animals were anesthetized by inhalation of isoflurane, decapitated, or bled by cardiac puncture, and thereafter killed by cervical dislocation.

Blood was collected and serum was separated by centrifugation and stored at −85°C. The collected livers were immediately frozen in liquid nitrogen and stored at −85°C. All animal studies were approved by the institutional Animal Care and Use Committee.

Lipid assays

Total plasma cholesterol and triglycerides were determined individually using a Monarch automated analyzer using commercially available kits (Instrumentation Laboratory Co., Lexington, MA). Separation of lipoproteins by FPLC and subsequent on-line assay of cholesterol was performed on 10 μl of plasma using a miniaturized system (23).

Synthesis of 7β-hydroxy-4-cholesten-3-one

7β-Hydroxy-4-cholesten-3-one was prepared from 5-cholesten-3β,7β-diol by enzymatic oxidation with cholesterol oxidase (Sigma) as described (24). The concentration of the internal standard was determined from the absorbance at 241 nm using 4-cholesten-3-one (Sigma) as standard.

Optimization of conditions for solid-phase extraction of serum and plasma samples

For the solid-phase extraction of C4, we tested different columns. Columns with C2, C4, C6, C8, and C18 as a functional group gave a pure C4 peak, whereas columns with phenyl or cyanopropyl sorbents resulted in a highly contaminated end product. To reduce ionic and polar secondary interaction, we selected an end-capped C8 column. The optimal temperature for sample workup was tested from 60.0°C to 70.0°C, and it was found that C4 had the highest binding capacity at a column temperature of 64.0°C (internal temperature, monitored using a thermistor);

<table>
<thead>
<tr>
<th>Storage Temp.</th>
<th>1 Day</th>
<th>2 Days</th>
<th>15 Days</th>
<th>3 Months</th>
<th>6 Months</th>
<th>10 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>+4°C</td>
<td>8.0 ± 0.2</td>
<td>7.5 ± 0.3</td>
<td>6.3 ± 0.1</td>
<td>3.7 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>−20°C</td>
<td>7.4 ± 0.2</td>
<td>6.8 ± 0.1</td>
<td>7.1 ± 0.1</td>
<td>7.4 ± 0.4</td>
<td>7.1 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>−85°C</td>
<td>7.5 ± 0.2</td>
<td>6.9 ± 0.1</td>
<td>7.2 ± 0.1</td>
<td>7.2 ± 0.5</td>
<td>7.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>N₂ (l)</td>
<td>7.7 ± 0.3</td>
<td>6.7 ± 0.1</td>
<td>7.4 ± 0.2</td>
<td>6.7 ± 0.4</td>
<td>6.8 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

Plasma samples were aliquoted and stored at +4°C, −20°C, −85°C, and in liquid nitrogen. Samples were analyzed concomitantly after the indicated storage times. Mean and SEM are shown.
other temperatures gave significantly lower concentrations of C4. Different elution conditions were also tested using various mixtures of hexane and chloroform (95:5, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, and 30:70; v/v). It was found that the lowest level of contaminants was obtained using hexane-chloroform at the proportion 95:5 (v/v).

**Specific procedure for solid-phase extraction of serum and plasma samples**

Blood serum (0.5 ml from rats and 1 ml from humans) was diluted with 1 ml of saline (plasma with 2 ml of saline) and 10 µl internal standard 7β-hydroxy-4-cholesten-3-one in H2O was added. In the experiment, presented in Fig. 5, we used 0.8 ml serum from each individual except for mice, where we used pools. C8 Isolute® SPE columns (500 mg, 3 ml, International Sorbent Technology Ltd, Hengoed, UK) were prewashed with 2 × 2 ml pure methanol and with 2 × 2 ml of water. Columns were then mounted in the Varian CEREX® SPE processor equipped with a block heater and heated to 64°C (internal temperature). The samples were sonicated in water for 15 min and incubated at 37°C for 15 min prior to loading onto the preheated columns. The samples were allowed to reside on the columns for 5 min to attain 64°C. Column stoppers were then removed and samples were passed through the columns, which were subsequently washed with 64°C water. The heating block was removed, and columns were washed with 2 × 2 ml 65% methanol at room temperature. Prior to elution with 2 × 2 ml of hexane-chloroform (95:5, v/v), columns were dried by a stream of nitrogen applied for 30 s. The eluted product was evaporated to dryness at 60°C under a stream of nitrogen and dissolved in 100 µl acetonitrile; 50 µl of this was injected into the HPLC system.

**HPLC assay**

As a mobile phase, acetonitrile-water (95:5, v/v) was used at a flow rate of 1 ml/min. The wavelength and column temperature were 241 nm and 20°C, respectively. C4 was quantitated from the areas of the respective peaks using the known amount of internal standard.

**Assay of C7αOH activity**

Microsomes were prepared from each rat liver by differential ultracentrifugation of liver homogenates as described earlier (25). The activity of C7αOH was determined as the formation of 7α-hydroxycholesterol (pmol/mg protein/min) from endogenous microsomal cholesterol by using isotope dilution mass spectrometry in duplicate samples from each individual (25). In each animal experiment presented, the enzyme assay was carried out on one occasion in order to eliminate interassay variation.

**Statistics**

Data are presented as means ± SD or means ± SEM. When multiple groups were analyzed, one-way ANOVA with Dunnett’s test was used (Statistica Software, Tulsa, OK). Measure of correlation was evaluated using the correlation coefficient.

**RESULTS**

We initially optimized the extraction procedure to obtain the best possible yield and purity for C4 as described in Materials and Methods. **Figure 1A** shows a typical HPLC chromatogram generated after processing 1 ml of human serum as described in the specific section for the solid-phase extraction. The retention time for C4 was ~16.5 min, whereas the internal standard (7β-hydroxy-4-cholesten-3-one) had a retention time of ~17.8 min. The internal standard and C4 were clearly separated from each other (inset Fig. 1A).

We then confirmed the specificity of the method by the addition of increasing amounts of synthetic C4 to aliquots of a human serum sample (Fig. 1B). The limit of detection for the method was evaluated from analysis of C4 in a serum sample that was diluted stepwise and analyzed in quadruplicate (Fig. 2). The assay was linear down to 1 ng of C4/ml. We therefore set the detection limit of C4 to 2 ng/ml. To evaluate whether blood plasma would give equivalent results as serum, we analyzed C4 in serum and plasma samples drawn from one subject. It was found that the C4 content was not significantly different when plasma was used: 16.5 ± 0.7 ng/ml (n = 4) as compared with 15.3 ± 0.4 ng/ml (n = 4) for serum.

We next evaluated the within-run precision of the method using 12 serum and 12 plasma samples prepared from two individuals. The coefficients of variation were 4.4% and 4.3% for the serum and plasma samples, respectively.

The reproducibility (between-runs precision) of the method was then studied by analyzing C4 in plasma samples obtained from one individual. Sample aliquots were
stored at four commonly employed conditions: +4°C, −20°C, and −85°C, and in liquid N2 from 1 day up to 10 months (Table 1). It was found that all storage conditions were satisfactory except refrigerated storage for more than 15 days. The reproducibility, as calculated from all other data with an interassay time span of up to 10 months, was satisfactory, with a coefficient of variation of 6.2%. In a parallel experiment using serum, similar results were obtained, with a coefficient of variation of 5.0%.

We then wanted to evaluate the crucial aspect of how well plasma C4 could relate to the enzymatic activity of hepatic C7αOH in the liver at steady-state conditions. For this purpose, we employed established pharmacologic interventions. Three groups of rats received regular rodent chow (n = 20), regular rodent chow supplemented with 2% cholestyramine (n = 12), or 0.8% cholic acid (n = 12). Animals were killed and serum and liver tissue were collected for assay of C4, lipids, and lipoprotein profiles in serum and for microsomal enzymatic activity of hepatic C7αOH as well as for HMG-CoA reductase. A very strong relation (P < 0.001) was found when the enzymatic activity of hepatic C7αOH was related to serum C4 levels (Fig. 3A). A significant relationship was also present among animals on normal and cholic acid diets (P < 0.001) (inset Fig. 3A). Also, when the 20 animals on normal diet were analyzed separately, a significant relation was observed (r = 0.732, P < 0.001).

Analyses of plasma lipids showed that cholic acid treatment increased total plasma cholesterol (P < 0.01), whereas plasma triglycerides were not altered (Table 2). Serum lipoprotein cholesterol profiles showed that the increase in cholesterol following cholic acid was within VLDL and LDL particles (Fig. 3B). Cholestyramine treatment did not alter plasma cholesterol, whereas plasma triglycerides were significantly increased (P < 0.01). The serum lipoprotein profile of cholestyramine-treated animals showed slight reductions within larger particles (VLDL and LDL), whereas HDL-C was increased.

The activity of the rate-limiting enzyme in cholesterol synthesis, HMG-CoA reductase, was strongly induced (about 10-fold) by cholestyramine treatment, while cholic acid did not significantly suppress the activity of the enzyme (Table 2).

Thus, it was clear that at steady-state conditions, assay of C4 in peripheral blood showed a very strong correlation to the enzymatic activity of C7αOH in the liver.

We then evaluated whether C4 in peripheral blood would also mirror the enzymatic activity of C7αOH in conditions when the activity of this enzyme is altered rapidly. It is well known that the enzymatic activity of hepatic C7αOH has a strong diurnal variation, with peak levels in the middle of the dark phase (26). We therefore sacrificed groups of rats at different hours of the day, and subsequently assayed microsomal hepatic C7αOH activity and the C4 concentration in blood serum. It was found that the level of C4 in the blood serum followed the same diurnal variation, as did the enzymatic activity of hepatic C7αOH (Fig. 4).

Finally, in order to compare the basal C4 levels in humans with those in commonly used laboratory and domesticated animals, we determined plasma C4 levels in eight different species. As can be seen in Fig. 5, C57BL6 mice and Sprague-Dawley rats had 1.8-fold and 5.8-fold higher plasma C4 levels when compared with humans. By far the lowest plasma C4 level was observed in New Zealand rab-

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Fig. 2. Determination of detection limit of the method. A serum sample was diluted and the concentration of C4 was determined in quadruplicates of each dilution.

Fig. 3. Significance of plasma C4 as a marker for the enzymatic activity of hepatic cholesterol 7α-hydroxylase (C7αOH). Rats received normal chow (n = 20) or 2% cholestyramine (n = 12) for 7 days, or 0.8% cholic acid (n = 12) for 15 days. A: Relation between the microsomal in vitro activity of C7αOH, and serum C4 levels. Controls (blue), cholestyramine (red), and cholic acid (orange). B: Separation of serum lipoproteins by FPLC. Controls (blue), cholestyramine (red), and cholic acid (orange). Broad lines show means, thin lines indicate SD.
The temperature during sample workup, 64.0°C and used commercially available end-capped C8 columns. We found that the separation of C4 from the internal standard (27) and can therefore be automated. The reproducibility of the method showed a coefficient of variation of <6% for both blood serum and plasma. Different freezing conditions did not alter the C4 level, whereas C4 declined in samples stored at +4°C for more than 15 days. The fact that the C4 level does not decline following freezing of samples, and that prolonged storage up to at least 10 months does not alter C4 levels indicates that C4 is a stable intermediate that is a prerequisite for the future use of this method in larger clinical studies.

Previous studies have shown a good relationship between plasma C4 and the 75SeHCAT test in patients with diarrhea (28–30) and also between plasma C4 and the enzymatic activity of C7aOH in the liver when comparing untreated gallstone patients with gallstone patients treated with ursodeoxycholic acid, chenodeoxycholic acid, or cholestyramine (21). To the best of our knowledge, there are no data available regarding the use and significance of C4 in laboratory animals. We found a very good agreement between plasma C4 and enzymatic activity of C7aOH in the liver of rats, both at steady-state conditions and during the rapid regulation of the enzyme, which occurs during the diurnal phases (26).

Although the plasma C4 level did follow the diurnal changes of hepatic C7aOH activity, there appeared to be a small delay in the plasma C4 response when the enzymatic activity was rising most rapidly (Fig. 4). Although the half-life for C4 in rat blood is unknown, the data from the diurnal changes would suggest that it should be less than 5 h. Also, the mode of entry of C4 into blood is unclear. It re-

### DISCUSSION

The present results show that quantitation of C4 in peripheral blood samples can be used as an accurate indirect measurement of hepatic microsomal C7aOH activity in rats. C4 in peripheral blood also mirrors the hepatic C7aOH enzyme activity when the enzyme is regulated rapidly, as occurs during the diurnal phases in the rat.

The first and most commonly employed HPLC method to separate C4 uses a straight-phase system and radiolabeled 25-hydroxy vitamin D$_3$, as an internal standard. For the HPLC analysis, we utilized a reversed-phase system previously described by Pettersson et al. because that system can conveniently employ 7B-hydroxy-4-cholesten-3-one as internal standard (27) and can therefore be automated. We found that the separation of C4 from the internal standard was improved when the mobile-phase acetonitril-water was used at proportions of 95:5 (v/v). In the present method, we developed a novel sample workup procedure and used commercially available end-capped C8 columns. The temperature during sample workup, 64.0°C, was essential and was controlled using a water-jacketed steel block. Previously described methods employ custom-made water-jacketed glass columns (20, 27) that are time-consuming to prepare. The combination of the current sample preparation procedure with reversed-phase HPLC significantly increases the number of samples that can be analyzed.

The present results show that quantitation of C4 in peripheral blood samples can be used as an accurate indirect measurement of hepatic microsomal C7aOH activity in rats. C4 in peripheral blood also mirrors the hepatic C7aOH enzyme activity when the enzyme is regulated rapidly, as occurs during the diurnal phases (26).

### TABLE 2. Analysis of total plasma cholesterol, triglycerides, and enzymatic activity of hepatic microsomal HMG-CoA reductase

<table>
<thead>
<tr>
<th></th>
<th>Controls n = 20</th>
<th>Cholestyramine n = 12</th>
<th>Cholic Acid n = 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mM)</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.7 ± 0.2*</td>
</tr>
<tr>
<td>Triglyceride (mM)</td>
<td>1.9 ± 0.4</td>
<td>2.6 ± 0.4*</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>HMG-CoA (pmol/min/mg protein)</td>
<td>150 ± 46</td>
<td>1600 ± 370*</td>
<td>78 ± 57</td>
</tr>
</tbody>
</table>

Rats received normal chow (n = 20) or 2% cholestyramine (n = 12) for 7 days, or 0.8% cholic acid (n = 12) for 15 days. Means and SD are shown.

* Statistically significant difference compared with controls, P < 0.01 (Dunnett).
mains to be explored if the entry into the circulation is direct from the hepatocyte, or if C4 is reabsorbed from the bile (within the liver or after absorption from the small intestine). The question of plasma entry of C4 might be important for data interpretation when species having gallbladders are investigated.

The comparison between different species showed that the Sprague-Dawley rat and the pig had the highest C4 levels, about five times higher than in human plasma (Fig. 4B). The C57BL6 mouse and the horse had about two times higher plasma C4 than humans, the latter having similar C4 levels as those found in guinea pigs. The lowest C4 level was found in New Zealand rabbits having C4 levels of only one third that found in humans. We obtained an average plasma C4 level of 12 ng/ml using human samples from five normal males. This level is in good agreement with previously published data where averages from 8 ng/ml to 19 ng/ml have been reported (21, 27, 28, 31, 32). Our data on the relative differences of the C4 levels in rats, mice, and humans in the current investigation is in agreement with previously published data on the enzymatic activity of C7αOH in these species. From our previous literature data (17, 33–38), it can be found that Sprague-Dawley rats have the highest C7αOH activity (23–41 pmol/min/mg protein), followed by C57BL6 mice (11–13 pmol/min/mg protein), while humans have the lowest activity (5–10 pmol/min/mg protein).

To conclude, a fast and reliable method that reflects hepatic C7αOH activity using a peripheral blood sample has been developed. Such a method is of interest for several reasons. First, since it monitors the activity of the enzyme in vivo, it should provide important complementary information to the commonly employed mRNA quantitations. This is of particular importance, since C7αOH enzymatic activity appears to also be regulated at the posttranscriptional level (35, 39), although the physiological significance of such regulation is yet unclear. Second, it will allow the dynamic study of enzyme activity and bile acid synthesis in individual laboratory animals. Third, it will improve the possibility of analyzing in more detail the role of the synthesis of bile acids in human diseases, such as hyperlipidemia and gallstone disease, and may be employed to monitor the response following treatment with drugs that interfere with bile acid circulation.

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Gälman et al. Monitoring cholesterol 7α-hydroxylase from peripheral blood


