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平成２８年度学位申請論文

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主論文の要旨

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【緒言】

ダビガトランに代表される直接抗トロンビン薬（direct thrombin inhibitors; DTIs）はワルファリンとは異なり、定期的なモニタリングを必要とせずに臨床的に利用が可能であると期待されていた。しかし、ダビガトラン服薬患者での重篤な出血性副作用が複数報告されたことから、血中のダビガトラン濃度と出血性リスクについて強い関心が集められている。DTIsであるダビガトランおよびアルガトロバンは合成基質であるS-2238を用いた酵素学的研究から、可逆的かつ競合的にトロンビン反応を阻害することが知られている。しかし、この阻害様式がそのまま抗凝固効果を反映しているかは定かではない。

クラウス法は古典的であるが、現在においても最も一般的な血中のフィブリノゲン濃度の定量方法である。この方法は、トロンビンによってフィブリン塊が形成されるまでの時間とフィブリノゲン濃度との間の定量性に基づいている。高濃度のトロンビンを希釈した血漿に加えるため、凝固時間はフィブリノゲン濃度にのみ依存し、他の凝固因子の影響は受けない。

酵素反応速度論のためのミカエリスメンテン式の古典的線形変換の一つであるラインウィーバー＝バークプロットは、基質濃度の逆数と反応速度の逆数の間の直線性を示す。酵

素阻害の種類を決定するために用いると、ラインウィーバー＝バークプロットは競合、反競合、非競合、混合型阻害剤を区別することができる。

本研究では、ダビガトランまたはアルガトロバンを添加した血漿におけるクラウス法による測定結果に基づき、ラインウィーバー＝バークプロットを作成し、酵素学的な視点からDTIsが試験管内凝固反応に与える影響を評価した。

【方法】

3つのフィブリノゲン濃度のプール血漿を作製し、様々な濃度でダビガトランまたはアルガトロバンを添加した。DTIsに対して感受性を示す濃度である40.25IU/mLのトロンビン試薬を用いて、各添加血漿のクラウス法による凝固時間を測定した。その後、検体のフィブリノゲン濃度の逆数を横軸に、凝固時間を反応速度の逆数とみなして縦軸にとり、ラインウィーバー＝バークプロットを作成した。各添加濃度におけるプロットについて一次近似式と相関係数を得た。また、無添加の凝固時間に対する各濃度の添加時の凝固時間の比、すなわち延長比とDTIsの濃度の関係性を検討した。

【結果】

ダビガトランのラインウィーバー＝バークプロットの結果、250 ng/mL (0.53 μM)まで良好な直線性を示し、一次近似式の傾きがほぼ一定で、切片のみが変化したことから、反競合阻害様式を呈した。これに一致して、血漿検体のフィブリノゲン濃度、すなわち基質濃度が高いほど、ダビガトランによる凝固時間の延長が大きく認められた。一方、250 ng/mL以上の濃度では抗凝固効果は混合型阻害様の阻害様式を示し、凝固時間の延長は曲線的に急峻化した。興味深いことに、今回阻害様式や凝固時間の延長に重大な影響が

観察された250 ng/mLは、臨床試験（RE-LY試験）の解析にて出血リスクを2倍にするダビガトラン血中濃度（210ng/mL）に近いものであった。

アルガトロバンを用いた検討でも、おおよそダビガトランと同様の結果だったことから、観察された特徴はダビガトランのみに限らないことが示唆された。

【考察】

DTIsの抗凝固効果は、フィブリノゲンの分子構造とトロンビン分子の動態のために複雑に発現すると考えられる。S-2238はトロンビンによる触媒作用のために1箇所だけ結合部位を持つ小さなペプチド分子である。これに対して、フィブリノゲン1分子は２つの同一モノマーからなる巨大分子である。フィブリンモノマーはトロンビンによって、フィブリノペプタイドA（FPA）とフィブリノペプタイドB（FPB）がそれぞれ放出される2箇所の切断部位を持つ。FPA切断部位のトロンビンとの結合反応性は、FPB切断部位のそれよりも極めて大きい。トロンビンはまずフィブリノゲンからのFPA切断を触媒し、その直後にフィブリン生成中間体であるdes-A フィブリノゲンから解離することなく次にFPB切断を触媒する。今回の結果から、フィブリノゲンのFPA切断部位とトロンビンとの結合に競合できない低濃度のDTIsは、des-A　フィブリノゲンと結合したままのトロンビンと結合して見かけ上、反競合的にFPBの切断を阻害するかもしれない。また、FPA切断だけでも観察可能な凝固を起こすことができることは、250 ng/mL以下のダビガトラン添加血漿における凝固時間が無添加血漿のそれと非常に近いことを説明できるように思われる。対照的に、高濃度のDTIsではトロンビンとdes-A フィブリノゲンの複合体に加えて、遊離型のトロンビンとも結合してFPA切断とFPB切断の両者を阻害することで、混合型のような阻害形式を示したと考えられる。

トロンビンによって生成されたフィブリンはトロンビン分子の一部を吸着する。その結果起こるトロンビンの固相化は、遊離トロンビンだけの場合と比べて酵素の活性動態に複雑な影響を及ぼし得る。このように、フィブリノゲンの分子構造やトロンビン分子の動態は、S-2238を用いたトロンビン反応に基づくDTIsの知見からの乖離に関係すると推察される。

【結語】

クラウス法に適用したラインウィーバー＝バークプロットに基づきDTIsの抗凝固効果を評価した結果、S-2238を基質とするトロンビン反応を利用して得られた過去の知見との乖離が明らかとなった。試験管内あるいは生体内での凝固反応に対してDTIsがどのように影響を与えるのかについて理解を深めることが、より安全で有効なDTIsの投薬方法を研究する一助になることが期待される。

Abstract

Introduction: Direct thrombin inhibitors (DTIs) represented by dabigatran were expected to be available for therapeutic use without the need for routine monitoring. However, clinical concerns regarding impacts of plasma dabigatran concentrations on the rate of major bleeding have been raised. Based on enzymatic examinations with a synthetic substrate, S-2238, it has been well recognised that dabigatran and another DTI argatroban reversibly and competitively inhibit thrombin reaction, while it is still uncertain whether such an inhibition pattern reflects anticoagulation effects. The Clauss assay is a method for quantification of plasma fibrinogen concentrations, based on the quantitative relationship between fibrinogen concentrations and time for fibrin clot formation by thrombin. The Lineweaver–Burk plot, which is one of classical linear transformations of the Michaelis–Menten equation for enzymatic reaction kinetics, presents curve-linearity between the reciprocal of the substrate concentration versus the reciprocal of the rate of reaction. When used for determining the type of enzyme inhibition, this plot can distinguish competitive, uncompetitive, non-competitive and mixed inhibitors. To assess how DTIs affect in vitro clotting from the enzymatic perspective, the Lineweaver–Burk plot was applied to the Clauss assay, regarding the clotting time as an inverse function of the rate of thrombin reaction.

Materials and Methods: The clotting time in pooled plasma samples of three different fibrinogen concentrations was examined in the presence of various concentrations of dabigatran or argatroban, using a thrombin concentration of concentration of 40.25 IU/mL, which is sensitive to DTIs.

Results: Dabigatran appeared to inhibit thrombin reaction uncompetitively roughly within dabigatran concentrations of 250 ng/mL (0.53 μM). Consistent with uncompetitive inhibition, dabigatran seemed to be effective in plasma samples containing high fibrinogen concentrations. At the concentrations of more than 250 ng/mL, the anticoagulant effects exhibited mixed inhibition-like patterns, and the prolongation of clotting time was curve-non-linearly accelerated. Analogous experiments using argatroban provided findings roughly similar to those from the experiments using dabigatran, suggesting that the observations were not limited to the case of dabigatran.

Conclusions: The results revealed the considerable discrepancy from well-known findings originated from previous studies using a thrombin assay with S-2238 as a substrate. Further understanding of how DTIs impact in vitro and in vivo coagulation reaction is expected to aid in studying how best to improve the medication.

主論文

Direct thrombin inhibitors (DTIs) represented by dabigatran were expected to be available for therapeutic use without the need for routine monitoring, in contrast to warfarin. However, clinical concerns regarding impacts of plasma dabigatran concentrations on the rate of major bleeding have been raised.[1] Not only clinical but also basic aspects of DTIs would aid in studying how best to address concerns regarding bleeding risk in therapeutic use. Based on enzymatic examinations with a synthetic substrate, S-2238, it has been well recognized that dabigatran and another DTI argatroban reversibly and competitively inhibit thrombin reaction.[2 3] However, it still remains unclear whether the type of inhibition by DTIs of thrombin reaction with S-2238 can be applied to the anticoagulant effects.

The Clauss assay is classical but most popular even now for quantification of plasma fibrinogen concentrations.[4] This is based on the quantitative relationship between fibrinogen concentrations and time for fibrin clot formation by thrombin. As a high concentration of thrombin ranging from 40.25 to 115 IU/mL is added to dilute test plasma, the clotting time depends exclusively on concentrations of fibrinogen but not of other coagulation factors.

The Lineweaver-Burk plot, which is one of classical linear transformations of the Michaelis-Menten equation for enzymatic reaction kinetics, presents curve-linearity between the reciprocal of the substrate concentration versus the reciprocal of the rate of reaction.[5] When used for determining the type of enzyme inhibition, this plot can distinguish competitive,

uncompetitive, non-competitive, and mixed inhibitors.

To assess how DTIs affect in vitro clotting from the enzymatic perspective, the Lineweaver-Burk plot was applied to the Clauss assay, regarding the clotting time as an inverse function of the rate of thrombin reaction. In brief, the clotting time in pooled plasma samples of three different fibrinogen concentrations was examined in the presence of various concentrations of dabigatran, using a thrombin concentration of 40.25 IU/mL, which is sensitive to DTIs .[6]

The dabigatran concentration of around 250 ng/mL (0.53 mM) seemed to critically affect the inhibition type as well as the prolongation of clotting time (figure 1). Dabigatran appeared to inhibit thrombin reaction uncompetitively roughly within dabigatran concentrations of 250 ng/mL. Consistent with uncompetitive inhibition, dabigatran appeared to be effective in plasma samples containing high fibrinogen concentrations. At the concentrations of more than 250 ng/mL, the anticoagulant effects exhibited mixed inhibition-like patterns, and the prolongation of clotting time was curve-nonlinearly accelerated. Interestingly, the concentration, 250 ng/mL, appearing to critically affect the inhibition type and the prolongation of clotting time was close to the concentration of 210 ng/mL, which doubled the rate of major bleeding as analyzed by the RE-LY trials.[1] Analogous experiments using argatroban provided findings roughly similar to those from the experiments using dabigatran, suggesting that the

observations were not limited to the case of dabigatran (figure 2).

Expression of anticoagulant effects of DTIs is thought to be complicated due to the molecular structure of fibrinogen and dynamics of thrombin molecules. S-2238 is a small peptide molecule possessing just one binding site for catalysis, while a fibrinogen molecule is a dimeric macromolecule composed of two identical monomers possessing two enzymatic cleavage sites to release the fibrinopeptide A (FPA) and fibrinopeptide B (FPB), respectively. The specificity constant for release of FPB from intact fibrinogen is less than 3% of that for release of FPA from intact fibrinogen.[7] It is plausible that the two cleavage sites are differently sensitive to DTIs because of the great differences in binding affinity for thrombin. Notably, thrombin catalyzes FPB cleavage without dissociation of the catalytic intermediate, des-A fibrinogen, immediately just after catalyzing FPA cleavage. In fact, the specificity constant for release of FPB from intact fibrinogen is less than 10% of that for release of FPB from des-A fibrinogen.[7] Low concentrations of DTIs, which cannot compete with intact fibrinogen to bind to thrombin, might bind to thrombin still associated with des-A fibrinogen to exhibit apparently uncompetitive but not competitive inhibition of FPB cleavage. It is of considerable interest that even only release of FPA from intact fibrinogen can initiate observable clotting. This might be why the clotting times within dabigatran concentrations of 250 ng/mL were much closer to those in the absence of dabigatran than those at the concentrations of more than 250

ng/mL. In contrast, high concentrations of DTIs could bind not only to a complex of thrombin and des-A fibrinogen but also to free thrombin to exhibit mixed inhibition-like patterns, preventing both FPA release and FPB release.

Fibrin once generated by thrombin adsorbs a considerable amount of thrombin molecules.[8] The resulting immobilization of thrombin affects the enzymatic activities and kinetics, likely changing rate of catalytic reaction more complicatedly than the case of only soluble thrombin. Thus, it seems that structural features of fibrinogen and dynamics of thrombin molecules are related to the discrepancy from findings based on thrombin reaction with S-2238.

Potential weakness of the present study is the use of plasma samples spiked with DTIs but not of samples from patients on medication. It is difficult to assess the inter-individual variation using spiked samples. In addition, about 20% of dabigatran gets metabolized to pharmacologically active glucuronide conjugates by glucuronosyltransferase in vivo. Observations from samples spiked with dabigatran cannot reflect effects of its metabolites. However, it is unjustifiable to designedly expose patients to potentially toxic dose and indefensible to collect such samples from patients. To evaluate in vitro anticoagulant effects of DTIs not including their metabolites, the use of spiked samples is a valid methodology.

To our knowledge, the present study is the first to evaluate anticoagulant effects of DTIs based on the Lineweaver-Burk plot applied to the Clauss assay. The results revealed the

considerable discrepancy from well-known findings originated from previous studies using a thrombin assay with S-2238 as a substrate. Further understanding of how DTIs impact in vitro and in vivo coagulation reaction is expected to aid in studying how best to improve the medication.

References

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Figure legends

Figure 1. Examination of dabigatran. (A) Lineweaver-Burk plot analysis for the data from pooled plasma samples spiked with dabigatran at the concentrations of the range from 0 to 700 ng/mL as indicated in the graph. The fibrinogen concentrations of samples were 150, 240, and 410 mg/dL. (B) Linear approximate equations and correlation coefficients in (A). (C) Prolongation of the clotting time of pooled plasma samples bearing a fibrinogen concentration of 150, 240, or 410 mg/dL in the presence of dabigatran. Ratios of clotting time were ratios of the clotting time in the presence of dabigatran to the clotting time in the absence of dabigatran.

Figure 2. Examination of argatroban. (A) Lineweaver-Burk plot analysis for the data from pooled plasma samples spiked with argatroban at the concentrations of the range from 0 to 1000 ng/mL as indicated in the graph. The fibrinogen concentrations of samples were 140, 240, and 440 mg/dL. (B) Linear approximate equations and correlation coefficients in (A). (C) Prolongation of the clotting time of pooled plasma samples bearing a fibrinogen concentration of 140, 240, or 440 mg/dL in the presence of argatroban. Ratios of clotting time were ratios of the clotting time in the presence of argatroban to the clotting time in the absence of argatroban.

Figure 1 A

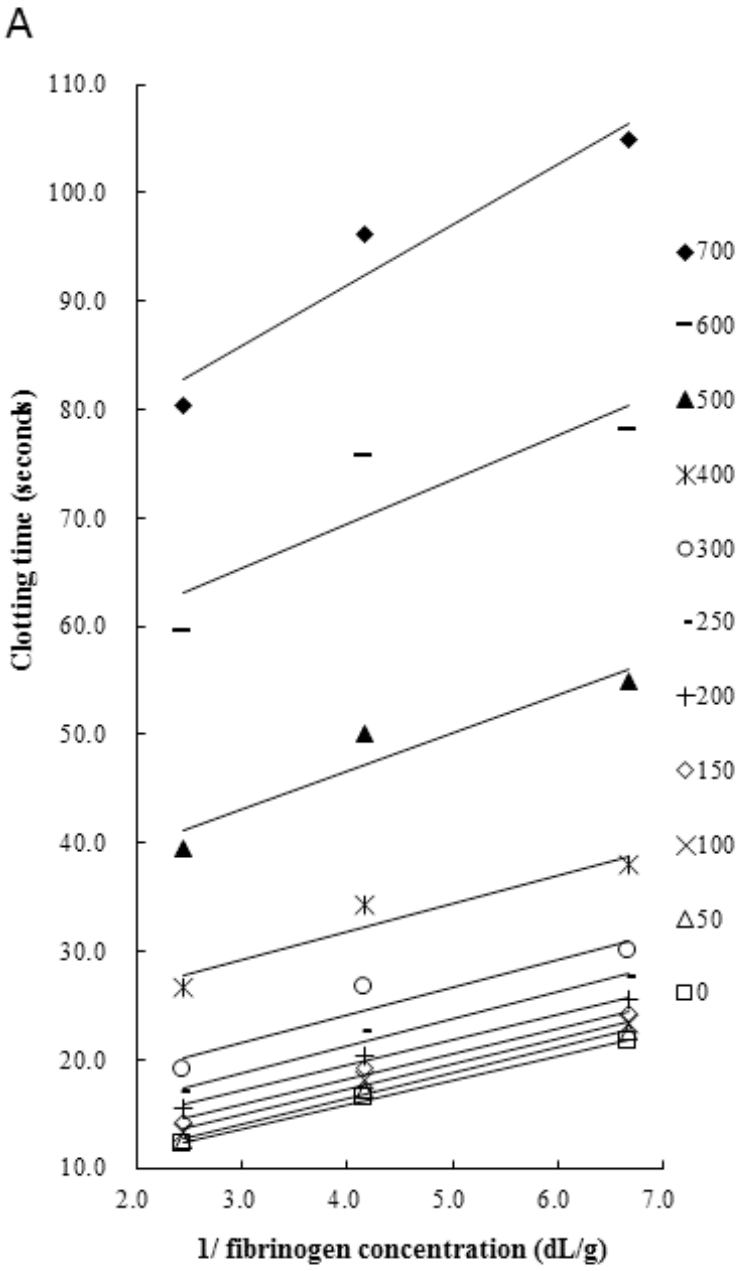
A
110.0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
Clotting time (seconds)
2.0
3.0
4.0
5.0
6.0
7.0
1/ fibrinogen concentration (dL/g)
700
600
500
400
300
250
200
150
100
50
0

Figure 1 B and C

B

Dabigatran (ng/mL)	linear approximate equation	correlation coefficient (R^2)
0	y = 2.26x + 6.78	0.9968
50	y = 2.35x + 7.04	0.9942
100	y = 2.35x + 7.87	0.9918
150	y = 2.34x + 8.85	0.9890
200	y = 2.36x + 10.0	0.9946
250	y = 2.50x + 11.3	0.9827
300	y = 2.52x + 14.1	0.8989
400	y = 2.59x + 21.5	0.9060
500	y = 3.52x + 32.6	0.9036
600	y = 4.13x + 52.9	0.7602
700 (1.48 μM)	y = 5.61x + 69.0	0.9281

C

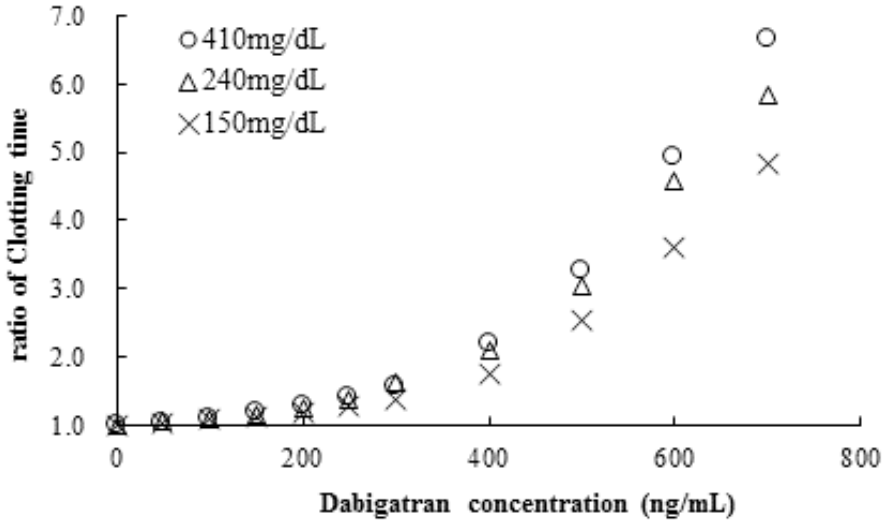

Figure 2 A

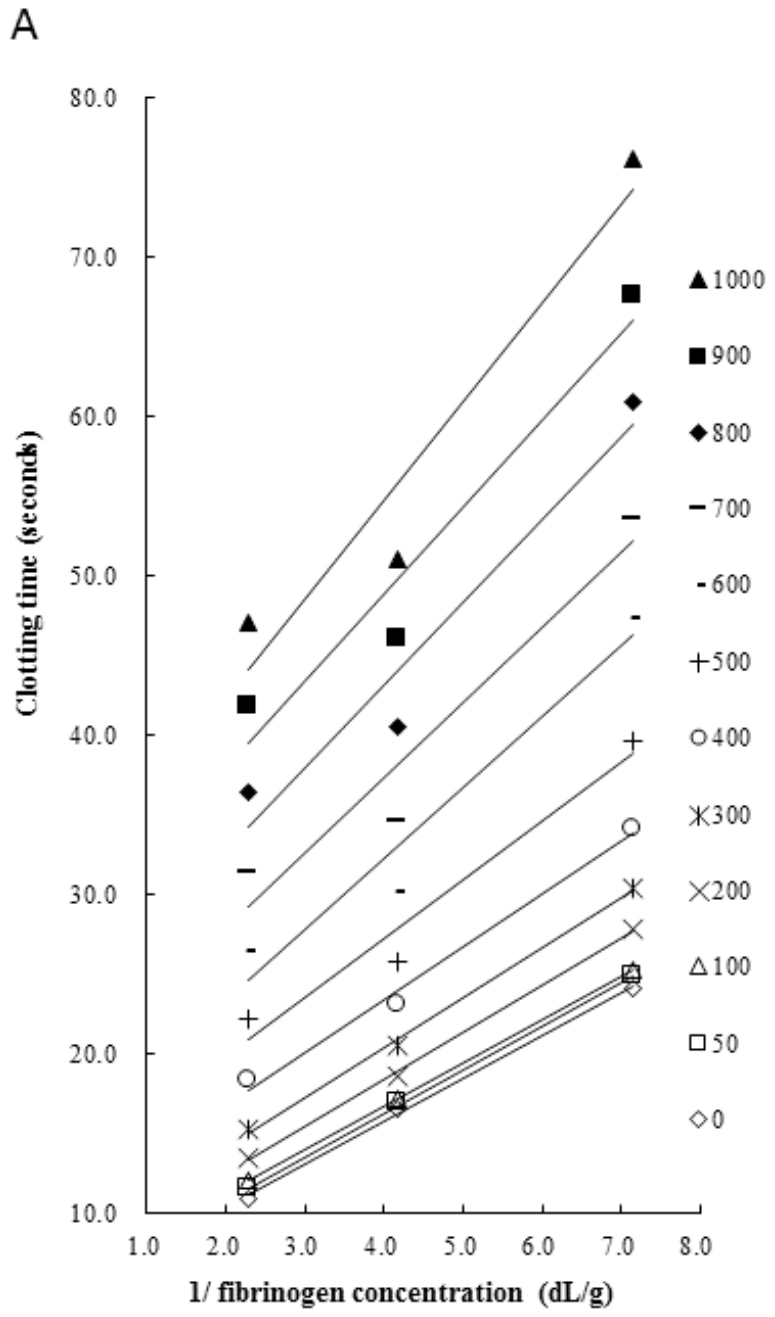
A
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
Clotting time (seconds)
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
1/ fibrinogen concentration (dL/g)
1000
900
800
700
600
500
400
300
200
100
50
0

Figure 2 B and C

B

Argatroban (ng/mL)	linear approximate equation	correlation coefficient (R^2)
0	y = 2.69x + 5.05	0.9988
50	y = 2.73x + 5.41	0.9998
100	y = 2.71x + 5.96	1.0000
200	y = 2.94x + 6.68	0.9985
300	y = 3.11x + 8.02	0.9980
400	y = 3.29x + 10.3	0.9907
500	y = 3.68x + 12.5	0.9623
600	y = 4.43x + 14.6	0.9492
700	y = 4.72x + 18.5	0.9334
800	y = 5.18x + 22.5	0.9456
900	y = 5.45x + 27.1	0.9431
1000 (1.90 μM)	y = 6.19x + 30.0	0.9299

C