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Journal of Chromatography B, 821 (2005) 210–214
JOURNAL OF CHROMATOGRAPHY B
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# Characterization of the urinary metabolites of dipetarudin

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Received 12 November 2004; accepted 8 May 2005

**Abstract**

Dipetarudin is a hybrid thrombin inhibitor composed of the N-terminal structure of dipetalogastin II and the exosite 1 blocking segment of hirudin. Pharmacokinetic studies demonstrated that it distributes in extravascular and intravascular spaces and is exclusively eliminated by the kidneys. Two active metabolites of dipetarudin with molecular masses of 6142 and 5395 Da, respectively, were isolated from rat urine. Analysis of their N-terminal sequences and molecular masses demonstrated that dipetarudin is cleaved in a first step at the peptide bond $Phe_{55}$–$Glu_{56}$ and then, at $Gly_3$–$Asn_4$. Nonmetabolized dipetarudin was not found in rat urine. Proteases localized in the proximal tubulus cells of kidneys might be responsible for its degradation.


*Keywords:* Dipetarudin; Renal metabolism; High performance liquid chromatography; Ecarin clotting time; Matrix assisted laser desorption/ionization time of flight

## 1. Introduction

The most investigated direct thrombin inhibitor is hirudin which was purified from the salivary glands of the leech *Hirudo medicinalis*. It is a 65-amino acid protein with a molecular mass of 7000 Da that forms a stoichiometric complex with human alpha-thrombin binding to the active site and to the fibrinogen binding site. The inhibition equilibrium constant ($K_i$) of this complex is 21 fM for native hirudin and 217 fM for its recombinant form, r-hirudin [1–3]. The pharmacokinetics of hirudin has been described using a two-compartment model. It has an elimination half-life of 0.75–1.3 h in all species studied including humans [4,5] and is exclusively eliminated by the kidneys. This naturally occurring thrombin inhibitor is degraded on the C-terminal end, following glomerular filtration in proximal tubulus cells [6,7].

Dipetarudin is a hybrid thrombin inhibitor composed of the N-terminal head structure of dipetalogastin II, the strongest thrombin inhibitor from the assassin bug *Dipetalogaster maximus* [8], and a fragment of the anion exosite 1 blocking segment of hirudin (comprising 55–65 amino acid residues), connected through a five glycine linker. After expression in *Escherichia coli* and purification, the amino terminus of dipetarudin is enlarged by the peptide GIPE and its C-terminus is completed by four additional amino acid residues AAAS, derived from the polylinker of the expression vector. Biochemical characterization of dipetarudin revealed that it is a slow, tight-binding inhibitor with a dissociation constant ($K_i$) of 446 fM and a molecular mass of 7560 Da [9].

Moreover, pharmacokinetic studies in rats demonstrated that after intravenous administration of dipetarudin, distribution phenomena are primarily responsible for the decrease of its blood level, the initial distribution was followed by an elimination phase with an elimination half life of 0.56 h. This behavior can be best described by an open two-compartment

*Abbreviations:* GST; glutathione S-transferase; ECT; ecarin clotting time; TFA; trifluoroacetic acid; ACN; acetonitrile; MALDI-TOF; matrix assisted laser desorption/ionization time of flight; RP-HPLC; reversed phase high performance liquid chromatography

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doi:10.1016/j.jchromb.2005.05.005

model with first-order elimination. On the other hand, administration of dipetarudin to nephrectomized rats was always followed by a higher blood level of this substance than found in animals with normal renal function. After the distribution phase, the blood level remained nearly constant, which speaks in favor of the exclusive renal elimination of this inhibitor and demonstrates that it does not undergo some metabolism in any other organ of the body [10].

We report here the purification of dipetarudin metabolites from rat urine and their structural characterization by amino acid sequence analysis and matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry.

## 2. Experimental

### 2.1. Materials

HPLC grade acetonitrile was purchased from J.T. Baker, Deventer, The Netherlands. Trifluoroacetic acid (TFA) was obtained from Acros Organics, New Jersey USA. Ethylurethane was purchased from Fluka, Seelze, Germany. Ecarin was purchased from Pentapharm Ltd, Basel, Switzerland. Human citrated plasma was collected by plasmapheresis of healthy donors, pooled and stored in portions at −80 °C. All other reagents were of the highest grade commercially available.

### 2.2. Dipetarudin cloning

Dipetarudin was cloned in our laboratories by use of recombinant DNA technology [9]. Briefly, dipetalogastin cDNA and specific primers were used to amplify the dipetarudin sequence by polymerase chain reaction. The PCR product was subcloned into the expression vector pGEX-5X-1 and transformed in *E. coli* JM105. The induction of expression and purification of dipetarudin were accomplished using the Glutathione S-transferase (GST) Gene Fusion System according to manufacturer's guidelines. Cleavage of the fusion protein was achieved using factor Xa. Finally, dipetarudin was purified by reverse phase HPLC.

### 2.3. Animal preparation and experimental protocol

Wistar rats (160–250 g body weight) were anaesthetized by a parenteral injection of 1.5 g/kg ethylurethane. A catheter was placed in the left jugular vein for a continuous infusion to ensure diuresis. For this purpose a 20% mannitol solution was infused at a flow of 12 mL/h for 5 min and then, the infusion was continued with 10% mannitol containing 5% bovine serum albumin at 3 mL/h for 10 h. Dipetarudin was administered intravenously as a single bolus injection of 1 mg/kg body weight. Urine was collected from all subjects immediately before dosing and at definite time intervals after dosing and frozen at −20 °C until further analysis.

### 2.4. Purification and characterization of dipetarudin metabolites

Following adjustment of pH to 4.0 using 40% acetic acid, the urine was heated at 65 °C for 10 min, neutralized to pH 7.0 with 2 M NaOH and then, centrifuged. The supernatant was desalinized by gel filtration chromatography on a PD-10 column, filtered through a 0.2 μm pore-sized membrane and injected into a Vydac C18 reverse-phase HPLC column equilibrated with 19% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA). The elution was performed with a gradient of acetonitrile that started with 19% ACN containing 0.1% TFA for 4 min and then, changed lineally from 19 to 35% ACN (with 0.1% TFA) until 28 min. Peaks were detected at a wavelength of 210 nm.

The eluate was collected every minute by an autocollector (FRC-10A, Shimadzu Europa). The collected fractions were lyophilized in a rotation vacuum dryer (Speed Vac Plus SC 110 A, Savant Instruments, Farmingdale, USA).

The anticoagulant activities of lyophilized HPLC-fractions were measured by ecarin clotting time. The active fractions were submitted to a second RP-HPLC purification step using the same conditions.

### 2.5. Ecarin clotting time

Antithrombin activity in urine samples or HPLC fractions was determined in a mechanical coagulometer (CL4) at 37 °C using ecarin clotting time [11]. Briefly, 20 μL of urine was added to a cuvette with 200 μL plasma and 60 μL of 0.05 M Tris/HCl buffer (pH 7.5) containing 0.154 M NaCl. The mixture was preincubated for 3 min at 37 °C. The reaction was started by addition of 20 μL ecarin solution (5 EU/mL in 0.154 M NaCl/0.05 M $CaCl_2$) and the time until clotting was measured.

### 2.6. Structural characterization of dipetarudin metabolites

N-terminal amino acid sequence analysis was performed by automated Edman degradation in a protein sequencer model 476A (Applied Biosystems, Germany). As cysteine was not modified and therefore not detectable, gaps in the resulting sequence were assessed as cysteine residues. Data were analyzed using the model 610A data analysis program.

The molecular mass of metabolites present in the active peaks was determined by matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry, using a Kompact Probe instrument (Kratos Analytical, UK) equipped with a pulsed nitrogen laser ($\lambda = 337$ nm, 3 ns pulse width and 3 Hz frequency). Ions generated by the laser desorption were introduced into the flight tube with an acceleration voltage of 5 kV for the linear positive ion mode. All spectra were collected by averaging 60 individual laser shots.

The matrix solution was prepared as a stock solution containing 10 mg α-Cyano-4-hydroxy-cinnamic acid in

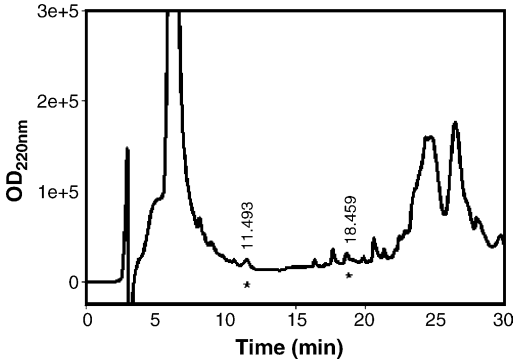


Fig. 1. RP-HPLC analysis of rat urine. Dipetarudin was administered intravenously as a single bolus injection of 1 mg/kg body weight. Urine was collected, precipitated changing its pH and temperature, desalted and applied on a Vydac C18 column. For chromatographic conditions, see methods. The asterisks indicate the peaks with antithrombin activity detected by ecarin clotting time (ECT).

1 mL 50% acetonitrile/50%water containing 0.1% TFA. 0.8 μL of the matrix, 0.8 μL sample and 0.8 μL matrix were dispensed on the sample slide by sandwich mode. Bovine insulin (5733.5 Da), bovine cytochrom C (12233 Da) and angiotensin II (1046 Da) were used as samples for mass calibration.

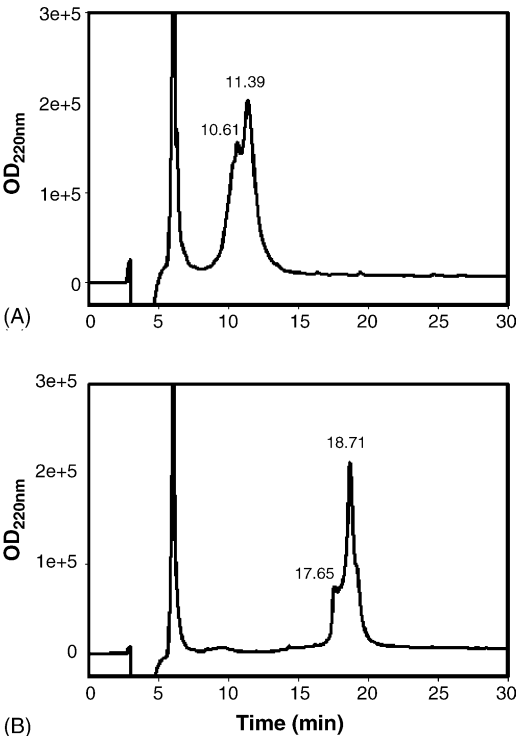


Fig. 2. RP-HPLC of the active dipetarudin metabolites purified from rat urine. The chromatography showed in Fig. 1 was performed several times in order to accumulate enough amount of both fractions with antithrombin activity, which were separately applied on a Vydac C18 column and eluted under the same conditions. (A) Fraction with retention time of 11.493 min. (B) Fraction with retention time of 18.459 min.

Molecular mass was also calculated according to the amino acid sequence by a computer program (Clone Manager for Windows. Version 4.01. SCI-ED Software, Bethesda, USA).

## 3. Results and discussion

Fig. 1 shows the elution profile of rat urine from a C18 Vydac column. Only two peaks were able to prolong the ecarin clotting time. These peaks have retention times of 11.493 and 18.459 min, respectively. Non-metabolized dipetarudin with a retention time of about 20.5 min was not found.

This chromatography was performed several times in order to accumulate enough amount of both fractions with antithrombin activity, which were separately applied on a Vydac C18 column and eluted under the same conditions. The resultant chromatograms are shown in Fig. 2. The active fractions with retention times of 11.387 and 18.710 min were used for further investigations.

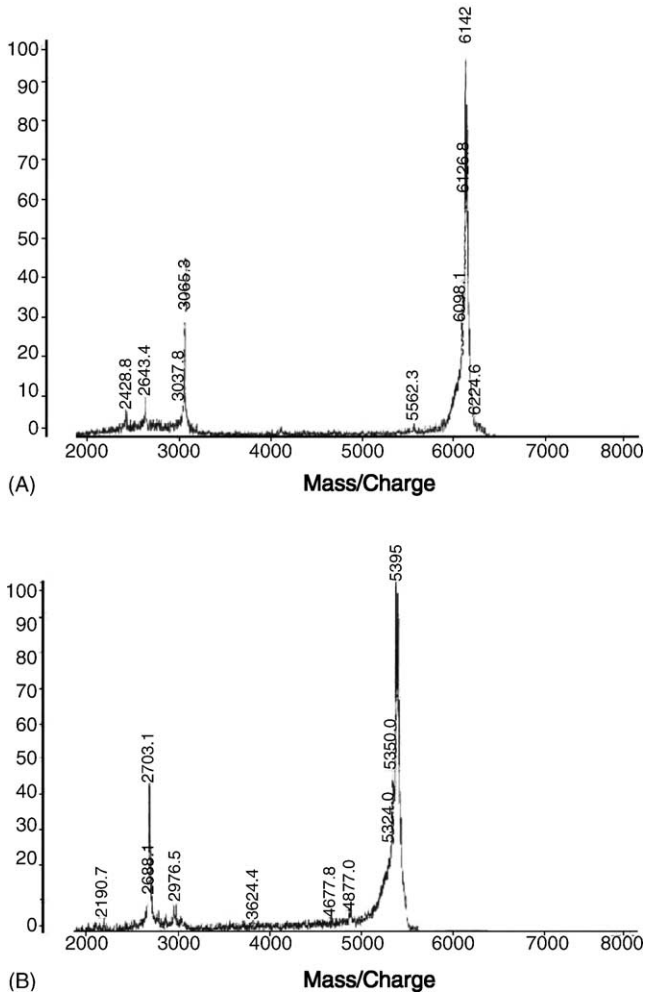


Fig. 3. MALDI TOF mass spectrometry of the dipetarudin metabolites with retention times of 18.459 min (A) and 11.493 min (B), respectively.

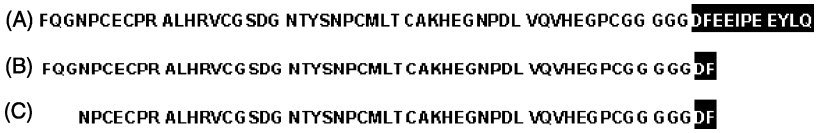


Fig. 4. Amino acid sequences of dipetarudin (A) and its rat urinary metabolites with retention times of 18.7 min (B) and 11.4 min (C). The amino acid sequences are described by one-letter codes. Amino acids identical to hirudin are indicated by the inversion of black and white. Residues derived from the polylinker of vector expression during the cloning are not represented.

To estimate the structure of these fractions, we firstly analyzed their amino acid sequences and found that the N-terminal sequence of the metabolite with a retention time of 18.710 min is GIPEFQGNP, that is identical to that of dipetarudin itself, suggesting that this fraction consists of a C-terminally truncated peptide of dipetarudin. Furthermore, as the molecular mass of this metabolite determined by the MALDI-TOF mass spectrometry is 6142 Da (Fig. 3B), the first cleavage site on the dipetarudin molecule must be localized at the peptide bond $Phe_{55}$–$Glu_{56}$ (Fig. 4).

On the other hand, the second cleavage site of dipetarudin is localized at the peptide bond $Gly_3$–$Asn_4$, as the N-terminal sequence of the metabolite with a retention time of 11.387 min was NPCECP (Fig. 4). This finding was also confirmed by comparison of the molecular mass for this metabolite of 5395 Da determined by MALDI-TOF mass spectrometry (Fig. 3A) with the molecular mass of 5401 Da calculated for the dipetarudin fragment consisting of the amino acids 4–55.

Like dipetarudin, hirudin is also eliminated exclusively by the kidneys. Investigation of the urinary metabolism of a recombinant hirudin analog (CX-397) in rats showed that unmodified hirudin was not found in rat urine after intravenous administration, but its pattern of cleavage by renal proteases is different to that of dipetarudin. Thus, only C-terminal peptides consisting of the first 49, 50, 51, 52, 54 and 55 amino acids were recovered [7]. Dipetarudin was not cleaved at the peptidic bond $Asp_{54}$–$Phe_{55}$ as expected if hirudin is digested at $Asp_{55}$–$Phe_{56}$. Moreover, dipetarudin was cleaved at the peptidic bond $Phe_{55}$–$Asp_{56,}$ which corresponds to $Phe_{56}$–$Asp_{57}$ in hirudin. This cleavage site on the hirudin molecule has not been reported previously.

On the other hand, hirudin and r-hirudin are excreted mainly as nonmetabolized form in human. However, C-terminally truncated metabolites consisting of the first 60, 61, 62, 63 and 64 amino acids were also found. The C-terminal degradation of hirudin is produced by extrinsic proteases located on the surface of the brushborder membranes of the proximal tubulus cells of human kidneys [6]. These extrinsic proteases can also be detected in urine, so that “metabolism/degradation” of hirudin can also be demonstrated ex vivo, by adding hirudin to urine. These postglomerular renal metabolites of hirudin have lower antithrombin activity compared with the original hirudin [5].

Degradation of several low-molecular weight proteins does not occur during glomerular passage, but in proximal tubulus cells by various membrane-bound enzymes [12,13]. One of these enzymes, meprin, a metalloprotease present in proximal tubulus cells of rats and some strains of mice [14], could be responsible for the dipetarudin metabolism observed in rats. This endoprotease exhibits a preference for peptide bonds that are flanked by hydrophobic or neutral amino acids. Like several other peptides, it is possible that small peptides of dipetarudin might be absorbed by the proximal tubulus cells of rats and undergo some metabolism [15].

## 4. Conclusions

Two metabolites of the potent thrombin inhibitor, dipetarudin, were isolated from rat urine. We have characterized these metabolites by amino acid sequence analysis and matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. We have also established the steps in which dipetarudin is metabolized in the kidneys. In a first step, it is cleaved in the carboxy terminus at the peptide bond $Phe_{55}$–$Glu_{56}$ and then, in the amino terminus at $Gly_3$–$Asn_4$.

The pattern of cleavage of the carboxy terminus of dipetarudin is different to that found for hirudin in rat urine, although both inhibitors have identical C-terminus sequence.

## Acknowledgements

We thank Dr. Katrin Mende for performing amino acid sequence analysis and Dr. Ute Lange for helpful discussions.

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