The Use of Snake Venom-Derived Compounds for New Functional Diagnostic Test Kits in the Field of Haemostasis

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Key Words

Snake venom · Haemostasis · Diagnostics · FV Leiden · APC resistance · Anti-coagulation monitoring · PiCT®

Abstract

Pentapharm Ltd. has a long tradition of producing and proceeding snake venom components as APIs and tools in pharmaceutical and diagnostic applications, beginning with the snake venom-derived product Reptilase® about 60 years ago. In recent years diagnostic test kits in the field of haemostasis have been developed, like Pefakit® APC-R Factor V Leiden and Pefakit® PiCT®, which make use of one or more snake venom compounds as key reactants. The venom-derived compounds are responsible for many outstanding properties of these tests and account for their top ranking performance qualities confirmed in non-clinical and clinical studies. Both tests are on the market in Europe. Pefakit® APC-R Factor V Leiden is also marketed in the US since January 2005. Research on snake venom-derived compounds for diagnostic and pharmaceutical use is ongoing.

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Introduction

For more than 50 years Pentapharm has been involved in the research on snake venoms. At several occasions the company has successfully brought new active compounds derived from snake venom to the market or used these compounds as tools in pharmaceutical and diagnostic applications. Some of them have been and still are commercial successes. Ongoing research by A. Perchuc, described elsewhere in this volume, has included an intensive investigation of the venom of the South American Lancehead *Bothrops moojeni*. Batroxobin, the main ‘thrombin-like’ enzyme from this species, has been isolated and used under its proprietary name Reptilase® for the production of Defibrase®, a defibrinogenating pharmaceutical active ingredient used for prevention and treatment of thrombotic events [1, 2]. Besides the well-known Batroxobin, the venom of this snake species contains over 100 further substances not yet identified and described. Most of the venom used for Batroxobin isolation is actually discarded as waste fractions. The research work therefore aims to identify possible yet unknown structures and functions with modern tools of proteomics and fast multiple functional screening methods. The venom of the closely related species *Bothrops atrox* is mainly used for the production of Reptilase® Reagent to be used as a tool in diagnostic procedures and test kits.

Fig. 1. Pefakit® PiCT® – test principle.

Snake Breeding and Use of Snake Venom Compounds

In order to fill the need for Batroxobin, Pentapharm maintains one of the largest snake breeding farms in the world, Pentapharm do Brazil, located in Minas Gerais, Uberlandia. This farm was built about 25 years ago to guarantee the supply with sufficient, high-quality, homogeneous venom from the two species *B. atrox* and *B. moojeni*. As many as 10,000 individuals can be housed in this field station. The company also purifies and sells compounds extracted from the venom of other snake species. The focus of these products is their activity in blood coagulation. For these products, Pentapharm relies on local breeders capable of collection and preparation of sufficient amounts of dried snake venom compliant with the same high quality and conformity standard, like Venom Supplies in Tanunda, South Australia, for the venom of the endemic Australian snakes [3].

Diagnostic Test Kits

For many years, Pentapharm only purified the venom components and sold them to third parties as tools for research and for the production of diagnostic reagents and kits. Examples for such products are Protac® and Ecarin. In the last 6 years, Pentapharm has intensively worked on the development of diagnostic test kits as final end products to be sold under the company's own label Pefakit®. Examples of kits in which venom-derived compounds play an essential role are Pefakit® Reptilase® Time, Pefakit® PiCT® and Pefakit® APC-R Factor V Leiden. Pefakit® Reptilase® Time, formerly known as Reptilase® Reagent, was the first kit to be sold in Europe

under the new CE certification label. The test is quite simple, but very efficient for the analysis of specific deficiencies in the last phase of coagulation [4, 5]. The following describes two practical examples, where snake venom-derived compounds have been used to develop new kinds of more complex diagnostic test kits with outstanding performance. Both tests have been CE-certified and introduced in the European market. For Pefakit® APC-R Factor V Leiden, FDA approval has been obtained early this year.

Pefakit® PiCT®

The test principle for Pefakit® PiCT, described by Calatzis et al. [6, 7], is given in figure 1. PiCT is the acronym for 'Prothrombinase induced Clotting Time'. The test kit is designed to be used to monitor all kinds of anticoagulants, like unfractionated heparin, low molecular weight heparin, direct thrombin inhibitors and direct or indirect FXa inhibitors. Oral anticoagulants acting on vitamin K-dependent coagulation factors are not detected by this test. In the incubation phase, the FV contained in the patient's plasma has the possibility to come into contact with the RVV-V contained in the activator reagent. RVV-V is a highly specific activator of factor V purified from the Russell's viper (*Daboia russelli russelli*) venom. Activated factor V in turn is an essential cofactor in the prothrombinase complex. It massively enhances the speed of the enzymatic activity of activated FX (FXa) and thus the activation of prothrombin to thrombin. The addition of RVV-V assures the complete activation of all available FV contained in the patient's plasma. FXa and phospholipids, necessary to form the prothrombinase complex, are also provided with the activator reagent. When Ca²⁺

Fig. 2. Pefakit® APC-R Factor F Leiden – test principle.

is added with the start reagent, the prothrombinase complex is formed very quickly, activating thrombin at optimal speed. Thrombin cleaves fibrinogen to fibrin which leads to a measurable endpoint, be it through mechanical or optical detection. The rapid build-up of prothrombinase complexes guarantees that the test has a very short, clear and repeatable reaction onset, giving a better reading signal especially when optical detection methods are used. Well-defined clear reagent solutions further add to its advantage when used in instruments with optical detection principle.

With exception of the oral anticoagulants dependent on vitamin K like Marcoumar or Warfarin, all anticoagulants used in medical practice at present or in the near future are acting on the level of FXa (anti-Xa activity) or on the level of thrombin (anti-IIa activity), or on both levels at the same time. The classical unfractionated heparin primarily has anti-IIa activity, but partially also anti-Xa activity. Low molecular weight heparins primarily act through their anti-Xa activity, but also have anti-IIa activity. The individual relation of both activities is dependent on the exact composition of the low molecular weight heparin, and the production method applied [8]. Direct thrombin inhibitors like hirudin and active substances derived thereof exclusively act on the thrombin level. A set of direct Xa inhibitors only acting on the Xa level are presently under development and can also be measured and monitored by this method, although in a slightly modified procedure. Since the PiCT® includes both levels, it actually measures the whole anticoagulant activity of a given anticoagulant or a combination of such anti-

coagulants. The Pefakit® PiCT® may therefore be regarded as a universal test for monitoring all these types of anticoagulants [6, 9].

A test that can directly be compared with the PiCT is the Heptest® from Haemachem [10]. It has a comparable test principle and comparable targets to be monitored. This test makes use of a reagent called Recalmix®, which is based on bovine plasma reported to be rich in FV and phospholipids, but depleted of prothrombin. This reagent contains a mixture of many different substances not further declared or explained by the manufacturer. It is a turbid solution strongly influencing optical measurements. In contrast, all PiCT® reagents are well-defined clear solutions. Increase of optical density as a result of fibrin clotting can be measured from its onset and the curves obtained are more easily interpreted and analysed. By using RVV-V the PiCT® test is independent of coagulation factors responsible for FV activation. It specifically focuses on the prothrombinase complex, thrombin activation and thrombin activity on fibrinogen. The test is therefore very insensitive to influencing or disturbing factors. Thus the RVV-V used in this test is crucial for its specific performance characteristics.

Pefakit® APC-R Factor V Leiden

Pefakit® APC-R Factor V Leiden is designed to screen for factor V Leiden mutation genotype in a patient's plasma [11]. FV Leiden is the most important single mutation of a coagulation factor increasing the thrombotic risk in

Fig. 3. Correlation of ratios obtained with Pefakit[®] APC-R Factor V Leiden and Coatest[®] APC Resistance in a study with plasma samples of different FV Leiden genotype.

a patient who is carrier of the heterozygous or homozygous type of this mutation [12]. The mutation leads to resistance of activated FV to inactivation by APC so that the feedback loop for thrombin generation inhibition cannot efficiently work. The risk is especially high when homozygosity for this mutation is combined with other prothrombotic deficiencies like the prothrombin mutation PT20210G/A and/or protein S deficiency. In western population of Caucasian origin the FV Leiden mutation has an incidence of 5–15%. Accordingly, there is good reason to check for this mutation when a higher risk of thrombotic event is expected or observed. Since the risk resides mostly with the relatively small group of homozygous carriers, it is clinically important to identify and confirm those carriers with high precision.

The test principle of the Pefakit[®] APC-R Factor V Leiden is shown in figure 2. The first step consists of the activation of factor V contained in the patient's plasma sample. In the wild-type situation, activated FV (FVa) is readily inactivated by the APC added with the +APC reagent. When –APC reagent (without APC) is used there is no inactivation, and FVa in the plasma sample remains active to serve as a cofactor in the second step. In this

step, Noscargin is added to start the thrombin generation which leads to the analytical endpoint already described for Pefakit[®] PiCT[®]. Noscargin is a prothrombin activator extracted from the venom of the Australian tiger snake *Notechis scutatus scutatus*. Noscargin is regarded as a prothrombin activator of group D since for enzymatic activity it is dependent on FVa as a cofactor [13]. The dependency on FVa makes this compound an excellent tool for use in a test focused on FV activity and its inhibition. Since only the activated form of FV can be inactivated by APC, the most important role of RVV-V in this test is to render the FV present in the patient's plasma accessible for cleavage by APC. Dependent on how much FVa remains in the plasma, the activity of Noscargin is strongly enhanced or not. Thus, in the normal wild-type situation, the +APC clotting time is much higher than the –APC clotting time, leading to a ratio of usually 2.5 and more. When the patient's FVa is resistant to the inactivation by APC due to homozygous factor V Leiden mutation, the added APC has no influence on FVa activity and the clotting times measured, which results in a ratio of about 1.0. In the heterozygous case, the ratio calculated lies in between these two extremes.

Fig. 4. Correlation of ratios obtained with Pefakit[®] APC-R Factor V Leiden and Coatest APC Resistance in a study with plasma samples of different FV Leiden genotype.

Fig. 5. Correlation of ratios obtained with Pefakit[®] APC-R Factor V Leiden and Coatest APC Resistance in a study with plasma samples of different FV Leiden genotype.

Table 1. Interference studies with Pefakit[®] APC-R Factor V Leiden

Factor	Interference of factor deficiency with test
I Fibrinogen	no relevant interference up to total deficiency
II Prothrombin	no relevant interference up to total deficiency
V	below 50% normal value marked influence on ratio
VIII	falling ratio below 50% normal value, but no influence on class formation and differentiation
X	falling ratio below 75% normal value, but no influence on class formation and differentiation
ATIII	falling ratio below 25% normal value, but no influence on class formation and differentiation
Protein C	no relevant interference up to total deficiency
Protein S	slight increase of ratio, but no influence on class formation and differentiation
Factor	Interference of factor surplus with test
I Fibrinogen	no relevant interference up to 5 times normal value
VIII	no relevant interference up to 5 times normal value
ATIII	no relevant interference up to 5 times normal value
TFPI	no relevant interference up to 5 times normal value

Being a prothrombin activator of group D, Noscarnin should also be dependent on the presence of free Ca ions. Experiments surprisingly have shown that Noscarnin can start the clotting reaction even in total absence of Ca ions. The FVa dependency of Noscarnin was even enhanced in these experiments, where free Ca ions were completely removed by EDTA.

Most factor deficiencies in patient's plasma are compensated in this test by using a dilution plasma which has normal content of all factors except for FV. Therefore, even massive deficiencies of factors I and II have no influence on the test outcome, as expected. Because this test, unlike most other functional clotting assays, does not need Ca ions, the test is not influenced by factors or reactions dependent on Ca ions. In addition, since the test starts at the prothrombin level, it is independent of all factors and their deficiencies upstream in the coagulation cascade. This has been demonstrated in internal (table 1) and external (table 2, fig. 4, 5) studies.

The results obtained with plasmas deficient in protein C and protein S deserves special attention. The activation of protein C by thrombin generated in the reaction is highly dependent on Ca²⁺. In the test situation free of cell surfaces and Ca ions, the protein C present in the patient's

Table 2. Comparison of the results obtained with lupus anti-coagulant (LA) patient samples using Coatest® and Pefakit®

	Coatest®				Pefakit®				FV-L genotype by PCR
	+APC	-APC	ratio	diagnosis	+APC	-APC	ratio	diagnosis	
AKH19	100.0	56.0	1.79	het	30.0	21.9	1.37	het	het
AKH143	70.5	48.9	1.44	het	34.2	24.0	1.43	het	het
AKH45	98.9	42.0	2.35	wt	131.2	22.4	5.86	wt	wt
AKH96	142.1	V>max	n.a.	n.a.	95.5	24.3	3.93	wt	wt
AKH98	145.4	V>max	n.a.	n.a.	88.5	20.4	4.34	wt	wt
AKH136	V>max	V>max	n.a.	n.a.	232.6	34.8	6.68	wt	wt
AKH139	179.7	V>max	n.a.	n.a.	160.7	26.8	6.00	wt	wt
AKH143	114.5	V>max	n.a.	n.a.	111.1	21.5	5.17	wt	wt
AKH147	142.9	V>max	n.a.	n.a.	82.1	20.9	3.93	wt	wt
AKH148	113.6	51.6	2.20	wt	87.5	22.6	3.87	wt	wt
AKH149	156.4	V>max	n.a.	n.a.	96.8	22.8	4.25	wt	wt

n.a. = Not applicable; wt = wild-type; het = heterozygous; +/- APC = reagent with or without addition of APC; n = 11.

plasma is not activated. Only the APC contained in the +APC reagent can interact with the test by inactivation of FVa. For this reason, even massive deficiency or excess of protein C does not influence the test. Protein S supports APC, as a cofactor, in its inhibition of FVa. The build-up of this complex between activated protein C and protein S is again dependent on Ca^{2+} . In the given test situation without free Ca ions the complex cannot be formed. Only the APC added with the +APC reagent reacts and cleaves FVa, without the help of protein S. Therefore, excess or deficiency of this protein has no influence on the test outcome.

Finally, lupus anticoagulant (LA) antibodies do not function without the presence of Ca ions and phospholipids and therefore cannot interfere. This is perhaps one of the most important advantages of the test, since all other clotting assays make use of the prothrombinase complex and are therefore more or less influenced by the presence of LA antibodies. In such tests, this can lead to results which are difficult to interpret or incorrectly classified.

All these characteristics make the Pefakit® APC-R Factor V Leiden both highly sensitive and highly robust. This feature was well demonstrated in a comparative clinical study, where the same set of plasmas genotyped by PCR technique were tested side-by-side on the same analytical instrument, an STA®-Analyzer, with the Pefakit® test kit and a comparable test, the Coatest® APC Resistance from IL [14]. The results of this comparison are

shown in figures 3–5 and table 2. Most other tests on the market can only differentiate between positive and negative carriers of the FV Leiden mutation. Thus, in order to distinguish the homozygous from the heterozygous carriers the positive carrier plasmas have to be tested by an expensive and time-consuming PCR test for confirmation of the genotype. With the Pefakit® APC-R Factor V Leiden, PCR verification may still be recommended for all homozygous carriers of the mutation, but for most of the carriers identified as being heterozygous this is no longer necessary.

Conclusion

Snake venom-derived enzymes have proven to be very useful tools in diagnostic test kits. In the last 6 years Pentapharm has developed, certified, registered and put on the market several new tests with excellent performance characteristics. The snake venom-derived enzymes used in these test kits are essential key ingredients responsible for the most important of these characteristics, and hence for their success. The development so far may only be a start. The potential of snake venom-derived products is not yet fully described and used. Research on them is therefore ongoing and may lead to further innovative diagnostic test kits.

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