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Separation of r-hirudin from similar substances by capillary electrophoresis

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Abstract

The thrombin inhibitor r-hirudin is a peptide of 65 amino acids and with a p*I* of 4.4. Capillary electrophoresis (CE) was used to separate r-hirudin from seven closely related substances which may be found as by-products or from degradation. Possibly two of these substances differ only in an isoaspartyl instead of an aspartyl binding. A baseline separation was possible with an acetate buffer (pH 4.4, 60 m*M*) containing 0.3% (m/m) PEG 20 000 and 0.1 m*M* Zn^{2+}. The possibilities to prevent wall adsorption are discussed.

1. Introduction

The peptide hirudin (Fig. 1) is a potent and specific thrombin inhibitor for anticoagulant therapy. Hirudin is used for post-operative venous thrombosis or prophylaxis of arterial thrombosis. It is applied subcutaneously or intravenously.

Natural hirudin is a peptide consisting of 65 amino acids (relative molecular mass ca. 7000) with an isoelectric point (p*I*) of about 4.4 (Fig. 1). The hydrophilic C-terminus is remarkable rich in acidic amino acids, which are printed bold in Fig. 1, including a tyrosin-O-sulfonate in position 63 in about 30% of the natural hirudin forms. The acidic C-terminus, three disulfide bridges and lysin in position 47 are essential for the therapeutical effects [1].

The natural source are the salivary glands of the bloodsucking leech (*Hirudo medicinalis*). A single leech contains about 10 μg of hirudin. Thus 1000 leeches will be necessary for one daily dosage, which is too much for a sufficient production of hirudin. More substance is available for medical research and treatment since the production of r-hirudin is possible by gene technology.

If synthesized proteins or polypeptides are used therapeutically, purity evaluation and characterization of minor impurities are important aspects for quality control. The structural differences between r-hirudin and the degradation products are minor. The purpose of this study was to examine whether r-hirudin can be separated from seven closely related artificial substances which may be found as by-products or after degradation of the product. Although all the seven substances will probably have the same effects as r-hirudin, a purity control gives information about the quality of the preparation process.

The isoforms of artificial r-hirudin are not sulfonated in position 63. Different variations exist, e.g. the two valines at the N-terminal are

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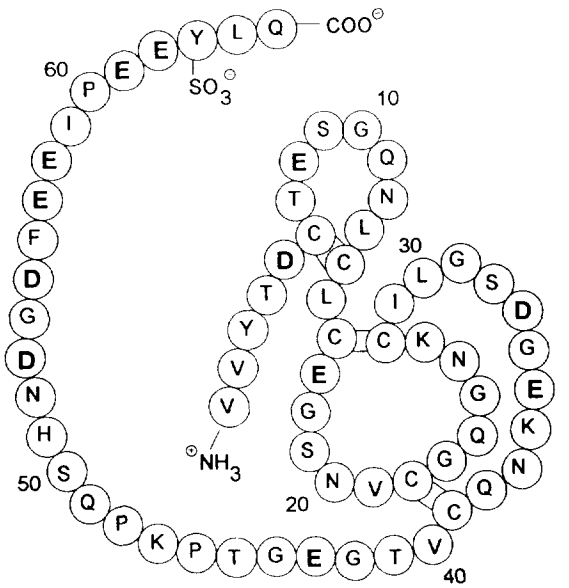

Fig. 1. Primary structure of natural hirudin, acids printed bold. C = Cys = Cysteine; D = Asp = aspartic acid; E = Glu = glutamic acid; F = Phe = phenylalanine; G = Gly = glycine; H = His = histidine; K = Lys = lysine; L = Leu = leucine; N = Asn = asparagine; P = Pro = proline; Q = Gln = glutamine; S = Ser = serine; T = Thr = threonine; V = Val = valine; Y = Tyr = tyrosine.

exchanged for variation 1 (r-HV1). Additionally two aspartic acids (Asp) in positions 33 and 53 are exchanged to asparagine (Asn) for variation 2 (r-HV2). Both show the same anticoagulant activity as native hirudin [2].

r-Hirudin (r-HH) and the seven analogous substances (aS1–aS7) all consist of 65 amino acids. r-HH under investigation could be r-HV1 or r-HV2. Structural information about the analogous substances was not available. However, they were characterized by their behavior in ion-exchange chromatography (IEC). Structural differences of possible variations to r-HV1 or r-HV2 are described. They are mainly caused by deamidation and isomerisation of Asn. Geiger and Clarke [3] explained that Asn–Gly structures easily undergo deamidation. First a succinimide (= imido binding) is built followed by hydrolysis to aspartyl or isoaspartyl in a ratio of 1:3 (Fig. 2). The succinimide is stable and can be isolated. Thus for r-HV2 seven similar products are possible [4]. More drastic conditions are necessary to form succinimide bindings from Asp–Gly structures [5]. Additionally deamidation of Asn^{52} [4] or O-phosphorylation, e.g. at Thr^{45} [6], are possible. Combinations of deamidation, isomerisation and phosphorylation lead to a variety of slightly different r-hirudin derivatives.

Capillary electrophoresis (CE) is chosen to separate these closely related proteins. Lüdi and Gassmann [7] and Paulus and Gassmann [8] separated r-hirudin from degradation products which consist of one or two amino acids less. Buffers are used of pH > 6, including several modifiers, e.g. N-[tris(hydroxymethyl)methyl]-glycine (Tricine), ethylendiaminetetraacetate (EDTA) or diaminobutane. These separation conditions were tested, but their selectivity was not sufficient to separate hirudin and the seven analogous compounds under investigation. Therefore another method was developed by optimizing the pH first.

2. Experimental

A Beckman P/ACE 2000 system was used, equipped with an UV detector operated at 214 nm. The electrophoretic experiments were performed in uncoated fused-silica capillaries with 300 mm effective length to the detector (370 mm total length) and 50 μm I.D. (Polymicro, Phoenix, AZ, USA) with 25 kV (25–26 μA) as applied voltage at a constant temperature of 25°C. The injection time was 4 s with 34.5 kPa (= 0.5 p.s.i.). The coated capillary was CElect-H2 with 600 mm effective length to the detector (670 mm total length) and 50 μm I.D. (Supelco Deutschland, Bad Homburg, Germany). If not stated otherwise, chemicals were of analytical-reagent grade, supplied by Merck (Darmstadt, Germany). The standard 60 m*M* acetate buffer, pH 4.4 with 0.3% polyethyleneglycol 20 000 (PEG 20 000; Hoechst, Frankfurt, Germany, pharmacopoeia quality) and 0.1 m*M* $ZnCl_2$ was prepared by dissolving 4.12 ml 1 *M* acetic acid, 154.4 mg sodium acetate, 300.0 mg PEG 20000

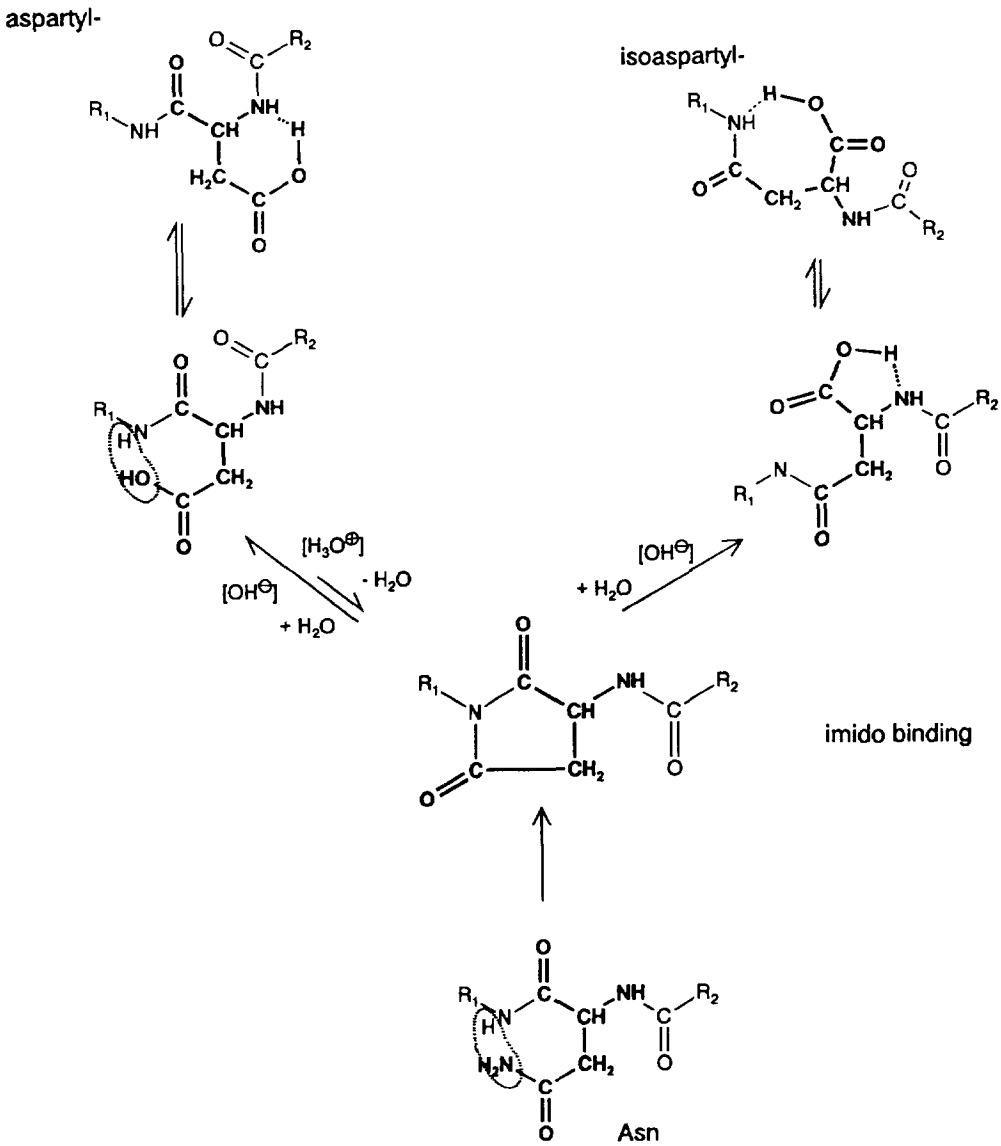

Fig. 2. Scheme of the formation of succinimide and isoaspartyl starting with aspartyl (printed bold), according to Refs. [3] and [4].

and 1.4 mg $ZnCl_2$ to 100.0 ml with HPLC-grade water (Millipore, Eschborn, Germany). r-Hirudin and related substances were kindly given by Hoechst. Usually about 1.8 mg/ml of the main compound and 0.4–1.2 mg/ml of the related substances were solved in HPLC-grade water.

3. Results and discussion

3.1. Method development

In general the method development in CE should start with optimizing the pH [9]. Usually the best pH is close to the pK_a of the compounds

involved, because there the differences of the charge/mass ratio are maximized. Using different acetate buffer the best results were found with 60 m*M* at pH 4.4 (Fig. 3), although the selectivity was still uncomplete.

The modifiers used in the literature [7,8] were now adapted to enhance selectivity. EDTA will complex all metal cations, so the proteins will be in their native conformations. The addition of a definite amount of metal cations, e.g. Zn^{2+} or Mg^{2+} will cause the opposite effects, defined protein–cation complexes. The mobility and thus the selectivity is changed by formation of complexes with Zn^{2+} or Mg^{2+}. While EDTA and Mg^{2+} give hardly, if any, improvement, Zn^{2+} slightly improved the separation.

Modifiers to manipulate the endoosmotic flow (EOF) were tried next to enhance efficiency. Tricine, also added in increasing amounts (up to 0.2 m*M*), showed no effects. Diaminobutane also gave no improvement and was worse to handle with. The addition of PEG with a relative molecular mass of 20 000 led to an optimal separation of hirudin and analogues (Fig. 4). However, shifting migration times show that adsorption still takes place.

3.2. Migration order

The resultant migration order (aS4, r-HH, aS1, aS2, aS3, aS5, aS7 and aS6, Fig. 4) and the structural differences described above should lead to some remarks about the possible structures of the r-hirudins analogous. All substances are negatively charged, so they migrate slower than the EOF. The longer they stay on the capillary the smaller is their gross mobility and the higher their electrophoretic mobility (μ_{ep}). A higher μ_{ep} means a higher charge/mass ratio. From migration order it should be expected that aS4 has the smallest, aS6 the greatest negative charge with the remaining products having intermediate charge. The same results were obtained by characterisation with preparative IEC.

aS4 (migration time 4.42 min, Fig. 4) is less negatively charged than r-HH, because it is migrating before r-HH (4.92 min, Fig. 4). A succinimide means for r-HV1 one negative charge less and a reduction of 18 ($=H_2O$) in relative molecular mass. Probably aS4 has one or two succinimide bindings.

The other substances are more negatively charged than r-HH. Three groups can be distinguished. Slightly more negative substances are possible because of one or two isoaspartyl bindings instead of an Asn. Isoaspartyl should be more acidic because of the better possibility for aspartyl to build intramolecular hydrogen bonds.

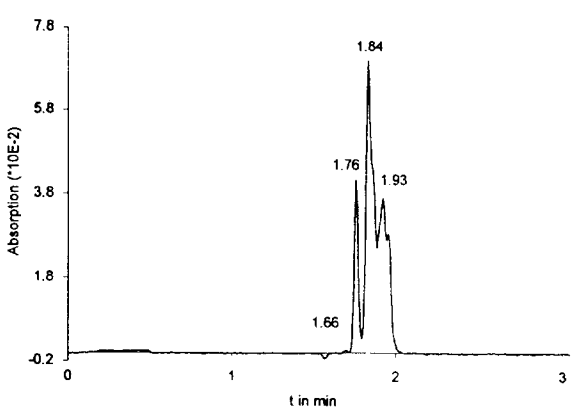

Fig. 3. Mixture of r-hirudin and seven related substances separated by an acetate buffer 60 m*M*, pH 4.4, 300 mm × 50 μm I.D. capillary, 25 kV (26 μA), 25°C. It was not possible to identify the peaks.

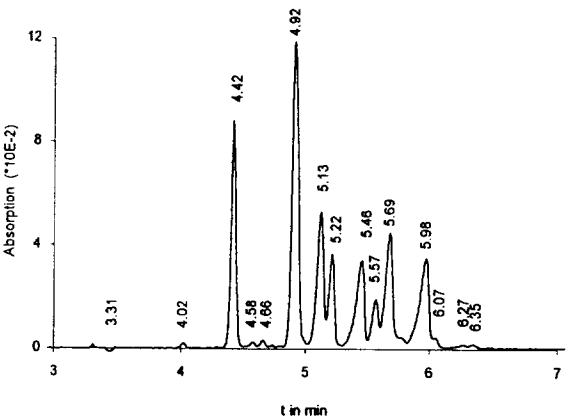

Fig. 4. Mixture of r-hirudin and seven related substances with an acetate buffer 60 m*M*, pH 4.4 + 0.3% PEG 20 000 + 0.1 m*M* Zn^{2+}; other conditions as in Fig. 3. Migration order: aS4 (migration time 4.42 min), r-HH (4.92 min), aS1 (5.13 min), aS2 (5.22 min), aS3 (5.46 min), aS5 (5.57 min), aS7 (5.69 min), aS6 (5.98 min); peaks at 4.02, 4.58, 4.66, 6.07, 6.27 and 6.35 min are unknown substances, probably degradation products from the reference substances.

Table 1
Migration times and electrophoretic mobilities of r-hirudin, seven analogous substances and acetanilide as the neutral marker in 15 consecutive runs

	EOF	aS4	r-HH	aS1	aS2	aS3	aS5	aS7	aS6
Migration times (min)									
	3.48	4.42	4.92	5.13	5.22	5.46	5.57	5.69	5.98
	3.46	4.4	4.9	5.11	5.2	5.44	5.55	5.67	5.96
	3.49	4.44	4.94	5.15	5.25	5.49	5.61	5.72	6.02
	3.5	4.45	4.95	5.17	5.26	5.5	5.62	5.74	6.03
	3.5	4.47	4.98	5.2	5.3	5.54	5.66	5.78	6.08
	3.53	4.49	5	5.23	5.32	5.57	5.69	5.8	6.11
	3.88	5.1	5.77	6.07	6.2	6.54	6.7	6.86	7.29
	3.85	5.06	5.72	6.02	6.11	6.47	6.64	6.79	7.22
	3.86	5.06	5.73	6.02	6.15	6.48	6.64	6.79	7.22
	3.86	5.07	5.73	6.03	6.15	6.49	6.65	6.8	7.23
	3.96	5.23	5.95	6.26	6.4	6.77	6.94	7.11	7.58
	3.93	5.18	5.88	6.19	6.33	6.69	6.86	7.02	7.48
	3.92	5.18	5.88	6.19	6.33	6.68	6.86	7.02	7.48
	3.93	5.19	5.89	6.2	6.34	6.7	6.87	7.03	7.49
	3.94	5.19	5.9	6.21	6.35	6.7	6.88	7.04	7.5
Mean	3.74	4.86	5.48	5.75	5.86	6.17	6.32	6.46	6.84
S.D.	0.21	0.36	0.45	0.5	0.52	0.57	0.6	0.62	0.7
R.S.D. (%)	5.61	7.41	8.21	8.7	8.87	9.24	9.49	9.6	10.23
Increase (%)	12.4	16.02	18.11	19.05	19.53	20.39	21.04	21.21	22.55
Electrophoretic mobilities ($cm^2\ kV^{-1}\ min^{-1}$)									
	0.00	−2.71	−3.73	−4.10	−4.25	−4.63	−4.79	4.96	−5.33
	0.00	−2.74	−3.77	−4.14	−4.29	−4.67	−4.83	−5.00	−5.38
	0.00	−2.72	−3.73	−4.10	−4.26	−4.63	−4.81	−4.96	−5.35
	0.00	−2.71	−3.72	−4.10	−4.24	−4.61	−4.79	−4.95	−5.32
	0.00	−2.75	−3.77	−4.15	−4.31	−4.67	−4.84	−5.00	−5.38
	0.00	−2.69	−3.70	−4.09	−4.23	−4.61	−4.77	−4.92	−5.31
	0.00	−2.74	−3.75	−4.13	−4.28	−4.65	−4.82	−4.97	−5.35
	0.00	−2.76	−3.77	−4.16	−4.27	−4.67	−4.85	−4.99	−5.38
	0.00	−2.73	−3.75	−4.13	−4.28	−4.65	−4.82	−4.96	−5.35
	0.00	−2.75	−3.75	−4.14	−4.28	−4.66	−4.83	−4.97	−5.36
	0.00	−2.72	−3.75	−4.12	−4.27	−4.65	−4.81	−4.97	−5.35
	0.00	−2.73	−3.75	−4.12	−4.28	−4.66	−4.83	−4.97	−5.36
	0.00	−2.76	−3.78	−4.15	−4.31	−4.68	−4.85	−5.00	−5.39
	0.00	−2.74	−3.76	−4.14	−4.29	−4.67	−4.83	−4.98	−5.37
	0.00	−2.71	−3.74	−4.12	−4.28	−4.64	−4.82	−4.96	−5.35
Mean	0	−2.73	−3.75	4.13	4.28	4.65	4.82	4.97	−5.36
S.D.	0	0.02	0.02	0.02	0.02	0.02	0.02	−0.02	−0.02
R.S.D. (%)	0	0.73	0.53	0.48	0.47	0.43	0.41	0.4	0.37

Separation conditions as in Fig. 4. The capillary was not washed with NaOH between runs. The R.S.D. of the migration times is high. The reason is a significant trend, which can be recognized by looking at the percental increase (difference between the first and last value divided by their mean). This trend is caused by adsorption at the capillary wall, which decreases the EOF. The electrophoretic mobility during the series is free of trends, the corresponding R.S.D. is only about 0.5%.

A strong increase in negative charge is possible because of deamidation, much more negatively charged substances are formed by phosphorylation or more than one deamidation or isomerisation.

3.3. Adsorption

The theoretical plate number of the main peak in Fig. 4 is about $N = 38\,000$. This is far below the obtained efficiency in other separations. In addition the migration time of consecutive separations shifts and the separation itself is not stable without prewashing the capillary with 0.1 *M* NaOH. These facts indicate adsorption. Migration times of the EOF and the substances increased during consecutive runs, if the capillary was not washed with NaOH between runs. However, μ_{ep} of the investigated compounds remained constant (Table 1). Moreover, peak tailing was not observed (Fig. 4). Both facts indicate that no chromatography-like process takes place, i.e. the adsorption is very strong.

Several possibilities are described to prevent adsorption. In this case the change of pH to an acidic [10] or basic [11] pH is not possible because the selectivity would be lost. Both high-ionic-strength buffers (0.2 *M* K_2SO_4) [12] and dynamic coating with cetyltrimethylammonium bromide (CTAB) [13] reduced the EOF so strongly that a switch of polarity became necessary. The separations achieved were worse in these cases. The use of coated capillaries was unsuccessful, too. Although the same amount was injected, UV absorption was insufficient. Probably the coating material shows an own absorption at the used detection wavelength. Nevertheless, it was not possible to prevent adsorption during the separation.

The only possibility to hold the migration times nearly constant is to wash the capillary with 0.1 *M* NaOH for a longer time (>30 min) before each run.

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References

[1] P. Walsmann, *Folia Haematol. (Leipzig)*, 116 (1989) 821.
[2] F. Markwardt, *Haemostasis*, 21 (1991) 11.
[3] T. Geiger and S. Clarke, *J. Biol. Chem.*, 262 (1987) 785.
[4] A. Tuong, M. Maftouh, C. Ponthus, O. Whitechurch, C. Roitsch and C. Picard, *Biochemistry*, 31 (1992) 8291.
[5] R. Bischoff, P. Lepage, M. Jaquinod, G. Cauet, M. Acker-Klein, D. Clesse, M. Laporte, A. Bayol, A. Van Dorsselaer and C. Roitsch, *Biochemistry*, 32 (1993) 725.
[6] M. Coulet, B. Domon, H. Grossenbacher, C. Guenat, W. Märki, D. Müller and W.J. Richter, *J. Mol. Struct.*, 292 (1993) 89.
[7] H. Lüdi and E. Gassmann, *Anal. Chim. Acta*, 213 (1988) 215.
[8] A. Paulus and E. Gassmann, *Applications DS-752*, Beckman, Palo Alto, CA, 1989.
[9] H. Wätzig and C. Dette, *Pharmazie*, 49 (1994) 83.
[10] R.M. McCormick, *Anal. Chem.*, 60 (1988) 2322.
[11] H.H. Lauer and D. McManigill, *Anal. Chem.*, 58 (1986) 166.
[12] J. Green and J. Jorgenson, *J. Chromatogr.*, 478 (1989) 63.
[13] J. Wiktorowicz and J. Colburn, *Electrophoresis*, 11 (1990) 769.