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Use of capillary electrophoresis and high-performance liquid chromatography for monitoring of glycosylation of the peptides dalargin and desmopressin[1]

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Abstract

Capillary electrophoresis (CE) and HPLC procedures have been developed and tested for monitoring of glycosylation of the peptide hormones dalargin and desmopressin, permitting identification and determination of the reaction products and establishing the reaction rate. Good separations have been obtained that can also be used for preparative purposes. Reversed-phase HPLC employs a gradient of methanol in a mobile phase of 0.1% trifluoroacetic acid, pH 2.2, with a diode-array UV detection. The CE systems involve 150 m*M* phosphoric acid, pH 1.9, or a micellar system containing 50 m*M* dodecyl sulfate and 20 m*M* sodium tetraborate, pH 9.2, both with UV photometric detection at 190 nm; the latter system is less suitable for glycoconjugate isolation. Whereas CE is superior to HPLC in analytical application, HPLC is preferable for preparative purposes.

Keywords: Glycosylation; Peptides; Dalargin; Desmopressin; Hormones

1. Introduction

The synthetic peptide hormones dalargin and desmopressin belong among the analogues of the natural hormones produced by the pituitary gland and have been obtained by modification of β-endorphin [1] and vasopressin [2]; their formulae are as follows:

Dalargin: Tyr – D – Ala – Gly – Phe – Leu – Arg

Desmopressin: Mpa – Tyr – Phe – Gln – Asn – Cys – Pro – D – Arg – Gly – NH_2

The biological activity of dalargin is being tested on various organisms, e.g. fish [3], and it seems that it also exhibits cancerostatic properties [4]. Desmopressin belongs among the most effective antidiuretic agents and is used to treat central diabetes insipidus [2,5,6]. Recently it has been found [7] that enkephalins, to which β-endorphin1 belongs, affect many immune functions.

In view of the biological importance of these

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substances, there a lot of attention is paid to the preparation of modified compounds, tailored for specialized applications in biology, medicine, animal breeding, etc. Glycosylation is one of several powerful techniques for modifying peptide hormones [8], as the attachment of a saccharide residue may improve the peptide stability toward proteolytic enzymes which facilitates application of the hormone to an organism, increase the peptide hydrophilicity which affects the hormone distribution within the organism, and may also considerably change the hormone interaction with the natural receptors. Non-enzymatic glycosylation also occurs in living organisms where a reducing saccharide directly reacts with the protein primary amino group with formation of a stable covalent product [9–11]; for the role of non-enzymatic glycosylation in vivo and in vitro see also Refs. [12–15].

The present paper deals with separation and characterization of the products of glycosylation of dalargin and desmopressin, obtained either by reductive amination [9–11] leading to the binding of reductive disaccharides to the amino group, or by the reaction with a thioglycoside that leads to glycosylation of both the amino and hydroxy groups [16,17].

It is well-known that CE and HPLC are suitable methods for analysis and purification of natural and synthetic peptides (see e.g. [18]), CE being highly efficient but having a low sample capacity and HPLC being a more universal technique, even if usually somewhat less efficient and more time- and cost-consuming. In CE, low-pH phosphate buffers have yielded good results [19], as the isoelectric point of the peptides is not attained and thus the analytes migrate toward the cathode and the detector [20], the electroosmotic flow and adsorption of the analytes on the capillary wall are suppressed owing to binding of the phosphate to the surface silanol groups [21]. Micellar systems are also efficient for peptide and protein separations using, e.g., sodium dodecyl sulfate (SDS) [22–24]. HPLC analyses of not very complex samples of peptides are best performed in reversed systems, employing ion-pairing reagents of which a common representative is trifluoroacetic acid [25]; such mobile phases have also been successfully applied to glycopeptides and glycoproteins [26–28].

The CE and HPLC procedures employed in the present work are based on the above principles and optimized for the studied analytes and their glycosylation products.

2. Experimental

2.1. Chemicals

The substances of dalargin and desmopressin were synthesized in the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic. To protect the amino group, *tert.*-butyloxycarbonyldalargin (Boc-dalargin) and phenylacetyldalargin (Pac-dalargin) were also prepared. Using the ISOELECTRIC program (Genetics Computer Group, WI, USA) and the tabulated dissociation constants for the appropriate amino acids, we calculated the isoelectric points of dalargin and desmopressin extrapolated to zero ionic strength, obtaining the values, p*I*(dalargin) = 9.59 ($\mu = 0$) and p*I*(desmopressin) = 11.48 ($\mu = 0$).

Highly pure methanol was obtained from Romil Chemicals, UK, trifluoroacetic acid for spectroscopy from Merck, Germany, SDS, analytical grade, from Serva, Germany and the other chemicals, analytical grade, from Lachema, Czech Republic.

The glycoconjugates of dalargin and desmopressin were prepared at our laboratories by reductive amination [9–11] and the reaction with 2-chloroethyl-1-thio-β-D-galactoside [16,17]. The products were purified by gel chromatography on a Bio-Gel P-2 column (45 × 1.5 cm), with 0.05 *M* ammonium hydrogencarbonate as the mobile phase at a flow-rate of 8 ml h^{-1}, collecting 2-ml fractions. The contents of neutral saccharides were determined in the fractions by the reaction with phenol in the presence of sulfuric acid [29], measuring the absorbance at 490 nm, the contents of peptide and glycopeptide together by measuring the absorbance at 280 nm and the fractions were then lyophilized. The existence of the appropriate dalargin glycoconjugates was confirmed by fast atom bombardment (FAB) MS at the Institute of Organic Chemistry and Biochemistry. All the prepared products are listed in Table 1.

Table 1
List of abbreviations of the glycoconjugates prepared

Reaction	Abbreviation
Reductive amination	
Dalargin + 1 molecule of D-galactose	Dalargin-gal
Dalargin + 2 molecules of D-galactose	Dalargin-digal
Dalargin + 1 molecule of D-lactose	Dalargin-lact
Dalargin + 2 molecules of D-lactose	Dalargin-dilact
Dalargin + 1 molecule of D-maltose	Dalargin-malt
Dalargin + 2 molecules of D-maltose	Dalargin-dimalt
Desmopressin + 1 molecule of D-lactose	Desmopressin-lact
Reaction with 2-chloroethyl-1-thio-β-D-galactose	
Dalargin monogalactoside	Dalargin-thiogal
Dalargin bisgalactoside	Dalargin-dithiogal
Pac-Dalargin monogalactoside	Pac-Dalargin-thiogal
Boc-Dalargin monogalactoside	Boc-Dalargin-thiogal
Desmopressin monogalactoside	Desmopressin-thiogal

3. Apparatus

A Crystal Model 200 liquid chromatograph was used with a Model 250 diode-array detector and a Dell datastation (all from ATI Unicam, UK) and a Rheodyne 7125 sampling valve with a 10-μl loop. A Hypersil Elite C_{18} column (5 μm, 150×4.6 mm; Life Sciences International, UK) was employed.

The CE measurements were performed on a Crystal CE System Model 310 (ATI Unicam) with a UV photometric detector and using pneumatic sample injection with an overpressure of 10–100 mbar. The fused-silica capillary, 75 μm I.D., was 770 mm long, with a distance to the detector of 600 mm.

All the measurements were carried out at laboratory temperature (22±2°C).

4. Results and discussion

4.1. Separation procedures

4.1.1. HPLC

On the basis of the literature [25–28], the classical mobile phase of aqueous trifluoroacetic acid (TFA) with methanol as the organic modifier was selected and its composition was optimized. Programming of the methanol content was found to be necessary to attain a high separation efficiency. The optimization led to three systems, all consisting of 0.1% (v/v) TFA in water and methanol containing 0.1% (v/v) TFA and differing in the parameters of the linear methanol gradient:

(A) From 30 to 50% (v/v) within 25 min.
(B) From 50 to 70% (v/v) within 20 min.
(C) From 30 to 50% (v/v) within 20 min.

The dead retention time was obtained by injecting a 0.1% solution of potassium nitrate: $t_M = 2.77$ min, R.S.D. = 1.15% (three measurements). The flow-rate was 0.5 ml min^{-1}.

System A is suitable for the separation of dalargin glycoconjugates. System B with the higher methanol content is required for the separation of Pac- and Boc-dalargin glycoconjugates, as the hydrophobic substituents cause a much higher retention (retention times around 40 min in system A). System C is then useful for the separation of desmopressin glycoconjugates. The capacity factors for the parent peptides and glycoconjugates are given in Table 2, together with their relative standard deviations.

An example of a three-dimensional chromatogram obtained with diode-array detection is given in Fig. 1. The wavelength, 205 nm, at which all the analytes

Table 2
HPLC characteristics of the glycoconjugates prepared

Compound	Separation conditions	Capacity factor k'	Relative standard deviation R.S.D.(%)
Dalargin	A	6.67	2.1
Dalargin-gal	A	6.02	0.8
Dalargin-digal	A	5.13	0.9
Dalargin-lact	A	5.82	1.0
Dalargin-dilact	A	4.63	1.0
Dalargin-malt	A	5.79	1.2
Dalargin-dimalt	A	5.51	0.9
Dalargin-thiogal	A	6.17	1.8
Dalargin-dithiogal	A	5.73	1.5
Pac-Dalargin	B	4.54	0.4
Pac-Dalargin-thiogal	B	0.47	0.6
Boc-Dalargin	B	4.45	1.1
Boc-Dalargin-thiogal	B	3.94	1.0
Desmopressin	C	5.61	3.3
Desmopressin-thiogal	C	5.56	1.9
Desmopressin-lact	C	3.09	1.3

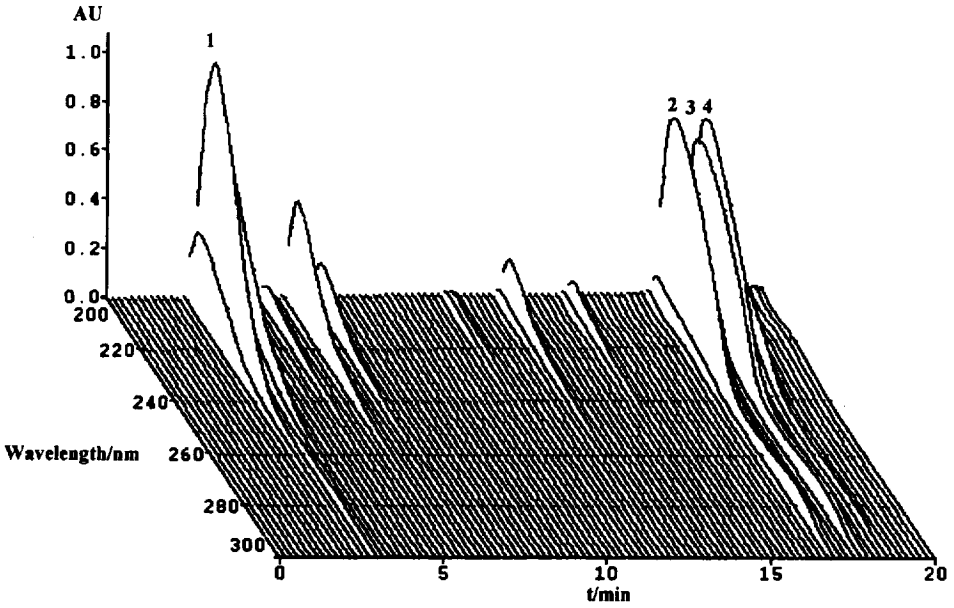


Fig. 1. Three-dimensional chromatogram (obtained with diode-array detection) of fraction No. 13 of desmopressin glycoconjugates prepared by reaction with 2-chloroethyl-1-thio-β-D-galactoside. For the conditions see the text, Section 2. Separation system C. 1 = mixture of free saccharides, 2 = unknown product, 3 = desmopressin-thiogal, 4 = desmopressin.

exhibit a sufficient absorbance, was then used for evaluation of the separations. Under these conditions, the quantitative measurements exhibit satisfactory analytical parameters, similar for all the analytes. The values for dalargin are as follows: the peak-area reproducibility, R.S.D. = 2.3% (5 measurements); the limit of detection (3 times peak-to-peak noise), $0.2 \cdot 10^{-5}$ mol l^{-1}; the linear dynamic range, from $6 \cdot 10^{-5}$ to $8 \cdot 10^{-2}$ mol l^{-1}, the linear regression coefficient of correlataion, 0.9915. Two examples of the separation of dalargin and desmopressin glycoconjugates are given in Fig. 2 Fig. 3.

The chromatograms of the individual fractions collected during gel chromatography after reductive amination of dalargin revealed certain reactivity of the arginine (Arg) residue and always indicated two products (reaction with one and two saccharide molecules). Two products were also found in the fractions after the dalargin reaction with 2-chloroethyl-1-thio-β-D-galactoside, containing one and two saccharide molecules; two monoglycosylated products of the reaction with either the amino or the hydroxy group, which might also be present, could not be distinguished. A glycoconjugate was also found in the fractions after reductive and non-reductive amination of desmopressin. Pac- and Boc-dalargin yield only a single product, similar to desmopressin, as the terminal amino group is blocked.

An advantage of HPLC lies in the fact that mono and diglycosylated products can readily be differentiated, as the latter generally exhibit a lower retention. The chromatograms clearly demonstrate a greater hydrophilicity of the glycoconjugates compared with the parent peptides.

The amounts of the products in the individual fractions after gel chromatography were determined using internal normalization (see Table 3 in which the contents are rounded to units of per cent). It follows that diglycosylated products (i.e. those with higher molecular masses) tend to accumulate in lower fractions, whereas higher fractions are enriched in the parent peptide and the monoglycosylated products; however, this trend is not completely general and the molecular shape also plays an important role. This is in agreement with the theory of gel chromatography.

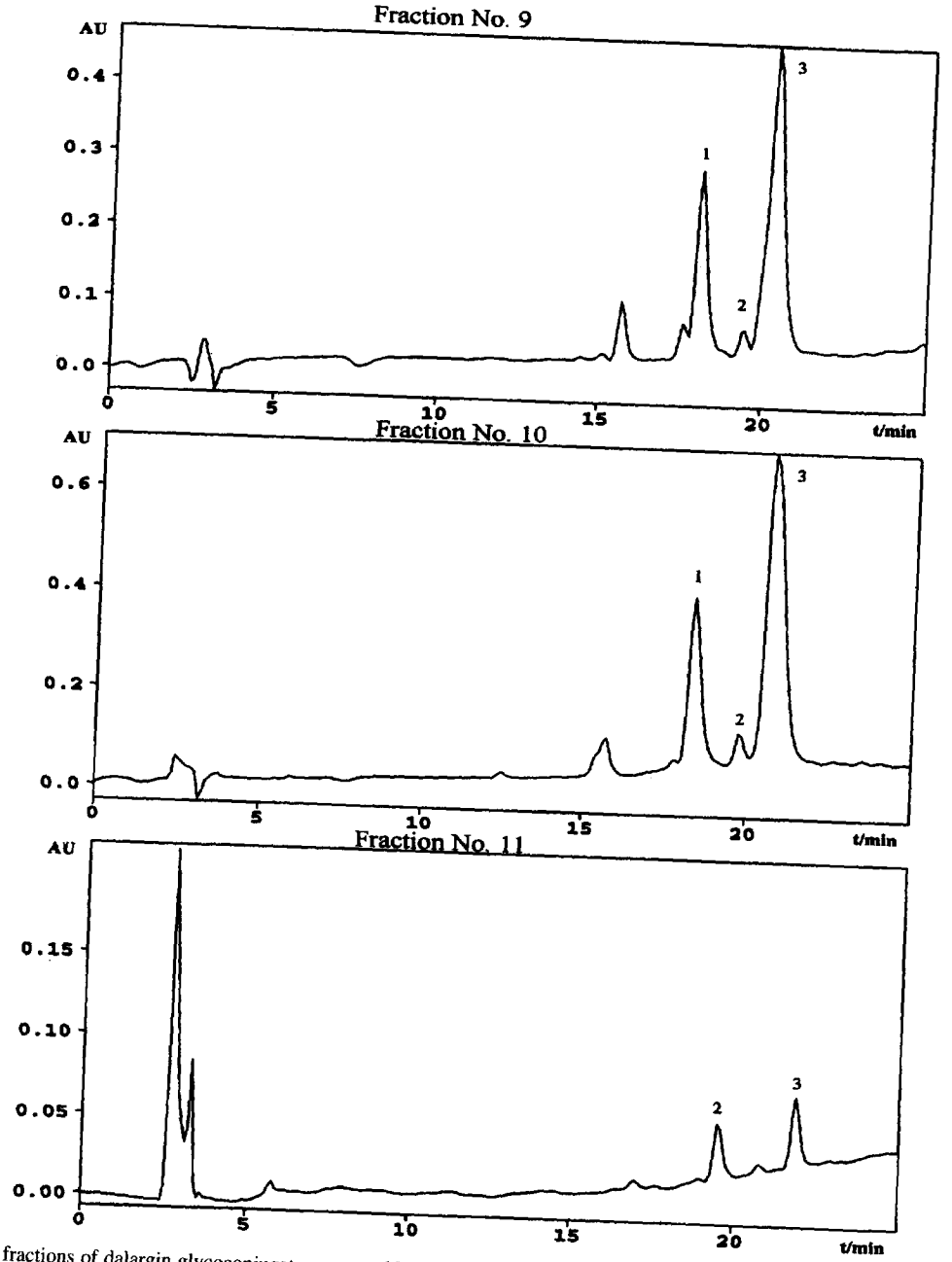


Fig. 2. HPLC of fractions of dalargin glycoconjugates prepared by reaction with 2-chloroethyl-1-thio-β-D-galactoside. For the conditions see the text, Section 2. Separation system A. Peaks: 1 = dalargin-dithiogal, 2 = dalargin-thiogal, 3 = dalargin.

5. Capillary electrophoresis

On the basis of previous works [19,22,23], two systems were selected and optimized: (A) 150 m*M* phosphoric acid, pH 1.9, applied voltage of 15 kV, and (B) 20 m*M* sodium tetraborate + 50 m*M* SDS,

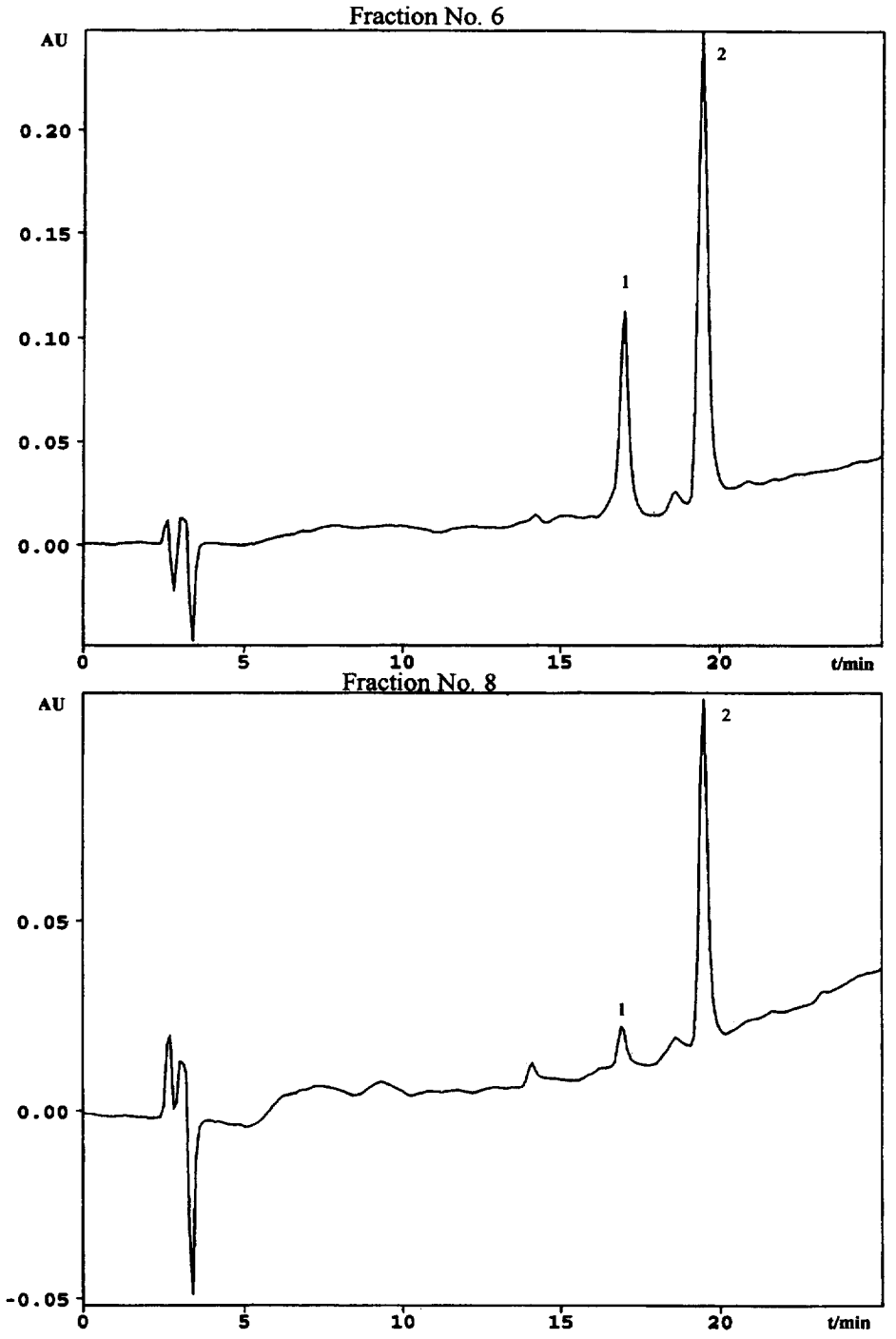


Fig. 3. HPLC of fractions of dalargin glycoconjugates prepared by reductive amination with D-galactose. For the conditions see the text, Section 2. Separation system A. Peaks: 1 = dalargin-digal, 2 = dalargin-gal.

Table 3
Average contents of the glycoconjugates found by HPLC and CE

Sample	Glycoconjugate	Content in HPLC (%)	Content in CE (%)
Reducive amination			
Dalargin + D-galactose	Dalargin-gal	75	78
	Dalargin-digal	15	15
Dalargin + D-lactose	Dalargin-lact	90	86
	Dalargin-dilact	8	8
Dalargin + D-maltose	Dalargin-malt	82	87
	Dalargin-dimalt	7	6
Desmopressin + D-lactose	Desmopressin-lact	29	22
Reaction with 2-chloroethyl-1-thio-β-D-galactose			
Dalargin + reagent	Dalargin-thiogal	6	6
	Dalargin-dithiogal	25	15
Pac-Dalargin + reagent	Pac-Dalargin-thiogal	44	35
Boc-Dalargin + reagent	Boc-Dalargin-thiogal	10	17
Desmopresin + reagent	Desmopressin-thiogal	21	15

pH 9.2, applied voltage of 20 kV. The solutes were detected at 190 nm.

The dead retention time was measured by injecting 1 m*M* thiourea and 1 m*M* benzylalcohol into system A; however, the retention was very high and thus the electroosmotic flow was close to zero. In system B, 1 m*M* thiourea as the marker yielded the value, t_M = 5.67 min., R.S.D. = 3.8% (five measurements) and $\mu_{eof} = 6.17 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}.

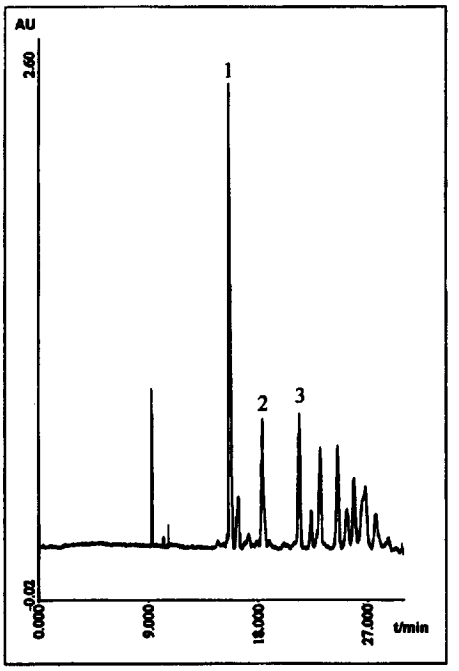
Two examples of separations in both systems are given in Fig. 4 Fig. 5. System A (elution order: peptide, monoglycosylated product, bisglycosylated product) separates the solutes on the basis of their charge and molecular mass and makes it also possible to adapt the procedure for preparative purposes; on the other hand, identification of the solutes is more difficult than in HPLC. Micellar system B (elution order is reversed compared to that in system A) is suited for more complex samples, the character of the separation is closer to that attained in HPLC and thus solute identification is easier. It can be seen that the separation efficiency is clearly superior to that of HPLC. Furthermore, very small samples required permit analyses even if the amount of the glycosylation reaction product is extremely small.

The CE characteristics of the studied substances are given in Table 4. The quantitative parameters are comparable to those obtained for HPLC (see previous section). The corresponding values for dalargin are as follows: R.S.D. = 2.1%; limit of detection, $3 \cdot 10^{-3}$ mol l^{-1}; linear dynamic range, $4 \cdot 10^{-5}$ mol l^{-1} to $5 \cdot 10^{-3}$ mol l^{-1}; the linear regression correlation coefficient, 0.9903. The contents of the solutes in the individual gel chromatography fractions were determined analogously to HPLC using internal normalization and are given in Table 3. It can be seen that the values obtained by the two methods are similar. The rest up to 100% corresponds to unidentified components and free saccharides; in system A saccharides do not migrate.

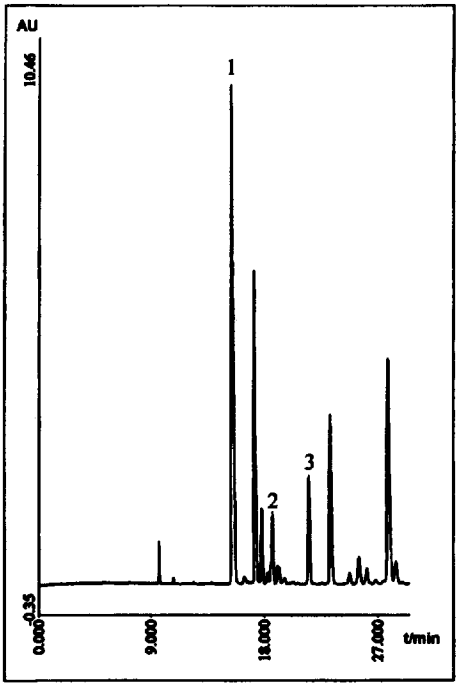
6. Kinetics of reductive amination

So far, reductive amination has been monitored purely empirically [9–11]. Therefore, it seemed useful to utilize the fact that glycosylation proceeds rather slowly and its extent is low with dalargin and to measure the reaction rate by CE; micellar system B has been found suitable for the purpose.

The reaction was carried out with an excess of the saccharide: c(dalargin) = $7.3 \cdot 10^{-3}$ mol l^{-1}, c(D-maltose) = 0.49 mol l^{-1}, $c(NaBH_3CN)$ = 0.34 mol l^{-1}. In view of a high rate of the reduction of the Schiff base formed, the reaction can be described by a simplified scheme:



Fraction No. 9



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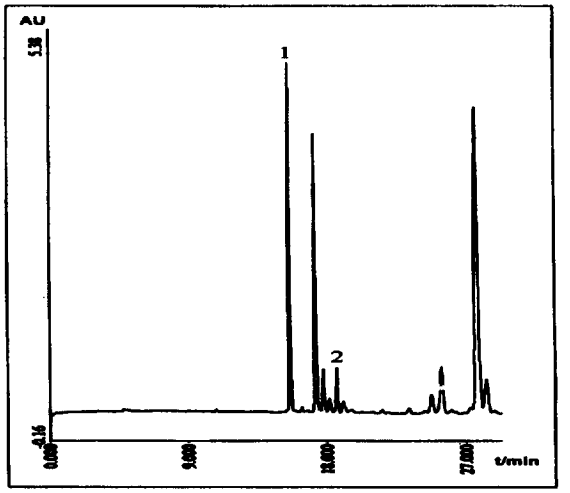
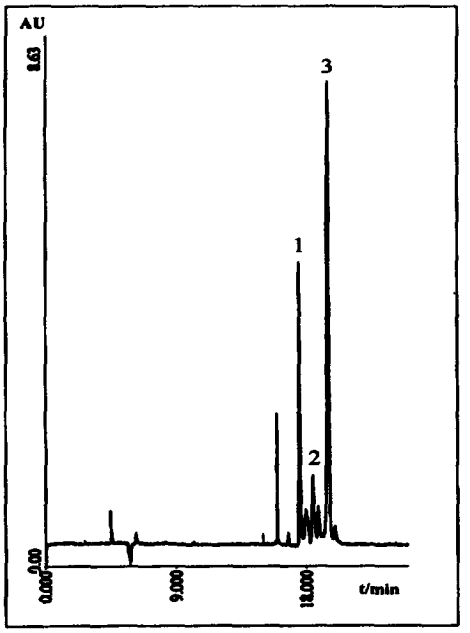


Fraction No. 11

Fig. 4. CE of fractions of dalargin glycoconjugates prepared by reaction with 2-chloroethyl-1-thio-β-D-galactoside, in 150 m*M* H_3PO_4, pH 1.9. For the conditions see the text, Section 2. Peaks: 1 = dalargin, 2 = dalargin-thiogal, 3 = dalargin-dithiogal.



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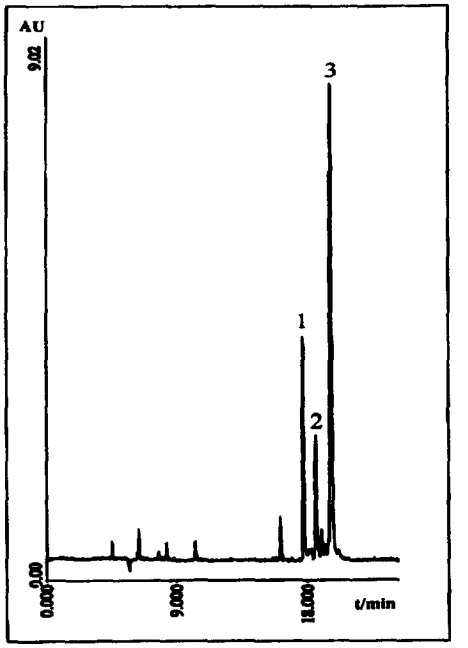

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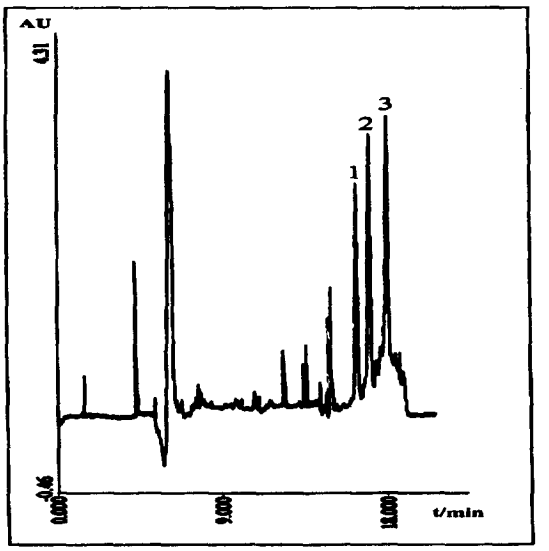


Fraction No. 11

Fig. 5. CE of fractions of dalargin glycoconjugates prepared by reaction with 2-chloroethyl-1-thio-β-D-galactoside, in 50 m*M* SDS + 20 m*M* sodium tetraborate, pH 9.2. For the conditions see the text, Section 2. Peaks: 1 = dalargin-dithiogal, 2 = dalargin-thiogal, 3 = dalargin.

Table 4
CE characteristics of the glycoconjugates prepared

Compound	Separation conditions	Mobility μ_c ($m^2/V\,s$)	Relative standard deviation R.S.D.(%)
Dalargin	A	$3.35 \cdot 10^{-8}$	1.4
	B	$-4.15 \cdot 10^{-8}$	2.1
Dalargin-gal	A	$3.13 \cdot 10^{-8}$	1.0
	B	$-3.40 \cdot 10^{-8}$	2.0
Dalargin-digal	A	$2.87 \cdot 10^{-8}$	1.0
	B	$-2.09 \cdot 10^{-8}$	1.9
Dalargin-lact	A	$3.10 \cdot 10^{-8}$	0.9
	B	$-2.92 \cdot 10^{-8}$	2.2
Dalargin-dilact	A	$2.79 \cdot 10^{-8}$	1.0
	B	$-1.82 \cdot 10^{-8}$	2.0
Dalargin-malt	B	$-3.00 \cdot 10^{-8}$	1.7
Dalargin-dimalt	B	$-2.18 \cdot 10^{-8}$	2.0
Dalargin-thiogal	A	$2.79 \cdot 10^{-8}$	0.8
	B	$-4.03 \cdot 10^{-8}$	1.9
Dalargin-dithiogal	A	$2.40 \cdot 10^{-8}$	0.8
	B	$-3.93 \cdot 10^{-8}$	2.0
Pac-Dalargin	A	$2.09 \cdot 10^{-8}$	1.0
	B	$-4.27 \cdot 10^{-8}$	1.5
Pac-Dalargin-thiogal	B	$-2.85 \cdot 10^{-8}$	1.6
Boc-Dalargin	A	$2.17 \cdot 10^{-8}$	0.9
	B	$-4.29 \cdot 10^{-8}$	2.2
Boc-Dalargin-thiogal	B	$-4.24 \cdot 10^{-8}$	2.0
Desmopressin	B	$-4.14 \cdot 10^{-8}$	1.5
Desmopressin-thiogal	B	$-4.07 \cdot 10^{-8}$	1.6
Desmopressin-lact	B	$-4.16 \cdot 10^{-8}$	1.5

$$R_1\text{: Dalargin} + \text{D} - \text{maltose} \xrightarrow[NaBH_3CN]{k_1} \text{Dalargin} - \text{malt}$$

$$R_2\text{: Dalargin} - \text{malt} + \text{D} - \text{maltose} \xrightarrow[NaBH_3CN]{k_2} \text{Dalargin} - \text{dimalt}$$

These two follow-up reactions were treated by the Ostwald isolation method. Reactions R_1 and R_2 are then pseudo-first order with respect to dalargin and the reaction rates are given by

$$v_1 = k_1[\text{dalargin}]^{\alpha}[\text{D} - \text{maltose}]^{\beta}$$

$$v_2 = k_2[\text{dalargin} - \text{malt}]^{\gamma}[\text{D} - \text{maltose}]^{\delta}$$

where α, β, γ, δ are equal to unity and the rate constants amount to

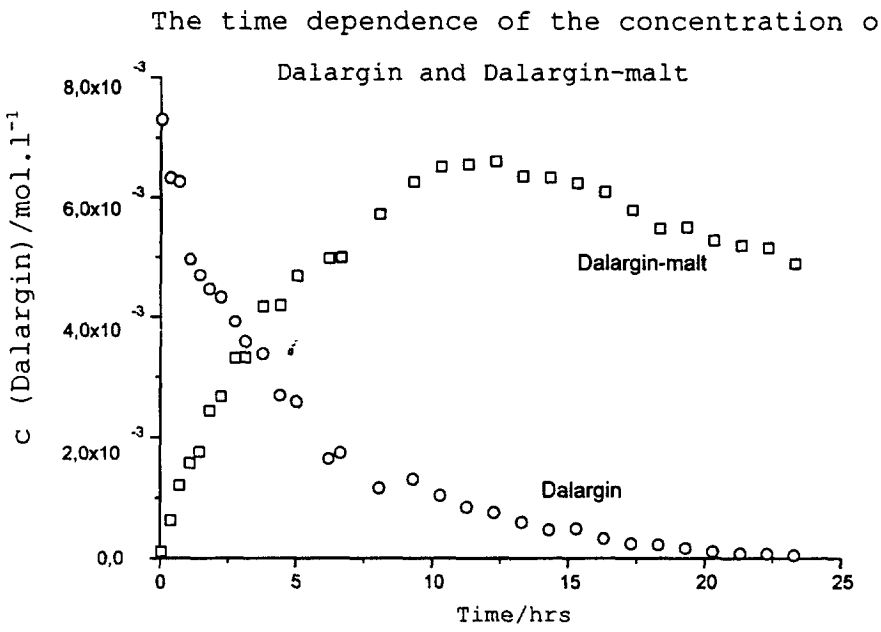
$$k_1 = 3 \cdot 10^{-4}\ \text{l mol}^{-1}\ \text{s}^{-1}$$

$$k_2 = 4 \cdot 10^{-5}\ \text{l mol}^{-1}\ \text{s}^{-1}$$

Fig. 6 depicts the time dependence of the concentrations of dalargin and dalargin-malt and the linearized concentration dependence for dalargin, from which the rate constants were obtained.

7. Conclusion

It can be seen that both HPLC and CE are suitable for monitoring of glycosylation reactions of peptide hormones of the enkephalin type. Whereas CE exhibits superior efficiency and requires extremely small samples, HPLC permits easier identification of the reaction products and is more simply adapted for preparative purposes. CE has been found useful for studying the kinetics of glycosylation reactions.



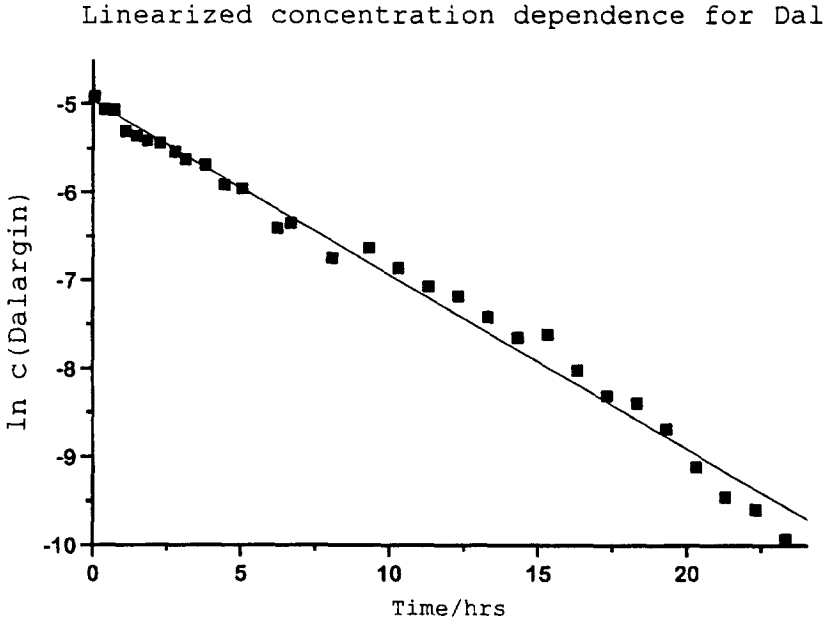


Fig. 6. Kinetics of reductive amination.

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