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Separation of antithrombin III variants by micellar electrokinetic chromatography

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Abstract

The characterisation of proteins is still one of the most challenging analytical tasks in modern bioanalysis. Due to the complex structure of proteins, several analytical techniques are often required to get sufficient information. Antithrombin III (AT III), a high-molecular-mass plasma glycoprotein which is an important protease inhibitor and the main modulator of thrombin activity, circulates in plasma in two isoforms, the so-called AT III-α (90–95%) and -β (5–10%). Micellar electrokinetic chromatography was used to analytically separate these AT III variants, which differ in their affinity to the polysaccharide heparin.

Keywords: Sample injection; Antithrombins; Glycoprotein; Proteins; Enzyme inhibitors; Heparin

1. Introduction

Antithrombin III (AT III) is a single-chain plasma glycoprotein that inhibits most proteinases of the coagulation cascade, such as thrombin which is its principal physiological target, and thus plays a major role in the regulation of blood clotting. In patients with a reduced AT III level there is increased risk of thrombosis, and therefore AT III is of high clinical importance. Inhibition of thrombin by AT III occurs through the formation of a stable equimolar complex between inhibitor and protease. The rate of thrombin inactivation and the broad inhibitory function of AT III, respectively, is strongly accelerated by the sulfated polysaccharide heparin, or by heparin-related structures on the endothelium. Located in the N-terminal domain of the protein the binding of heparin results in a conformational change in antithrombin [1,2]. By binding to glycosaminoglycans, like heparin sulfate, AT III protects the vessel wall and mediates antiinflammatory effects as well. There is experimental evidence concerning the heparin affinity of the AT III variants, i.e., especially AT III-α and -β.

For performing studies on α/β proportions in AT III concentrates and later on in plasmas, first of all a suitable analytical method needs to be established. The separation of AT III using capillary isoelectric focusing (cIEF) yields a few not well resolved broad peaks [3], but only the size-dependent separation of proteins denatured in sodium dodecyl sulfate (SDS) by capillary electrophoresis (CE) using a replaceable sieving matrix (SDS-protein CE) leads to an acceptable separation, in consequence of the difference in molecular mass of the antithrombin variants α and β [4]. Both methods are disadvantegeous in several ways, the most important are the influence of the sample matrix and the high UV absorbance of the

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ampholyte in cIEF as well as the sample pretreatment and the anomalous behaviour of glycoproteins like AT III in SDS-protein CE. The carbohydrate moieties of glycoproteins do not bind SDS, thereby causing lower than expected charge-to-mass ratios, requiring a specific evaluation [5]. Besides, both methods (cIEF and SDS-protein CE) are not easy to perform and thus, an easy-to-use method has therefore been established that could be used in any circumstances.

In particular, immuno-adsorption of AT III from complex protein solutions requires an analytical method to quantify the isoforms. As the α and β variants of AT III have similar size-to-charge ratios, making their electrophoretic mobilities virtually identical, micellar electrokinetic chromatography (MEKC) proved to be a powerful technique for the desired separation [6,7].

In this paper, we demonstrate the baseline separation of the antithrombin variants α and β via cyclodextrin-modified MEKC (CD-MEKC) as the method of choice.

2. Experimental

2.1. Separation of isoforms from AT III concentrates

Antithrombin III isoforms were prepared from AT III concentrate (Kybernin P, Aventis Behring, Germany) by adsorption to immobilised heparin and subsequent fractionated elution with NaCl. The α-isoform was eluted by increasing the ionic strength up to 0.8 *M* NaCl, whereas AT III-β was eluted with 2 *M* NaCl. Both preparations were dialysed against 0.01 *M* sodium citrate+0.15 *M* NaCl, pH 7.4. Concentrations were adjusted according to the respective heparin cofactor activity. The purified isoforms as well as defined mixtures were used to validate the MEKC conditions.

2.2. Isolation of AT III from human plasma

Prior to MEKC analysis, AT III was purified from the plasma of healthy subjects by immuno-adsorption to a monoclonal antibody (mAb) linked to BrCN-Sepharose (Amersham Pharmacia Biotech, Sweden). In pre-experiments the mAb had been demonstrated to bind both isoforms with identical affinity. A 3-ml volume of mAb-Sepharose (5 mg mAb per 1 ml of matrix) was used to isolate AT III from 1 to 3 ml plasma samples, which had been 10-fold diluted in 0.15 *M* NaCl beforehand. After extensive washing of the column with 25 column volumes each of 0.01 *M* Tris+2 *M* NaCl, pH 8.5, and water, AT III was eluted with a solution of 0.1 *M* glycine, pH 2.5. The eluate was then concentrated by ultrafiltration (M_r= 5000 cut-off filter) to 0.1 mg/ml of total protein. In parallel, the buffer was exchanged to 0.01 *M* sodium citrate+0.15 *M* NaCl, pH 7.4, and subjected to MEKC thereafter.

2.3. CE equipment

The instrumentation used for the majority of the measurements was an Applied Biosystems (ABI, Foster City, CA, USA) 270A-HT CE system, equipped with a monochromatic UV detector. A fused-silica capillary purchased from Supelco (Bellefonte, PA, USA) was attached to the system. According to heat dissipating and resolution perspectives the size of the capillary was 72 cm (50 cm to the detector)×50 μm I.D.×363 μm O.D. The detection window was created by glowing off the polyimide coating using a CE capillary burner purchased from Elektrokinetic Technologies (Broxburn, UK). Data collection were recorded via a Perkin-Elmer (San Jose, CA, USA) 900 series interface. All peak information was obtained through the PE Nelson PC-integrator software, version 5.1.

Concerning the CE system compatibility even using different sample injection modes, runs were also performed on a second instrument, the $HP^{3D}CE$ system from Hewlett-Packard (Waldbronn, Germany), equipped with a diode array detector. In contrast to the set-up described above, the capillary length was 70 cm (61.5 cm effective length). The standard Hewlett-Packard Chemstation Software running under Windows NT 4.0 was used for instrument control and data analysis.

2.4. Reagents

Sodium borate (boric acid), purchased from Riedel-de Haën (Seelze, Germany) was used as a

background electrolyte. The anionic surfactant SDS, the chiral additive β-cyclodextrin, both purchased from Merck (Darmstadt, Germany) as well as the cationic divalent amine 1,5-diaminopentane were used as modifiers in the migrating solution. Sodium hydroxide, purchased from Riedel-de Haën was used for pH adjustment. The distilled–deionized water was obtained from a Milli-Q laboratory water processing system ELIX 3 from Millipore (Eschborn, Germany).

2.5. Separation conditions

Prior to use a new capillary was first flushed with 1 *M* NaOH for 30 min, with 0.1 *M* NaOH for 15 min, and finally with Milli-Q water for another 15 min (application of a vacuum of approx. 650 mbar at outlet end of the capillary in the case of the ABI 270A-HT instrument and approx. 900 mbar pressure at inlet vial in the case of the HP^{3D}CE instrument, respectively). Capillary conditioning between runs was effected by flushing with water for 2 min, with 0.1 *M* NaOH for another 2 min, and with running buffer for 6 min to prepare the capillary for the immediate separation.

The optimised running buffer was composed of 60 m*M* borate, 40 m*M* SDS, 20 m*M* β-cyclodextrin and 1 m*M* diaminopentane (pH 9).

Solute detection was effected by UV absorbance at 200 nm and in consequence of the small dimensions of the capillary resulting in nanolitre sample volumes an adequate sample concentration of about 1 mg/ml, but not less than 0.1 mg/ml in the case of AT III samples was therefore necessary, presuming an ideal injection time of 0.5 s and 5 s (extended to 1 s and 10 s for the HP^{3D}CE instrument), respectively. All samples were introduced on the anode side of the capillary by vacuum injection at 169 mbar (ABI 270A-HT apparatus) and an applied pressure of 50 mbar (HP^{3D}CE instrument), respectively. The separation voltage was 20 kV (generating a current of approx. 22 μA), with the capillary temperature set to 30°C [8].

3. Results

The primary structure of human AT III (antithrombin α) leads to an isoelectric point (p*I*) in the range of 4.5 to 5.4 and a relative molecular mass of 58 000. Deprivation of a carbohydrate side chain results in the AT III variant β (p*I*=4.8 to 5.4; M_r=55 000).

An approximate 1:1 mixture of AT III-α and -β was used to establish a suitable buffer system. The α and β variants of antithrombin III were not properly resolved by customary free zone electrophoresis. Certainly two broad peaks were obtained, requiring a

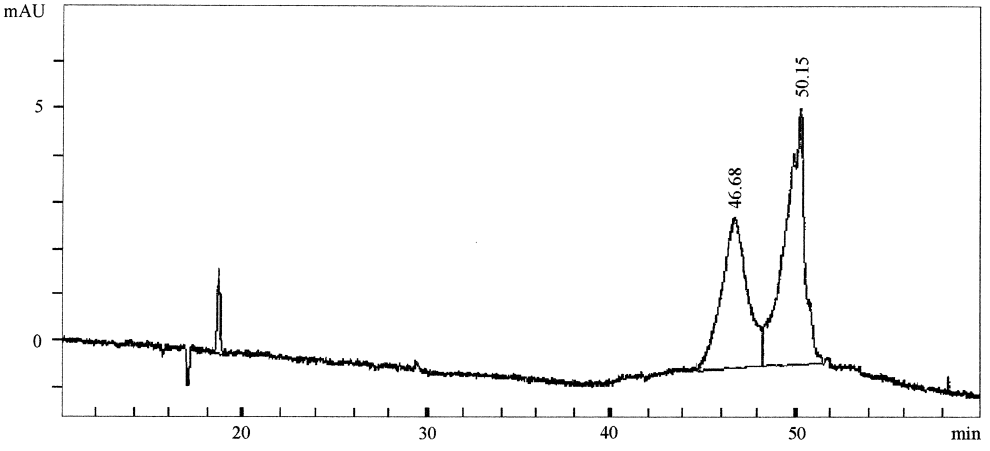

Fig. 1. CZE analysis of a mixture of AT III-α and -β, 1 mg/ml. Conditions: buffer, 50 m*M* sodium phosphate, pH 7; instrument, Applied Biosystems 270A-HT; capillary, 72 cm (50 cm effective length)×50 μm; detection, UV 200 nm; injection, 0.5 s vacuum 169 mbar; current, 20 kV.

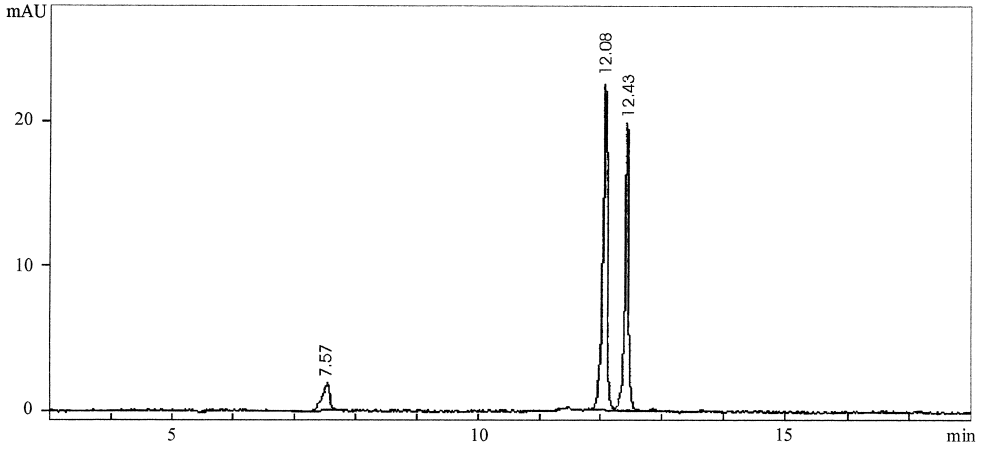

Fig. 2. CD-MEKC analysis of a mixture of AT III-α and -β, 1 mg/ml. Conditions: buffer, 60 m*M* sodium borate, 40 m*M* SDS, 20 m*M* β-cyclodextrin, 1 m*M* diaminopentane, pH 9; instrument, Applied Biosystems 270A-HT; capillary, 72 cm×50 μm; detection, UV 200 nm; injection, 0.5 s vacuum 169 mbar; current, 20 kV (22 μA).

rather long analysis time (Fig. 1). However, a buffer containing micelles and cyclodextrins as moving phases in capillary electrophoresis enables improved separation, resulting in two baseline-separated peaks at shorter migration times (Fig. 2). Both compounds, AT III-α and -β exhibit excellent peak shape, and the migration order was found to be α prior to β. Whereas the purity of the AT III-α sample is nearly 100% (Fig. 3), the AT III-β sample contained an impurity of approx. 10% of AT III-α (Fig. 4).

The isoelectric range of AT III (p*I* 4.5–5.4) and the relative molecular mass of the AT III variants (M_r 55 000 and 58 000, respectively) are close to those of human serum albumin (p*I* 4.9, M_r 67 000). In further experiments it could be shown that plasma proteins like albumin, which can interfere with the AT III analysis, were also solubilised by the micelle employed but eluted later than the AT III variants. Besides plasma sample impurities of smaller size which are able to absorb UV light, migrate much faster than AT III (Fig. 5).

Ruggedness was examined using a HP^{3D}CE in-

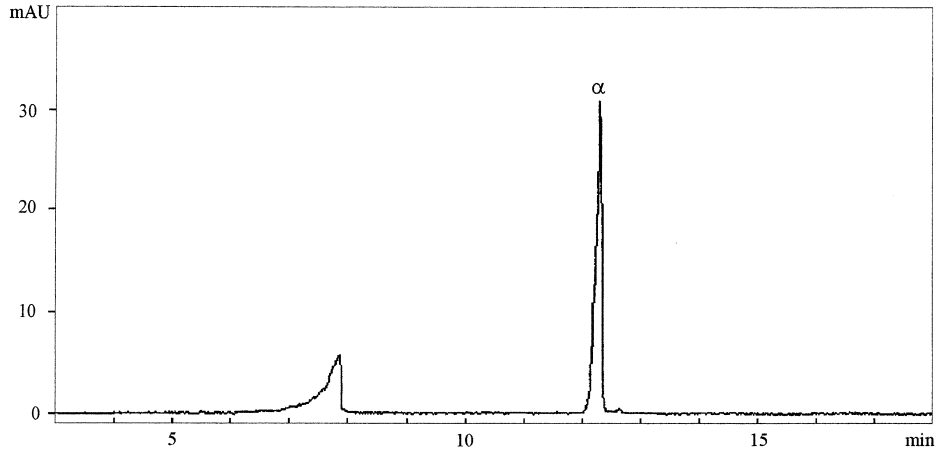

Fig. 3. CD-MEKC analysis of AT III-α, 1 mg/ml. Conditions as in Fig. 2.

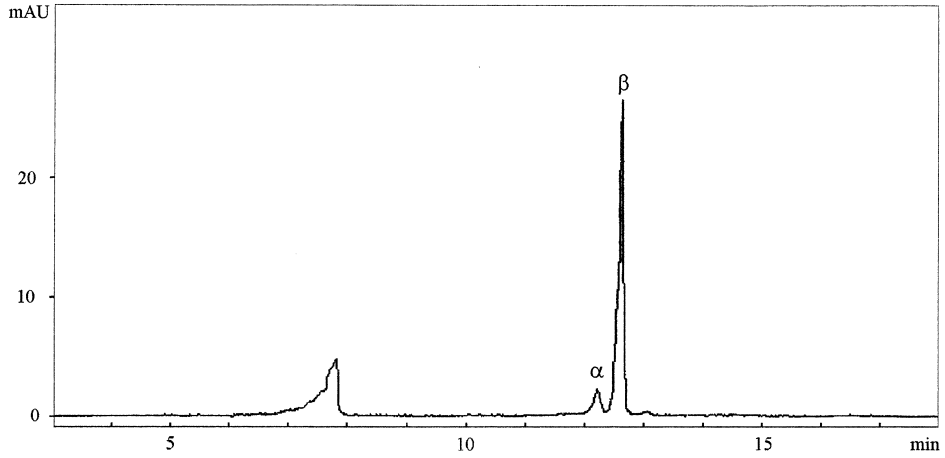

Fig. 4. CD-MEKC analysis of AT III-β, 1 mg/ml. Conditions as in Fig. 2.

strument. Both CE systems were shown to provide comparable separation although the operational conditions are slightly different due to differences in construction and function. Comparison experiments were performed using a sample mixture consisting of one part AT III-α (see Fig. 3) and three parts AT III-β (see Fig. 4). Baseline separation was likewise achieved but was concomitant with increased migration time caused by the enlarged effective length of the capillary (Fig. 6). Moreover, the established method enables the measurement of samples of lower AT III concentration. Finally, even a plasma sample consisting of only 0.1 mg/ml AT III was analysed (Fig. 7). As a consequence of the 10-fold lower AT III content, requiring a longer injection time and the full scale adapted ordinate (related to the AT III peaks), a large plasma impurities peak exists in the range of 9 min followed by AT III-α (94.4%) and -β (5.6%).

4. Discussion

To successfully separate the antithrombin III variants α and β from each other, CD-MEKC based on borate as the basic buffer was established in

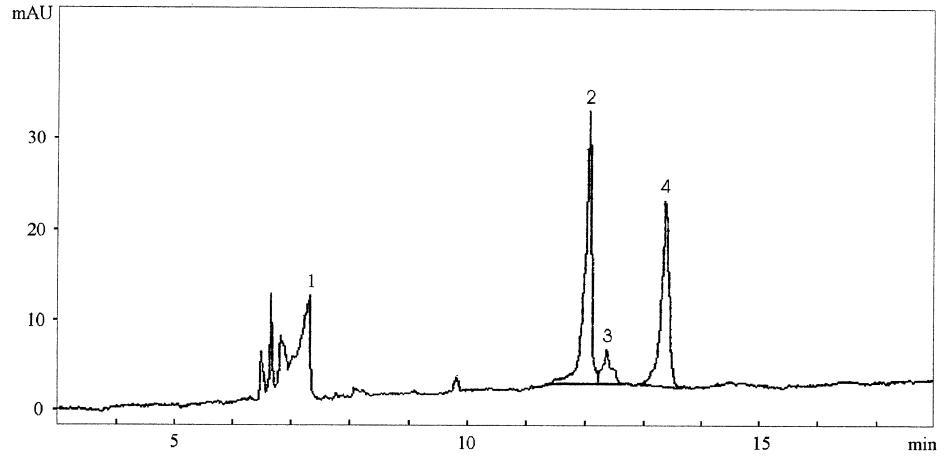

Fig. 5. CD-MEKC analysis of AT III and albumin. Conditions as in Fig. 2. Migration order: (1) sample matrix impurities, (2) AT III-α, (3) AT III-β, (4) albumin.

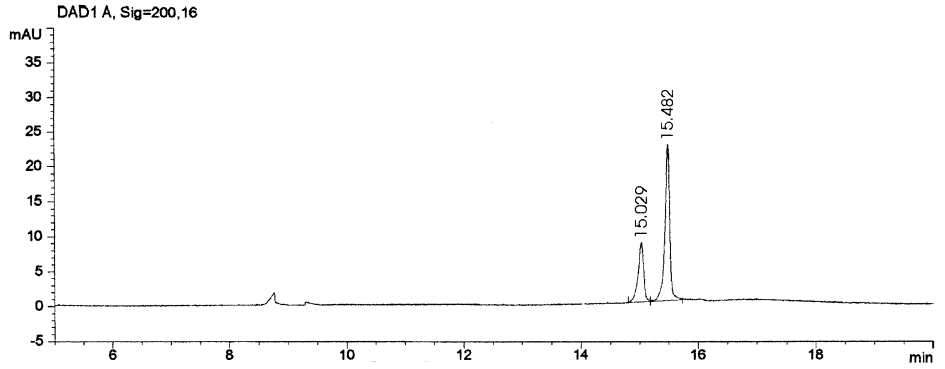

Fig. 6. CD-MEKC analysis of a mixture of AT III-α and -β. Conditions: buffer, 60 m*M* sodium borate, 40 m*M* SDS, 20 m*M* β-cyclodextrin, 1 m*M* diaminopentane, pH 9; instrument, Hewlett-Packard HP^{3D}CE; capillary, 70 cm (61.5 cm effective length)×50 μm; diode array detection 200 nm; injection, 1 s pressure 50 mbar; current, 20 kV (23 μA).

which SDS acts as an anionic surfactant providing sample solubility and β-cyclodextrin additionally introduces selectivity behaviour [9]. In conclusion a small amount of the cationic divalent amine 1,5-diaminopentane was added to prevent any adsorption effects between solute and capillary wall. Concerning this, SDS is also of advantage because of the ionic repulsion between the hydrophilic moieties of the surface-active molecules and the negatively charged silanol groups [10]. Furthermore small ion pairing effects between anionic surfactant and cationic amine are also present. As neither SDS micelles nor β-cyclodextrin are sufficiently large entities to allow insertion of the AT III variants, a complex separation mechanism appears likely, regarding factors such as solubility, conformational flexibility, hydrophobicity, hydrogen bonding, charge, size, shape, and structure.

As the AT III variants show different affinities towards heparin, the role of these isoforms should be further clarified. The predominant α-form, which accounts for about 90% of the inhibitor in plasma, is glycosylated on all four potential glycosylation sites (Asn-96, Asn-135, Asn-155, and Asn-192), whereas the minor β-form lacks the carbohydrate side chain on Asn-135 [11]. Antithrombin β has been shown to bind heparin more tightly than the fully glycosylated α-form, probably due to its higher conformational

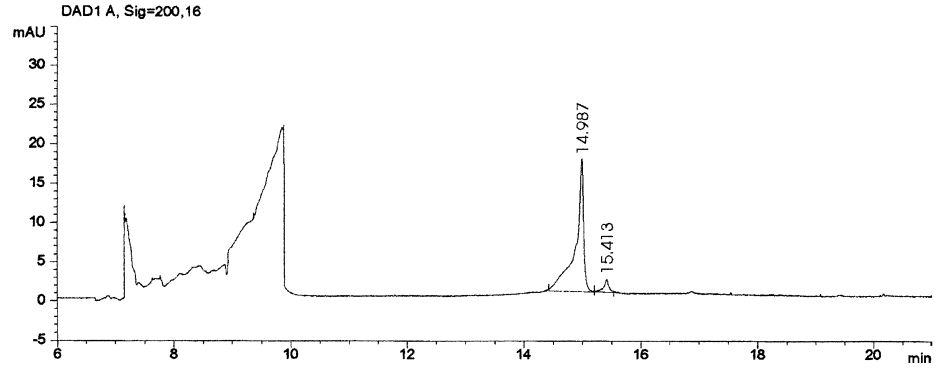

Fig. 7. CD-MEKC analysis of an AT III sample with lower antithrombin concentration. Conditions: buffer, 60 m*M* sodium borate, 40 m*M* SDS, 20 m*M* β-cyclodextrin, 1 m*M* diaminopentane, pH 9; instrument, Hewlett-Packard HP^{3D}CE; capillary, 70 cm (61.5 cm effective length)×50 μm; diode array detection 200 nm; injection, 10 s pressure 50 mbar; current, 20 kV (23 μA).

flexibility. Potential different in vivo functions of both isoforms are still a matter of discussion. On the account of its stronger binding to surfaces, including the vessel wall, β-antithrombin has been suggested to be the physiologically most important antithrombin form and might represent the first shield against proteolytic attack [12]. Due to the lack of a suitable quantification method studies on α/β proportions in plasmas of healthy or ill patients have not yet been reported. Further experiments are needed in order to evaluate the clinical function of these isoforms.

5. Conclusions

We have shown that the AT III isoforms α and β are readily separated by MEKC in the presence of additives. Therefore, this procedure is regarded superior to existing high-performance capilalry electrophoresis methods occasionally used for AT III analysis. As it is well known that optimal conditions for the CE separation are extremely protein specific, the reported fast and inexpensive method demonstrates that MEKC can be successfully applied to the separation of the antithrombin III variants α and β. In particular, immuno-adsorption of AT III from complex protein solutions prior to MEKC constitutes a suitable method to quantify the isoforms of AT III even from complex protein solutions. Thus, the separation method presented in this paper may be used to further evaluate the clinical function of the AT III-α and -β isoforms.

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References

[1] I. Björk, K. Ylinenjärvi, S.T. Olson, P. Hermentin, H.S. Conradt, G. Zettlmeissl, Biochem. J. 286 (1992) 793.
[2] L. Mourey, J.P. Samama, M. Delarue, J. Choay, J.C. Lormeau, M. Petitou, D. Moras, Biochimie 72 (1990) 599.
[3] O.W. Reif, R. Freitag, J. Chromatogr. A 680 (1994) 383.
[4] A. Buchacher, P. Schulz, J. Choromanski, H. Schwinn, D. Josic, J. Chromatogr. A 802 (1998) 355.
[5] W.E. Werner, D.M. Demorest, J. Stevens, J.E. Wiktorowicz, Anal. Biochem. 212 (1993) 253.
[6] H. Nishi, T. Fukuyama, M. Matsuo, S. Terabe, J. Chromatogr. 515 (1990) 233.
[7] M.A. Evenson, J.E. Wiktorowicz, Clin. Chem. 38 (1992) 1847.
[8] P. Hermentin, R. Doenges, R. Witzel, C.H. Hokke, J.P.K. Vliegenthart, H.S. Conradt, M. Nimtz, D. Brazel, Anal. Biochem. 221 (1994) 29.
[9] J.E. Noroski, D.J. Mayo, M. Moran, J. Pharm. Biomed. Anal. 13 (1995) 45.
[10] J.C. Jacquier, P.L. Desbène, J. Chromatogr. A 718 (1995) 167.
[11] S.O. Brennan, P.M. George, R.E. Jordan, FEBS Lett. 219 (1987) 431.
[12] B. Turk, I. Brieditis, S.C. Bock, S.T. Olson, I. Björk, Biochemistry 36 (1997) 6682.