Journal of Pharmaceutical and Biomedical Analysis
30 (2003) 1531–1537

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

www.elsevier.com/locate/jpba

Quantification of hyaluronic acid fragments in pharmaceutical formulations using LC–ESI–MS

Andrea V. Kühn [a,*], Klaus Raith [a], Volker Sauerland [b], Reinhard H.H. Neubert [a]

[a] *Institute of Pharmaceutics and Biopharmaceutics, Martin-Luther-University, Halle-Wittenberg, Wolfgang-Langenbeck-Street 4, 06120 Halle/Saale, Germany*

[b] *Bruker Saxonia Analytik GmbH, Permoserstr. 15, 04318 Leipzig, Germany*

Received 8 August 2002; received in revised form 16 September 2002; accepted 16 September 2002

Abstract

Three different hyaluronic acid fragment preparations (HAF) derived from hyaluronic acid (HA) by hyaluronate lyase digestion have been investigated. The amount of these fragment mixtures in pharmaceutical formulations was determined by liquid chromatography-electrospray tandem mass spectrometry (LC/MS/MS). HAF analysis was performed in less than 8 min using a Nucleosil® 100-7 C2 column. Based on the assumption that the mass distribution is kept constant, which is confirmed by the calibration results, quantification can be carried out relating to the most intense fragments. For that purpose, the ratios of the peak areas of product ions of $m/z = 378$ (tetramer, hexamer, octamer) to the peak area of $m/z = 83$ ([2 × maltose-H^+], internal standard) were calculated. Calibration was done for each HAF and good linearity from 5 to 80 μg/ml has been shown. To evaluate the molecular weight distribution of the fragment preparations used in this approach MALDI-TOF, mass spectra have been collected.

Keywords: Hyaluronic acid; Hyaluronate lyase; Mass spectrometry; LC/MS; MS/MS

1. Introduction

Hyaluronic acid (HA) is a naturally occurring linear polysaccharide. The repeating disaccharide units consist of glucuronic acid and *N*-acetylglucosamine via a β-1,4 linkage. HA is used as pharmaceutical excipient (hydrogel formation, drug attachment) as well as drug (wound healing, anti-inflammation).

Oligosaccharides resulting from enzymatic degradation with hyaluronate lyase (HA lyase, bacterial hyaluronidase, E.C. 4.2.2.1) have been used for characterization purposes [1]. HA lyase is an *endo*-hexosaminidase, producing even-numbered fragments of structure β-D-4en-thrHex*p*A-(1→3)-[β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A]$_n$-(1→3)-D-Glc*p*NAc (Fig. 1). As described in [1], these are accompanied by minor amounts of odd-numbered oligomers of structure β-D-4en-

* Corresponding author. Tel.: +49-345-552-5215; fax: +49-345-552-7292

E-mail address: kuehn@pharmazie.uni-halle.de (A.V. Kühn).

0731-7085/02/$ - see front matter
PII: S0731-7085(02)00544-7

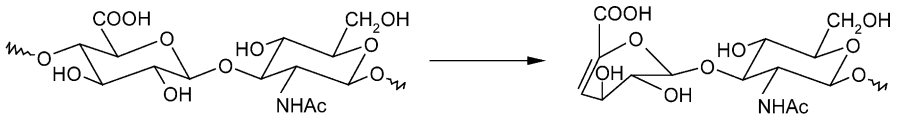

Fig. 1. Enzymatic cleavage of HA by bacterial hyaluronidases.

thrHex*p*A-(1→3)-[β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A]$_n$.

The application of hyaluronic acid fragment (HAF) in various drug delivery systems is currently under investigation. UV-light-induced radical formation has been reported [2]. Three items make the application of HAF instead of native HA favorable in pharmaceutical formulations: firstly, smaller molecules should better penetrate into the skin, secondly, the presence of a double bond in the fragments is considered to be important for a radical scavenger effect which may protect human epidermal keratinocytes against UV-induced oxidative damage [3,4] and thirdly, HAF is much more accessible to mass spectrometric characterization and quantification, which is mandatory for quality control. Recently a method for the determination of high molecular weight HA by means of CE has been presented [5]. Further reading on the analysis of hyaluronic acid and its fragments is given in literature [6–12].

The more or less complex nature of the HAF mixtures obtained by hyaluronate lyase digestion makes the specific quantification a challenge. Dependent on the digestion time the maximum of the molecular weight distribution is different. Therefore, the first step must be the evaluation of chain length distribution, which is best possible with the help of MALDI-TOF MS [13]. Thereafter, the major fragments can be quantified using LC/ESI–MS. In this study, we developed a new method using high performance liquid chromatography with electrospray tandem mass spectrometry detection for the measurement of HAF in pharmaceutical formulations.

2. Experimental

2.1. Materials

Methanol was obtained from J.T. Baker (Deventer, The Netherlands). Tetrahydrofuran was from Merck (Darmstadt, Germany). D-(+)-Maltose and carboxymethylcellulose (CMC, sodium salt) were purchased from Sigma (Taufkirchen, Germany). Ungt. emulsificans aquosum (DAB 1999) was supplied by MainoPharm (Frankfurt/Main, Germany). HA fragments (HAF, derived from HA by hyaluronate lyase digestion) were kindly provided by J.H. Ozegowski, Friedrich-Schiller-University of Jena, Germany. SDHB (2,5-dihydroxybenzoic acid/2-hydroxy-5-methoxy-benzoic acid, 9:1, m/m), peptide standard (angiotensin II, angiotensin I, substance P, bombesin, ACTH 1–17, ACTH 18–39, somatostatin 28) and protein standard (insulin, ubiquitin I, cytochrome *C*, myoglobin) were from Bruker (Leipzig, Germany).

2.2. Enzymatic degradation

For the digestions, 25 g of HA (Aqua Biochem GmbH, Dessau, Germany) were suspended in 4.975 l 0.01 M sodium acetate buffer pH 7, and the mixture was stirred overnight at 4 °C. The clear solution thus formed was warmed with stirring to 30 °C. Seven hundred milliliter of this solution were mixed with 10 ml of 0.01 M sodium acetate buffer pH 7 containing 280 U/ml HA lyase (EC 4.2.2.1, *Streptococcus agalactiae* [14]). Enzymatic depolymerization was carried out for 60 min (HAF 4), 90 min (HAF 5) and 120 min (HAF 6). The mixtures were then boiled for 5 min, ultrafiltered and lyophilized.

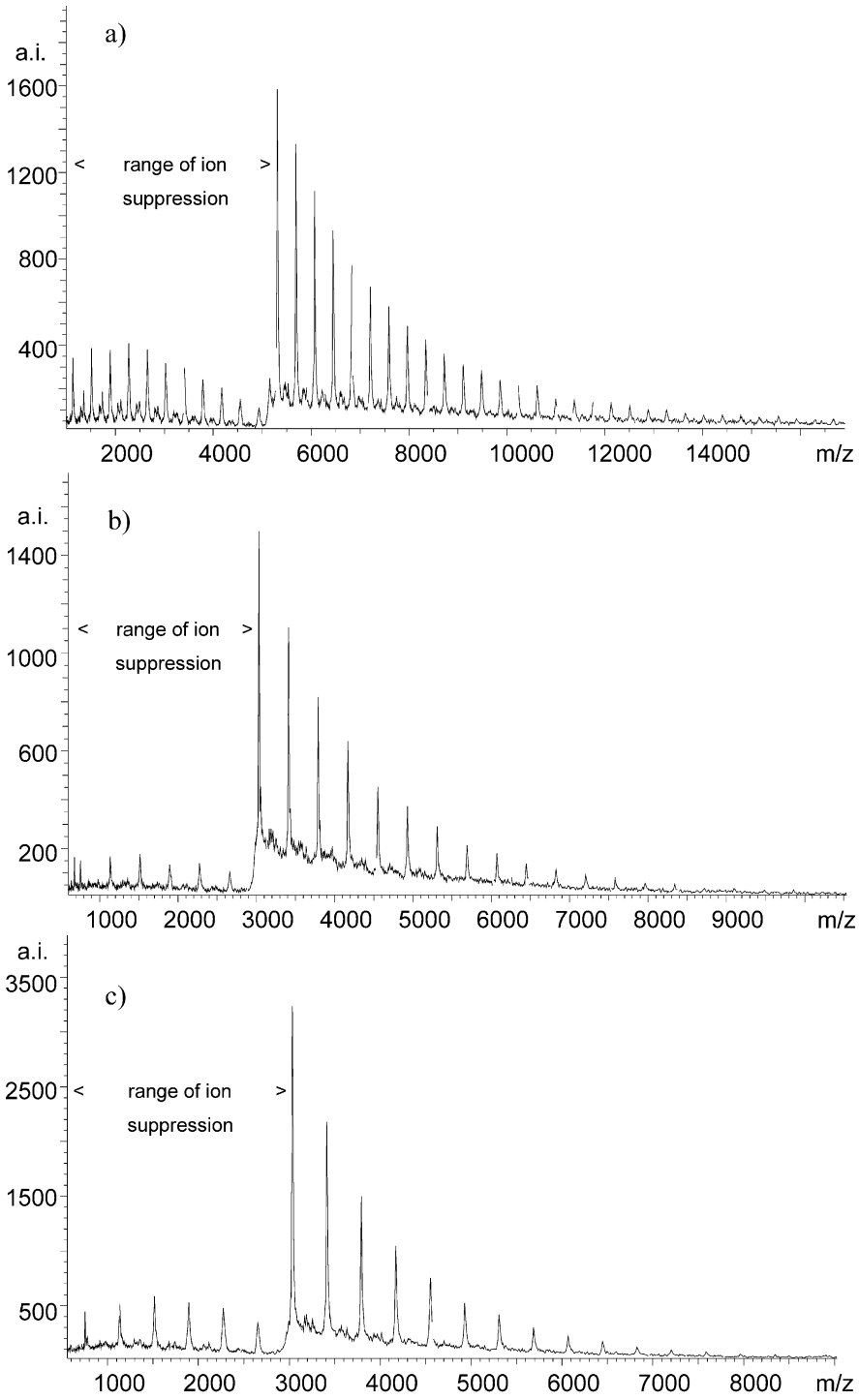

Fig. 2. MALDI-TOF-mass spectra of (a) HAF4, (b) HAF5 and (c) HAF6, linear negative mode, please note the different axis scale; a.i., arbitrary intensity.

Table 1
Scheme of fragmentation for even- and odd-numbered oligomers, $m/z = 174.8 = \mathrm{Glc}p\mathrm{A} - \mathrm{H_2O}$, $m/z = 201.8 = \mathrm{Glc}p\mathrm{NAc} - \mathrm{H_2O}$, in brackets: charge

ESI–MS		ESI–MS/MS observed (charge) m/z	ESI–MS^3 observed (charge) m/z
Possible oligomer	Observed m/z		
4-*mer*	378.2	554.1 (−1)	377.8 (−1), 174.8 (−1)
4-*mer*		276.5 (−2)	377.8 (−1), 174.8 (−1)
6-*mer*		466.1 (−2)	757.1 (−1), 174.8 (−1)
8-*mer*		437.1 (−3)	567.7 (−2), 466.1 (−2), 174.8 (−1)
10-*mer*		422.3 (−4), 201.8 (−1)	504.5 (−3)
7-*mer*	437.3	567.8 (−2), 174.8 (−1)	466.2 (−2)
5-*mer*	466.3	757.1 (−1), 174.9 (−1)	554.0 (−1)
8-*mer*	504.5	655.9 (−2)	567.8 (−2)
8-*mer*		437.0 (−3)	–
16-*mer*		565.4 (−5)	–
8-*mer*	511.9[a]	667.0 (−2)[a]	1158.1 (−1)[a], 955.1 (−1)[a]
8-*mer*		444.4 (−3)[a]	578.7 (−2)[a], 477.2 (−2)[a]
16-*mer*		574.1 (−5)[a]	673.7 (−4)[a], 622.9 (−4)[a]
3-*mer*	554.1	377.9 (−1), 174.9 (−1)	–
6-*mer*	567.7	466.2 (−2)	757.1 (−1)
12-*mer*		517.0 (−4)	–
6-*mer*	578.6[a]	477.1 (−2)[a]	779.1 (−1)[a]
12-*mer*		528.1 (−4)[a]	645.6 (−3)[a]
12-*mer*		704.5 (−3)[a]	968.9 (−2)[a]
10-*mer*	645.8[a]	577.9 (−3)[a], 867.4 (−2)[a]	779.1 (−2)[a], 677.8 (−2)[a]
10-*mer*	638.5[a]	570.5 (−3)[a], 856.3 (−2)[a]	768.2 (−2)[a], 666.7 (−2)
4-*mer*	757.2	554.1 (−1)	377.8 (−1), 174.8 (−1)
8-*mer*		655.7 (−2)	1136.1 (−1)
12-*mer*		689.6 (−3)	–
4-*mer*	779.2[a]	576.2 (−1)[a]	399.9 (−1)[a]
8-*mer*		677.7 (−2)[a]	1180.2 (−1)[a], 977.2 (−1)[a]
12-*mer*		711.6 (−3)[a]	–
6-*mer*	1136.1	933.1 (−1)	757.0 (−1), 553.9 (−1) (MS^4)
6-*mer*	1180.2[a]	977.2 (−1)[a]	779.1 (−1)[a]
12-*mer*		1078.7 (−2)[a]	–

[a] Even-numbered oligomers, sodium salts.

2.3. Preparation of formulations with HAF

The three fragment preparations were mixed with Unguentum emulsificans aquosum in different concentrations (1, 3 and 5%, m/m). For preparing the carboxymethylcellulose gel the fragments were first dissolved in distilled water. Afterwards 6% (m/m) CMC (sodium salt) have been added under stirring. The gels were kept in refrigerator (4 °C) overnight. For quantitative analysis of HAF in these gels and formulations defined amounts were extracted with internal standard solution (methanol/water (80:20, v/v), Maltose 10 μg/ml).

2.4. MALDI-TOF-MS

MALDI-TOF mass spectra were collected according to [13]. Hundred milligram of sample were mixed with 10 ml of TA (acetonitrile/0.1% trifluoroacetic acid, 1:2, v/v). One microliter of sample solution was mixed with 10 μl of a 10 mg/ml solution of SDHB in TA. This preparation (0.2 μl) were placed onto a MALDI-sample plate. Mass analysis was carried out in negative linear and reflector mode using an Omniflex™ (Bruker Daltonics, Billerica, MA, USA) equipped with a 337 nm nitrogen laser. The acceleration voltage was set to 19 kV and the delay time was 450 ns. A

total of 200 mass spectra were acquired and summed for each sample spot. Mass calibrations were performed over several m/z ranges, using protein and peptide standards.

2.5. LC/MS and LC/MS/MS

An HPLC pump SpectraSystem P4000 equipped with an autosampler AS 3000 and a membrane degasser was coupled via an electrospray interface to an ion trap mass spectrometer Finnigan LCQ (ThermoFinnigan, San Jose, CA, USA) with a nominal mass range to 2000 Da. For optimal electrospray conditions of HA fragments with this interface, the tip of the fused silica capillary had to be positioned exactly at the end of the ESI needle. The electrospray voltage applied to the ESI needle was −4.5 kV. The running eluent was 2.5% (m/v) THF in methanol at a flow-rate of 0.2 ml/min. Fifteen microliter of HAF dissolved in internal standard solution were injected onto a Nucleosil® C2 column (CC 250/2 100-7, Macherey-Nagel, Düren, Germany). Detection was performed in negative ESI-mode over 8 min. The resulting peak areas were calculated as average from three consecutive chromatograms ($n = 3$).

3. Results and discussion

The MALDI-TOF mass spectra (Fig. 2) show singly charged pseudomolecular ions ($[M-H]^-$) indicating a molecular weight distribution from 757 to 14401 Da for HAF4, from 757 to 8337 Da for HAF5 and from 757 to 7579 Da for HAF6. Throughout these patterns one large peak (even-numbered oligomers) is accompanied by a much smaller peak (odd-numbered oligomers) following at a distance of 176 u, which refers to an additional glucuronic acid monomer in the molecule. As commonly noticed, the detection of high mass molecules is discriminated in presence of small molecules. Therefore, the small m/z range has been suppressed to improve the response of higher masses.

Under ESI conditions the molecules are multiply charged and the spectra are more difficult to interpret. Molecules consisting of four to 16-

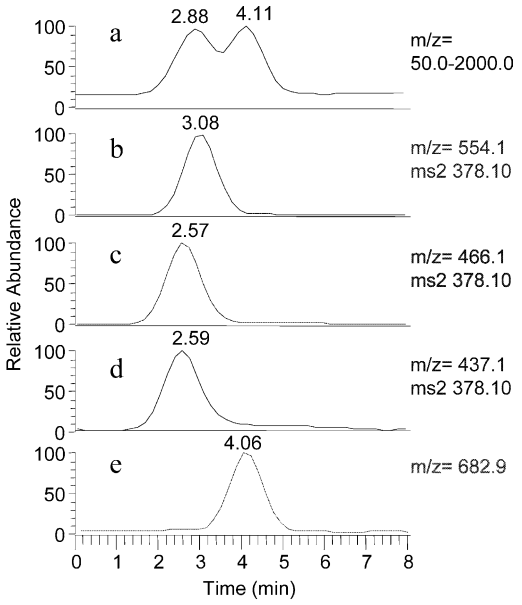

Fig. 3. LC/MS-chromatogram of HAF6 (20.9 μg/ml) + Maltose (10.3 μg/ml); (a) total ion current chromatogram; (b) m/z = 554.1 originating from the tetramer, (c) $m/z = 466.1$ originating from the hexamer, (d) $m/z = 437.1$ originating from the octamer, (e) $m/z = 682.9$ ([2 × maltose-H^+]), all measured in single ion monitoring.

monomer units have been found. Electrospray tandem MS shows in negative-ion mode a loss of one molecule *N*-acetylglucosamine (as $[M-H-H_2O]^-$, $m/z = 202$) for the even-numbered oligomers (Table 1), while odd-numbered oligomers split off glucuronic acid (as $[M-H-H_2O]^-$, $m/z = 175$). The next step of fragmentation according to this scheme can be observed in MS^3. Since the carboxy functions of glucuronic acid are partially present as sodium salts, the corresponding sodium adduct ions have been detected in addition to the pseudomolecular ions. Under MS/MS and MS^n conditions, these sodium ions are also found in the corresponding fragments. All obtained fragment masses have the β-D-4en-thrHex*p*A-structure.

In case of HA fragments the response is very dependent on all operation ranges and conditions, e.g. the position of the fused silica capillary in the ESI needle. Therefore, it is necessary to calibrate for each fragment preparation and to use an internal standard in order to obtain reliable quantification. Based on the assumption that the

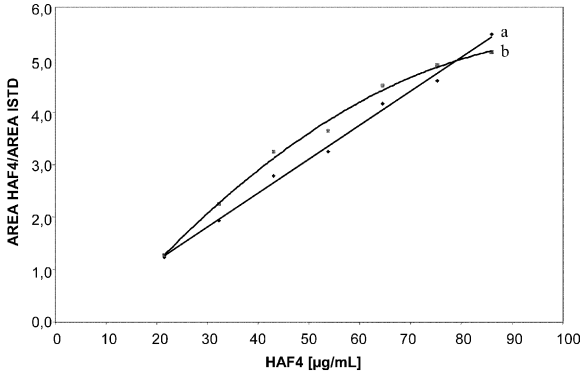

Fig. 4. (a) Linear fit for $m/z = 554.1$ ($A = 0.0646c - 0.1239$, $R = 0.9953$) (b) second-order polynomial fit for $m/z = 466.1$ ($A = -0.0006x^2 + 0.1235x - 1.1026$, $R = 0.9949$) where A is the ratio of the peak areas of HAF to ISTD (internal standard) and c is the concentration of HAF (μg/ml).

mass distribution is kept constant, which is confirmed by the calibration results, quantification can be carried out relating to the most intense fragments. For that purpose, the ratios of the peak areas of $m/z = 554$, 466 and 437 (product ions of $m/z = 378$) to the peak area of $m/z = 683$ ([2 × maltose-H^+]) were calculated. Different retention times indicate that these fragments originate from different precursors, which contribute to $m/z = 378$, e.g. in case of $m/z = 554$ and 466 (Fig. 3). The addition of thf to the eluent is required to improve separation. The retention on the Nucleosil® C2 column is considered to be due to interactions with underivatized silanolic groups. In contrast on reversed phase C8 and C18 no retention is observed since steric hindrance prevents this interaction.

Fig. 4 shows that under these ESI conditions for $m/z = 554$ a linear calibration curve was found. For $m/z = 466$ a second-order polynomial curve fits best, due to a response drop at higher concentrations [15]. Linearity has been shown over a range from 5 to 50 μg/ml for HAF6 and from 20 to 80 μg/ml for HAF5 and HAF4. Rating these parameters, one has to take into account that HAF is a mixture of different chain lengths and not a single substance. Up to now no calibration standards are available. Only one of the oligosaccharides in each preparation has been chosen for quantification purposes.

Table 2
Recovery rates of HAF found by LC/MS/MS

	CMC		Ungt. emulsificans aquosum (DAB 1999)	
	Recovery rate (%)	R.S.D. (%)	Recovery rate (%)	R.S.D. (%)
HAF4 1%[a]	105.99	2.51	88.75	7.47
HAF4 3%[a]	112.53	12.66	97.83	4.39
HAF4 5%[a]	114.49	12.75	86.67	9.93
HAF5 1%[a]	118.17	18.55	100.19	2.45
HAF5 3%[a]	101.28	1.65	105.97	2.51
HAF5 5%[a]	99.82	2.79	112.54	13.43
HAF6 1%[a]	107.12	1.84	103.72	9.39
HAF6 3%[a]	121.33	3.85	100.45	0.76
HAF6 5%[a]	105.69	3.18	107.07	9.25
HAF4 1%[b]	91.04	17.96	46.76	18.27
HAF4 3%[b]	103.95	3.38	45.44	5.93
HAF4 5%[b]	98.84	14.42	63.23	12.89
HAF5 1%[b]	117.78	18.64	64.42	17.12
HAF5 3%[b]	113.53	8.94	56.00	8.57
HAF5 5%[b]	94.64	11.43	81.38	28.22
HAF6 1%[b]	109.32	5.85	58.39	3.10
HAF6 3%[b]	122.19	3.05	74.40	3.61
HAF6 5%[b]	108.29	7.97	86.32	4.33

[a] Referring to $m/z = 554$.
[b] Referring to $m/z = 466$.

As already pointed out in the introduction, HAF are of growing interest in dermatology. Therefore, semisolid formulations containing HAF have to be developed and characterized. The method described above has been applied to the quantification of HAF in two representative formulations, namely Unguentum emulsificans aquosum and carboxymethylcellulose gel.

The chromatograms of blank samples, diluted ointment and gel show no peak at $m/z = 554$ and 466. The recovery rates obtained with $m/z = 554$ and 466 are shown in Table 2. It is evident that in CMC-gel too high recovery rates have been found. This finding may result from water evaporation. Poor recovery rates and correspondingly high R.S.D.s are considered to result from matrix effects due to physicochemical interferences. Referring to $m/z = 466$ the recovery rates in Ungt. emulsificans aquosum are lower than calculated with $m/z = 554$. Possibly fragments with longer chains show a more marked tendency to interact with ingredients of the formulation, e.g. surfactants.

4. Conclusions

The present LC/MS/MS method allows the quantification of several HAF preparations based on the determination of the 4-, 6-, 8- and 10-*mer* in pharmaceutical formulations such as hydrogels and creams. Tandem MS implies a higher specificity compared with other methods, e.g. capillary electrophoresis [5,16]. Complementary structural information (chain-length) can be obtained simultaneously.

Acknowledgements

The authors wish to thank M. Woigk for excellent technical assistance. Furthermore, the financial support of the Land Sachsen-Anhalt for the stipend of Andrea V. Kühn is gratefully acknowledged.

References

[1] K.N. Price, A. Tuinman, D.C. Baker, C. Chisena, R.L. Cysyk, Carbohydr. Res. 303 (1997) 303–311.
[2] L. Lapčík, P. Chabreček, A. Stačko, Biopolymers 31 (1991) 1429–1435.
[3] D. Gerlach, T. Klapperstueck, C. Huschka, W. Wohlrab, Arch. Pharm. Pharm. Med. Chem. 334 (Suppl. 2) (2001) 61.
[4] D. Gerlach, A. Kuehn, C. Huschka, T. Klapperstueck, R. Neubert, W. Wohlrab, Hyaluronic acid and fragments of hyaluronic acid diminish ultraviolett-induced oxidative damage in human keratinocytes, J. Invest. Dermatol. May (2002) submitted for publication.
[5] M. Plätzer, J.H. Ozegowski, R.H.H. Neubert, J. Pharm. Biomed. Anal. 21 (1999) 491–496.
[6] J. Schiller, J. Arnhold, S. Bernard, S. Reichl, K. Arnold, Carbohydr. Res. 318 (1999) 116–122.
[7] K. Takagaki, K. Kojima, M. Majima, T. Nakamura, I. Kato, E. Masahiko, Glycoconjug. J. 9 (1992) 174–179.
[8] R.M. Pope, C.S. Raska, S.C. Thorp, J. Liu, Glycobiology 11 (2001) 505–513.
[9] D.C. Armstrong, M.R. Johns, Biotechnol. Tech. 9 (1995) 491–496.
[10] N. Volpi, Anal. Biochem. 277 (2000) 19–24.
[11] D.M. Whitfield, S. Stojkovski, H. Pang, J. Baptista, B. Sarkar, Anal. Biochem. 194 (1991) 259–267.
[12] D.J. Mahoney, R.T. Aplin, A. Calabro, V.C. Hascall, A.J. Day, Glycobiology 11 (2001) 1025–1033.
[13] B. Yeung, D. Marecak, J. Chromatogr. A 852 (1999) 573–581.
[14] H. Rodig, J.H. Ozegowski, G. Peschel, P.J. Müller, Zentralblatt für Bakteriologie 289 (1999) 835–843.
[15] J.A. Alkrad, Y. Mrestani, R.H.H. Neubert, New approaches for quantifying hyaluronic acid in pharmaceutical semisolid formulations using HPLC and CZE, J. Pharm. Biomed. Anal., in press.
[16] R. Willoughby, E. Sheehan, S. Mitrovich, A Global View of LC/MS, first ed., Global View Publisher, Pittsburg, PA, 1998.