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Quantification of hyaluronan in pharmaceutical formulations using high performance capillary electrophoresis and the modified uronic acid carbazole reaction

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Abstract

The amount of hyaluronan (HA) in pharmaceutical formulations was determined by high-performance capillary electrophoresis (HPCE) and the results were compared with the carbazole reaction established by Bitter and Muir (T. Bitter, H.M. Muir, Anal. Biochem. 4 (1962) 330–334), HA analysis was performed in less than 10 min by using an untreated fused silica capillary with bubble detection cell. The influence of several buffers and pH values was examined. Calibration curve shows good linearity from 0.01 to 5.0 mg/ml. The lower limit of detection by monitoring the absorbance at 195 nm was 10 μg/ml at a signal to noise ratio of 5. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Hyaluronan; Capillary electrophoresis; Carbazole reaction

1. Introduction

Hyaluronan (HA) is an acidic linear mucopolysaccharide, having regularly alternating units of *N*-acetylglucosamine and glucuronic acid linked by 1,4- or 1,3-β-glycosidic bonds. HA as well as its salt hyaluronate is a component of skin, the vitreous humour of the eye and other tissues. It plays an important role in stabilisation of the vitreous gel structure. Other physiological functions are discussed for HA [1]. The interest in pharmaceutical as well as cosmetic application of HA grows remarkably, e.g. in ophthalmology, arthrosis treatment, diagnostics, drug delivery and cosmetic products [2].

Former capillary electrophoresis (CE) studies detect HA as copper(II) complexes at 240 nm [3] or after being labelled at the reducing end with chromophores like 7-amino-1,3-naphthalene disulfonic acid [4] or 1-aminopyrene-3,6,8-trisulfonic acid [5]. By reacting with the reducing end group the intensity is low unless a former hydrol-

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ysis was done. CE has been used in analysis of glucosaminoglycan oligosaccharides prepared by lyase–catalysed depolymerisation [6]. Detection took place at 232 nm because of absorption of unsaturated uronic acid residues. HA from vitreous humour has been analysed using phosphate buffer pH 9.0 with SDS as additive [7]. Hayase et al. [8] used pullulan, a polysaccharide with a specified molecular mass, as an additive in buffer and determined amount and molecular mass. Suzuki and Honda [9] wrote a tabulated review on general trends of carbohydrate analysis by CE dealing with problems of separation and detection. Extensive reviews on capillary electrophoresis of glycosaminoglycans (HA, heparan, keratan and galactosaminoglycans) were published by Karamanos and Hjerpe [10] and by Grimshaw [11].

In this study, CE and a modified carbazole reaction were used to determine the amount of HA in semisolid pharmaceutical formulations. Caused by complex structure of formulations a hydrolysis or derivation of HA without side products could not be used. Therefore, a rather simple determination method has to be developed. The analytical parameters of the CE method should be compared with the well-established photometric method of Bitter and Muir [12] with regard to specificity, sensitivity and reproducibility.

2. Experimental

HA (MWavg 1.1 Mill) was a gift from Friedrich-Schiller-University Jena. Glycerol was purchased from Roth (Karlsruhe, Germany) and propylene glycol from Fluka (Buchs, Switzerland). Reagent grade dipotassium phosphate, monopotassium phosphate and ethanol were supplied by Merck (Darmstadt, Germany). Tris(hydroxymethyl)–aminomethan (Tris) was supplied by Serva (Heidelberg, Germany). Sulfuric acid, sodium tetraborate·10 H_2O and hydrochloric acid were obtained by Riedel–de Haën (Seelze, Germany). Carbazole, sodium hydroxide and *p*-hydroxybenzoic acid were purchased from Sigma (Deisenhofen, Germany). The ointments Basiscreme DAC [13] (Wasserfuhr, Bonn, Germany) and Unguentum alcoholum lanae SR 90 [14] were supplied by PKH (Halle, Germany).

A stock solution of HA was prepared by dissolving 5 mg/ml freeze-dried HA in distilled water. The samples were kept in refrigerator (4°C) overnight. Afterwards dilutions in the range from 0.01–5 mg/ml (m/V) were prepared. These dilutions were measured with CE and Bitter and Muir method to construct calibration curves.

2.1. Preparation of formulations with HA

At first three standard gels were prepared using a composition shown in Table 1. The samples were kept in refrigerator (4°C) overnight.

These gels were mixed with Basiscreme DAC[1] as well as Unguentum alcoholum lanae SR 90[2]

Table 1
Composition of standard gels

	HA I	HA II	HA III
Hyaluronic acid (g)	3.00	3.00	3.00
p-Hydroxybenzoic acid (g)	0.25	0.25	0.25
Glycerol (g)	–	5.00	–
Propylene glycol (g)	–	–	5.00
Distilled water (g)	96.75	91.75	91.75

[1] Basiscreme DAC

Glycerylmonostearate	4.0
Cetyl alcohol	6.0
Medium chain triglycerides	7.5
White petrolatum	25.5
Macrogol 1000 monostearate	7.0
Propylene glycol	10.0
Water	40.0

[2] Ungt. alc. lanae SR 90

Wool alcohol's	2.5
Sorbitan trioleate GOT	3.0
Petrolatum	ad 100.0

in different concentrations (20, 50 and 80% gel, m/m). For quantitative analysis of HA in these gels and formulations definite amounts (80–160 mg) were weighed into test tubes and extracted with 5.0 ml of distilled water. The tubes with Ungt. alc. lanae SR 90 were subjected to a water bath (80°C, 30 s) to suspend the formulation. All tubes were than shaken for 1 h and stored in refrigerator for 2 h.

2.2. Capillary electrophoresis

The phosphate buffers were prepared from reagent grade K_2HPO_4 and KH_2PO_4 (20 mM; pH 6.0 and 7.4). The borate buffer was prepared by dissolving sodium tetraborate (50 mM) in distilled water and mixing this with 0.1 N HCl (3:1, V/V). This solution was diluted 1:10 to prevent the detrimental effects of a high current in CE. The resulting pH value was 8.8. The Tris buffer solutions were prepared by mixing 25 ml of 0.2 M Tris with 0.1 N HCl (39.9/26.8/14.4/5.0 ml) and diluting with distilled water to 100 ml. All buffer solutions were filtered through membrane filters of 0.45 μm pore size and degassed by ultrasound for 10 min before the first use. The samples were only degassed to prevent influence of filtration on quantification.

A Hewlett Packard ^{3D}CE system (Waldbronn, Germany) fitted with an on-column diode array detector (190–600 nm) was used. Fused-silica capillaries 645(560) × 0.05 mm [$l_T(l_D)$ × I.D.] (extended lightpath, I.D. 150 μm) were obtained from HP. The capillaries were preconditioned for 15 min with 1 M NaOH before first run. Prior to each subsequent run the capillaries were rinsed with 0.1 M NaOH and actual buffer solution for 3 min. The separation conditions used were as follows: + 30 kV voltage (U, inlet), 250 mbar s pressure injection and 25°C capillary temperature. The detection took place 8.5 cm before the cathodic end ($\lambda = 195$ and 200 nm). The effective mobilities of HA were calculated using the following equation:

$$\mu_{\text{eff}} = \frac{l_T l_D}{U}\left(\frac{1}{t_{HA}} - \frac{1}{t_{EOF}}\right)$$

In this equation t_{HA} and t_{EOF} are the measured migration times of HA peak and negative peak of electroosmotic flow. The resulting electrophoretic mobilities μ and peak areas are average values from three consecutive electropherograms.

2.3. Carbazole reaction

For photometric determination of HA the modified uronic acid carbazole reaction of Bitter et al. [12] based on DISCHE's carbazole reaction [15] was used.

2.3.1. Reagents

1. 0.025 M sodium tetraborate·10 H_2O in sulphuric acid
2. 0.125% carbazole in absolute ethanol (W/W)
3. HA standards in distilled water

2.3.1.1. Procedure.

1. 3 ml of A placed in tubes were cooled to 4°C,
2. 0.5 ml of C or sample were carefully layered above the acid,
3. Closed tubes were shaken gently at first and then vigorously by constant cooling,
4. Tubes were then heated for 10 min in boiling water bath and cooled to room temperature,
5. 0.1 ml of B was added and the tubes were shaken again,
6. Tubes were heated for 15 min in a boiling water bath, cooled down to room temperature and
7. Absorption of samples was measured at 530 nm in 1 cm cell against blank (distilled water instead of reagent C).

Steps (1)–(4) serve to cut the HA into monomers. The following steps include the reaction of glucuronic acid with carbazole, resulting in a violet product. The colour is stable for at least 16 h. A Spectronic 601 spectrophotometer (Milton Roy, Ivyland, PA, USA) was used to measure the absorption.

3. Results and discussion

The following calibration curve was obtained by photometric determination of HA standards:

Table 2
Electrophoretic mobilities of HA in different buffer solutions

Buffer, pH value	Mobility of HA (cm^2/V s)	RSD (%)	N
Phosphate 20 mM, 6.0	-3.118×10^{-4}	1.429	14
Phosphate 20 mM, 7.4	-3.118×10^{-4}	0.553	25
Borate, 8.8	-3.317×10^{-4}	1.572	10
Tris, 7.5	-2.514×10^{-4}	0.895	10
Tris, 8.0	-2.664×10^{-4}	0.834	10
Tris, 8.5	-2.932×10^{-4}	0.997	10
Tris, 9.0	-3.621×10^{-4}	0.438	10

$$E = 0.000468c - 0.0228 \quad (R = 0.996)$$

where E is the measured absorption and c is the concentration of HA (μg/ml).The reaction is sensitive for uronic acids and glucose. If there are other carbohydrates in the formulations the determination of HA could be influenced. As Bitter et al. described the photometric detection of HA can be suffered from impurities such as potassium, sodium and ammonium chloride. These substances lower the intensity of the colour.

Before quantification of the HA using the method of capillary electrophoresis the electrophoretic behaviour of HA in several buffer systems were tested. The electrophoretic mobility, μ_D, of a molecule in free solution is proportional to its electrical charge, q, and inversely proportional to the hydrodynamic radius, r, determined by molecular mass, M. The HA is in anionic form at pH above 6. The calculated mobilities summarised in Table 2 show only slight differences by comparing the used pH values. The increase in buffer pH causes an increase in negative mobility of HA because of the dissociation of the carboxyl groups and reduced protonation of amine groups. An increase in negative net charge of HA results. A 20 mM phosphate buffer pH 7.4 turned out to be best suitable with regard to peak shape and analysis time and was used in further experiments. Using HA standards the following calibration curve was obtained for CE experiment (A represents the obtained peak area)

$$A = 417.608c + 6.959 \quad (R = 0.999)$$

The amounts of HA in standard gels and ointments found with both methods are compared in Table 3. The recovery rates obtained with CE are significantly higher than those obtained by photometric analysis. This result was ascribed to a higher robustness against matrix effects caused by components of the ointments. The electropherograms of diluted ointments (blind sample), and the additives propylene glycol and glycerol, show some peaks around the EOF but no peaks at migration time of the HA. The separation of HA from all other components of ointments is demonstrated in Fig. 1 (absolute injected amounts of HA were estimated using the plug volume (5.94 nl (Hewlett Packard data) using 250 mbar s injection)). Other carbohydrates cannot influence the detection of HA because of having different migration times. Most pharmaceutically used carbohydrates are uncharged at the chosen pH value and would be detected at EOF. Another advantage of CE is the possible simultaneous determination of p-hydroxybenzoic acid used as preservative ($\mu = -2{,}9499\times10^{-4} \pm 3.235\times10^{-6}$ cm^2/V s) which can be used as internal standard. As expected, there is a general decrease in recovery rates by decreasing amounts of HA in the ointments. This phenomenon is caused by the simple extraction method. Little amounts of HA can be included in lipid components. This effect seems to be more significant in Ungt. alc. lanae samples than using Basiscreme as ointment.

Enzymatic digested hyaluronan (MW_{avg} from 223 000 to 1 000 000) was also used. The mobility of HA at a given pH value didn't change because of the constant charge to mass ratio. The peaks of digested HA became a bit broader caused by the variation in hydrodynamic radius of the molecules (data not shown).

A comparison of the analytical parameters obtained by the two methods shows that the

Table 3
Amounts of HA found by photometric analysis and CE

Gel	Addition of ointment (%)	Theoretical amount HA (%)	Photometric determination		CE	
			Measured amount (%)	Recovery rate (%)	Measured amount (%)	Recovery rate (%)
HA I	–	3	3.18 ± 0.11	106.0	3.14 ± 0.12	104.8
HA II	–	3	1.62 ± 0.09	54.0	3.14 ± 0.25	104.6
HA III	–	3	2.92 ± 0.19	97.3	2.95 ± 0.26	99.8
HA I	BC[a](20	2.4	0.94 ± 0.02	39.2	2.52 ± 0.16	105.0
	BC 50	1.5	0.28 ± 0.04	18.6	1.34 ± 0.07	89.1
	BC 80	0.6	0.04 ± 0.02	6.6	0.47 ± 0.03	77.2
HA II	BC 20	2.4	0.73 ± 0.04	30.4	2.41 ± 0.22	100.5
	BC 50	1.5	0.35 ± 0.03	23.3	1.27 ± 0.04	84.4
	BC 80	0.6	0.13 ± 0.09	21.7	0.51 ± 0.07	84.7
HA III	BC 20	2.4	0.29 ± 0.02	12.1	2.48 ± 0.14	103.6
	BC 50	1.5	0.14 ± 0.02	9.3	1.58 ± 0.14	105.6
	BC 80	0.6	0.04 ± 0.02	6.7	0.40 ± 0.06	66.9
HA I	UL[b](20	2.4	1.64 ± 0.26	68.3	2.62 ± 0.21	109.1
	UL 50	1.5	1.24 ± 0.11	82.7	0.98 ± 0.17	65.4
	UL 80	0.6	0.13 ± 0.06	21.7	0.45 ± 0.09	75.0
HA II	UL 20	2.4	1.63 ± 0.18	67.9	1.87 ± 0.03	78.1
	UL 50	1.5	1.13 ± 0.04	75.3	1.03 ± 0.15	68.5
	UL 80	0.6	0.16 ± 0.04	26.7	0.25 ± 0.07	42.4
HA III	UL 20	2.4	1.71 ± 0.16	71.3	1.56 ± 0.33	65.0
	UL 50	1.5	0.65 ± 0.10	43.3	0.87 ± 0.17	57.7
	UL 80	0.6	0.07 ± 0.02	11.7	0.29 ± 0.08	47.5

[a] Basiscreme DAC.
[b] Unguentum alcoholum lanae SR 90.

detection limit of photometric method is better than that of CE. Using a signal to noise ratio of 5 the detection limit of HA is 10 μg/ml. Such a low detection limit was supported by the use of the bubble detection cell. The detection limit of photometric method is 7.5 μg/ml. The relative standard deviation of CE method is twofold better than that of photometric determination (9.8 vs. 18.4%). Very good reproducibility of the migration times (RSD < 1.5%) and peak areas (RSD < 3%) of HA was obtained for both ointments using CE.

4. Conclusions

In this study a simple but reliable CE method has been developed to determine the amount of HA in pharmaceutical formulations. Additional components are separated by this method and a simultaneous quantification is possible. The photometric method is advantageous for the quantification of HA in aqueous solution. Under the conditions of semisolid formulations it does not reach the robustness of CE because of interactions with the ointment matrix. CE is shown to

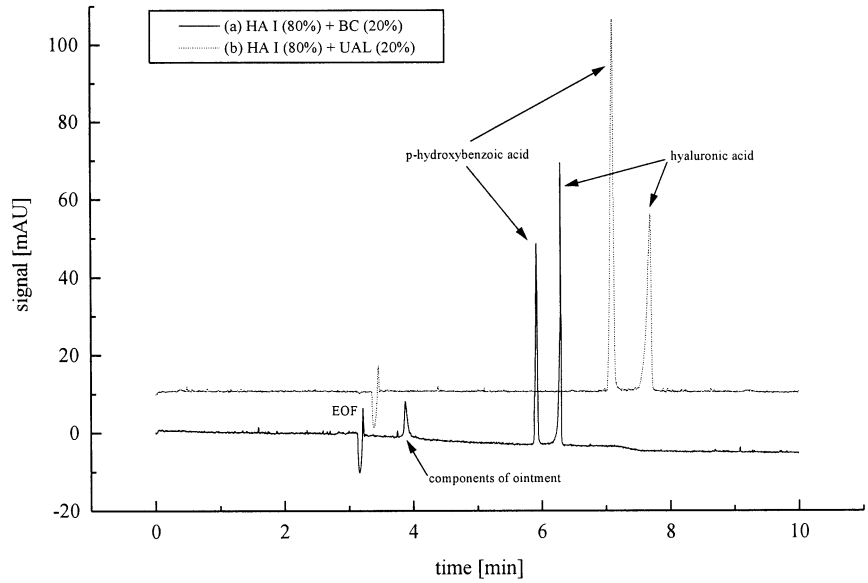

Fig. 1. Electropherogram of ointments including HA. 20 mM phosphate buffer (pH 7.4); $\lambda = 195$ nm; other conditions as specified before; (absolute injected amount of HA was 1.19 ng in (a) and 2.71 ng in (b))

be an attractive tool for the analysis of pharmaceuticals because of their high separation efficiency, easy operation and low running cost.

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