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New approaches for quantifying hyaluronic acid in pharmaceutical semisolid formulations using HPLC and CZE

Jamal Alyoussef Alkrad, Yahya Merstani *, Reinhard H.H. Neubert

Institute of Pharmaceutics and Biopharmaceutics, Martin-Luther-University Halle-Wittenberg, Wolfgang-Langenbeck-Strasse 4, 06120 Halle/S., Germany

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Abstract

HA was quantified in pharmaceutical formulations using HPLC–UV-detector and spectrophotometrically after digestion with concentrated H_2SO_4. Intact HA was quantified by capillary zone electrophoresis (CZE) using direct and indirect methods. The results were compared with the carbazole reaction established by Bitter et al. (Anal. Biochem. 4 (1997) 330) and with established method from Pläzer et al. (J. Pharm. Biomed. Anal. 21 (1997) 491) regarding detection limits, linearity, reproducibility and simplicity. The present results show that the investigation using HPLC and CZE led to a considerable improvement of the detection limit [0.3 ng/ml (HPLC1), 1 μg/ml (HPLC2) and 5 μg/ml (CE-D1)] compared with other methods (10 μg/ml).

Keywords: Hyaluronic acid; HPLC; CZE; Pharmaceutical semisolid formulations

1. Introduction

Hyaluronic acid was isolated for the first time from vitreous humor of the eyes by Meher in 1934 [1]. It is a linear polysaccharide formed from disaccharide units containing *N*-acetylglcosamine and glucoronic acid. Its molecular weight is usually in the order of 10^6–10^7 [2]. In aqueous solution, HA has a secondary structure because of hydrogen bonding (Fig. 1), and it behaves as an expanded random coil with a diameter of 500 nm. The chain is entangled at concentrations in the order of 1 g/l [3]. HA is distributed in the body, in the skin, vitreous humor, cartilage and synovial fluid [1–3]. It is used as a diagnostic factor for many diseases such as tumor, rheumatoid arthritis and liver diseases [4]. HA is also used in ophthalmological and ontological operations and in skin care [5]. It is, therefore, important to quantify HA in the body as well as in pharmaceutical formulations some times with low concentration as by studying the HA pharmacokintics. HA as glucoseamineglycan (GAG) is usually enzymatically digested and can be detected with HPLC or

* Corresponding author. Tel.: +49-345-552-5108; fax: +49-345-552-7292
E-mail address: mrestani@pharmazie.uni-halle.de (Y. Merstani).

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HPCE as complex with Cu^{2+} with UV-detector at a wavelength of 235 nm [6] or as a complex with 2-aminopyridine, 2-aminoacridone or 1,3-naphtlendisolfon acid in the presence of cyanoborhydrid with fluorescence detector [7–10] or directly with UV-detector, HPLC [11] HPCE [12], or GFC–MS, HPLC–MS, HPCE–MS [13–16]. HA was quantified as a complex with carbazole at a wavelength of 530 nm after being digested with concentrated H_2SO_4 and tetrahydroborate [17]. Intact HA was also measured with HPCE with detection limit of 10 μg/ml [18]. In this study, an attempt was made to find new method cheaper and simple regarding to the limited detection and reproducibility and sensitivity. Due to its high molecular weight, it is impossible to run HA through HPLC-column without degradation. Since enzymatic degradation of HA is expensive and time consuming, in this study it was desired to investigate the possibility of using sulfuric acid for the degradation of HA instead of hyluronidase. In this study new analytical approaches have been developed to quantify HA with HPLC and capillary zone electrophoresis (CZE) directly and indirectly. The results have been compared with terms of linearity, reproducibility and detection limits with other methods, namely, modified carbazole reaction, HPLC and CZE after enzymatic digestion. These methods were also used to determine amounts of HA in pharmaceutical semisolid formulations (SF).

2. Experimental

2.1. Apparatus

2.1.1. HPLC

A chromatography LC-10 AD SCHIMADZU, (Schimadzu, Kyoto, Japan) coupled with UV Spectrophotometric Detector: SPD-10 A SCHIMADZU (Schimadzu) was employed. The substances were separated with column1, Nucleosil-100 C 18 (VERTX Column 4 mm ID) (Packing Material 5 μm) (Knauer, Berlin, Germany) and with column 2, LichroCHRT® 125-4, Lichrospher 100 RP-8 (LiChroCART 125-4) (5 μm) (Merck, Darmstadt, Germany).

2.1.2. CZE

Capillary electrophoresis experiments were performed on a Hewlett–Packard Model G1600A (Waldbronn, Germany) ^{3D} CE system. A standard capillary (fused-silica) and a capillary with bubble cell (optical path length is 150 μm) from Hewlett–Packard (Waldbronn, Germany) were used for the determination of HA.

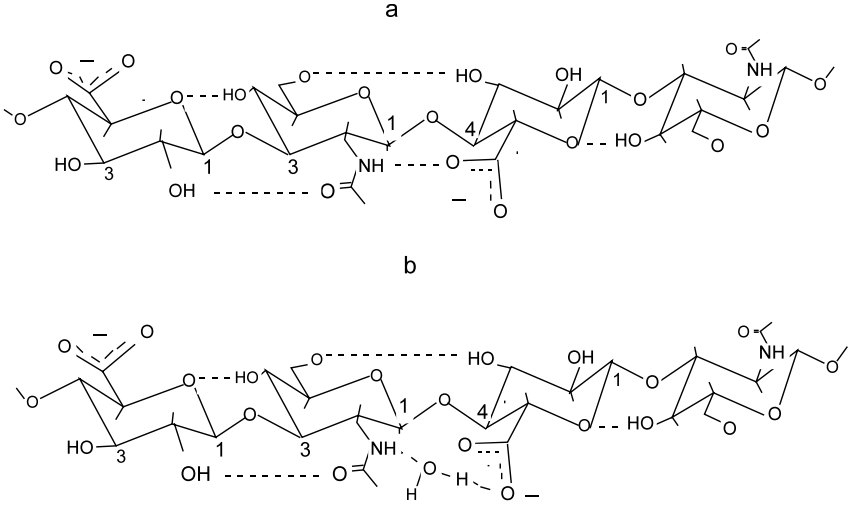

Fig. 1. The secondary structure of hyaluronic acid in (a) dimethyl sulfoxide and (b) dimethyl sulfoxide containing water.

Table 1
Composition of the SF

SF	Concentrations of HA and the corresponding formulations		
	0.5%	2%	5%
Water containing hydrophilic ointment	1	2	3
Amphipilic cream	4	5	6
Water containing wool wax alcohol ointment	7	8	9

2.1.3. UV-spectrophotometer

UV-120-02 Photometer (Schimadzu) was used for the determination of HA.

2.2. Materials

HA1200 and 31 kDa were obtained from Hans-Knöll-Institut (Jena, Germany). Sulfuric acid, sodiumborate decahydrate and carbazole were obtained from Fluka (Buchs, Switzerland). Methanol, acetonitril were obtained from (J-T-Baker, Deventer, Netherlands). NaOH: Roth (Karlsruhe, Germany). Dipotassium phosphate, monopotassium phosphate and ascorbic acid were obtained from Merk (Darmstadt, Germany). Basis creme DAC was obtained from Wasserfuhr (Bonn, Germany).

2.3. Solutions preparation

2.3.1. Phosphate buffer preparation

A 40 mM phosphate buffer solution (pH 7.7) was prepared by dissolving 63.03 g potassium hydrogenphosphate and 5.13 g potassium dihydrogenphosphate in water, filling up to a volume of 1000 ml. The pH of the buffer was measured at 25 °C using a HI 9321 microprocessor pH meter (Hana-Instrument).

2.3.2. HPLC solutions preparation

2.3.2.1. Water:methanol 96:4 (v/v). The mobile phase was fresh prepared by adding 40 ml of HPLC-grade methanol to 960 ml of discharged water. This solution was degassed with ultrasound for 30 min and kept in a tightly closed bottle.

2.3.2.2. Water:acetonitrile: (96:4) (v/v). Water:acetonitrile was prepared similar to Section 2.3.2.1.

2.3.3. Preparation of HA

Stocks solutions of either 1200 and 31 kDa-freeze-dried HA were prepared by dissolving 150 mg of HA in 100 ml distilled water. The solutions were kept in refrigerator at 4 °C over the night (to make homogenate solution). Afterwards dilutions in the range of 1–1500 μg/ml were prepared using distilled water. The samples should be kept in the refrigerator at 4 °C (stable for 2 weeks). By room temperature they are stable for 3 days (bacterial growth).

2.3.4. Semisolid formulations preparation

Table 1 summarizes the SF preparation.

2.4. Methods

2.4.1. HA determination by modified carbazole method (MCM)

The samples were prepared using the method described by Bitter et al. [17].

2.4.2. HA determination by HPLC method (HPLC1)

The series of solutions from HA1200 kDa were dried in an autoclave at 100 °C for an hour. Then each sample was dissolved in 0.25 ml concentrated H_2SO_4 for 10 min. The samples were kept for 5 min at room temperature and then adjusted to pH 5 with 1 N NaOH using pH-meter. For the measurements of HA the column1 was used. The mobile phase, water/methanol 96:4 (v/v) was run at a flow rate of 0.8 ml/min. Detection was done at 280 nm.

2.4.3. Spectrophotometric determination of HA

The samples were obtained similar to HPLC1 (Section 2.4.2) and were measured using UV-spectrophotometry (UV-120-02 Photometer, Schimadzu) at a wavelength of 280 nm.

2.4.4. HA determination by HPLC-method (HPLC2)

The series of solutions of enzymatically digested HA31 kDa were injected directly into the column 2. The mobile phase, water/acetonitrile 96:4 (v:v) was run at a flow rate of 0.5 ml/min. Detection was carried out at a wavelength of 195 nm.

2.4.5. CZE-methods

2.4.5.1. HA determination by direct methods

HA determination using CZE (CZE-D1). The series of aqueous solution of HA1200 kDa was injected into a capillary with a total length of 65 cm (length to detector 56 cm) and internal diameter of 50 μm with a 150 μm extended light path (bubble cell). The samples were injected at 50 m bar pressure for 10 s (hydrodynamic injection). Running phosphate buffer (40 mM; pH 7.7) and a separation potential of 22 kV were used. Detection was carried out at a wavelength of 194 nm.

HA determination using CZE (CZE-D2). HA samples were measured similar to Section 2.4.5.1. using standard capillary with window detection (length of 48.5/40 cm, internal diameter of 50 μm). Separation potential of 12 kV were used.

HA determination using CZE (CZE-D3). A 40 mM phosphate buffer (pH 6.2), separation potential of 22 kV, injection time of 30 min and a bubble capillary (optical path length is 150 μm) were used for the determination of HA (31 kDa).

2.4.5.2. HA determination by indirect CZE method (CZE-IN). HA1200 was measured indirectly using ascorbic acid at a wavelength of 250 nm. A 40 mM phosphate buffer (pH 7.7), 10 mM ascorbic acid, separation potential of 13 kV and standard capillary (total length of 65 cm, internal diameter of 50 μm) were used for the measurements.

2.5. Determination of 1200 kDa HA in ointments and creams

HA1200 kDa was incorporated in different SF, namely, water containing hydrophilic ointment and amphipilic cream and water containing wool wax alcohol ointment [19], in concentrations of 0.5, 2 and 5% HA. The various formulations are numbered from 1 to 9 as shown in Table 1.

In the quantification of HA from each SF, 100 mg of each semisolid containing HA was taken and extracted as follows: 100 mg of the sample was dissolved in 5 ml hot distilled water at 90 °C. Upon separation, the lipophilic phase was extracted with 10 ml ether while the aqueous phase was diluted to 10 ml of distilled water. 0.5 ml of the diluted aqueous phase was measured with MCM. 5 ml of the aqueous phase was dried in the autoclave for 1 h and measured with HPLC1. Finally, 1 ml of the same phase was measured using HPCE equipped with bubble capillary.

3. Results

3.1. Quantification of HA using HPLC

The measurement of HA digested with sulfuric acid through HPLC showed a good linearity, ($Y = -0.072 + 0.0314X$, $r = 0.9998$) for concentrations ranging from 5 to 1500 μg/ml versus area under the curve. The method also showed good reproducibility with a procedure standard deviation (S.D.) of 5.98 μg/ml and a detection limit of 1 μg/ml. In the measurement of enzymatically digested HA (Fig. 2), HPLC showed good linearity ($Y = 1.65 + 1.66X$, $r = 0.9998$) for concentrations ranging from 10 to 100 μg/ml, low procedure S.D. of 0.58 μg/ml, and very good detection limit of 0.3 ng/ml.

3.2. Quantification of HA by MCM

The photometric measurement for digested HA with sulfuric acid after reaction with carbazole exhibited linear relationship between concentration ranging from 10 to 350 μg/ml and color intensity, $Y = 0.158 + 0.0035X$ ($r = 0.9972$). A high

procedure S.D. of 36 μg/ml and poor reproducibility was observed. The detection limit was 10 μg/ml.

3.3. Quantification of HA by photometric method (NPM)

The photometric measurement of digested HA using sulfuric acid directly at 260 nm showed linearity for concentrations ranging from 5 to 1500 μg/ml, $Y = 0.0187 + 0.0013X$ ($r = 0.9972$). The procedure S.D. was high, 7 μg/ml which is an indication of poor reproducibility. The detection limit of the method was 5 μg/ml.

3.4. Quantification of HA using CZE (direct method)

In order to overcome the disadvantages associated with digestion, HA1200 kDa was directly measured by capillary electrophoresis with standard capillary. HA showed negative electrophoretic mobility ($\mu_e = -2.3 \pm 0.0126$ cm^2/V per s) migrating in the direction of the cathode (Fig. 3). HA also showed an increase in mobility with decreased pH of the phosphate buffer used. In this method, the detection limit was 30 μg/ml.

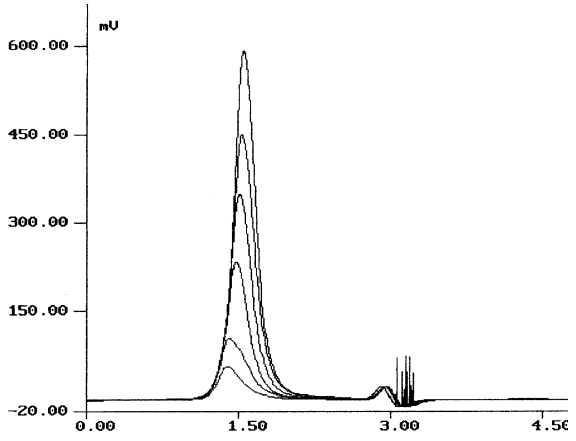

Fig. 2. Chromatogram of enzymatically digested HA using column RP-8; water/acetonitrile 96:4; flow rate: 0.5 ml/min; detection, 194 nm; temperature, 25 °C.

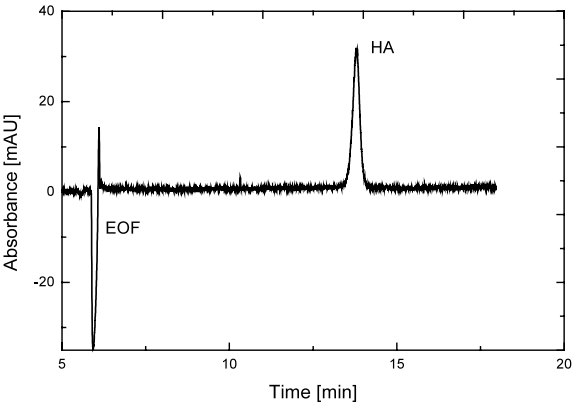

Fig. 3. Electrophorogram of HA using 40 mM phosphate buffer (pH 7.7); detection, 194 nm; field strength, 22 kV; a capillary with bubble cell, 64.5 (56 cm to detector) X 50 μm i.d. (optical path length is 150 μm); temperature, 25 °C.

Linearity was observed for concentrations of HA ranging from 30 to 500 μg/ml, $Y = 10.192 + 2.006X$ ($r = 0.998$) with a procedure S.D. of 4.55 μg/ml. To improve the detection limit of undigested HA the bubble capillary was used. Thus, it was possible to reach the detection limit as low as 5 μg/ml maintaining the linear relationship of HA concentration ranging from 12.5 to 500 μg/ml versus mAU, $Y = 0.14108 + 1.315X$ ($r = 0.9880$). The procedure S.D. was 2.33 μg/ml. For comparison purposes, enzymatically digested HA was also measured with bubble capillary. HA showed linearity for the concentrations ranging from 10 to 500 μg/ml, $Y = 32.18 + 5.879X$ ($r = 0.9994$) and procedure S.D. of 5.42 μg/ml. The lowest detection limit was 10 μg/ml.

3.5. Quantification of HA using CZE (indirect method)

An attempt was also made to improve the detection limit of CZE by measuring HA indirectly using UV active ascorbic acid. Although an acceptable linearity for concentrations ranging from 25 to 500 μg/ml, $Y = -27.9 + 2.12X$ ($r = 0.9033$) and a detection limit of 30 μg/ml was obtained, a high procedure S.D. of 14.41 μg/ml was also observed in this measurement.

Table 2
Percent of HA determined from different formulations using different methods

Formulations	Recovery rate (%)		
	MCM	HPLC1	CE-D2
1	9.8	94.4	86.9
2	7.0	85.0	89.4
3	45.8	91.0	95.4
4	–	105.0	83.2
5	–	83.2	76.4
6	–	93.3	97.0
7	73.0	85.4	92.8
8	104.3	87.5	83.5
9	74.4	86.5	89.9

3.6. Determination of HA from semisolid formulations

To determine HA in pharmaceutical SF, various concentrations of HA were incorporated in three different systems and then extracted. The extracts were analyzed with four methods, namely, MCM, HPCE (bubble capillary), HPLC1 and photometric method. The measurement made with photometric method showed large scattered results due to the contamination of the system particles. As shown in Table 2, the precision of MCM for the determination of HA is less in comparison with other methods (Table 2).

4. Discussion and conclusions

All measurements were done in triplicate and the results were analyzed using ORIGIN Program. The detection limits were calculated according to Weinberger [20]. The determination of HA concentration in pharmaceutical formulations plays an important role in pharmaceutics. In this study HPLC and HPCE methods have been developed to determine the amount of HA in pharmaceutical SF. Table 3 summarizes the estimated quantitative analytical parameters of HA.

HPLC exhibited low procedure S.D. and good detection limits particularly in measuring enzymatically digested HA. However, digesting HA enzymatically is costly and time taking. The use of H_2SO_4 to degrade HA is, therefore, a good alternative in terms of cost and simplicity. In addition, it is not specific as an enzyme and, hence, can be used to digest other GAG such as heparin.

CZE was found to offer the best opportunity in quantifying undigested HA. It allows high resolution separation. Moreover, the low amount of sample required and the relatively short analysis time are the main advantages of using CZE method. By increasing the concentration of the phosphate buffer from 20 mM used by Plätzer et al. [18] to 40 mM, it was possible to improve the detection limit from 10 to 5 μg/ml. The problem with photometric measurements was well described by Bitter et al. [17]. In our study it was

Table 3
Analytical parameters for the determination of HA

Method	LOD	Sxo ± μg/ml	*R*	Range
HPLC1	1 μg/ml	5.98	0.9998	5–1500 μg/ml
HPLC2	0.3 ng/ml	0.52	0.9998	10–100 μg/ml
		0.81	0.9978	1–10 μg/ml
		0.66 ng/ml	0.9975	0.05–10 μg/ml
		0.42 ng/ml	0.8759	1–100 ng/ml
MCM	10 μg/ml	36.75	0.9972	10–350 μg/ml
NPM	5 μg/ml	7.061	0.9953	5–1500 μg/ml
CE- DI1	5 μg/ml	2.33	0.9880	5–500 μg/ml
CE- DI2	30 μg/ml	4.55	0.9981	30–500 μg/ml
CE- DI3	10 μg/ml	5.42	0.9994	10–500 μg/ml
CE-IN	30 μg/ml	14.41	0.9033	25–500 μg/ml
CE [18]	10 μg/ml		0.996	100–5000 μg/ml

Sx_o, procedure standard deviation; *R*, linearity; LOD, limits of detection.

also observed that because of the interaction of HA with the ointment matrix, separations were poor with measurements done by photometric and MCMs. Hence, it is difficult to use them to quantify HA in pharmaceutical SF. In conclusion, the precision of MCM in quantifying HA in pharmaceutical SF is less as compared with both HPLC and CZE methods. In the determination of undigested HA, CZE is the best method but HPLC produced better measurement results than CZE in determining digested HA.

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