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Determination of hyaluronic acid in pharmaceutical products by spectrophotometry and HPLC coupled to fluorescence or mass spectrometric detection

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In a daily basis, the pharmaceutic and cosmetic industry makes effort in advertising their products with a promise to avoid diseases or health problems. Among one of the most popular substances is nowadays, without doubts, hyaluronic acid (HA). Due to its natural origin allowing its biodegradability and non-toxicity, HA is widely used as a component of joint supplements or as a substance ensuring moisturizing properties. Here, interest is focused on the determination of hyaluronic acid in commonly available non-prescription products using different analytical techniques followed by comparison of the determined content with that declared by manufacturers.

Keywords: Hyaluronic acid; Supplements; High-performance liquid chromatography; Spectrophotometry; Mass spectrometry

Introduction

In recent years, great attention is focused on hyaluronic acid (HA), a natural polysaccharide which is often used in pharmaceutical or cosmetic products. Its importance is given by its natural origin, biodegradability, immunosuppressive

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and moisturizing properties. Due to a very readily binding of water, hyaluronic acid is beneficial in products with lubrication or space-filling function. Today, HA is used as joint supplements, eye drops, nose spray, shampoos, face creams and not only in other products so-called "anti-aging" [1–3]. The structure of HA is built up by disaccharide units connected to polysaccharide chains up to 8000 kDa which is called a high-molecular weight HA (HMW-HA). This type of HA has an immunosuppressive effect, whereas the lower-molecular weight has the opposite effect leading to activation of the immune system [4–7].

Determination of hyaluronic acid can be performed using different techniques, e.g. planar electromigration separation (gel electrophoresis), capillary electrophoresis (CE) or high-performance liquid chromatography (HPLC). For detection, modern instrumental tools, such as highly sensitive fluorescence detection (FL) or highly specific mass spectrometric (MS) detection are frequently used [8–11].

This work is focused on optimization of the methods suitable for determination and quantification of HA in selected pharmaceutical products. For this purpose, the spectrophotometric technique and HPLC coupled with fluorescence and mass spectrometric detection were selected and the determined amount of HA compared to that declared by manufacturer(s).

Materials and methods

Low-molecular weight hyaluronic acid in tablets (Hepatica, Poland), nose drops (Hysan®; Ursapharm, Germany), nose spray (Olynth® HA, 0.1 %; Ursapharm, Germany) and eye drops (Hyal-Drop® multi) were purchased in a local pharmacy. Separation column Ascentis Express C18 (150 × 3 mm; 2.7 μm particles, Supelco, USA), high-molecular weight hyaluronic acid from *Streptococcus equi* (HMW-HA, 1.5–1.8 MDa), bacterial hyaluronan lyase, 2-aminoacridon, cyanoborohydride, dimethylsulfoxid were purchased from Sigma-Aldrich (USA). Hyaluronic acid (30 kDa) was produced by Contipro (Czech Republic). All other reagents were of reagent grade.

Spectrophotometric assay

HA standards, as well as real samples, were enzymatically fragmented before further analysis. For this reason, hyaluronan lyase immobilized onto magnetic macroporous bead cellulose was used (with enzyme activity 216 mU mL^{-1}, settled beads). Hyaluronic acid of the HMW-HA type (1.5–1.8 MDa) was fragmented in a ratio of 225 μg of HMW-HA to 20 μL (equal to 4.3 mU) of applied settled magnetic beads with the enzyme. The total reaction volume was 1 mL, all reagents were dissolved in 0.1 M phosphate buffer (pH 7.0).

After fragmentation of HA, the product of enzymatic cleavage was monitored using UV spectrophotometer (model "Libra S22"; Biochrom, UK) at 230 nm, which corresponds to the absorption maximum of the detected unsaturated bond at the produced HA fragments. For quantification, the calibration curve was measured by fragmentation of high-molecular weight HA (1.5–1.8 MDa).

Derivatization of hyaluronic acid fragments

Hyaluronic acid (30 kDa, 5 mg mL^{-1}) or pharmaceutical products were fragmented with soluble bacterial hyaluronan lyase (5 U, corresponding to 104.7 μL). Total fragmentation of substrate was verified by repeating the spectrophotometric measurements in time (with sufficient period: 4 min). Calibration points were prepared by aliquoting the different volumes (0.5–14 μL) from this solution. The solutions were evaporated by nitrogen to dryness at 30 °C for 45 min. and derivatized. This operation was performed according to the published protocol with some slight modifications (for details, see [12]). 10 μL of 2-aminoacridon — i.e.: 0.1 M solution in acetic acid and dimethylsulfoxide in a ratio 3:17 (v/v) — was used as derivatization agent, vortexed properly and after 15 min incubation at room temperature, 10 μL 1 M sodium cyanoborohydride solution was added, again vortexed and the resultant mixture centrifugated for 2 min. (at 14000 rpm). After derivatization (for 4.5 hours at 45 °C), the samples were diluted by 50 % dimethylsulfoxide to the final volume of 0.5 ml and analyzed by HPLC.

HPLC and HPLC-MS

The HPLC system equipped with a LC-30AD binary gradient pump, a DGU-20 degassing unit A, a RF-20A XS fluorescence detector (all Shimadzu; Kyoto, Japan) and a LCO 102 single thermostat column (Ecom, Prague) and a six-port valve with external loop (5 μL; Valco-Vici, Schenkon, Switzerland) was used to analyse the derivatized HA fragments. For HPLC-MS analysis, the HPLC system equipped with a LC-20AD binary gradient pump, a DGU-20 degassing unit A, a SIL-20A autosampler (all Shimadzu, Kyoto, Japan) and a LCO 102 Single thermostat column (Ecom, Prague) was coupled to a QTRAP 4500 mass spectrometer (AB SCIEX, USA).

The separation was performed on Ascentis Express C18 (150 mm × 3 mm i.d. and 2.7 μm particle size) at 30 °C. The mobile phase consisted of 60 mM ammonium acetate, pH 5.6 (A) and acetonitrile (B), when the isocratic elution of 20 % B at a flow rate of 0.5 mL min^{-1} was used. The wavelengths for fluorescence detection were λ_{ex} = 428 nm and λ_{em} = 525 nm. The conditions of MS analysis in the negative-ion mode were as follows: declustering potential: –100 V, entrance potential: –10 V, curtain gas: 20 psi, temperature: 500 °C, ion source gas 1: 50 psi,

ion source gas 2: 50 psi, ion spray voltage: –4500 V. Selected reaction monitoring mode (m/z: 571.9–208.9) at collision energy of –45 V and collision cell exit potential of –8 V with the dwell time of 100 ms was used for quantification.

Results and discussion

Hyaluronic acid (HA) standards and samples of pharmaceutical products underwent the fragmentation by specific bacterial hyaluronan lyase to achieve fragments for further analysis by spectrometry assay or HPLC methods. Each sample was prepared in a ratio of 225 µg HA and 20 µL of settled magnetic beads (enzyme activity 216 mU mL^{-1} settled beads) in total volume of 1 mL (0.1 M phosphate buffer was used as solvent). Fragmentation efficiency of the whole samples was confirmed by monitoring of fragmentation absorption spectra in an interval of 190–300 nm. Enzymatic fragmentation allows easy detection at 230 nm due to the formation of HA fragments with unsaturated end bonds. The absorption spectra of samples after fragmentation were compared with the spectra of HMW-HA and low-molecular weight (30 kDa HA) hyaluronan standards (Fig. 1). When assuming the complex matrices, pharmaceutical products before fragmentation were used as blanks.

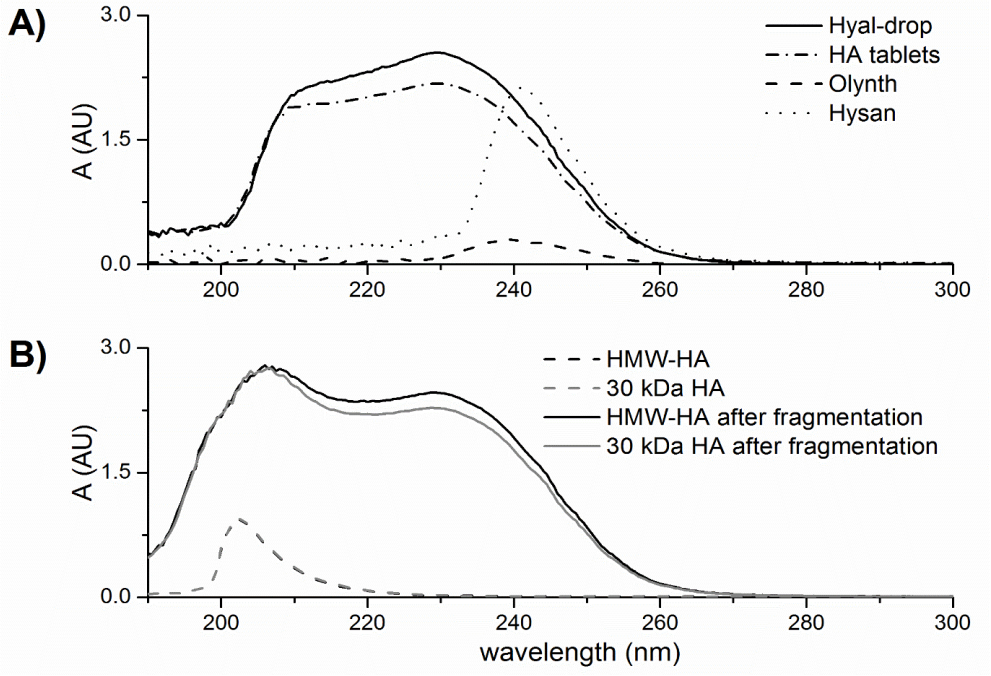

Fig. 1 *Absorption spectra of hyaluronic acid standards and of the samples of pharmaceutical products after fragmentation*

A) Pharmaceutical products; B) Hyaluronic acid standards (HMW-HA – high molecular weight hyaluronic acid in range of 1.5–1.8 MDa, 30 kDa HA).

In case of Hyal-drop® and HA tablets, the fragmentation was performed successfully, in case of Olynth® nose spray and Hysan® nose drops, the fragmentation spectra showed a different trend with a shift in absorption maximum which could be caused by some interaction with additional substances in both pharmaceutical products. Nevertheless, if HA is presented in these both products, it should be fragmented as well.

In case of the spectrophotometric assay, the fragmentation products were prepared from HMW-HA and measured directly without any derivatization step. HPLC with fluorescence detection or HPLC coupled with MS was used as an alternative method to quantify the HA fragments. The 30 kDa HA was selected for preparation of the respective calibration curve due to the best formed signals after derivation step with 2-aminoacridon. Using all the methods, the HA pharmaceutical products and standard HA samples were prepared by the same procedure using bacterial hyaluronan lyase for its total fragmentation (with sufficient time of 4 min). This enzyme cleaves the HA down to the unsaturated disaccharides units regardless the initial size of the HA molecule. For HPLC-FL method, the limit of detection was 2.716 ng and limit of quantification 9.053 ng. The mass spectrometric conditions used for quantification of HA had to be first optimized. The declustering potential was optimized for ion m/z 571.9 in the range from –40 V to –200 V. The highest peak area and peak high were obtained with the declustering potential of –100 V (data not shown). Further parameters, the collision energy (CE) on Q2 and collision cell exit potential (CXP) was optimized in the range from –5 V to –55 V and from –7 V to –12 V, respectively. With respect to the maximal intensity of monitoring transitions, the optimal CE was set to –45 V. The fragmentation spectrum of ion m/z 571.9 at optimal collision energy is shown in Fig. 2.

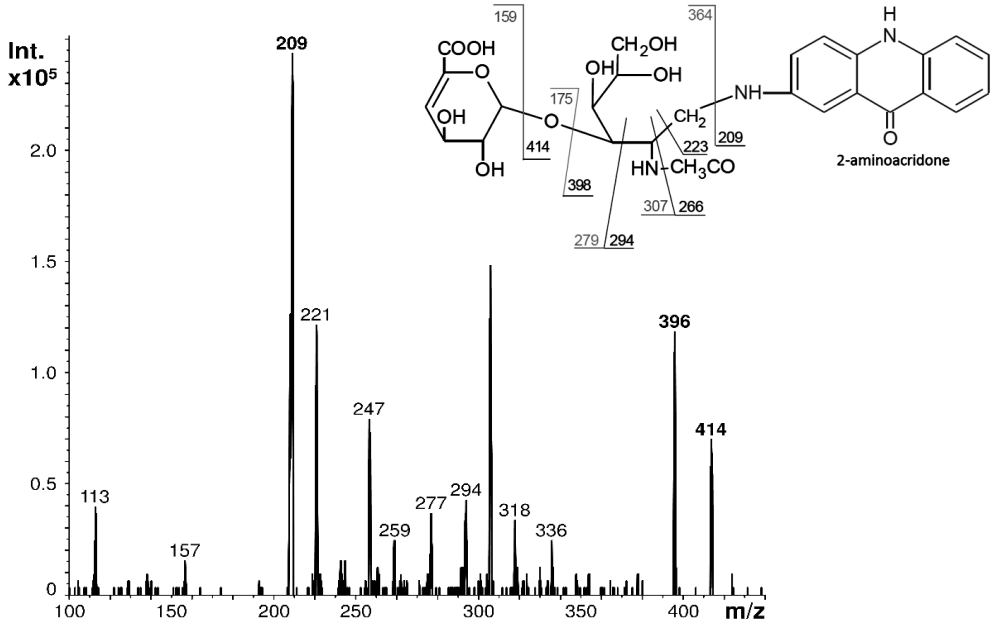

Fig. 2 *Fragmentation mass spectrum of m/z 571.9*

The optimal CXP for transition 571.9–208.9 was –8 V. Apart from quantification transition, the confirmation transition was selected (for m/z 571.9–395.9) to be CE –35 V. Optimized conditions for MS detection are summarized in Table 1; the limit of detection for HPLC-MS technique being 22.44 ng and limit of quantification 74.81 ng.

Table 1 *Determined amount of HA in pharmaceutical products by various methods*

Pharmaceutical product	Spectrophotometry	HPLC-FL	HPLC-MS	Declared amount*
HA tablets	64.27 mg	69.17 mg (SD 2.82)	73.67 mg (SD 2.43)	70 mg per tab.
Hyal-drop®	1.067 mg mL^{-1}	1.116 mg mL^{-1} (SD 0.043)	1.259 mg mL^{-1} (SD 0.057)	2.4 mg mL^{-1}
Hysan®	0.217 mg mL^{-1}	0.273 mg mL^{-1} (SD 0.015)	0.306 mg mL^{-1} (SD 0.028)	0.25 mg mL^{-1}
Olynth®	0.012 %	0.023 % (SD 0.001)	0.0023 % (SD 0.002 %	0.1 % solution

* According to manufacturer

Quantification of HA in pharmaceutical products by all the techniques (spectrophotometry, HPLC-FL, and HPLC-MS) was performed using the calibration curve method (Fig. 3). The content of HA in selected pharmaceutical products determined using all three determinations is summarized in Table 1. All values were compared with the declared content of HA provided by manufacturers of these pharmaceutical products. Evident fluctuations were observed for all the determinations in samples of Hyal-Drop® and Olynth HA®. Here, the determined amount was about 50 % lower (Hyal-Drop®) then that declared by manufacturer or even about 80 % lower in the case of Olynth HA®. Analyses of the samples of HA tablets and Hysan® nose spray revealed almost same or higher amounts than those declared by manufacturer. The biggest fluctuations between the methods used were observed for samples of HA tablets, which could be caused by the complex matrix and content of some excipients. The comparison among the individual techniques and the corresponding methods show that the spectrophotometry has provided the lowest determined amounts of HA in all the samples, which could be due to its lower sensitivity. The highest measured values were obtained using HPLC-MS, which was probably associated with too high specificity of detection. On the other hand, the best sensitivity was observed with HPLC-FL.

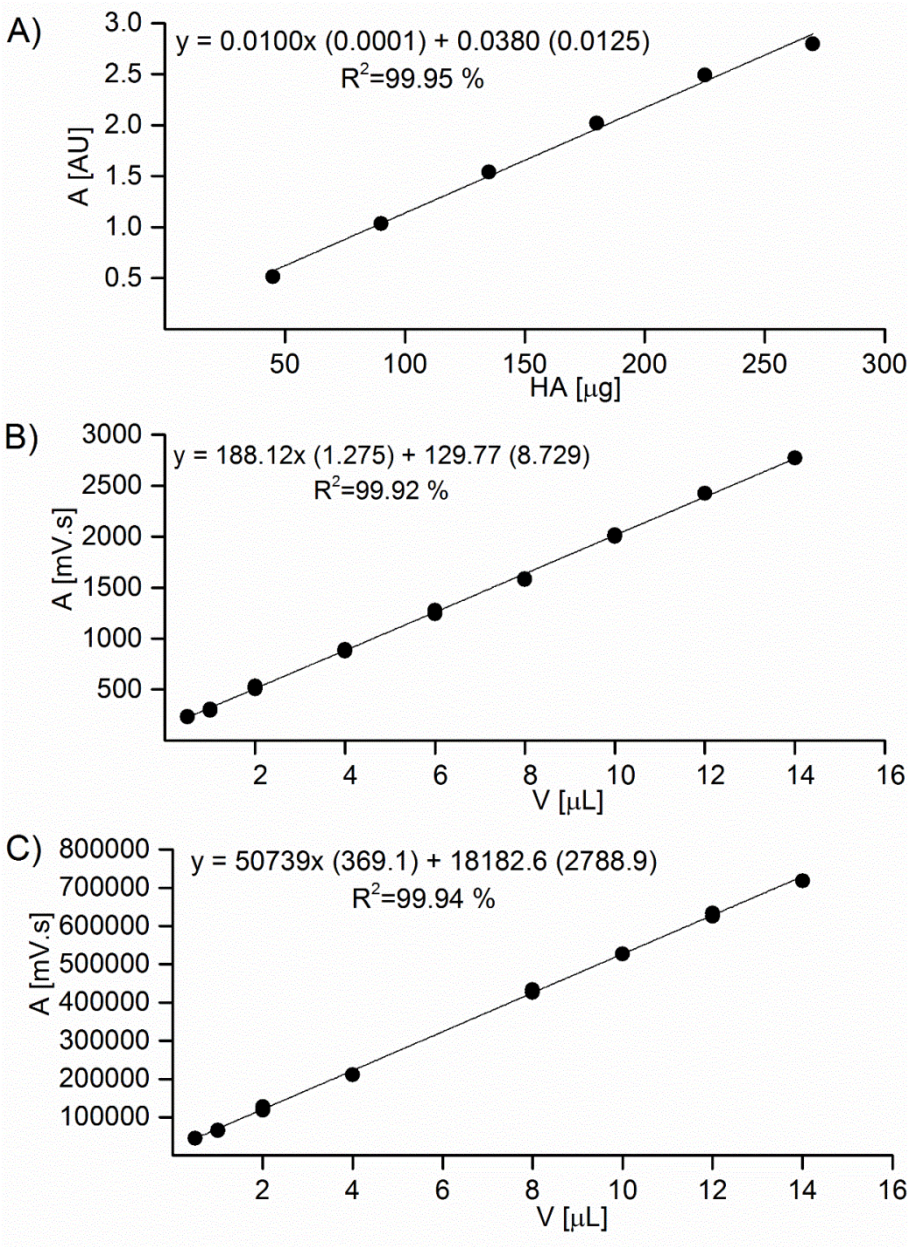

Fig. 3 *Calibration curves for hyaluronic acid quantification*

A) Spectrophotometric measurement (230 nm). Direct measurement without derivatization. Total HA amount range: 50–250 μg, B) HPLC-FL analysis; and C) HPLC-MS analysis of AMAC derivatives, total HA amount range: 1.64–46.95 μg (3.282 μg μL^{-1} HA).

Conclusions

Hyaluronic acid became very interesting in recent years for its moisturizing and immunosuppressive properties to human health. Nowadays, the HA is used mainly as a lubricant or space-filling agent to joint supplements, face creams or eye and nose sprays. This work is focused on the determination of the HA content in commonly available pharmaceutical products and its comparison to the amount of HA declared by manufacturers.

All the samples were firstly treated with specific hyaluronan lyase, an enzyme being used for the specific cleavage of HA substrates. For quantification, the spectrophotometric assay and HPLC were used. The spectrophotometric method allows detection without the necessity of further derivatization. HPLC was used with fluorescence detection or coupled with mass spectrometry using electrospray ionization, both needing the previous derivatization with 2-aminoacridon. In case of Hysan® nose spray and HA tablets, the determined content of HA corresponded to that declared by the manufacturers. In case of Hyal-drop® eye drops, the determined content was almost 2-times lower than the declared value. The most pronounced difference was achieved for Olynth® nose spray, in which the determined amount was just about 20 % of the declared. Highest contents were obtained for all the analyzed samples by the HPLC-MS method.

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