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Comparative studies of HPLC-fluorometry and LC/MS method for the determination of N-acetylneuraminic acid as a marker of deteriorated ophthalmic solutions

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ABSTRACT

Methods for determining the deterioration of ophthalmic solutions using both high-performance liquid chromatography (HPLC) with fluorescence detection and liquid chromatography coupled with selected ion monitoring mass spectrometry (LC/MS) are described. The methods are based on the determination of N-acetylneuraminic acid (NeuAc) released by the hydrolysis of foreign bodies that contaminate eye drops during use. The released NeuAc was either labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB) for fluorometric detection or detected without derivatization by mass spectrometry. The calibration curves for NeuAc showed good linearity between 1.2 ng/mL and 39 ng/mL for fluorometric HPLC and 5.0 ng/mL and 100 ng/mL for LC/MS, respectively. Detection limits for fluorometric HPLC and LC/MS were 0.20 ng/mL and 0.88 ng/mL, respectively. The NeuAc content of some model glycoproteins determined by LC/MS method were 62–78% of those determined by fluorometry. The differences are attributed to matrix effects but the LC/MS method afforded sufficiently high sensitivity that NeuAc in the foreign bodies could be determined in eight of nine test samples.

1. Introduction

N-acetylneuraminic acid (NeuAc) is present at the non-reducing ends of the glycans attached to glycoproteins and glycolipids that are widely distributed in human tissues. NeuAc is also found in several fluids such as serum, urine, saliva, cerebrospinal fluid and breast milk [1].

Both tear fluids and eye mucus materials, agglomerates of the waste products displaced from the surface of the eye, also contain NeuAc [2]. The preocular tear film consists mainly of a mucin-containing gel and the ocular mucins are known to be sialoglycoproteins which contain NeuAc [3,4]. These compounds belong to a family of glycoproteins containing a large number of O-glycosidic carbohydrate chains linked through the hydroxyl groups of Ser/Thr residues [5].

Colorimetric methods such as thiobarbituric acid assay for sialic acids including NeuAc have been used for the analysis of mucin in salivary glands [6]. Hara et al. reported fluorometric high-performance liquid chromatography (HPLC) of NeuAc and its analogous compound, N-glycolylneuraminic acid (NeuGc) after precolumn derivatization with 1,2-diamino-4,5-methylenedioxybenzene (DMB) [7,8]. Recently, Klein et al. applied this method to the analysis of various sialic acid analogues in bovine submaxillary mucin and described the nature of the fluorophore including its structure in detail [9]. Liquid chromatography–tandem mass spectrometry assay was used for the quantification of free and total sialic acid in human cerebrospinal fluid [10]. Morimoto et al. proposed a general strategy for the simultaneous determination of NeuAc and its analogues using HPLC coupled with electrospray ionization mass spectrometry and validated their method for the determination of NeuAc concentrations in various tissue samples from rats and mice [11].

Several studies have proposed NeuAc as a marker for the presence of mucus proteins because the non-reducing termini of mucus carbohydrate chains are often occupied with NeuAc. For example, during the investigation of the role of protease-activated receptor-2 (PAR-2) in the rat salivary glands, Kawabata et al. described the use of NeuAc as an indicator of salivary mucin secretion triggered by PAR-2 activation [12]. Our group has also shown, using NeuAc as a marker, that mucin concentrations were significantly decreased in tear fluids of contact lens wearers [13].

In the case of ophthalmic solutions, consumer dissatisfaction is often linked to the presence of foreign bodies in the container. Pharmaceutical manufacturers therefore have an interest in identifying

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such contaminants and determining if they may have been introduced by the consumer by inappropriate handling of the solution. Contamination by secretions such as eye mucus flowing backward into the ophthalmic container when touching it to the eyelid is often observed, and approximately one thousand cases of such contaminations are recorded in Japan every year [14].

To avoid unnecessary product recalls, a rapid and effective technique is required to differentiate contamination of ophthalmic solutions by users from other causes. Eye mucus can be briefly identified by visual inspection. However, if the quantity of contaminant is too small, such inspection is often ineffective. In cases where foreign body contamination is detected, ninhydrin tests for protein, epidermal cell observation using Giemsa-stain or methylene blue-stain or detection of mucopolysaccharides using Alcian blue-stain are employed [14].

In general, observation of epidermal cells, which are released from cornea, is commonly used for confirming the presence of eye mucus [14]. However, a more robust, rapid and quantifiable technique is needed for effective screening of potentially contaminated products. As indicated above, the authors previously reported a method for mucin determination in tear fluids by HPLC with fluorometry using NeuAc as a marker [13]. In the present report, a technique for NeuAc determination using LC/MS method was compared with the previously reported method.

2. Experimental

2.1. Reagents

1,2-Diamino-4,5-methylenedioxybenzene (DMB) was obtained from Dojin (Kamimashiki-gun, Kumamoto, Japan). Samples of NeuAc, mucin (pig stomach) and Giemsa-stain solution, acetic acid, sodium hydrosulfite, β-mercaptoethanol and ammonium formate were obtained from Wako Chemical (Chuo-ku, Osaka, Japan). Fetuin (fetal calf serum) was obtained from Sigma–Aldrich, Japan (Shinagawa-ku, Tokyo). Other solvents were of HPLC grade and used without further purification.

2.2. Eye mucus samples

Eye mucus samples (less than 1 mg as wet weight from two volunteers) were collected from the nasal side of the eye using a cotton bud after awakening in the morning and were transferred to a petri dish and stored at −15 °C until assay. The samples were obtained under the permission of the Ethics Committee of Kinki University and used in accordance with the tenets of the Declaration of Helsinki. The samples of deteriorated ophthalmic solutions which were sent by end users, were received by Senju Customer Center (Chuo-ku, Osaka, Japan) and used in the present studies.

2.3. Sample preparation

2.3.1. Release of NeuAc from eye mucus by acid hydrolysis

An eye mucus sample (one third portion of the sample as the suspended mixture, 50 μl) was mixed with 4 M aqueous acetic acid solution (500 μL), and the mixture was kept at 80 °C for 3 h to release NeuAc. After cooling and centrifugation of the mixture, a portion (25 μL) of the supernatant liquid was transferred to a polypropylene tube for derivatization with DMB as described below. Another portion (50 μL) of the supernatant liquid was diluted 10-fold by the addition of water (450 μL), mixed and transferred to an autosampler vial for LC/MS analysis. Standard solutions of NeuAc were prepared by dissolution of NeuAc (1.0 mg; 3.2 μmol) in water (100 mL), and used after appropriate dilution with water. The solution was stable at least one month when stored at 5 °C.

2.3.2. Fluorescent derivatization of NeuAc with DMB

Derivatization of NeuAc with DMB was completed using the method of Kawabata et al. [12]. Briefly, 0.7 M DMB solution (200 μL) containing 0.018 M sodium hydrosulfite and 0.75 M β-mercaptoethanol in 1.4 M acetic acid solution (200 μL) were added to the sample solution (25 μL), and the mixture was kept at 50 °C for 150 min. After cooling, a portion (5 μL) of the mixture was injected manually and analyzed by HPLC.

2.3.3. Analysis of NeuAc in the glycoprotein samples

A glycoprotein sample (each 1.0 mg for pig stomach mucin, fetuin and apo-transferrin, respectively) was mixed with 4 M aqueous acetic acid solution (500 μL), and the mixture was kept at 80 °C for 3 h to release NeuAc. After cooling and centrifugation of the mixture, a portion (25 μL) of the supernatant liquid was transferred to a polypropylene tube for derivatization with DMB as described above. Another portion (50 μL) of the supernatant liquid was diluted 10-fold by the addition of water (450 μL), mixed and transferred to an autosampler vial for LC/MS analysis. To confirm the matrix effects in LC/MS analysis, standard solutions (1 μl) of NeuAc were spiked to the supernatant liquids (50 μL), and analyzed.

2.4. Instruments

2.4.1. High-performance liquid chromatography with fluorescent detection

After fluorescent labeling with DMB, NeuAc was analyzed on an octadecyl-silica column (Shim-pack VP-ODS, 4.6 mm i.d. × 150 mm, Shimadzu, Nakagyo-ku, Kyoto, Japan) using a Shimadzu Prominence LC-20 HPLC apparatus with an RF 10AXL fluoromonitor (Shimadzu). The separation was carried out by a stepwise elution with a binary solvent system comprising solution A: water and solution B: acetonitrile–methanol (7:1, v/v). The elution was initially performed with 75% solution A for 7 min, and then with 20% solution A until 12 min. Finally, the column was conditioned with 75% solution A for 5 min. The flow rate was fixed at 0.9 mL/min and the column temperature was 40 °C. A 5-μL portion was injected by manual injection, and the column effluent was monitored by the fluorescence detector set at an excitation wavelength of 375 nm and 448 nm for emission.

2.4.2. Liquid chromatography coupled with mass spectrometry (LC/MS)

NeuAc released from eye mucus was analyzed with an LCMS-2010EV (Shimadzu) using an amino column (Unison UK-Amino, 2.0 mm i.d. × 100 mm, Imtakt Corp., Shimogyo-ku, Kyoto, Japan). Isocratic elution was carried out at a flow rate of 0.2 mL/min at 40 °C with 36% (v/v) acetonitrile in water containing 10 mM ammonium formate as the mobile phase. The injection volume was 1 μL. The electrospray ionization source was set to the negative ion mode. Selected ion monitoring was conducted using the deprotonated molecular ion for NeuAc at m/z 308 $[M-H]^-$. MS operating conditions were as follows: drying gas flow rate was 1.5 L nitrogen/min, the CDL temperature was 250 °C, the heat block temperature was 200 °C, and the ionization voltage was −3.5 kV.

2.5. Tests for foreign bodies in ophthalmic solutions returned by complaints

Ophthalmic solutions, in which foreign bodies were present and suspected to be the result of contamination with eye mucus, were selected as test samples. The foreign bodies in the ophthalmic solution were separated from the solution using a micropipette and fixed to a microscope slide for 30 s with two-three drops of

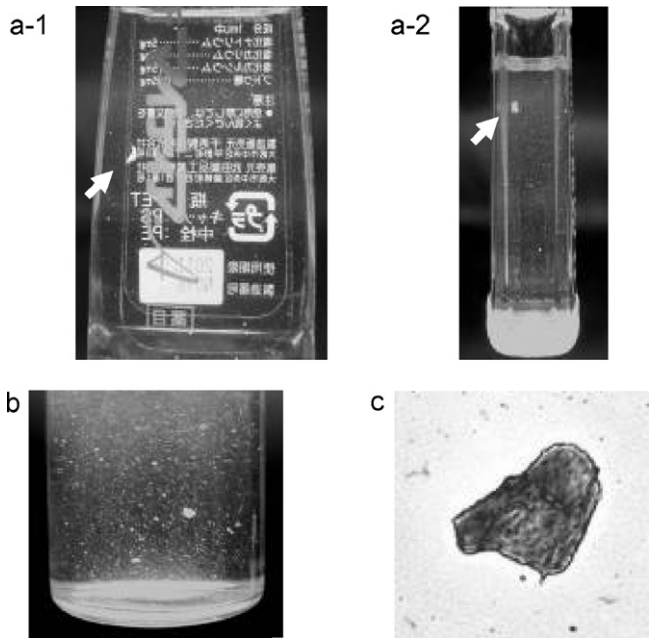

Fig. 1. Microscopic observation of the contaminating foreign body of complaint sample 2. (a) Foreign body in an ophthalmic container (1, front view; 2, side view), (b) floating foreign bodies transferred to a glass vial, and (c) microscopic observation after Giemsa-stain (×400).

methanol. The slide was immersed in a freshly prepared Giemsa-stain solution for 20–30 min, then flushed with water (30 mL) and left to dry. The presence of eye mucus including the epidermal cells was indicated by the foreign body staining red to blue-purple. Collected foreign bodies were further tested by LC/MS analysis after releasing NeuAc by acid hydrolysis, as described above in Section 2.4.2.

3. Results and discussion

3.1. Observation of epidermal cells by Giemsa staining

In general, observation of epidermal cells which are released from the cornea is used for confirmation of eye mucus [14]. We demonstrated this method in 9 cases for the identification of contaminating foreign bodies in ophthalmic solutions. Initially, foreign bodies were examined by the naked eye (complaint sample 2, Fig. 1a-1 and a-2). Floating foreign bodies were more easily confirmed by putting the mixture into a glass vial (Fig. 1b). A portion of the foreign body was also investigated by Giemsa staining (Fig. 1c), which shows positive reactions toward epidermal cells from cornea or conjunctiva.

All samples were examined in the same manner and the presence of foreign bodies due to eye mucus was confirmed in the samples 1, 2, 3, 4, 5 and 6. However, samples 7, 8 and 9 showed negative results by Giemsa staining.

3.2. HPLC-fluorometry analysis of NeuAc in model glycoproteins

Fig. 2a shows a typical example for the analysis of the standard sample of NeuAc (30 ng/mL) as the DMB derivative.

NeuAc was observed at 6.0 min, and the calibration curve showed good linearity between the concentrations of 1.2 ng/mL and 39 ng/mL. The equation for the regression analysis was $y = 12929x - 6522$ ($R = 0.998$), where y is the response (μV s) and x is the concentration of NeuAc (ng/mL). The limit of quantitation and limit of detection for NeuAc were 0.61 ng/mL and 0.20 ng/mL at the level of S/N = 10 and 3.3, respectively.

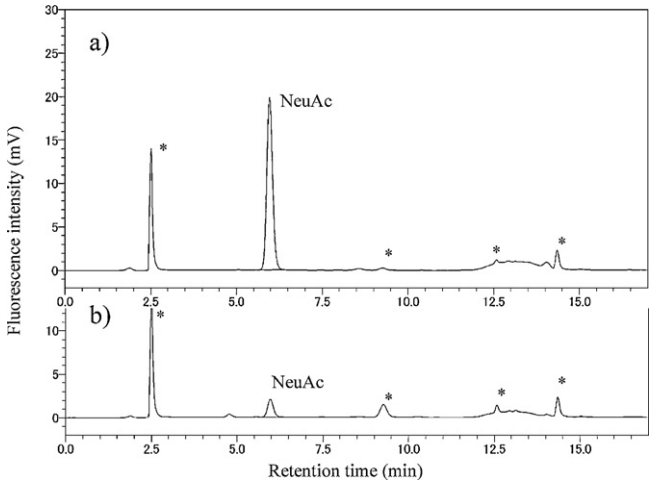

Fig. 2. Analysis of NeuAc as DMB derivative by HPLC-fluorometry. (a) Standard solution of NeuAc (30 ng/mL) and (b) pig stomach mucin. The peaks (asterisks) are due to DMB reagent.

NeuAc was analyzed in some sialoglycoprotein samples. The results are summarized in Table 1 and the analysis of NeuAc in pig stomach mucin is shown in Fig. 2b. NeuAc contents of other sialoglycoproteins are also summarized in Table 1. As shown from these data, the concentrations obtained by HPLC-fluorometry in this work shows good accordance with the values from the previous reported data [15,16].

3.3. LC/MS analysis of NeuAc in model glycoproteins

The mass spectrum of NeuAc generated by infusion of a standard sample solution is shown in Fig. 3a.

NeuAc showed an abundant pseudo-molecular ion at m/z 308 corresponding to $[M-H]^-$ and the ion due to a dimeric form was observed at m/z 617 $[2M-H]^-$.

An amino silica column was successfully employed for the analysis of NeuAc, and the peak due to NeuAc was observed at 7.0 min (Fig. 3b). The calibration curves for NeuAc showed good linearity between the concentrations of 5.0 ng/mL and 100 ng/mL. The equation for the regression analysis was $y = 534x - 696$ ($R = 0.999$), where y is the response (ion intensity) and x is the concentration of NeuAc (ng/mL). The limit of quantitation and limit of detection for NeuAc were 2.66 ng/mL and 0.88 ng/mL at the level of S/N = 10 and 3.3, respectively.

The NeuAc contents in some sialoglycoprotein samples were determined by LC/MS (Table 1). As an example, the result on the analysis of pig stomach mucin is shown in Fig. 3c. In the present study, LC/MS method afforded approximately 70% recoveries relative to those observed by HPLC-fluorometry. Each of the standard solutions of 10 ng/μL and 20 ng/μL of NeuAc was spiked in the

Table 1
Amount of NeuAc in sialoglycoprotein samples.

Sample	Amount of NeuAc (μg/mg protein)[a]		Reported data (μg/mg protein)
	HPLC-fluorometry	LC/MS	
Pig stomach mucin	2.9 (9.1)	1.8 (11.2)	3.3 [16]
Fetuin	26.8 (4.8)	21.0 (8.7)	26.8 [15]
Apo-transferrin	17.2 (3.2)	12.9 (3.9)	16.5 [15]

[a] RSD ($n = 3$) in parentheses.

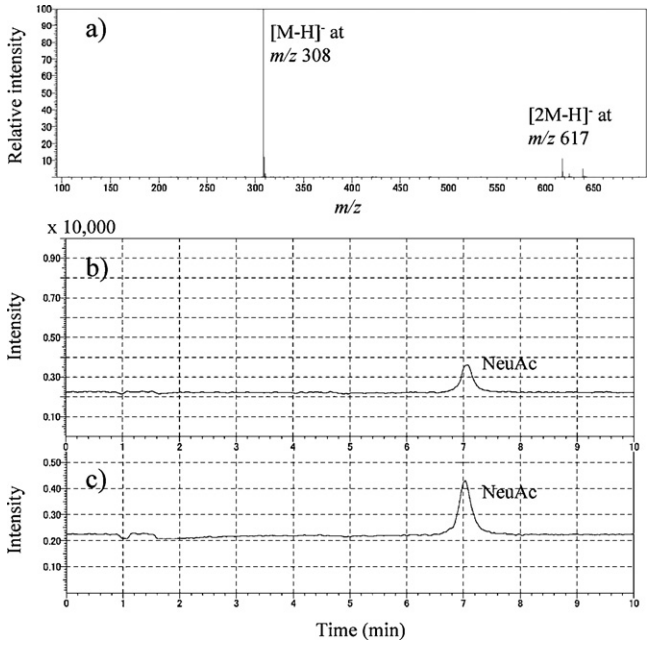

Fig. 3. Analysis of NeuAc by LC/MS. (a) MS spectrum of the standard solution of NeuAc as determined by flow injection. (b) Standard solution of NeuAc (100 ng/mL) and (c) pig stomach mucin. Analysis was performed by LC/MS as described in Section 2.

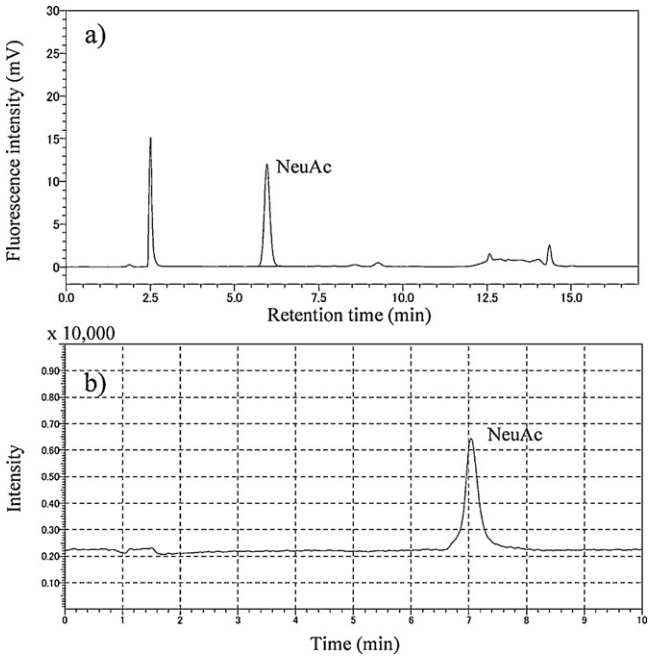

Fig. 4. Analysis of NeuAc in an eye mucus sample. (a) HPLC-fluorometry (eye mucus sample-1) and (b) LC/MS (eye mucus sample-1). Analytical conditions for both samples are shown in Section 2.

solutions of pig stomach mucin. And when the amounts of NeuAc were determined by LC/MS as described in the Section 2, we found that recoveries were 7.3 ng and 13.6 ng, respectively (data not shown). These values corresponded to 73% and 68% of the added amount of NeuAc. From these data, LC/MS of NeuAc in hydrolyzed products show lower values than the predicted ones probably due to matrix effects [17,18].

The objective of the present study is to confirm the presence of NeuAc due to eye mucus materials in tear fluids, and to estimate the reason for the deterioration of ophthalmic solutions. We found that the sensitivity of LC/MS method is satisfactory for identification of NeuAc. In addition, it should be noticed that no interfering peaks were observed in the chromatogram obtained by LC/MS analysis (see Section 3.4).

3.4. Comparison of HPLC-fluorometry and LC/MS analysis of NeuAc in eye mucus samples

Eye mucus samples collected from two donors were analyzed using HPLC-fluorometry and LC/MS methods as described above (Fig. 4).

The results indicate that NeuAc was abundant in the eye mucus samples. As shown in Fig. 4, LC/MS allowed highly selective analysis and unequivocal identification of the analyte (NeuAc) (Table 2).

We would like to emphasize that LC/MS method gives only the peak due to NeuAc and no other interfering peaks are observed, although the amounts showed somewhat lower values than those by HPLC-fluorometry. In any cases, these methods demonstrate that NeuAc is a good indicator for identification of eye mucus in deteriorated ophthalmic solutions, because pure ophthalmic solution did not give any peaks on the chromatograms by the present methods (data not shown). As mentioned above, LC/MS method affords higher specificity, because no peaks due to reagents were observed. In addition, the LC/MS method does not need a derivatization step and therefore it is more desirable than HPLC-fluorometry for routine analysis in a manufacturer's laboratory.

Table 2
Amount of NeuAc in eye mucus samples.

Sample	Average amount of eye mucus (mg)	Amount of NeuAc (μg/mg)[a]	
		HPLC	LC/MS
Eye mucus sample 1	0.82	18.9 (3.6)	14.5 (8.1)
Eye mucus sample 2	0.53	23.6 (3.9)	15.6 (9.2)

[a] RSD ($n = 3$) in parentheses.

3.5. Analysis of NeuAc in deteriorated ophthalmic solutions

Samples of contaminated ophthalmic solutions ($n = 9$) were analyzed by LC/MS after examining the foreign bodies by microscopic observation. The results are summarized in Table 3.

Samples (1–6) showed positive reactions to Giemsa staining. Samples 1–4 also showed the presence of NeuAc but unequivocal results were not obtained for samples 5 and 6 using the LC/MS method. In contrast, samples (7–9) showed no reaction to Giemsa staining but LC/MS provided evidence of significant levels of NeuAc.

Table 3
Detection of the cornea epithelial cells by Giemsa staining and the results on the analysis of NeuAc in the contaminated foreign bodies in ophthalmic solutions returned by complaints.

	Giemsa staining (detection of epithelial cells)	Amount of NeuAc (ng/sample)
Complaint sample 1	Positive	118.6
Complaint sample 2	Positive	14.3
Complaint sample 3	Positive	11.7
Complaint sample 4	Positive	28.2
Complaint sample 5	Positive	2.1 (ULOQ)
Complaint sample 6	Positive	<0.176 (ULOD)
Complaint sample 7	Negative	2534.5
Complaint sample 8	Negative	18.0
Complaint sample 9	Negative	1.2 (ULOQ)

ULOD, under limit of detection; ULOQ, under limit of quantitation.

Sample 8 also clearly showed the presence of NeuAc, although the amount was small. No NeuAc was detected for sample 9. These findings are most important because they demonstrate that when an ophthalmic solution contains soluble materials from tear fluids, Giemsa satin cannot be applied. However, NeuAc determination using the LC/MS method affords a positive reaction to soluble glycoproteins in tear fluids.

These results indicate that a combination of microscopic observation and LC/MS analysis is important for accurate confirmation of foreign bodies in contaminated eye drops.

4. Conclusions

Combinations of several analytical methods are necessary to identify eye mucus in ophthalmic solutions [14]. We previously reported that NeuAc which is the characteristic monosaccharide component in tear fluids is a good marker for dry eyes [13]. Based on these previous studies, contaminating substances due to tear fluids are considered to be confirmed by detecting NeuAc.

In the present study, HPLC-fluorometry of the DMB derivative of NeuAc and LC/MS for direct detection of NeuAc were compared for the analysis of eye mucus materials. We found that both methods showed satisfactory abilities for the detection and confirmation of NeuAc derived from eye mucus or tear fluids in deteriorated ophthalmic solutions. The LC/MS method gave values of 62–78% less than those obtained by HPLC-fluorometry probably due to matrix effects as indicated in Tables 1 and 2.

The objective of the present method is to identify NeuAc, but not to quantify NeuAc. To quantify the amount of NeuAc, further studies (sample extraction, improvement chromatographic separation, use of the internal standard) are required to remove or minimize matrix effects [17,18].

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