Sample Preparation and Determination of Acetylcholine in Corneal Epithelium Cells Using Liquid Chromatography–Tandem Mass Spectrometry

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Abstract

A sample preparation method with subsequent liquid chromatography (LC)–mass spectrometry (MS)–MS analysis for acetylcholine in corneal epithelium is developed. The sample preparation is developed with a focus on compatibility with the LC–MS–MS system and the stability of acetylcholine because acetylcholine esterase is present in the tissue. It appears that both acetylcholine as well as the internal standard (IS) used (acetyl-β-methylcholine) have fragments at *m/z* values in the tandem MS spectrum, which correspond with the *m/z* values of fragments of endogenous substances. Acetylcholine and (3-carboxypropyl)triethylammonium both have 146→87 and 146→60 transitions. Acetyl-β-methylcholine and an unknown compound both have 160→101 and 160→60 transitions. This makes it necessary to use a chromatographic step, which has a baseline separation between these endogenous compounds, acetylcholine, and the IS. The analytical procedure has linearity from 1 ng/mL (30 pg/mg corneal epithelium tissue) to at least 250 ng/mL (7.55 ng/mg corneal epithelium tissue). The limits of detection and quantitation are 15 and 45 pg oncolumn, respectively. Relative standard deviation and bias values are within the range of acceptance for all concentration levels.

Introduction

Acetylcholine is well-known as a neurotransmitter in the central and peripheral nervous systems, and extensive research has been carried out concerning its distribution and functional aspects. The functional mechanism in this context is principally mediated by the binding of neurally derived acetylcholine to subtypes of nicotinic and muscarinic receptors in adjacent tissues. In contrast to this neuronal acetylcholine, our information about the nonneuronal acetylcholine is scant, and their function is not well understood (1,2). A crucial point has, of course, been to show that acetylcholine really occurs in cells independently of neurons. This has been demonstrated beyond a doubt through the appearance of significant amounts of acetylcholine in human placentas, horse spleens, circulating blood cells, unicellular organisms, sponges, and plants. Regarding the functional role of this nonneuronal acetylcholine, however, opinions are divergent and suggest broad involvement, such as in the regulation of mitosis, trophic and immune functions, locomotion, and intercellular contacts (i.e., activities with no obvious common denominator).

The presence of acetylcholine in the corneal epithelium has been known for decades; in fact, the reported values account for the highest concentrations among all tissues examined to date (3). But again, its functional significance is unknown. This epithelium is a homogeneous tissue; easy to collect for analytical procedures; and because of its superficial location, is ideal for in vivo experiments. We therefore decided to use this tissue as a model to study fundamental biological functions of nonneuronal acetylcholine. This presupposes a reliable analytical procedure.

A thorough review of the methods used to determine acetylcholine and related substances has been carried out by Tsai (4). Acetylcholine determination after a chromatographic step requires specific detection techniques because it does not have any functional groups that can be detected easily with UV or fluorescence. Electrochemical detection can be performed. This type of detection gave good sensitivity, however (besides its common disadvantages) it is an indirect method because it requires the use of a postcolumn immobilized enzymatic reactor (IMER)(5–9). The use of single MS (10) and tandem MS (11) after high-performance liquid chromatography (HPLC) separation showed lower detection limits. Reversed-phase separation before MS detection necessitated the use of volatile counter ions (12,13).

Sample preparation required specific precautions becuase acetylcholine is unstable in both enzymatic and chemical degradation (14). To validate the method, the entire procedure needed to be carried out in the matrix of interest. For these reasons, the focus was on the specific sample preparation needed to validate the method in the bovine corneal epithelium.

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Experimental

Chemicals

The acetylcholine, acetyl-β-methylcholine, and acetylcholine esterase were purchased from Sigma-Aldrich Norway AS (Oslo, Norway). All of the other chemicals used were analytical grade.

Sample preparation/final method

Bovine eyes were obtained from the local abattoir (Fatland Oslo AS Oslo, Norway), which treated the animals according to Norwegian law. The eyes were removed after death, transported on ice to the laboratory, and the corneal epithelium immediately rubbed off by a glass spatula. The cells were homogenized using a glass piston (Heidolph, type RZR50, tür/Bayern, Germany) for 10 min in a 20mM ammoniumformiate buffer (pH 2.8), which contained 3% methanol and 20mM heptafluorobutyric acid solution (250 ng/mL acetyl-β-methylcholine) in the ratio of approximately 35 mg cells/1 mL liquid phase. In this way, the sample was compatible with the LC–MS–MS system, could be quantitated, and acetylcholine esterase was inactivated. The samples were centrifuged at 2500 rpm for 10 min. A 15-µL aliquot of the clear supernatant was injected directly into the chromatographic system.

Sample preparation for validation samples

Degradation of endogenous acetylcholine

Four hundred milligrams of corneal epithelium cells were homogenized in 800 µL water. This solution was kept at room temperature and samples were, after various timepoints, diluted with the mobile phase to a cell concentration of 50 mg/mL. The samples were centrifuged, and the supernatant was analyzed for acetylcholine content.

Stop of esterase activity

To 1 mL water, 1 µL acetylcholine esterase (1×10^{-3} U) was added. This solution was spiked with 10 µL acetylcholine so that the final concentration was 100 ng acetylcholine/mL. This mixture was analyzed for its acetylcholine content at 0, 11, 22, 33, 44, and 55 min.

To 1 mL of mobile phase [20mM ammoniumformiate buffer (pH 2.8), which contained 3% methanol and 20mM heptafluorobutyric acid], 1 µL acetylcholine esterase (1×10^{-3} U) was added. This solution was spiked with 10 µL acetylcholine so that the final concentration was 100 ng acetylcholine/mL. This mixture was also analyzed for its acetylcholine content at 0, 11, 22, 33, 44, and 55 min.

Final method for sample preparation in validation procedure

The cell material (517.7 mg) was homogenized in 1.5 mL water. This sample was placed at room temperature for at least 48 h to allow enzymatic breakdown by (endogenous) acetylcholine esterase of all endogenous acetylcholine. This homogenate was diluted by adding 11 mL of the mobile phase solution. This solution served as a pool to perform the validation procedure. An aliquot of 80 µL of this solution was mixed with 10 µL of acetylcholine standard (for calibration curve or for accuracy and precision experiments or both) and 10 µL of a 2.5-µg/mL acetyl-β-methylcholine solution. The samples were centrifuged at 2500 rpm for 10 min. A 15-µL aliquot of the clear supernatant was injected directly into the chromatographic system.

Chromatographic conditions

The HPLC system consisted of a Tsp SCM1000 vacuum degasser, Tsp SpectraSystem P4000 quaternary gradient pump, and a Tsp SpectraSystem AS3000 auto-sampler. Detection was carried out by a Finnigan LCQ ion trap mass spectrometer. Xcalibur version 1.0 software was used to control this system and to perform data acquisition. All of the equipment was purchased from Instrument-Teknikk AS (Østerås, Norway).

The separation was performed on a 50- × 2.0-mm (100 Å, 3-µm) Inertsil ODS-3 column from Varian (Holger, Oslo, Norway) at a flow rate of 200 µL/min. The isocratic elution was carried out using a 20mM ammoniumformiate buffer (pH 2.8), which contained 3% methanol and 20mM heptafluorobutyric acid.

MS–MS conditions

The HPLC was connected to the MS detector with an atmospheric-pressure-ionization electrospray interface. The detector was operated in the positive ion mode. The sheath gas (N_2) flow was set at 60 units, and the auxiliary gas (N_2) flow was 20 units. The spray voltage was set at 5 kV, and the capillary temperature was 250°C. Both acetylcholine and acetyl-β-methylcholine were detected in the MS–MS mode by monitoring the 146→87 and 146→60 transitions for acetylcholine during the first 5 min of the chromatogram and monitoring the 160→100 and 160→60 transition for acetyl-β-methylcholine during the last 5 min of the chromatogram. The isolation width was *m/z* 2 in both cases. Helium gas was used to cause collision-induced dissociation at 25% relative collision energy for both ions.

Validation procedure

The validation was carried out according to reported guidelines (15). Table I shows the experimental design. Each run was carried out separately to determine the interrun variance and bias. Each point on the calibration curve was prepared in duplicate. For interrun variance and bias, each

Table I. Design of the Validation Experiments

	Calibration curve									Accuracy and precision		
Sample number	1	2	3	4	5	6	7	8	9	10	11	12
Run 1	2×	2×	2×	2×	2×	2×	2×	2×	2×	5×	5×	5×
Run 2	2×	2×	2×	2×	2×	2×	2×	2×	2×	2×	2×	2×
Run 3	2×	2×	2×	2×	2×	2×	2×	2×	2×	–	2×	2×
Run 4	2×	2×	2×	2×	2×	2×	2×	2×	2×	2×	2×	2×
Run 5	2×	2×	2×	2×	2×	2×	2×	2×	2×	2×	2×	2×
Concentration acetylcholine (ng/mL)	0	1.0	5.0	10	25	50	70	100	250	6.22	13.6	70.0
Concentration internal standard (ng/mL)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5

point for accuracy and precision was carried out in duplicate. For intrarun variance and bias, each point for accuracy and precision was carried out five times. The samples used to determine accuracy and precision were prepared independently from the calibration curve samples.

From this design, the accuracy, precision, linearity, limit of detection (LOD) (3× signal-to-noise ratio), and limit of quantitation (LOQ) (10× signal-to-noise ratio) could be determined.

Quantitation of the standards and samples was based on area measurement of the ratio between the multiple reaction monitoring signals for acetylcholine (146→87 and 146→60) and acetyl-β-methylcholine (160→101 and 160→60).

Results and Discussion

There were several pitfalls in the determination of acetylcholine using acetyl-β-methylcholine as the internal standard (IS). From earlier published work by other authors, it was clear that acetylcholine has at least one endogenous structural isomer which, in addition to the *m/z* value, also has similar transitions in the MS–MS spectra (11). This study also showed that acetyl-β-methylcholine has at least one structural isomer, which, in addition to the *m/z* value, also has similar transitions in the MS–MS spectra. When corneal epithelium cells were treated as described under the Sample Preparation section, two peaks for 146→87 and 146→60 and two peaks for 160→101 and 160→60 were measured.

Figure 1 shows the chromatograms of a sample that was spiked with IS. The first chromatogram (Figure 1A) shows the sample when it was analyzed only for acetylcholine (transition 146→87 and 146→60). The presence of the substance that elutes at 2.3 min was described by Zhu et al. (11) and is identified as (3-carboxypropyl) triethylammonium. The MS–MS fragmentation spectrum of this substance showed different intensities than the MS–MS fragmentation spectrum of acetylcholine, which elutes at 3.4 min (Figure 2).

The second chromatogram (Figure 1B) shows the sample when it was analyzed only for acetyl-β-methylcholine (transition 160→101 and 160→60). Here, two peaks also occurred. The peak that eluted at 6.5 min is the IS, and although the peak that eluted at 3.1 min showed the same transitions, it is more hydrophilic

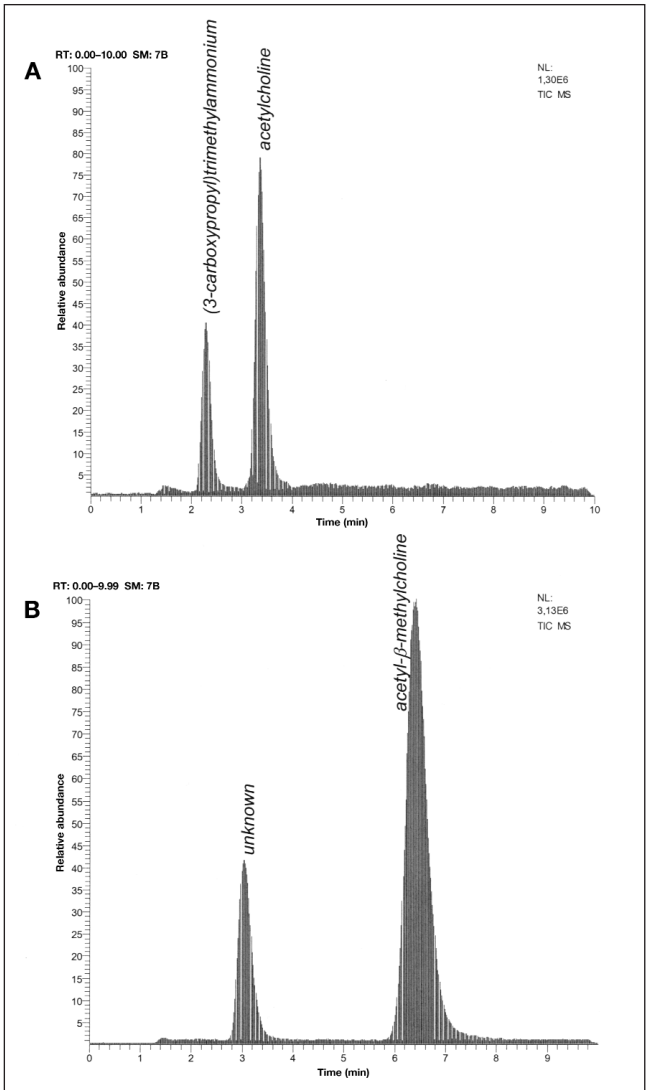

Figure 1. (A) chromatogram of (3-carboxypropyl)trimethylammonium and acetylcholine. Detection mode: MRM of transitions 146→87 and 146→60. (B) chromatogram of acetyl-β-methylcholine and an unknown. Detection mode: MRM of transitions 160→101 and 160→60.

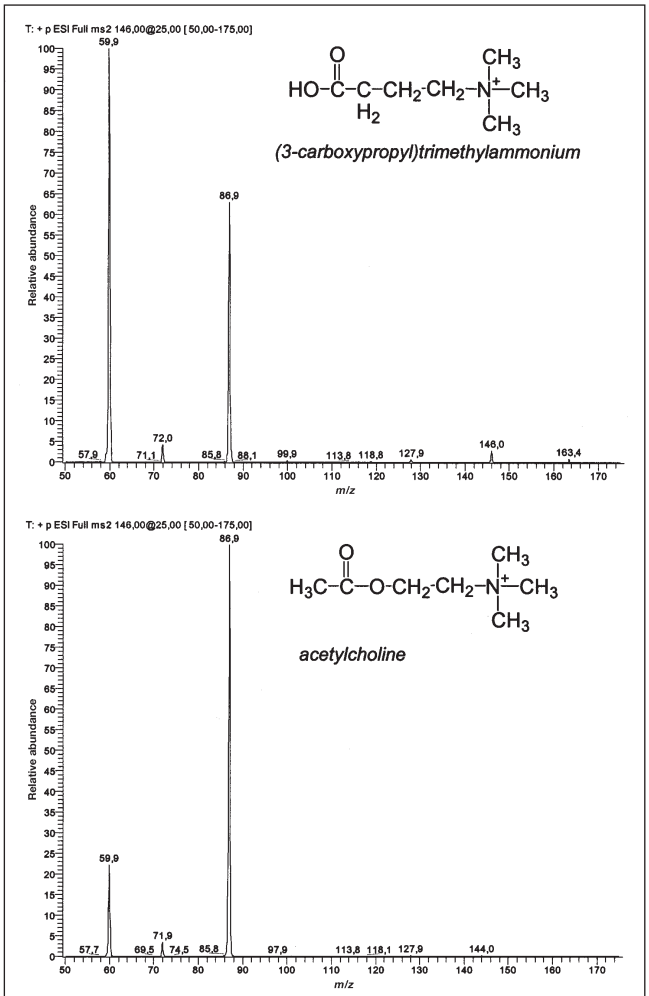

Figure 2. MS–MS spectra of (3-carboxypropyl)trimethylammonium and acetylcholine at 25% relative collision energy.

than the IS. This compound was present endogenous and was observed when analyzing a sample that was not spiked with the IS. Comparing the MS–MS fragmentation spectrum of this substance (3.1 min) with the MS–MS fragmentation spectrum of acetyl-β-methylcholine showed that not only are the intensities of the main fragments different, but that other fragments also occur (Figure 3). Although the nature of this compound was not investigated, it is concluded that this compound is not acetyl-β-methylcholine.

The nature of all the detected fragments was unclear because their investigation was beyond the scope of this paper. However, fragmentation probably involved a heterolytic cleavage of the bond adjacent to the *N*-atom. This might result in fragments in which the positive charge is located either on the alkyl or the ammonium moiety.

Sample preparation for validation samples

In order to validate this method, samples (corneal epithelium cells) spiked with acetylcholine and the IS were required. Here, another pitfall occurred; the unspiked sample already contained a reasonable amount of acetylcholine, which varied from sample to sample. The presence of variable amounts of acetylcholine would interfere with the validation of the method. In order to avoid this problem, the sample prepared to validate the method underwent some additional pretreatment.

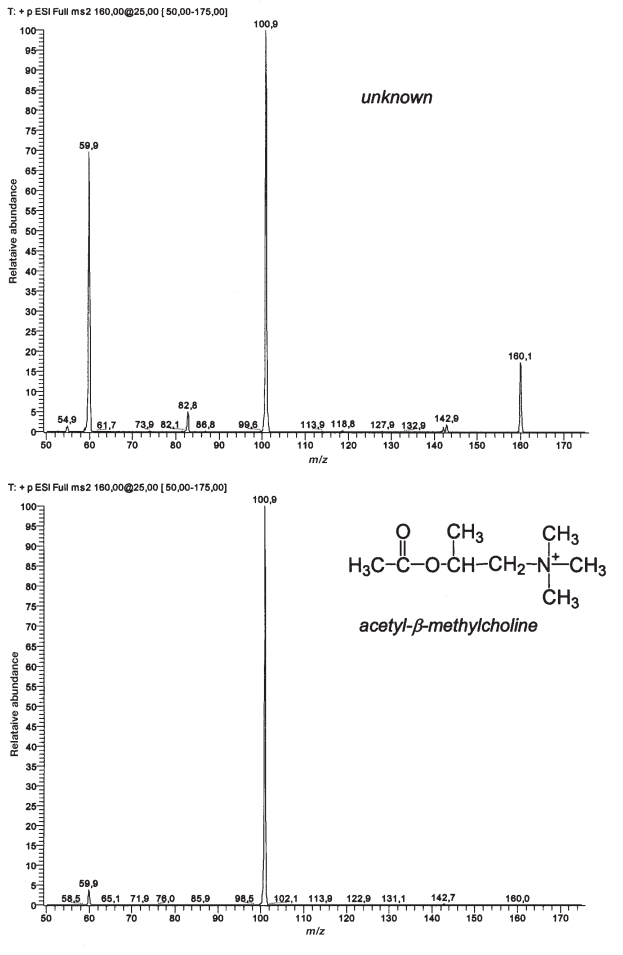

Figure 3. MS–MS spectra of acetyl-β-methylcholine and the unknown at 25% relative collision energy.

Degradation of endogenous acetylcholine

To ensure the absence of endogenous acetylcholine in the sample needed for validation, the hydrolysis of acetylcholine in corneal epithelium cells was monitored over time. Figure 4 shows the decrease of acetylcholine in the sample over a three-day period. At least 40 h were needed to obtain quantitative degradation.

Stop of esterase activity

After having hydrolyzed all endogenous acetylcholine, it was important to stop the activity of the acetylcholine esterase, otherwise the acetylcholine, which would be added to perform the validation, would also be hydrolyzed. This would result in too-low signals for acetylcholine.

Figure 5 shows the amount of acetylcholine in an aqueous solution containing 1×10^{-3} U acetylcholine esterase over a course of time. From this figure, it became clear that adding the mobile phase [20mM ammoniumformiate buffer (pH 2.8), which contained 3% methanol and 20mM heptafluorobutyric acid] to acetylcholine esterase decreased the enzyme activity to such a level that the acetylcholine amount stayed constant. This contrasted to the amount of acetylcholine in the sample to which water was added. Here, the acetylcholine peak had disappeared between 22 and 33 min.

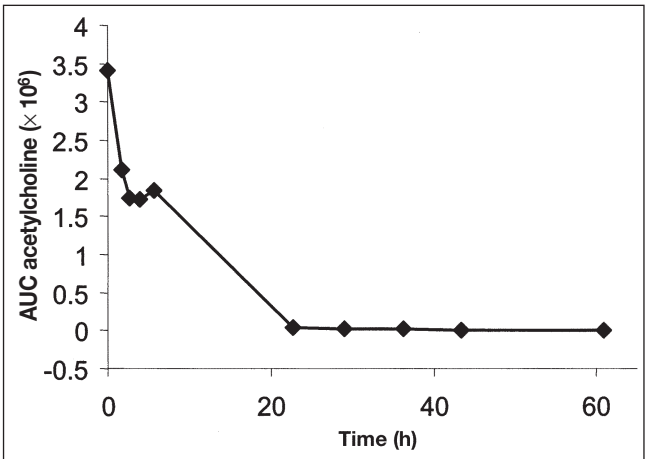

Figure 4. Decrease of acetylcholine in corneal epithelum at room temperature.

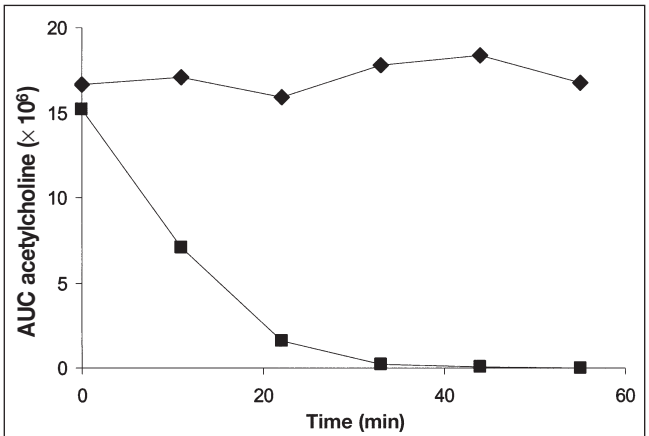

Figure 5. Stability of acetylcholine in the presence of acetylcholine esterase and water (■) and acetylcholine esterase and mobile phase (◆).

An additional advantage of stopping the enzyme activity with the mobile phase was its compatibility with the LC–MS–MS system.

Degradation of endogenous acetylcholine was only important for the samples used in the validation of the method, whereas the esterase activity should be stopped in all samples.

Table II. Correlation and Regression Coefficients for the Linear Calibration Line ($y = bx + a$) of acetylcholine ($n = 5$)

	r*	b†	a
Average	0.998	2.65×10^{-3}	-8.22×10^{-3}
Standard error		0.17×10^{-3}	
Standard deviation	0.0025		7.54×10^{-3}
$t_{calculated}$ ($n = 5$)			2.4
t_{table} ($n = 5$, $\propto < 0.05$)			2.776

* r, correlation coefficient.
† b, regression coefficient.

Table III. Accuracy (Bias) and Precision Data for Intra- and Interrun Validation

	Actual concentration acetylcholine (ng/mL)	Measured concentration acetylcholine (ng/mL)	RSD (%)	Bias (%)
Intrarun	6.22	6.55 ± 0.466	7.11	5.32
	13.6	11.9 ± 1.38	11.6	–12.4
	70.0	65.4 ± 6.39	9.77	–6.60
Interrun	6.22	7.56 ± 1.31	17.3	21.7
	13.6	14.8 ± 1.31	8.84	9.40
	70.0	59.6 ± 8.58	14.4	–14.8

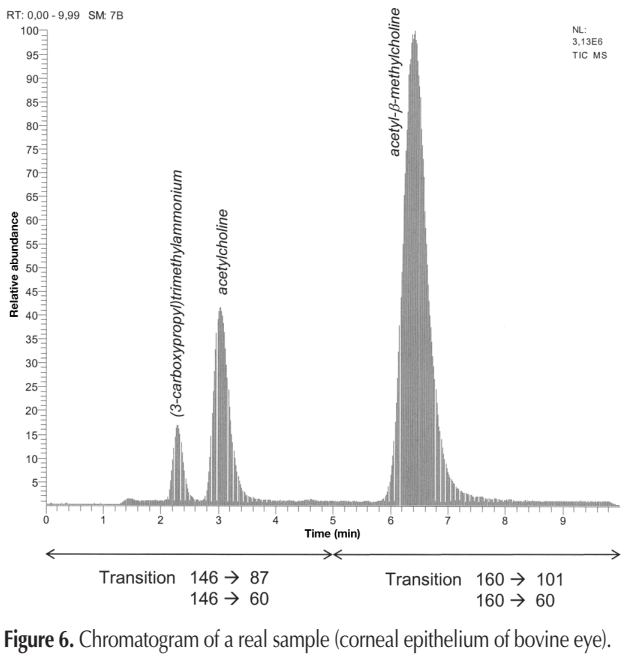

Figure 6. Chromatogram of a real sample (corneal epithelium of bovine eye). Detection mode from 0 to 5 min; the MRM of transitions 146→87 and 146→60, from 5 to 10 min; and MRM of transitions 160→101 and 160→60. The calculated concentration for acetylcholine was 153 ng/mL.

Conclusion

Validation results

Table II shows the statistical data of the standard calibration curve of acetylcholine where y is the ratio between acetylcholine and the IS, and x is the concentration in ng/mL. From the student t-test, it was clear that the intercept (a) of the calibration curve does not differ significantly from zero ($t_{calculated} < t_{table}$ when $n = 5$ and $\alpha < 0.05$).

The calibration line showed its linearity from 1 to at least 250 ng/mL. This corresponded with the linearity of corneal epithelium tissue from 30 to at least 7.55. The LOD and LOQ were 1 and 3 ng/mL, respectively. This corresponded with 30 and 91 pg/mg corneal epithelium tissue.

Table III shows the validation data for the intra- and interrun accuracy and precision at three concentration levels. The relative standard deviation (RSD) and bias values were within the range of acceptance for all concentration levels. However, the interrun bias for the lowest concentration was slightly higher than the acceptance level of 20% (21.7%, see Table III).

A real sample (corneal epithelium from bovine eye) spiked with acetyl-β-methylcholine is shown in Figure 6. It was found that the concentration of acetylcholine in this sample was 153 ng/mL. This is in the expected order of magnitude.

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