Identification of Unknown Quaternary Ammonium Compounds in Corneal Epithelium and Aqueous Humor

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Abstract

Bovine corneal epithelium and bovine aqueous humor are investigated for their content of quaternary ammonium compounds. In total, four compounds are found. Three of these are identified. For the fourth compound, a proposal for its structure is made on the basis of tandem mass spectrometry fragmentation spectra. The compounds investigated have *m/z* values of 146, 160, and 174. The compounds with *m/z* 146 are confirmed as acetylcholine (in corneal epithelium) and (3-carboxypropyl)-trimethylammonium (in both corneal epithelium and aqueous humor). The compound with *m/z* 174 is identified as butyrylcholine (in corneal epithelium). The compound with *m/z* 160 is probably acetyl-γ-homocholine (in both corneal epithelium and aqueous humor). For both butyrylcholine and acetyl-γ-homocholine, it is the first time the presence of these compounds in corneal epithelium or aqueous humor (or both) is described. Both acetylcholine and butyrylcholine are unstable compounds, which are probably susceptible to enzymatic degradation by acetylcholine-esterase and butytrylcholine-esterase, respectively.

Introduction

Although the high content of acetylcholine in the corneal epithelium has been known for decades (1), its physiological significance is still unclear. Influenced by the classical concept of acetylcholine as an efferent neurotransmitter, it has been deduced that perhaps the corneal acetylcholine is needed for pain reception on the surface of the eye (2). However, attempts to settle this question by corneal denervation experiments have failed because of inconsistent results diverging from no (3), to moderate (1), to significant acetylcholine reduction (2) in the corneal epithelium after surgery. On top of that, there is disagreement about whether or not cholinergic receptors are present in this epithelium (4–6). These fairly confusing results have made many researchers look for alternative functions for acetylcholine in the corneal epithelium.

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In fact, not only neuronal, but also non-neuronal acetylcholine is widely expressed in the animal and plant kingdom (7), indicating that non-neuronal acetylcholine may be involved in different basic cell functions. As to the corneal epithelium, acetylcholine has been said to participate in maintenance of the corneal transparency (8), adaptation to postnatal corneal environment (9), epithelial mitotic rate control (10), and ion transport (11). It should be noted that the acetylcholine itself has been found mainly within the epithelial cells (3,12), whereas the acetylcholinesterase seems to reside intercellularly (13).

It has recently been shown that the previously reported findings of high acetylcholine concentrations in the corneal epithelium are confined to diurnal mammals only (14), an overall pattern reminiscent of that reported for ascorbic acid in the same tissue (15). Furthermore, injection of acetylcholine subcutaneously in rabbits caused reduced concentrations of acetylcholine in the corneal epithelium (16). How this "message" of acetylcholine loading in the neck connective tissue is mediated to the eye is unknown. Both neurogen and humoral pathways are conceivable, but acetylcholine itself as a humoral carrier seems unlikely because of its unstable nature.

During development of the bioanalytical method published earlier in this journal (17) and the projects that followed (14,16), several substances with tandem mass spectrometry (MS–MS) spectra similar to acetylcholine have been detected. The study reported here deals with the identification of these substances because there is limited knowledge on the presence of other quaternary ammonium compounds in corneal epithelium and aqueous humor.

Experimental

Chemicals

Acetylcholine, acetyl-β-methylcholine, (3-carboxypropyl)-trimethylammonium, butyrylcholine, propionylcholine, and muscarine were purchased from Sigma-Aldrich (St. Louis, MO). These compounds were dissolved in water at a final concentration of 500 ng/mL. These solutions were injected into the chromato-

graphic system to obtain retention times and MS–MS spectra. All other chemicals used were of analytical grade.

Sample preparation

Corneal epithelium

Bovine eyes were obtained from the local slaughterhouse (Oslo, Norway), treating 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 with a glass spatula. The cells were homogenized using a glass piston (Heidolph, type RZR50, tür/Bayern, Schwabach, Germany) for 10 min in a 20mM ammoniumformiate buffer (pH 2.8), containing 3% methanol and 20mM heptafluorobutyric acid solution. This was carried out in the ratio of approximately 35 mg cells/1 mL liquid phase. In this way, the sample was compatible with the liquid chromatography (LC)–MS–MS system, could be quantitated, and acetylcholine-esterase was inactivated. The samples were centrifuged at 2500 rpm for 10 min. A 20-µL aliquot of the clear supernatant was injected directly into the chromatographic system.

Samples of corneal epithelium needed to investigate the stability of the compounds were treated as described previously with the difference that instead of the buffer–methanol–HFBA solution, water was used to homogenize the corneal epithelial cells.

Aqueous humor samples were diluted 1:1 with 20mM ammoniumformiate buffer (pH 2.8), containing 3% methanol, and 20mM heptafluorobutyric acid solution and centrifuged at 2500 rpm for 10 min. A 20-µL aliquot of the clear supernatant was injected directly into the chromatographic system.

Stability of the known and unknown compounds

Corneal epithelium homogenized in water was divided into 15 portions containing 100 µL each. These portions were stored at room temperature for various time intervals within a 3-day period. The reaction was stopped by adding 100 µL 20mM ammoniumformiate buffer (pH 2.8), containing 3% methanol, and 20mM heptafluorobutyric acid solution. A 20-µL aliquot was injected after centrifuging the samples.

Chromatographic conditions

The HPLC system consisted of a Tsp SCM1000 vacuum degasser, Tsp SpectraSystem P4000 quaternary gradient pump, and Tsp SpectraSystem AS3000 autosampler. Detection was made with a Finnigan LCQ^{duo} ion trap MS. Xcalibur version 1.0 software was used to control this system and to perform data acquisition (all from Instrument-Teknikk AS, Østerås, Norway).

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. Gradient elution was carried out. Mobile phase A consisted of 20mM ammoniumformiate buffer pH 2.8 containing 2% methanol and 20mM heptafluorobutyric acid; mobile phase B consisted of 20mM ammoniumformiate buffer pH 2.8 containing 80% methanol and 20mM heptafluorobutyric acid. From t = 0 min to t = 20 min, the mobile phase composition changed linearly from 100% mobile phase A to 100% mobile phase B. After an isocratic part with 100% mobile phase B (from t = 20 min to t = 22.5 min) the mobile phase composition changed from 100% mobile phase B to 100% mobile phase A from t = 22.5 min to t = 23 min. The start–end composition was held for at least 5 column volumes to re-equilibrate the stationary phase.

MS and 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. Sheath gas (N_2) flow was set at 60 units and auxiliary gas (N_2) flow at 20 units. The spray voltage was set at 5 kV and the capillary temperature at 250°C.

To screen the samples, a full scan was carried out from 50–500

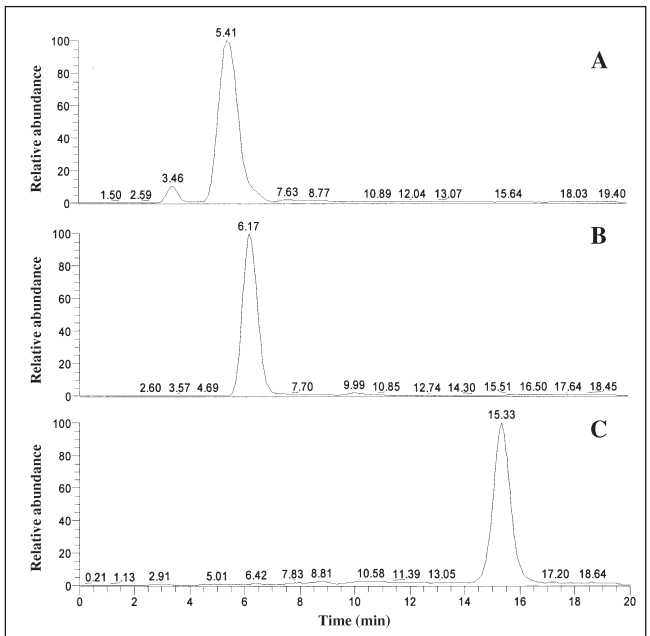

Figure 1. Ion chromatograms of *m/z* 146 (A), 160 (B), and 174 (C) in bovine corneal epithelium.

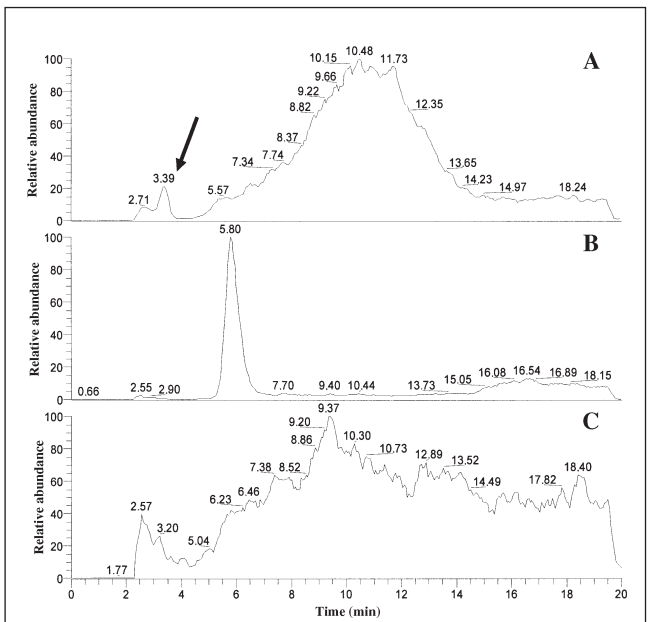

Figure 2. Ion chromatograms of *m/z* 146 (A), 160 (B), and 174 (C) in bovine aqueous humor.

amu in the single MS mode. To identify the new substances, MS–MS detection was carried out by monitoring the fragments from *m/z* 146, 160, and 174. The isolation width was *m/z* 1 in all cases. Helium gas was used to cause collision-induced dissociation at 25% relative collision energy for all ions.

Both the chromatographic separation and MS–MS detection is a slightly modified method from a previously published method for detection and quantitation of acetylcholine. (17)

Results and Discussion

Screening of corneal epithelium and aqueous humor using single MS detection

Both corneal epithelium and aqueous humor samples were injected into the LC–MS system as described previously. Figure 1 shows the ion chromatograms of the most abundant ions in corneal epithelium; Figure 2 shows the same ion chromatograms in aqueous humor. It is clear that in corneal epithelium *m/z* 146 is detected twice; *m/z* 160 and *m/z* 174 were each detected once. In aqueous humor, *m/z* 146 as well as *m/z* 160 were only detected once. In order to identify these signals, reference compounds of the possible components were injected. Figure 3 shows the ion chromatograms of these compounds. It appears that for *m/z* 146, both acetylcholine and (3-carboxypropyl)-trimethylammonium have a similar retention time as the compounds with *m/z* 146 in Figure 1 and that the retention time of the compound with *m/z* 146 in Figure 2 corresponds with that of (3-carboxypropyl)-trimethylammonium. For *m/z* 160, no similarity in retention time between the reference compounds and compounds with *m/z* 160 in both corneal epithelium and aqueous humor was observed. For *m/z* 174, butyrylcholine seems to have a similar retention time (see Figure 3) as the compound with *m/z* 174 detected in corneal epithelium (Figure 1). Spiking corneal epithelium with acetylcholine, (3-carboxypropyl)-trimethylammonium, and butyrylcholine shows that they coelute with the endogenous compounds. Spiking corneal epithelium with (3-carboxypropyl)-trimethylammonium shows that it coelutes with the endogenous compound. In both corneal epithelium and aqueous humor, the compound with *m/z* 160, which elutes at approximately 6 min, does not coelute with any of the reference compounds.

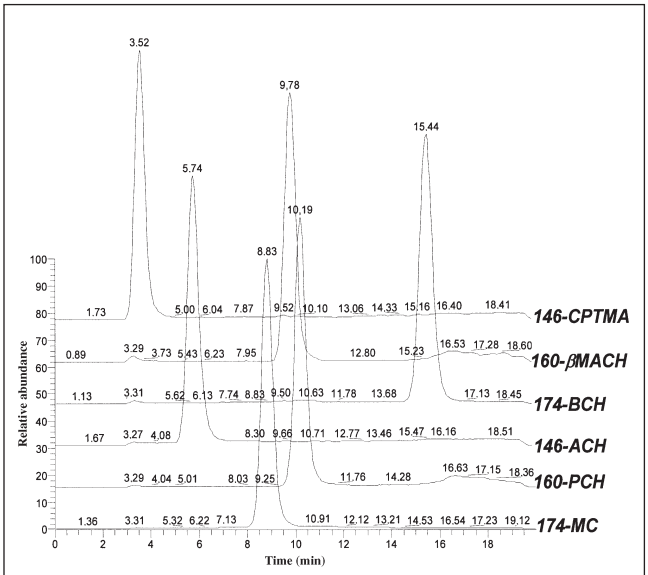

Figure 3. Ion chromatograms of (3-carboxypropyl)-trimethylammonium (146-CPTMA), β-methyl acetylcholine (160-βMACH), butyrylcholine (174-BCH), acetylcholine (146-ACH), propionylcholine (160-PCH), and muscarine (174-MC) in bovine corneal epithelium.

Identification and confirmation of the unknown compounds using MS–MS

MS–MS spectra of the reference compounds were compared with the MS–MS spectra of the endogenous compounds eluting at the same retention time. In this way, the nature of the endogenous compounds is identified or confirmed (or both). Figure 4 shows the MS–MS spectra of the reference compounds, endogenous compounds in corneal epithelium, and aqueous humor. These spectra were acquired at different retention times.

MS–MS spectra in Figure 4A–4C were recorded after chromatographic analysis of the reference compounds: Figure 4A, fragmentation of *m/z* 146 between retention times 3.3 and 3.7 min; Figure 4B, fragmentation of *m/z* 146 between retention times 5.0 and 6.0 min; and Figure 4C, fragmentation of *m/z* 174 between retention times 15.1 and 15.8 min.

MS–MS spectra in Figure 4D–4F were recorded after chromatographic analysis of the endogenous compounds in corneal epithelium: Figure 4D, fragmentation of *m/z* 146 between retention times 3.3 and 3.7 min; Figure 4E, fragmentation of *m/z* 146 between retention times 5.0 and 6.0 min; and Figure 4F, fragmentation of *m/z* 174 between retention times 15.1 and 15.8 min.

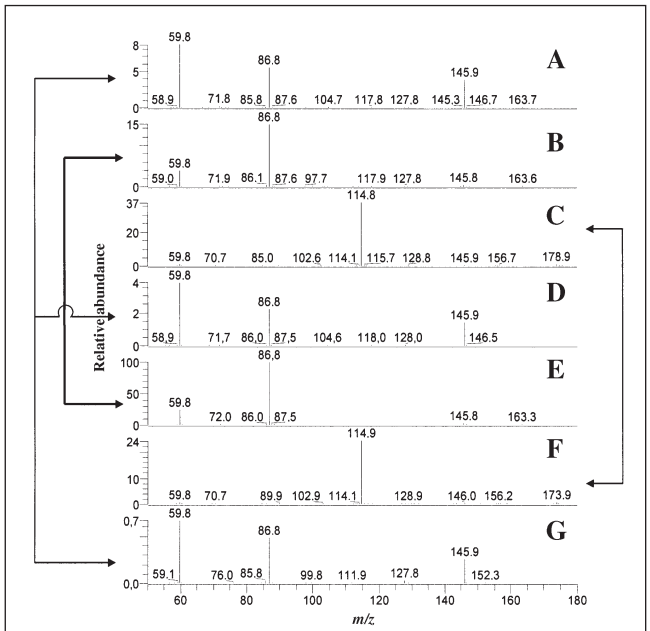

Figure 4. References: MS–MS spectra of (3-carboxypropyl)-trimethylammonium (A), acetylcholine (B), and butyrylcholine (C). Corneal epithelium: MS–MS spectra of *m/z* 146 between retention time 3.3 and 3.7 min (D), *m/z* 146 between 5.0 and 6.0 min (E), and *m/z* 174 between 15.1 and 15.8 min (F). Aqueous humor: MS–MS spectrum of *m/z* 146 between 3.3 and 3.7 min (G).

The MS–MS spectrum in Figure 4G was recorded after chromatographic analysis of the endogenous compounds in aqueous humor: fragmentation of *m/z* 146 between retention times 3.3 and 3.7 min.

m/z 146

From the spectra in Figure 4 it becomes clear that compounds in corneal epithelium with *m/z* 146 are acetylcholine (compare Figure 4D with 4A) and (3-carboxypropyl)-trimethylammonium (compare Figure 4E with 4B). The presence of the latter in corneal epithelium was suggested earlier (17) and is in this way confirmed. The same compound also appears in aqueous humor. This is the first time the presence of (3-carboxypropyl)-trimethylammonium in aqueous humor is shown.

m/z 174

Spectrum C and spectrum F (Figure 4) reveals that the endogenous compound eluting at 15.4 min is butyrylcholine. This is the first time that the presence of butyrylcholine in corneal epithelium is shown.

m/z 160

None of the reference compounds have the same MS–MS spectrum and retention time as the endogenous compound eluting at approximately 6 min in corneal epithelium as well as in aqueous humor. Figure 5 shows MS–MS fragmentation spectra of *m/z* 160 in both corneal epithelium (spectrum H) and aqueous humor (spectrum I) eluting between 5.5 and 6.5 min. These spectra are identical, confirming that the endogenous compound with *m/z* 160 is present in both corneal epithelium and aqueous humor. On the basis of its MS–MS spectrum, the nature of this compound is suggested to be acetyl-γ-homocholine (see Figure 5). This is a false neurotransmitter, which is seldom published in literature. Its presence in subcellular stores in the human brain is described. The lack of reference compound (not commercially available) prevented the confirmation of its structure.

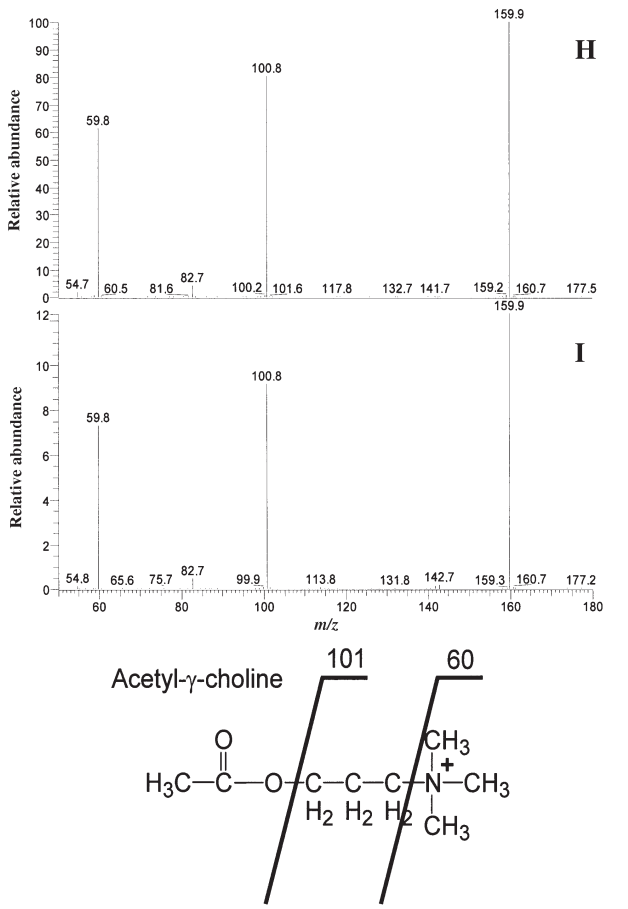

Figure 5. MS–MS spectra of *m/z* 160 in corneal epithelium (H) and aqueous humor (I) together with structure of acetyl-γ-homocholine.

Stability of m/z 146, 160, and 174 in corneal epithelium

Corneal epithelium homogenized in water was tested to determine if it contained *m/z* 146, *m/z* 160, and *m/z* 174 as described in the Experimental section (Figure 6A). It appeared that acetylcholine and butyrylcholine were susceptible to degradation. Both decrease to levels lower than 10% compared with their start level within 24 h. This is probably because of enzymatic degradation because corneal epithelium homogenized with 20mM ammoniumformiate buffer (pH 2.8), containing 3% methanol and 20mM heptafluorobutyric acid solution shows no decrease (data not shown) in acetylcholine and butyrylcholine levels. Both acetylcholine-esterase as well as butyrylcholine-esterase are known enzymes. From Figure 6B, it appears that both (3-carboxypropyl)-trimethylammonium and acetyl-γ-homocholine are stable compounds over the period tested. These compounds are also stable in aqueous humor.

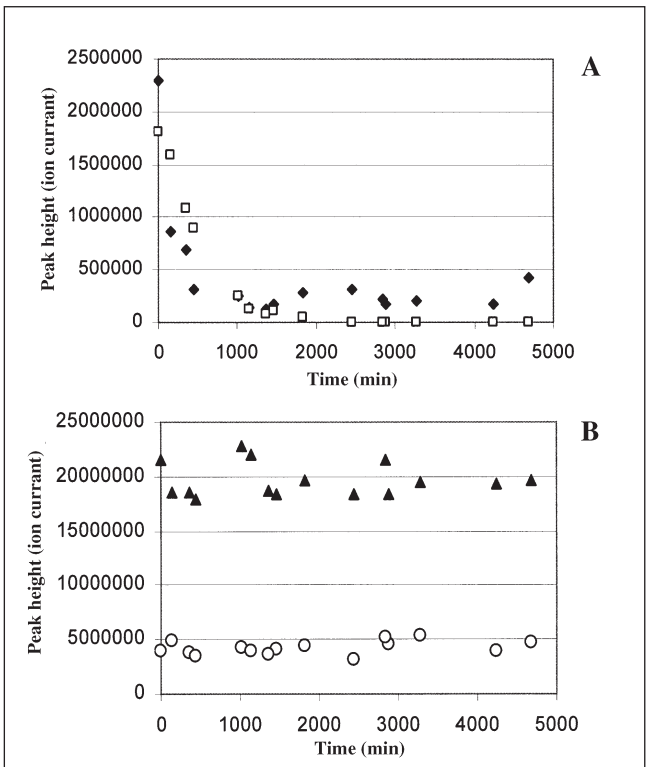

Figure 6. Decrease of acetylcholine (◆) and butyrylcholine (□) in course of time (A). Levels of (3-carboxypropyl)trimethylammonium (O) and acetyl-γ-homocholine (▲) in course of time (B).

Conclusion

Bovine corneal epithelium and bovine aqueous humor were investigated to determine the content of quaternary ammonium compounds. Four compounds were found in corneal epithelium and two compounds in aqueous humor. The compounds with *m/z* 146 were confirmed as acetylcholine (in corneal epithelium) and identified as (3-carboxypropyl)-trimethylammonium (in both corneal epithelium and aqueous humor). The compound with *m/z* 174 was identified as butyrylcholine (in corneal epithelium). This is the first time the presence of this compound in corneal epithelium is described. The compound with *m/z* 160 is probably acetyl-γ-homocholine, a false transmitter, which is present in both the corneal epithelium and aqueous humor and is described for the first time here.

Both acetylcholine and butyrylcholine are unstable compounds that are probably susceptible to enzymatic degradation by acetylcholine-esterase and butytrylcholine-esterase, respectively.

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