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Journal of Pharmaceutical and Biomedical Analysis
31 (2003) 905–915
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Detection of choline and acetylcholine in a pharmaceutical preparation using high-performance liquid chromatography/electrospray ionization mass spectrometry

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Received 22 April 2002; received in revised form 8 October 2002; accepted 15 November 2002

Abstract

A sensitive, rapid, and specific method for the detection of choline and acetylcholine in a pharmaceutical preparation is described. The method employs a perfluorinated carboxylic acid as ion-pairing reagent, post-column addition of a surface tension reducing agent and mass spectrometric detection using either selected ion monitoring (SIM) or selected reaction monitoring (SRM) modes. The resulting chromatographic performance is comparable or superior to methods reported previously in both quality of the separation and sensitivity when using mass spectral detection, with the added advantage of reduced cycle time. Acetylcholine is easily and rapidly separated from its major decomposition product choline. The method was able to detect acetylcholine and its primary degradation product choline at the 30 fmol level, with an analysis time of less than 6 min.

Keywords: Ophthalmic preparation; Analysis; HPLC; Electrospray ionization; Mass spectrometry; Tandem mass spectrometry; MS/MS; Choline; Acetylcholine; Perfluorobutyric acid; Ion-pairing reagent; Post-column addition; Surface tension reducing agent

1. Introduction

Sensitive and specific analytical methods are important for the analysis of aqueous solutions of acetylcholine, **1** (e.g. Miochol-E®), or other direct-acting drugs such as bethanechol, methacholine, and carbachol [1,2]. These preparations have therapeutic utility as intraocular irrigating fluids when administered via subconjunctival or intracameral injection, since they induce a number of desirable physiological responses including prompt short-term miosis and decreased intraocular pressure [3]. Such effects are of particular value during surgical procedures such as cataract removal. Chemical assays for acetylcholine (and choline, **2**) vary widely and for the most part depend on indirect means of detection [4] because of absence of a strongly absorbing UV chromophore in the molecule(s).

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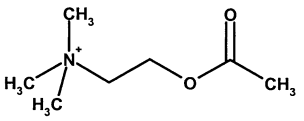
0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(02)00674-X



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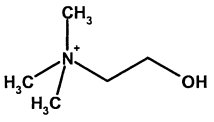

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Representative examples of these indirect methods include electrochemical [5–9] or spectrophotometric detection [10] (both requiring prior enzymatic reaction), and various methods involving radioisotopically labeled material [11–13]. A variety of mass spectrometric methods have also been utilized to detect quaternary ammonium species. The detection of acetylcholine using mass spectrometry was first carried out in conjunction with gas chromatography. A considerable improvement over previously available methods such as bioassay [14], this approach was still indirect, since the quaternary ammonium species had to be converted to a volatile tertiary amine by means of a demethylation reaction, achieved either chemically [15] or by pyrolysis [16]. Ionization methods such as thermospray ionization [17] and fast-atom bombardment [18] found immediate application in the analysis of acetylcholine and related compounds [19,20], because they enabled the combination of on-line high-performance liquid chromatographic (HPLC) separations with a very sensitive mode of mass spectrometric detection, and required no pre-treatment of non-volatile or thermally fragile analytes. The improvements described in those initial accounts were extended in subsequent work that employed electrospray ionization (ESI) [21]. In this study, the investigation of mass spectrometry as a sensitive and specific mode of detection for acetylcholine has been refined through the use of a modified (and mass spectrometrically compatible) HPLC separation method used in combination with ESI and either single-stage (MS) or tandem mass spectrometry (MS/MS). The new method exhibits excellent chromatographic performance (e.g. good peak shape and short analysis time) and detection limits that equal or surpass those reported previously, while providing the enhanced specificity characteristic of multiple reaction monitoring analyses.

2. Experimental

2.1. Materials

Water (Millipore Milli-Q UF purification system), methanol (EM Science, Hawthorne, NY), heptafluorobutyric acid (HFBA; Aldrich, Milwaukee, WI), ammonium hydroxide (Mallinckrodt, St. Louis, MO), Luna C_{18} HPLC column, 3 μ particles, 2.0 mm × 150 mm (Phenomenex, Torrence, CA), acetylcholine chloride drug substance and Miochol-E® drug product (lyophilized preparation of acetylcholine chloride with mannitol and potassium chloride; OMJ Pharmaceuticals, Inc., San German, P.R.).

2.2. Equipment and method

The solvent delivery system was either a Hewlett-Packard HP1100 (Agilent Technologies, Palo Alto, CA) or Perkin–Elmer Series 410 (Norwalk, CT). The HPLC method was a modified isocratic reverse-phase ion-pairing procedure. The mobile phase (Method A) was prepared by adding 1 ml of HFBA to 980 ml of water followed by 20 ml of methanol. The resulting solution was mixed thoroughly and degassed by sonication for 35 min. A variation (for faster separations) of the method (Method B) used a mobile phase having a slightly higher organic modifier content (water, methanol, and perfluorobutyric acid: 90/10/0.1, v/v/v). Evaluation of ammonium ion content on method performance (i.e. reduction of peak tailing) was conducted using a water/methanol mobile phase that contained 0.1% ammonium hydroxide in addition to HFBA (90/10/0.1/0.1, v/v/v/v). Sample solutions of Miochol-E® drug product were prepared by diluting the therapeutic preparation (2 ml water for injection containing 20 mg of acetylcholine chloride, 56 mg mannitol, and either sodium

or potassium chloride as electrolyte) by a factor of 1000 using Milli-Q water. A series of standard solutions was prepared by serial dilution of a stock solution that contained equimolar quantities of choline and acetylcholine dissolved in a water/methanol mixture (90/10). The approximate concentration of the stock solution was 30 mM in each analyte. Five dilutions were carried out to yield solutions having final approximate concentrations of 300 μM, 3 μM, 30 nM, and 300 pM. Injection volumes for the standard solutions were 1, 5, and 10 μl. The injection volume for the Miochol-E® sample solution was 1 μl. The column was eluted isocratically at a flow rate of 0.3 ml/min and maintained at a temperature of 60 °C. The mass spectrometer was operated either in the selected ion monitoring (SIM) mode or in the selected reaction monitoring (SRM) mode. The SRM experiments monitored a collision-induced dissociation (CID) transition for each of the compounds. The transition producing the most abundant fragment ion was selected for each of the analytes ($146^+ \rightarrow 87^+$ for acetylcholine and $104^+ \rightarrow 60^+$ for choline; see Fig. 1). The collision gas was xenon at a measured pressure of approximately 1.5 mTorr (multiple collision conditions). The collision energy in the laboratory frame of reference (E_{lab}) was 15 eV and a dwell time of 0.5 s was used for each transition. SIM experiments monitored the m/z values of the intact cations for the two analytes (m/z: 104 and 146) using dwell times of 0.2 s. Detection was accomplished using a Finnigan TSQ7000 triple quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with the manufacturer's heated capillary atmospheric pressure ionization interface operating in the ESI mode. The electrospray conditions were as follows: 4.5 kV electrospray voltage, 300 °C heated capillary temperature, and 0.6 ml/min eluent flow to the source (0.3 ml/min from the column and 0.3 ml/min make-up flow supplied by an ABI Model 400 pump (Foster City, CA)).

3. Results and discussion

The first reported reverse-phase HPLC method [1] for the analysis of Miochol employed refractive index detection because of the lack of a suitable UV–visible chromophore. While exhibiting sensitivity appropriate for the levels of acetylcholine encountered in bulk drug substance or formulated drug products, this mode of detection lacks the requisite sensitivity for the determination of low levels of related compounds such as decomposition products or synthetic impurities. Refractive index detection also is generally inadequate for the low levels of acetylcholine that are encountered when analyzing for the presence of this endogenous neurotransmitter in biological matrices such as cerebrospinal fluids or tissue homogenates. Improved analytical methods based on enzymatic conversion of acetylcholine to hydrogen peroxide, with subsequent electrochemical detection, display lower detection limits (when compared with refractive index detection), but suffer from the shortcoming of being laborious and requiring non-standard HPLC columns. The most prevalent HPLC methods for the determination of acetylcholine employ alkylsulfonate ion-pairing reagents [22–25], and include the previously reported mass spectrometric methods [19,21,26,27]. While some of these ion-pairing methods exhibited acceptable chromatographic performance, the use of non-volatile mobile phase additives is not generally recommended with mass spectrometric interfaces. These conventional ion-pairing reagents (e.g. alkylsulfonates) have limited volatility and can deposit on various electrostatic ion source or interface elements resulting in poor performance or catastrophic clogging of small-diameter orifices.

The HPLC analysis of acetylcholine and choline in cerebrospinal fluids and tissue homogenates using thermospray ionization [19] demonstrated detection limits in the range of 30 pmol using 4.6 mm i.d. columns. Subsequently, a similar HPLC method [20] reported detection limits of 2 and 5 pmol for acetylcholine and choline, respectively, using continuous-flow FAB ionization and a 1.7 mm i.d. column operating at flow rates between 40 and 150 μl/min. Both these efforts represented a substantial lowering of the limits of detection (LODs) compared with those achieved by refractive index detectors, but they still lagged substantially behind the results (50 fmol) achieved by enzymatic electrochemical methods of detection.

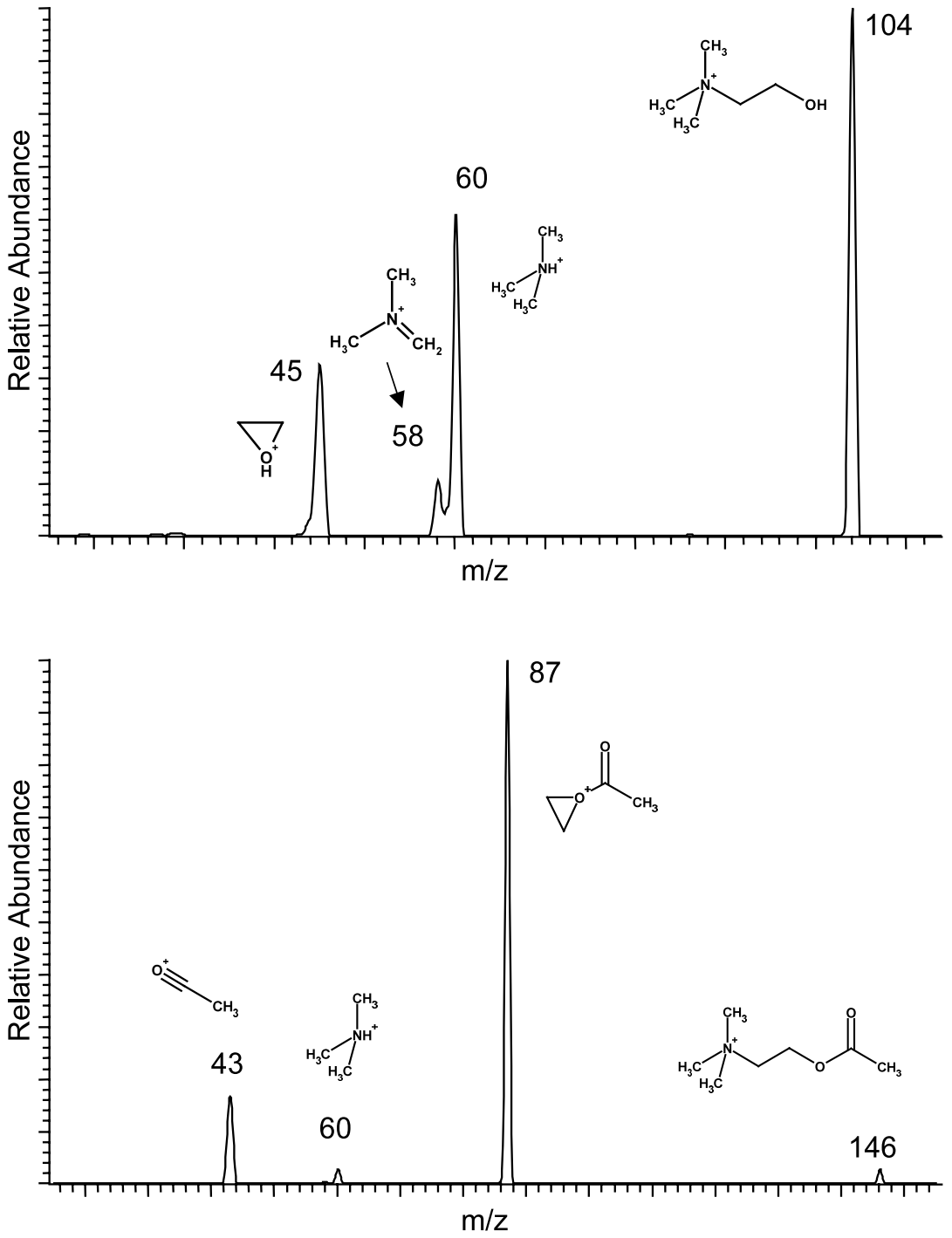

Fig. 1. Product-ion CID spectra for the cations of (top panel) choline (**1**) and (bottom panel) acetylcholine (**2**) illustrating the decomposition processes used for the SRM experiments.

The determination of acetylcholine and other small organic cations using ESI has been reported in the study of complexation behavior of lipophyllic cyclodextrins with small substrate molecules [28] for quantitation in cell culture extracts [21] and as substrates for stopped-flow kinetic studies [29]. An objective of the current work was to develop a chromatographic method that was simple, specific, rapid, and completely compatible with LC/MS interfaces. A combination of elevated column temperature (60 °C), a modest amount of organic modifier, and the use of a perfluorinated organic acid ion-pairing reagent yielded the desired chromatographic performance. The method displayed peak symmetry that was comparable or superior to other recently reported methods and provided an acceptable compromise between k' and analysis cycle time (less than 6 min). HFBA performed as well as the alkylsulfonates (either heptane or octane sulfonic acid or its sodium salt) employed in previous methods and possessed appropriate volatility to preclude potential problems associated with the deposition of nonvolatile salts, such as clogging of the electrospray interface elements. The use of perfluorocarboxylic acids as ion-pairing reagents in HPLC analyses was first reported for the separation of peptides [30]. Since that time, these compounds have found utility in the reversed-phase chromatographic separation of a variety of polar analytes including herbicides [31], aminoglycoside antibiotics [32], leukotrienes [33], and nucleotides [34].

In order to evaluate the current chromatographic methods (referred to subsequently as Methods A and B) against some representative examples of previously reported methods, five figures of merit were calculated for acetylcholine and its main decomposition product (or metabolite), choline. In each of the methods selected for comparison, the following quantities were determined from the available data: asymmetry (A_s), efficiency (N_{Whh}), resolution (R_s), separation factor (α), and cycle time. The values of merit for the five methods are summarized in Table 1. Method B compared favorably in peak symmetry with Method II, which used an ion-pairing method with an exhaustively end-capped stationary phase and a nitrogen-containing mobile phase additive such as pyridine or some ammonium salt to reduce peak tailing. Method A used a mobile phase based on that employed by Acevedo et al. [21]. Notable differences between the two methods (A and II) were the substitution of the perfluorinated organic acid (HFBA) for the conventional ion-pairing reagent (octane sulfonic acid), the presence of a small percentage of an organic modifier (2 vol.% methanol), and the elimination of any nitrogen-containing additives (such as ammonium acetate and pyridine) introduced to eliminate peak tailing. These conditions produced a chromatogram that was quite similar in appearance to that reported in the literature. However, the 16-min cycle time allowed only modest sample throughput (three samples per hour). Increasing the organic modifier content to 10% and heating the column to 60 °C (Method B), reduced retention times for both analytes (and consequently analytical cycle time), improved peak symmetry, and lowered the detection limits for both compounds. As can be seen in Fig. 2, the retention time of acetylcholine was reduced from 11.4 to 4.7 min and the retention time of choline decreased from 4.5 to 3.2 min. More importantly, the peak heights (and areas) of the two analytes increased by 135% (111%) and 200% (63%), respectively, for choline and acetylcholine. The post-column additive (methanol at 0.3 ml/min) was the same for these comparative experiments. Of the previously reported methods, Method II displayed the best efficiency, resolution, and separation factor of the five methods evaluated, but at the expense of analysis time (16 min). Method B showed values for the figures of merit that were comparable to or surpassed those of Methods I, III, and IV, and possessed the added advantage of a total cycle time of 6 min, with an effective throughput rate of 27 analyses per hour (the low value of α allows injections to be "stacked" and made every 2 min). It should be noted that the reduction of analysis time comes at the expense of chromatographic separating power. The sensitivity and the time saving resulting from an abbreviated chromatographic separation and sample "stacking" can only occur in "clean" samples (i.e. those containing no significant interfering components or sample matrix). If the methods were to be applied for the analysis of

Table 1
Comparison of chromatographic parameters for five HPLC methods used for the determination of acetylcholine and choline

	A_s[a]	N_{Whh}[a]	R_s	k'_{AcCh}	k'_{Ch}	α	Cycle time (min)
Method B (present study)	1.2	1690	4	2.0	1.0	2.0	6[b]
Method I (Tao et al. [1])	1.5	1640	4	5.8	3.1	1.9	9
Method II (Liberato et al. [19])	1.0	2070	11	5.3	1.1	4.8	16
Method III (Ishimaru et al. [20])	2.7	700	2	10	7.5	1.3	16
Method IV (Acevedo et al. [21])	1.6	350	3	5.4	1.5	3.6	18

Asymmetry, $A_s = B/A$, where A is the width of the front half of the chromatographic peak measured at 10% height and B the width of the back half of the chromatographic peak measured at 10% height; efficiency, $N_{Whh} = 5.54(t_R/W_{hh})^2$, where t_R is the retention time of the peak of interest and W_{hh} the peak width at half height; resolution, $R_s = (t_2 - t_1)/((W_1 + W_2)/2)$, where t_2 and t_1 are retention times of adjacent chromatographic peaks, and W_1 and W_2 are widths of adjacent chromatographic peaks; separation factor, $\alpha = k'_2/k'_1$, where $k' = (t_R - t_0)/t_0$, where t_0 is the retention time of unretained material.

[a] Average value for the two peaks.

[b] "Stacking" of injections (i.e. each subsequent injection is made before the total analysis time has elapsed) allows for an injection to be made every 2 min (27 analyses per hour).

samples such as tissue homogenates or other biological fluids, it would be a completely different story. In such cases, the methodology would have been rigorously re-evaluated with respect to achievable LOQ and analytical cycle time.

The role of ammonium ions for minimizing peak tailing was evaluated in Method A, but revealed no improvement in peak shape, presumably due to high-quality stationary phase. The inclusion of ammonium ions in the mobile phase did result in an unexpected decrease in sensitivity for both analytes. Consequently, no ammonium ion additive was included in the final version of Method B. The impact of column temperature on the figures of merit for Method A also was evaluated. Increasing the kinetics of analyte mass transfer in the stationary phase by operating the column at an elevated temperature was expected to improve the efficiency of separation. Increasing the column temperature from ambient (approximately 25 °C) to 60 °C resulted in no discernable change in the separation efficiency, but column back-pressure was reduced. In order to maximize the performance characteristics of Method B, a column operating temperature of 60 °C was adopted.

In addition to the optimization of chromatographic performance (as reflected by A_s, N_{Whh}, R_s, and α), the detector response was also maximized in order to achieve the lowest possible LODs. It is well known that eluent composition is a significant aspect of mass spectrometric detector optimization. The composition of the solvent plays an important role in gas-phase ion formation under ESI conditions. Kebarle and coworkers [35] have shown that solution surface tension impacts the formation of gas-phase ions in the ESI process. The release of ions from a sprayed droplet (due to ion evaporation) is more efficient when the surface tension of the eluent solution is low. Thus, eluents comprised of 100% water form ions under electrospray conditions far less efficiently than aqueous solutions containing an organic modifier such as methanol or other organic compound. The presence of an ion-pairing reagent is another factor recognized to diminish ESI signal intensity. In order to evaluate the impact of the high-aqueous content (90% water) ion-pairing mobile phase used in Method B on LODs for choline and acetylcholine, the eluent from the column was supplemented with a make-up flow (methanol) just prior to introduction into the ESI interface. The make-up solvent flow rate was 0.3 ml/min. Addition of the methanol make-up flow to the isocratic (90:10 water/methanol) eluent stream coming from the column (also at 0.3 ml/min) resulted in a total flow rate of 0.6 ml/min into the ESI interface with an effective composition of 45% water and 55% methanol. The experiment was repeated using a make-up flow of water (again at 0.3 ml/min) resulting in an effective ESI eluent composition of 95% water and 5% methanol. The results of this comparison are shown in Fig. 3. The two traces are

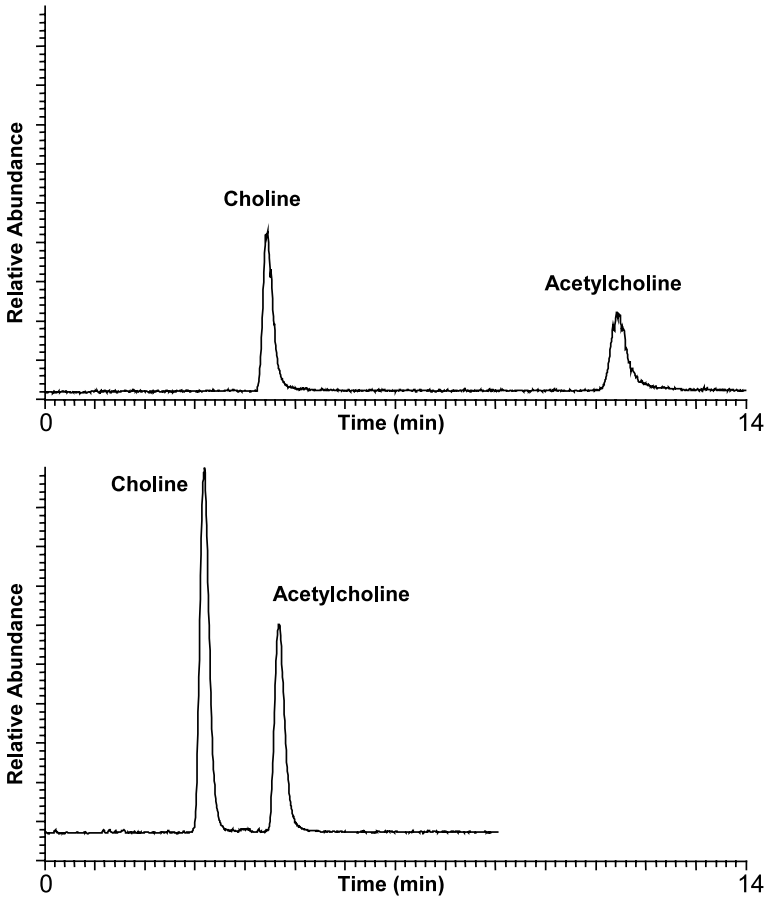

Fig. 2. Ion chromatograms from SIM acquisition (m/z: 104 and 146) obtained for 3 pmol each of choline and acetylcholine injected on-column. (Top panel) LC/MS chromatogram obtained using a mobile phase comprised of water/methanol/HFBA (98/2/0.1, v/v/v) with column at ambient temperature—Method A. (Bottom panel) LC/MS chromatogram obtained using a mobile phase comprised of water/methanol/HFBA (90/10/0.1, v/v/v) with column temperature of 60 °C—Method B. The vertical axis scales for the two traces are identical.

displayed using the same vertical axis scale and demonstrate the increase in signal intensity when a high-aqueous composition mobile phase is supplemented with methanol. The average increase in peak heights with addition of methanol to the eluent stream was 165% (160% for choline and 169% for acetylcholine) and the average increase in peak areas was 183% (171% for choline and 195% for acetylcholine). In a recent report by Yamaguchi et al. [36], the compound 2-(2-methoxyethoxy)ethanol (2-MEE) was found to have a profoundly positive impact on the signal intensity of the negative ions generated from the analyte ibuprofen (and its metabolites). Other post-column additives have been reported to either reduce surface tension or counteract the deleterious impact of ion-pairing reagents [37] or other signal-suppressing agents in HPLC mobile phases.

As expected, the ESI sensitivity for the pre-charged quaternary ammonium ions of choline and acetylcholine was excellent. Shown in Fig. 4 are replicate injections for each of three of the serial dilution standard solutions. The respective amounts of choline and acetylcholine injected for each set of replicates were 0.3 fmol, 1.5 fmol, 3 fmol, 30 fmol, 150 fmol, 300 fmol, 3 pmol, 15 pmol, and 30 pmol. The mass chromatograms exhibit some differential response between choline

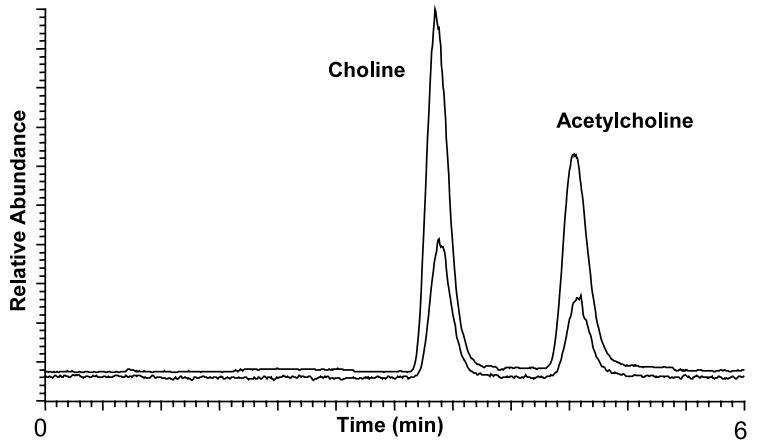

Fig. 3. Ion chromatograms from SIM acquisition (m/z: 104 and 146) obtained for 3 pmol each of choline and acetylcholine injected on-column. (Top trace) LC/MS chromatogram obtained using a mobile phase comprised of water/methanol/HFBA (90/10/0.1, v/v/v) with column temperature of 60 °C and a post-separation make-up flow of methanol (0.3 ml/min). (Bottom trace) LC/MS chromatogram obtained using a mobile phase comprised of water/methanol/HFBA (90/10/0.1, v/v/v) with column temperature of 60 °C and a post-separation make-up flow of water (0.3 ml/min). The vertical axis scales for the two traces are identical.

and acetylcholine. As can be seen in the figure, choline generated detectable responses at the 0.3 fmol level while acetylcholine was only detectable down to 1.5 fmol. These data were obtained with the mass spectrometer operating in SIM mode with sample being introduced directly to the ESI interface using no chromatographic column (flow injection analysis, FIA). Fig. 5 shows the same analysis repeated with the same sample solutions being introduced onto the analytical HPLC column. Not unexpectedly, the signal-to-noise ratio (S/N) for the on-column experiment is poorer, the peak widths are representative of an on-line chromatographic separation (wider than the profiles generated from the FIA experiment), and the sensitivity is diminished due to unavoidable losses

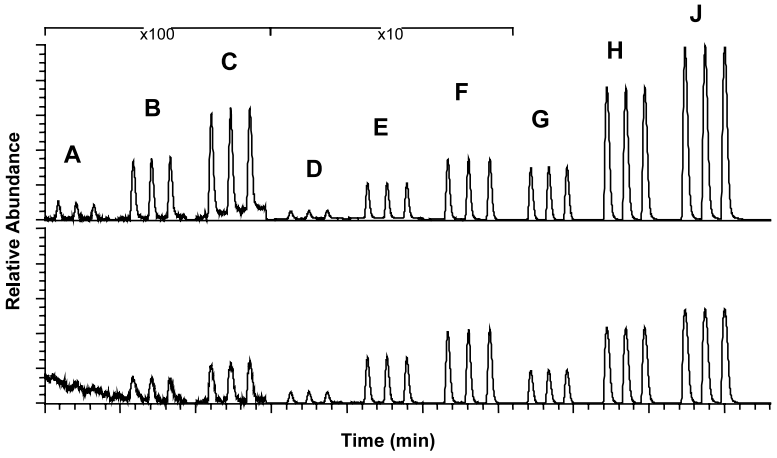

Fig. 4. Series of replicate injections of standard solutions containing equimolar quantities of choline (top panel) and acetylcholine (bottom panel). Samples were injected directly into the electrospray interface (FIA using 50/50/0.1 water/methanol/HFBA, v/v/v) and detection was accomplished using the SIM mode (m/z: 104 and 146, dwell time: 200 ms). On-column quantities of material for the nine sets of injections were as follows: A = 0.3 fmol; B = 1.5 fmol; C = 3 fmol; D = 30 fmol; E = 150 fmol; F = 300 fmol; G = 3 pmol; H = 15 pmol; and J = 30 pmol.

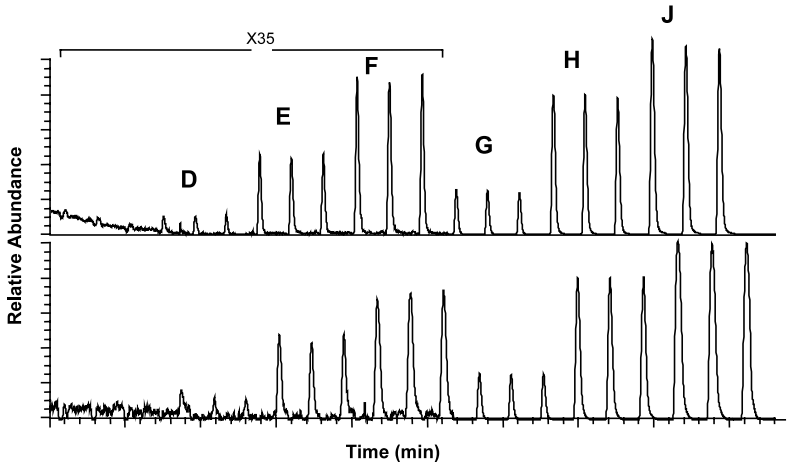

Fig. 5. Series of replicate injections of standard solutions containing equimolar quantities of choline (top panel) and acetylcholine (bottom panel). Samples were injected onto a 2 mm i.d. reverse-phase HPLC column and detection was accomplished using the SIM mode (m/z: 104 and 146, dwell time: 200 ms). The mobile phase was comprised of water/methanol/HFBA (90/10/0.1, v/v/v) with column temperature of 60 °C and a post-column make-up flow of methanol (0.3 ml/min). On-column quantities of material for the six sets of injections were as follows: D = 30 fmol; E = 150 fmol; F = 300 fmol; G = 3 pmol; H = 15 pmol; and J = 30 pmol.

of material due to interactions with the stationary phase. Despite the shortcomings, these results are in agreement with those reported by Acevedo et al. [21]. The FIA results for the standard solutions exhibit lower detection limits than any of the previously reported methods (30 pmol for thermospray [19], 2 pmol for fast-atom bombardment [20], and 0.3 pmol for a previous ESI study [21]) including electrochemical detection [9]. Although this direct means of sample introduction may be suitable for samples having extremely simple matrices (such as Miochol), it would most likely be inappropriate for the analysis of complex samples such as tissue homogenates or biological fluids due to various interferences.

The presence of other species at varying concentrations in even simple samples such as the Miochol formulation (species such as the electrolytes or excipients such as mannitol) can have a deleterious impact on detection limits of low-level compounds due to signal suppression phenomena. Chromatographic separation in combination with a tandem mass spectrometry scheme (either single or multiple reaction monitoring, SRM or MRM) is the most obvious means of achieving the requisite selectivity. By observing only specific unimolecular decomposition transitions for the analyte(s) of interest, a high degree of specificity can be achieved and some of the negative factors arising from the simultaneous presence of multiple compounds may be overcome. Fig. 6 shows the outcome of the SRM experiments obtained for series of replicate injections onto an analytical column. The use of a column again diminishes the sensitivity of the analysis for the lowest concentration sample solutions. However, as before, the detection limits are comparable or superior to previous methods. The performance of the SRM experiment that appears adequate even if additional specificity been required (due to chemical matrices of greater complexity than this simple ophthalmic solution).

4. Conclusions

This account describes a simple ion-pairing reversed-phase HPLC method for the determination of choline and acetylcholine using a mobile phase additive that is completely compatible with

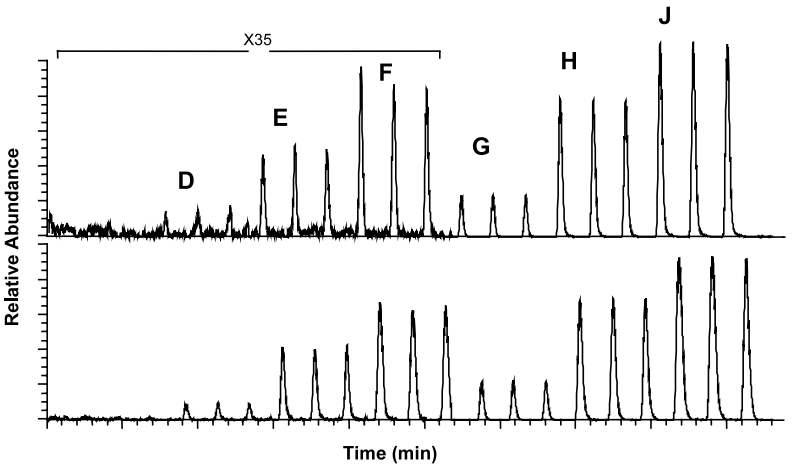

Fig. 6. Series of replicate injections of standard solutions containing equimolar quantities of choline (top panel) and acetylcholine (bottom panel). Samples were injected onto a 2 mm i.d. reverse-phase HPLC column and detection was accomplished using the multiple reaction monitoring mode ($146^+ \rightarrow 87^+$ for acetylcholine and $104^+ \rightarrow 60^+$ for choline with a dwell time of 200 ms). The mobile phase was comprised of water/methanol/HFBA (90/10/0.1, v/v/v) with column temperature of 60 °C and a post-column make-up flow of methanol (0.3 ml/min). On-column quantities of material for the six sets of injections were as follows: D = 30 fmol; E = 150 fmol; F = 300 fmol; G = 3 pmol; H = 15 pmol; and J = 30 pmol.

ESI mass spectrometry interfaces. HFBA provides for a separation that displays good efficiency, peak symmetry, and resolution, all in a cycle time that makes the method very attractive for high-throughput analyses. When used in conjunction with ESI and SIM, the method exhibits excellent sensitivity for these compounds, rivaling or surpassing previously reported methods of detection.

Acknowledgements

The authors acknowledge A.R. Oyler and L. Acosta for their contributions to this work.

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