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Review

Separation methods used in the determination of choline and acetylcholine

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Abstract

Cholinergic neurotransmission has been the subject of intensive investigations in recent years due to increasing recognition of the importance of its roles in physiology, pathology and pharmacology. The fact that the disposition of a neurotransmitter may reflect its functional status has made the measurement of acetylcholine and/or its precursors and metabolites in biological fluids an integral part of cholinergic research. With evolving complexity in experimental approaches and designs, and correspondingly increasing demand on sensitivity, specificity and accuracy matching advancements in sophistication in analytical methods have been made. The present review attempts to survey the array of analytical techniques that have been adopted for the measurement of acetylcholine or its main precursor/metabolite choline ranging from simple bioassays, radioenzymatic assays, gas chromatography (GC) with flame ionization detection, GC with mass spectrometry (GC–MS) detection, high-performance liquid chromatography (HPLC) with electrochemical detection (ED), HPLC with MS (HPLC–MS) to the sophisticated combination of micro-immobilized enzymatic reactor, microbore HPLC and modified electrode technology for the detection of ultra-low levels with particular emphasis on the state of the art techniques.

Keywords: Reviews; Choline; Acetylcholine

Contents

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1. Introduction

That neurotransmission was chemical in nature was unequivocally established and acetylcholine became the very first neurotransmitters identified when the Austrian pharmacologist Otto Loewi demonstrated in 1921 that stimulation of the vagus nerve not only slowed the beating of an isolated frog heart but also the aqueous humor from it slowed the beating of a second heart [1]. Dale and Dudley subsequently identified acetylcholine in the central nervous system in 1929 [2]. The cholinergic system is an important neurotransmission system in both the peripheral and central nervous systems and is implicated, along with other regulatory systems, in the functional regulation of the heart, blood vessels, glands, visceral organs, skeletal and smooth muscles, and various parts of the brain.

Because of the relative simplicity, roles of acetylcholine in the peripheral system are relatively known. These include regulation of muscular contraction in the motor system, ganglionic transmission in the autonomic nervous system as well as transmission at the effector organs of the parasympathetic branch of the autonomic nervous system [3]. The fact that it has an excitatory effect on the membrane of skeletal muscle fibers [4] and an inhibitory effect on the membrane cardiac muscles illustrates the multiplicity of actions by a single agent [5]. Acetylcholine receptors on skeletal muscle fibers control sodium ion channels and produce depolarization (excitation), whereas acetylcholine receptors on cardiac muscle fibers control potassium ion channels and produce hyperpolarizations (inhibition) [6]. Despite intensive research, the roles of acetylcholine in the central nervous system is much less delineated although it is generally recognized that it is involved in temperature, blood pressure regulation, motor coordination, learning and memory [7] and in controlling the stages of sleep [8]. Together, pathological conditions that have been attributed to cholinergic malfunctions include myasthenia gravis [9], coronary artery disease [10], aging, cognition [11], Hirschsprung's disease [12], Tourette's syndrome, Huntington's disease, Schizophrenia [13], Parkinson's disease, Alzheimer's disease [14], and other mental diseases.

In both the peripheral and the central nervous systems, the level of acetylcholine at the biophase, believed to be immediately responsible for the intensity of its actions, is the result of a dynamic equilibrium resulting from interactions between a number of factors including synthesis, storage, release, and inactivation to provide optimal levels of neurotransmission.

Acetylcholine is synthesized from choline and acetyl-coenzyme A (acetyl-Co A), with the reaction being catalyzed by the enzyme choline acetyl-transferase in the presynaptic terminal. Acetyl-CoA is produced by the mitochondria, with an acetate attached to it [15]. Most choline is synthesized in the liver, although some is obtained directly from the diet. Choline is partly obtained by reuptake from the synaptic cleft and partly from the blood, which may cross the blood–brain barrier through a specific membrane transporter. In the presence of the catalyzing choline acetyltransferase located in the mitochondria, the acetate ion is transferred from the CoA molecule to the choline molecule, yielding one molecule of acetylcholine and one of CoA [16].

Upon synthesis, acetylcholine is stored in vesicles of nerve terminals [17,18]. Following stimulation, the vesicles can fuse with the presynaptic membrane and, in the process, discharge their contents into the synaptic cleft. The release of a few hundred packets or quanta of acetylcholine is carried out in about 1 millisecond. This released acetylcholine is rapidly hydrolyzed and inactivated by a specific enzyme, acetylcholine esterase (AChE), present on the pre- and post-junctional membranes of the cholinergic nerve [17–19]. Unlike other neurotransmitter systems in which excess neurotransmitters in the synaptic cleft are taken up again into the presynaptic vesicles through transport systems specific for the neurotransmitters, the specificity of the cholinergic reuptake system is towards choline, which is then recycled. Choline thus serves both as the precursor and metabolite. The physiological actions of acetylcholine in the biological system are mediated through nicotinic and muscarinic cholinergic receptors, which transduce signals via a distinct mechanism [20]. The basic mechanism at the cholinergic synapse is summarized in Fig. 1.

A major difficulty in the definition of cholinergic functions in the central nervous system is the fact that the cholinergic system is anatomically and

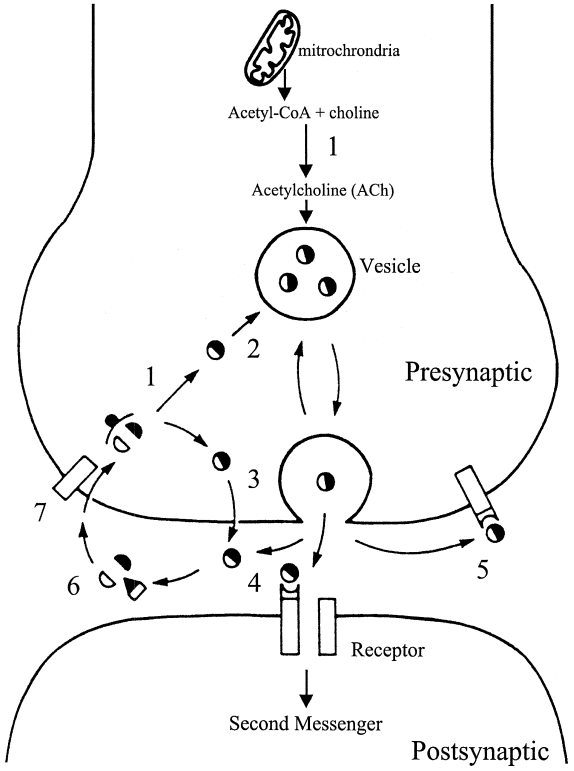

Fig. 1. The main steps of molecular mechanisms of cholinergic synapse are indicated by numbers from (1) to (7) in the above diagrams: (1) synthesis of acetylcholine from acetate and choline in the cell body or the synaptic terminal; (2) transport and storage in vesicles; (3) release by exocytosis and diffusion in the synaptic cleft; (4) binding of acetylcholine to the post-synaptic receptor, which opens the channel and triggers the following biological functions; (5) binding of acetylcholine to the presynaptic receptor; (6) hydrolysis of acetylcholine into acetate and choline by acetylcholinesterase; (7) reuptake of choline into the presynaptic terminal.

functionally very closely intertwined with other neurotransmission system, making delineation of functions very difficult, if not impossible. Based on the premise that intensity of action is proportional to concentration, assays specific for acetylcholine among a slew of other substances in the biological milieu can provide valuable information. In addition, because compensatory mechanisms exist to provide a stable and optimal level of neurotransmission, disposition dynamics rather than the static levels of neurotransmitters are much more informative. Techniques such as the push-pull cannula, microdialysis and various ways of studying turnover are designed to follow the dynamics of acetylcholine in various physiological, pathological and pharmacological contexts.

Analysis methods for acetylcholine can be broadly classified into general categories of bioassay and chemical methods. Bioassays include the isolated tissue of leech dorsal muscle [21], the frog rectus abdominis muscle [22], the guinea pig ileum [23], the serosal strip of the rabbit fundus [24], and the clam heart [25]. Acetylcholine content is estimated from the degree of contraction or the extent of relaxation. However, bioassay methods appear neither specific nor sensitive enough for the determination of acetylcholine [26,27]. Moreover, the reproducibility of the bioassay method is quite poor, necessitating multiple analyses to obtain an average response [28]. Numerous chemical methods have been developed, including radioenzymatic assays, gas chromatography (GC) and mass spectrometry (MS), as well as techniques utilizing high-performance liquid chromatography (HPLC). Indeed, HPLC–ED and GC–MS methods are the most frequently used methods for the analyses of acetylcholine and choline.

2. Gas chromatography–mass spectrometry (GC–MS)

A specific requirement of GC is that the substance to be analyzed is volatile. Volatilization of acetylcholine is achieved by demethylation of the quaternary *N*-atom with sodium benzenethiolate to form the tertiary amine analog. This reaction is relatively selective in converting quaternary ammonium compounds to tertiary amines even at low temperatures [29,30]. The demethylated product, dimethylaminoethyl acetate, is determined by GC with flame ionization detector (FID) [30,31]. The detection limit for acetylcholine by GC–FID method is 0.02 nmol [32]. Acetylcholine is also converted to its volatile dimethylamino derivative by pyrolysis of a halide salt. A quantitative value of 1 ng (7×10^{-12} mole) acetylcholine for pyrolysis GC–FID has been reported [33]. In addition, demethylation by pyrolysis is also suitable for the simultaneous determination of

deuterium-labeled variants of acetylcholine by GC–MS [34].

Quantitation of acetylcholine can be achieved through pyrolytic demethylization of the choline derivatives, thereby allowing the convenient GC separation of the esters of dimethylaminoethanol. Coupling to mass fragmentometric analysis of the $(CH_3)_2NCH_2^+$ ion (m/e 58) permits their quantitation [35,36]. Using this method, it was found that choline was rapidly incorporated into brain acetylcholine following intravenous injection of radiolabeled choline [37,38]. Determination of acetylcholine, choline, and their deuterium labeled moieties using GC–MS and deuterated internal standards was applied to the investigation of acetylcholine turnover in the brain of mice. To prevent postmortem changes in acetylcholine and choline, the mice were killed by focused microwave irradiation to rapidly inactivate the metabolizing enzyme [39]. Deuterated internal standards were also used to correct for variations in recovery. Choline and acetylcholine were measured in rat liver, heart, muscle, kidney, plasma, red blood cells, and brain and in human plasma by GC–MS [40].

To improve the sensitivity in the determination of choline and acetylcholine in biological samples, a capillary column GC–MS was used for canine brain and blood samples. The detection limit was approximately 0.5 pmol/ml for both choline and acetylcholine [41]. The metabolism and blood–brain transport of choline were investigated in perfused canine brain under control conditions and for 60 min after inhibition of brain cholinesterase by organophosphate compounds. Choline and acetylcholine in blood and brain samples were analyzed using GC–MS methods [41,42]. The balance between acetylcholine synthesis and degradation and the availability of choline were studied in the neuromuscular junction of rats by GC–MS [43].

Using GC–MS for determination, acetylcholine turnover rate was used as an index of cholinergic neuronal activity in rats [44]. Intracerebral microdialysis was combined with a sensitive and specific GC–MS assay to measure the release of endogenous acetylcholine in the rat striatum in vivo [45]. A capillary GC–MS assay for the simultaneous quantitation of acetylcholine, and choline in biological tissue has also been developed. The compounds were extracted from tissues by ion-pairing using sodium tetraphenylboron in 3-heptanone. The method easily detected 25 pmol of these chemicals taken through the assay, and was linear through 50 nmol [46].

3. High-performance liquid chromatography (HPLC)

Several disadvantages, however, are associated with GC–MS and radiometric assays [47–49]. These include time-consuming sample clean-up procedures, relatively inadequate sensitivity or specificity, and requirement of expensive equipment [50]. By comparison, HPLC provides the advantages of simplicity, stability, sufficient sensitivity, specificity and also the use of a device common in general laboratories. The use of HPLC–ED [51] for the determination of acetylcholine and choline was originally reported by Potter et al. [52]. This milestone clearly demonstrated the general usefulness of this approach. Subsequent modifications, including column packing optimization, enzyme immobilization and modifications of the electrochemical detector, have improved the methodology to enable the detection of basal levels of acetylcholine in rat brain dialysates. A detection limit of 20 fmol for acetylcholine was obtained for the routine analysis of acetylcholine release by microdialysis in the presence and absence of esterase inhibitors [53]. HPLC coupled to electrochemical detection (ED) is based on the separation of acetylcholine and choline by a reversed-phase column and then mixing/reacting with a solution containing AChE and choline oxidase in a reaction coil to generate electrochemically detectable hydrogen peroxide, as discussed by Potter et al. [52]. Choline and acetylcholine were first separated by reversed-phase chromatography; acetylcholine was then hydrolyzed to choline in post-column allowed to mix/react with a solution containing AChE and choline oxidase. Choline was oxidized enzymatically by choline oxidase to betaine and hydrogen peroxide (Fig. 2). The resulting hydrogen peroxide can be detected electrochemically. The sensitivity of this procedure is 1 pmol for choline and 2 pmol for acetylcholine [52]. Later, the solution enzymes were improved with an immobilization enzyme reactor

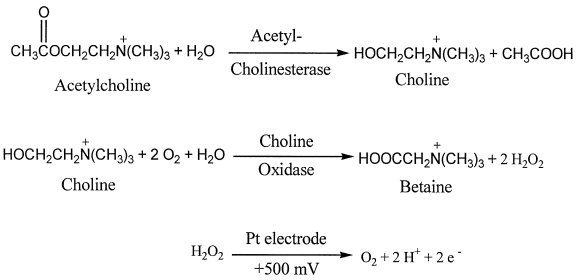

Fig. 2. Enzymatic and electrochemical conversion scheme for acetylcholine and choline to hydrogen peroxide.

column by Damsma et al. [54]. In their extended study, AChE and choline oxidase are covalently bonded to a post-column immobilized enzyme reactor (IMER) which generates hydrogen peroxide according to specific reactions (Fig. 3). The immobilization of AChE and choline oxidase significantly increases the sensitivity of acetylcholine and reduces the waste of expensive enzymes [53,55,56]. The sensitivity of post-column IMER method is 50 fmol [54] and 10 fmol [57] for acetylcholine.

The enzyme reactor was subsequently improved by immobilizing the enzymes through physical binding to an anion-exchanger [58]. By this method, AChE and choline oxidase were found to retain their activity for 1–2 weeks at room temperature while adsorbed to an anion-exchanger cartridge [59]. A

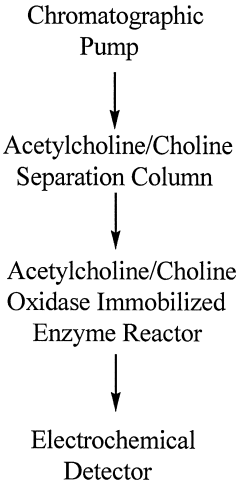

Fig. 3. Flow chart for the assay of acetylcholine and choline by the sequence of analytical column and post-column enzyme reactor (from Ref. [68]).

sandwich-type enzyme reactor was developed in which enzymes were physically immobilized in a minimal dead space between two cellulose membranes. This design improves sensitivity for the determination of acetylcholine and choline, providing determination limits of acetylcholine and choline at 15 and 10 fmol, respectively [60].

In addition, commercial acetylcholine and choline assay kits with microbore separation column and IMER are available from the Bioanalytical System Inc. (BAS, West Lafayette, IN, USA) [61–65] (Fig. 4), and from Eicom (Kyoto, Japan) [66].

A problem, in that the acetylcholine peak overlaps with that of choline, arises when the performance of an analytical column deteriorates. The choline peak is so large that it may interfere with the acetylcholine measurement. To remove the interfering choline, a pre-column IMER has been adopted for the selective assay of acetylcholine. Placing a pre-column of immobilized choline oxidase in the flow sequence has the effect of converting choline to hydrogen peroxide. However, although hydrogen peroxide is not retained in the analytical column, this signal appears on the chromatogram. The use of combination enzymes of choline oxidase and catalase IMER in a pre-column reaction to eliminate choline can

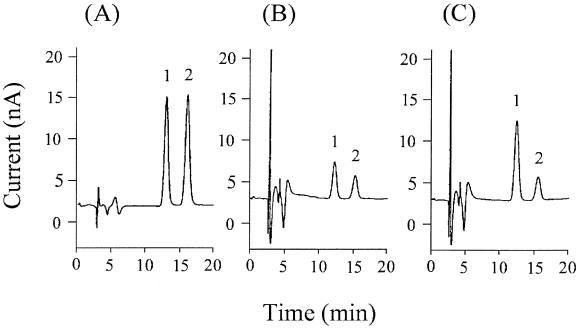

Fig. 4. Typical chromatogram of acetylcholine (1) and choline (2) assay. BAS microbore polymeric column (530×1 mm I.D., particle size 10 μm); mobile phase (pH 8.5) consisting of 28 m*M* Na_2HPO_4 and 0.5% antimicrobial solution Kathon (1.0%); flow-rate 0.1 ml/min; post-column reactor (BAS, acetylcholinesterase/choline oxidase IMER, 55×1 mm, particle size 10 μm); platinum electrode set at a potential of +0.5 V *vs.* Ag/AgCl. A: standard mixture containing acetylcholine and choline; B: baseline dialysate sample from striatum; C: dialysate sample collected 40 mm after methamphetamine treatment (2 mg/kg, s.c.) (from Ref. [65]).

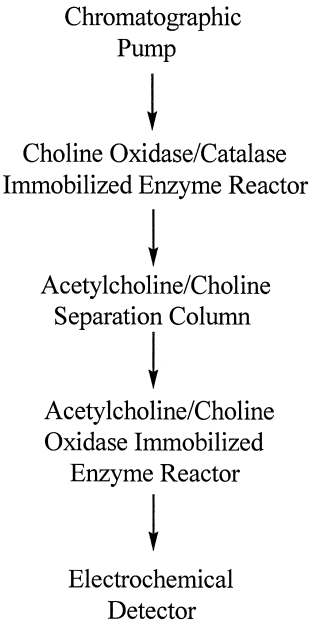

Fig. 5. Flow chart for the assay of acetylcholine following the sequence of pre-column enzyme reactor, analytical column and post-column enzyme reactor (from Ref. [68]).

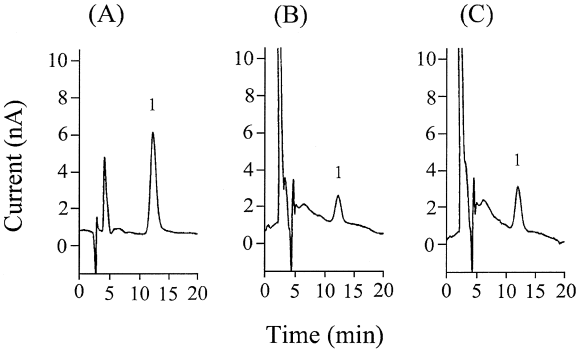

Fig. 6. Typical chromatogram of acetylcholine (1) assay. BAS microbore polymeric column (530×1 mm, particle size 10 μm); mobile phase (pH 8.5) consisting of 28 m*M* Na_2HPO_4 and 0.5% antimicrobial solution Kathon (1.0%); flow-rate 0.1 ml/mm; pre-column reactor (BAS, choline oxidase and catalase combination IMER, 55×1 mm I.D., particle size 10 μ*M*); post-column reactor (BAS, acetylcholinesterase/choline oxidase IMER, 55×1 mm I.D., particle size 10 μm); platinum electrode set at a potential of +0.5 V *vs*. Ag/AgCl. A: standard acetylcholine; B: baseline dialysate sample from striatum; C: dialysate sample collected 40 mm after physostigmine treatment (0.5 mg/kg, s.c.) (from Ref. [68]).

significantly reduce void response due to choline conversion (Fig. 5). This improvement results from the digestion of the hydrogen peroxide by catalase [67,68] (Fig. 6).

A rapid drop from the initial sensitivity of a newly installed platinum electrode or coating electrode is frequently observed. The successive decrease of acetylcholine response over time makes the quantification of acetylcholine problematic. This is a serious limitation of HPLC–ED methodology, especially for on-line coupling of microdialysis to HPLC–ED. To overcome this problem, internal standardization or frequent calibrations could be used to improve the accuracy of the assay. Kehr et al. used an internal standard in the perfusion solution in their microdialysis system [69]. This approach eliminates the volume errors caused by pipetting and volume loss during microdialysis sampling. Using an internal standard also permits the determination of its in vivo recovery, which can be used as a measure of acetylcholine recovery in vivo.

Recently, a horseradish peroxidase–osmium redox polymer-modified glassy carbon electrode (HRP-GCE) has been employed instead of the conventional platinum electrode used in HPLC–ED [70]. This technique could detect less than 10 fmol of standard acetylcholine and basal acetylcholine levels in the dialysate from a conventional concentric design microdialysis probe without the use of cholinesterase inhibitors. This technique also may facilitate physiological investigation of cholinergic neuronal activity in the central nervous system [71,72]. A modified glassy carbon electrode, the plastic formed carbon (PFC), has been prepared by mixing various amounts of pure graphite with an organic binder and pyrolysing the mixture to a ''glassy carbon'' for the direct determination of acetylcholine and choline. These PFC pre-columns are useful for the direct determination of acetylcholine and choline in brain tissue homogenates and other biological samples [73].

Acetylcholine and choline retained in the reversed-phase column is a function of the percentage acetonitrile, salt concentration, concentration of the ion

pairing reagent, and its carbon chain length i.e. C_{18} or C_8 [52,74–77]. Ion-pairing and hydrophobic interactions are two separate retention mechanisms for acetylcholine assay by reversed-phase column. Increasing salt concentration in the mobile phase causes a sharper acetylcholine peak for the reversed-phase column [57,78]. Cation-exchange liquid chromatographic system for the determination of acetylcholine and choline provide several advantages over the conventional silica-based cation-exchange chromatography: better pH stability; shielded active sites of the packing material; as well as interaction with anionic groups, allowing better optimization of the composition of the mobile phase for enzymatic reaction [78–80]. A styrene polymer as packing material for liquid chromatographic separation under weak alkaline condition (pH 8.3) achieved a good separation of acetylcholine in brain tissue extraction. Minimal detectable amounts of both acetylcholine and choline were 1 pmol [81].

In order to improve the determination sensitivity of acetylcholine and choline in biological samples, a microbore liquid chromatographic was developed. The advantages of the microbore system are greater sensitivity, shorter analysis time, and slower flow-rate of the mobile phase [71]. The system of microbore HPLC provides a good linear relationship between the peak height and the concentration of acetylcholine over the range of 0.05–10 pmol/5 μl. The limit of quantitation has been reported to be 0.05 pmol/5 μl or 10 n*M* [65,69,82].

Thermospray liquid chromatography–mass spectrometry (TS-LC–MS) and HPLC continuous-flow fast atom bombardment– mass spectrometry (HPLC-FAB–MS) with the detection limit of 2–5 pmol have also been developed. The advantage of HPLC-FAB–MS is that it requires minimal sample preparation and no derivatization [83,84]. A chemiluminescent procedure has also been developed and used to continuously detect the release of acetylcholine from stimulated electric organ slices and synaptosomes [85–87]. The sensitivity for the determination of acetylcholine is similar to the immobilized enzyme methods, although cholinergic drug interference with the chemiluminescent assay is minimal [88]. A survey of detection limits of various HPLC methods is summarized in Table 1.

4. Biological applications

4.1. Brain tissue and smooth muscle samples

A large number of analytical techniques have been developed for the determination of acetylcholine and choline in biological tissues, employing bioassays, enzyme assays, chemical procedures or instrumental methods [36,38]. To prevent postmortem changes of acetylcholine and choline in brain tissues, the animals are killed by microwave irradiation [89]. The basic extraction method is based on the procedure of Maruyama et al., [90]. Dilute formic acid (15%) is used for the homogenization solution to precipitate tissue proteins. To minimize the solvent front and interference peaks in the chromatogram, potassium triiodide solution (KI and I_2) is added to the homogenate supernatant for the oxidation of unwanted compounds. The limits of detection for acetylcholine and choline are approximately 10 pmol [91]. Alternatively, brain tissues could be extracted by acidic deproteinization [92–94].

Ikarashi et al. from 1985 to 1998 attempted a number of modifications designed to completely remove the interference from biological amines and their metabolites in HPLC–ED analysis [95]. The incorporation of a glassy carbon pre-column dramatically decreases the size of the solvent front as well as interfering catecholamine peaks, resulting in decrease in sample preparation time and shortening of chromatographic elution time and possibly improvement in sensitivity for acetylcholine and choline [91,95,96]. Alternatively, plastic formed carbon (PFC) can be inserted as pre-column to completely eliminate biological amine peaks. The solvent front was dramatically reduced by the PFC pre-column [73].

The investigation for release of acetylcholine during cholinergic stimulation of rabbit trachea was carried out using HPLC–ED [97]. The results indicate that post-synaptic M3 muscarinic receptor antagonists inhibit smooth muscle contraction in rabbit trachea without increasing the release of acetylcholine [98]. Increased release of acetylcholine from airway parasympathetic nerve endings is one mechanism that may contribute to increase in airway responsiveness in immunoglobulin B (IgE)-immune

Table 1
HPLC assays for the determination of choline and acetylcholine

Specimen	Column	Reaction	Detector	Detection limit	References
Plasma	Reverse-phase	Soluble enzyme	ED, Pt electrode		[51]
Brain tissue	Reverse-phase	Soluble enzyme	ED, Pt electrode	2 pmol	[52]
Microdialysate	Reverse-phase	Post-column IMER	ED, Pt electrode	20 fmol	[53]
Brain tissue	Reverse-phase	Post-column IMER	ED, Pt electrode		[55]
Plasma	Reverse-phase	Post-column IMER	ED, Pt electrode	10 pmol	[57]
Brain tissue	Polymeric	Post-column IMER	ED, Pt electrode	5 pmol	[59]
Salivary gland	Reverse-phase				
Brain tissue, microdialysate	Microbore Aminex A-9	Post-column IMER	ED, Pt electrode	15 fmol	[60]
Brain tissue, microdialysate	Polymeric	Post-column IMER	ED, Pt electrode		[61,62]
Microdialysate	Polymeric	Pre- and post-column IMER	ED, Pt electrode	1–8 pmol	[63,64]
Microdialysis	Microbore Polymeric	Post-column IMER	ED, Pt electrode	25 fmol	[65]
Cerebrospinal fluid	Shodex RSpak	Post-column IMER	ED, Pt electrode	0.3 pmol	[66]
Microdialysis	Microbore Polymeric	Pre- and post-column IMER	ED, Pt electrode	25 fmol	[68,82]
Brain tissue	Polymeric Styrene-based	Post-column IMER	ED, Pt electrode		[96]
Microdialysate	Microbore cation-exchange	Post-column IMER	ED, Peroxidase-redox polymer coated glassy carbon	10 fmol	[69,71]
Microdialysate	Microbore	Pre- and post-column IMER	ED, Horseradish peroxidase-osmium redox polymer glassy carbon electrode	10 fmol	[72]
Cerebrospinal fluid	Reverse-phase	Post-column IMER	ED, Peroxidase-redox	50 fmol	[75]
Microdialysate	Reverse-phase	Post-column IMER	ED, Peroxidase-redox		[76]
Brain tissue	Styrene polymer	Post-column IMER	ED, Peroxidase-redox	1 pmol	[81]
Brain tissue	Reverse-phase		Fast atom bombardment MS	2 pmol	[83]

allergen-exposed animals [99]. The effect of neuropeptides on cholinergic neurotransmission has been evaluated by directly measuring acetylcholine released from nerve endings [100]. That acetylcholine released from horse airway cholinergic nerves is modulated by specific adrenoceptors is derived from data from HPLC–ED determination of acetylcholine in a tissue bath HPLC–ED [101,102].

4.2. Blood and cerebrospinal fluid (CSF)

Despite modern HPLC–ED and GC–MS analytical methods, the quantification of acetylcholine in blood and CSF remains difficult. Two major reasons are its very low concentrations (ca. <20 pmol/ml) in blood and CSF and its very fast hydrolysis by esterase. Concerning the reliability of analytical

methods, HPLC–ED and GC–MS are both suitable for the determination of acetylcholine and choline in blood and CSF. There is no consensus concerning the correct actual value of acetylcholine concentration in blood. Okonek and Kilbinger were unable to detect acetylcholine in blood plasma [103], but Hanin et al. reported 28 pmol/ml of acetylcholine in plasma using GC–MS [104]. Four different methods (TS–MS, HPLC–MS, HPLC–ED, and GC–MS) to measure acetylcholine and choline concentrations in CSF were compared by Frolich et al. [105]. A solid-phase extraction of human CSF was carried out. Only GC–MS and HPLC–ED were considered adequate for the detection of acetylcholine in human CSF extracts. However, their data indicated that methodological limitations precluded some samples below detection limit. Further work for the improvement of detection limit of these methods in the determination of acetylcholine for clinical applications is warranted.

4.3. Microdialysate

Microdialysis is an in vivo brain perfusion method, originally used by Ungerstedt and his group at the beginning of the 1980s, to measure dopamine release from the rat striatum [106]. The microdialysis technique for the determination of acetylcholine was first developed by Damsma et al. [54,107–110], Westerink et al. [111–114], and Ajima and Kato [115–117].

The semi-permeable dialysis membrane permits bi-directional movements of small molecules (e.g. neurotransmitters and their metabolites) between the extracellular fluid and the perfusate. Unlike open-ended perfusion methods (for example push-pull perfusion), neurotransmitters in the brain extracellular fluid diffuse down the concentration gradient into the perfusion solution via the semi-permeable membrane and is constantly being swept away, allowing steady but dynamic extraction of the neurotransmitters. This simple innovation minimizes the disturbance of brain tissues and avoids direct washing, pressurizing and traumatizing of the tissue. An important advantage of this method is that it can be applied to small laboratory animals for the neurochemical research. However, because of limitations in recovery, the sensitivity of the current available analytical systems is insufficient for reliable quantification of acetylcholine in microdialysates on a routine basis [118].

This problem has been partially solved by the addition of an AChE inhibitor in the perfusion solution to enhance the extracellular levels of acetylcholine. Typically, 50 fmol of acetylcholine could be detected with microbore HPLC–ED. Many investigators have employed AChE inhibitors, such as physostigmine or neostigmine, in the measurement of acetylcholine [65,68,69]. Unfortunately, the use of AChE inhibitors is less than ideal as they affect neuronal activity and the pharmacology of the neuronal circuits under investigation. Detection of basal acetylcholine in brain microdialysates without using AChE inhibitors has been reported by Huang et al. [71], and Kato et al. [72]. A peroxidase-redox polymer modified glassy carbon electrode operated at +100 mV vs. Ag/AgCl has been used to detect the reduction of hydrogen peroxide. With this method, a detection limit of 10 fmol (injected) for acetylcholine ($S/N=3:1$) was obtained and the basal acetylcholine concentration in striatal microdialysate was determined without using esterase inhibitors [71]. Typically, a detection limit of ca. 10 fmol acetylcholine could be obtained in microbore HPLC–ED methods with modified electrodes.

5. Conclusion

Based on current understanding that the intensity of action is proportional to the concentration of an active agent and that the functional level of acetylcholine is normally regulated through a number of biochemical processes, the reliable determination of acetylcholine and choline concentrations in biological samples is indeed valuable in investigations of cholinergic neurotransmission, particular in a complex organ such as the brain where multiple neurotransmission systems exist and interact in intimate manners. With ever-increasing demands on sensitivity, specificity and reliability, the simplistic bioassays and variable radioenzymatic assays have largely been superseded. Due to the heterogeneity of biological samples, the objectives in modern assay develop-

ments are separation and positive identification through chemical recognition. Thus, the high resolution power of GC and HPLC is utilized and coupled to sensitive and selective detectors such as MS and ED. While both techniques offer high degrees of sensitivity, specificity and accuracy, each possesses certain advantages and disadvantages. Appropriate combination could be complementary. Thus HPLC–MS could circumvent the need for volatility and therefore derivatization while retaining positive identification of the compounds being investigated. On the other hand, HPLC–ED provides a much less expensive alternative. Further modifications such as micro IMER, microbore HPLC and modified ED greatly enhances the sensitivity and selectivity. In conclusion, GC–MS, HPLC–MS and HPLC–ED provide the mainstream of modern analytical techniques for acetylcholine and choline in biological fluids with additional modifications extending their limits of usefulness. Depending on experimental designs, objectives and availability of funds and equipment, these techniques or variations thereof are adequate for most experimental purposes. However, there is still room for improvement as clinical reliability is still unsatisfactory.

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