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Review

Biologically active components of *Physostigma venenosum*

Bin Zhao*, Shabbir M. Moochhala, Su-yin Tham

Centre for Biomedical Sciences, Defence Medical and Environmental Research Institute, DSO National Laboratories (DMERI @ DSO), 27 Medical Drive, #09-01, Singapore 117510, Republic of Singapore

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Abstract

Physostigmine is a major alkaloid found in the seeds of the fabaceous plant *Physostigma venenosum*. It is a powerful and reversible acetylcholine esterase inhibitor which effectively increases the concentration of acetylcholine at the sites of cholinergic transmission. It exerts its cholinesterase inhibitor effect in both the periphery and central nervous system. Many studies on physostigmine have involved the reliance on techniques that extract and quantify physostigmine in biological samples. This paper presents an overview of the currently applied methodologies for the determination of physostigmine and its metabolites in various biological samples. Papers published from January 1980 to December 2003 were taken into consideration for the discussion of the metabolism and analytical method of physostigmine. HPLC methods have been discussed and used in most of the references cited in this review. A few CE and RIA methods that have been recently reported are also mentioned in this paper. Basic information about the sample assayed, sample preparation, chromatographic column, mobile phase, detection mode and validation data are summarized in a table.

Keyword: Physostigma venenosum

Contents

Abbreviations: AChE, acetylcholinesterase; ATP, adenosine triphosphate; BSA, bovine serum albumin; CE, capillary electrophoresis; CNS, central nervous system; CSF, cerebrospinal fluid; CZE, capillary zone electrophoresis; DPS, sodium alpha-(3,4-dimethoxyphenyl) cinnamonitrile-2′-sulfonate; ECD, electrochemical detection; EK, electrokinetic injection; FL, fluorescence; GC, gas chromatography; HPLC, high-performance liquid chromatography; HI, hydrodynamic injection; LC, liquid chromatography; LLE, liquid–liquid extraction; LOD, limit of detection; LOQ, limit of quantitation; NLM, National Library of Medicine; ODS, octadecylsilanized silica gel; PAD, photodiode-array detection; PC, paper chromatography; PK, pharmacokinetics; RFD, radioactive flow detector; RIA, radioimmunoassays; R.S.D., relative standard deviation; SDS, sodium dodecyl sulphate; SPE, solid-phase extraction; TLC, thin-layer chromatography; UV, ultraviolet

* Corresponding author. Tel.: +65 6485 7202; fax: +65 6485 7226.
E-mail address: zbin@dso.org.sg (B. Zhao).

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1. Introduction

Physostigma venenosum (Calabar) is one of the numerous woody climbers, which inhabit the tropical forests of Africa, sometimes reaching the length of 50 ft, with a stem 2 in. in diameter. The flowers are borne in pendulous racemes similar to the garden bean, but are of a darker color. The fruit is a thick brown pod, each containing two or three seeds that are familiar to us as "Calabar beans" [1].

Physostigmine (eserine), calabarine, eseridine, eseramine and phytosterin were some of the components of calabar bean that were discovered in the 19th century [1]. Although other chemicals were discovered later, physostigmine still remains as the most valuable alkaloid of the Calabar bean [2–4]. It is soluble in alcohol, ether, chloroform, benzol and carbon disulphide; and slightly soluble in water. Physostigmine is highly unstable, and it turns red and converts into rubreserine upon exposure to light, air and especially heat [5,6].

Physostigmine is a powerful sialogogue and stimulates almost all involuntary muscles in the body. It is a powerful and reversible acetylcholine esterase inhibitor, which effectively increases the concentration of acetylcholine at the sites of cholinergic transmission [7]. Its carbamate functional group binds to the active site of cholinesterase in a similar manner to the binding of organophosphorous cholinesterase inhibitors to cholinesterase [8,9]. The carbamylation of the active site of cholinesterase ensures that the active site is protected from attack by organophosphorous nerve agents, which binds to the enzyme permanently. The result is a continual stimulation of receptor cells that causes intense spasms of muscles due to the prevention of a breakdown of acetylcholine. This reversible carbamylation accounts for the prophylactic properties of physostigmine against poisoning by nerve agents. It has recently been shown that physostigmine is more effective than pyridostigmine for protection against the detrimental effects of organophosphorus nerve agent in human [10].

Physostigmine has been used mainly for the treatment of glaucoma and myasthenia gravis as it is capable of constricting the pupil and hence facilitates the outflow of aqueous humor [11]. Its capability of contracting the eye pupil was discovered in the late 19th century [1]. Clinically, it can also be used to reverse the effect upon the central nervous system, caused by clinical or toxic dosages of drugs capable of producing the anticholinergic syndrome [12,13]. The ability of physostigmine to increase acetylcholine levels allows it to be used for treatment from over dosages of tricyclic antidepressants, antihistamines, antipsychotics and benzodiazepines [7]. More recent studies have shown that physostigmine may aid and improve the performance of everyday working memory when administered to people by infusion in laboratory tests [14]. In a study conducted in 1978 by Davis et al., it was found that physostigmine significantly enhanced storage of information into long-term memory in 19 normal, male subjects who received 1 mg of the drug on two non-consecutive days. There was also an improvement in retrieval of this long-term memory [15].

Eseroline, the major degradation product of physostigmine, possesses morphine-like effects and produces untoward stimulation of the CNS [16,17]. The hydrolysis product is comparatively more toxic than the parent compound, physostigmine. It seems that eseroline causes neuronal cell death by a mechanism involving loss of cell ATP [18]. Hence, formation of eseroline may contribute to the toxic effect of physostigmine.

Calabarine, an ether-insoluble alkaloid, resembles strychnine in its physiological action more nearly than physostigmine and is believed to be a decomposition product of physostigma alkaloids. Eseridine is closely related to physostigmine, and is convertible to the latter by warming with dilute acids. Phytosterin is a substance that is related to cholesterin in animal fats [1].

Few published reports have been made on calabarine, eseridine, eseramine, phytoserin and other chemicals and their potential as medicines have not been established as compared to physostigmine. Hence, our review will concentrate on the identification and quantification of physostigmine and its degradation products by high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) and other methods. A structured literature search will be performed to identify articles of chromatographic and electrophoretic methods pertaining to physostigmine analysis.

2. Degradation products of physostigmine

2.1. Decomposition path

Physostigmine is not stable and hydrolyzed enzymatically or in aqueous medium at high pH, particularly at pH >5 [19]. Hydrolysis initially removes the urethane group to produce eseroline, a colorless compound. Subsequent oxidation yields rubreserine, a red compound, which is in turn converted into eserine blue or eserine brown (Fig. 1) [5,21].

The in vitro rates of decomposition of physostigmine in plasma at ambient temperature (22 °C) have been determined with and without the presence of stabilizing agents [20]. The half-life of physostigmine in fresh human plasma was approximately 30 min at 22 °C.

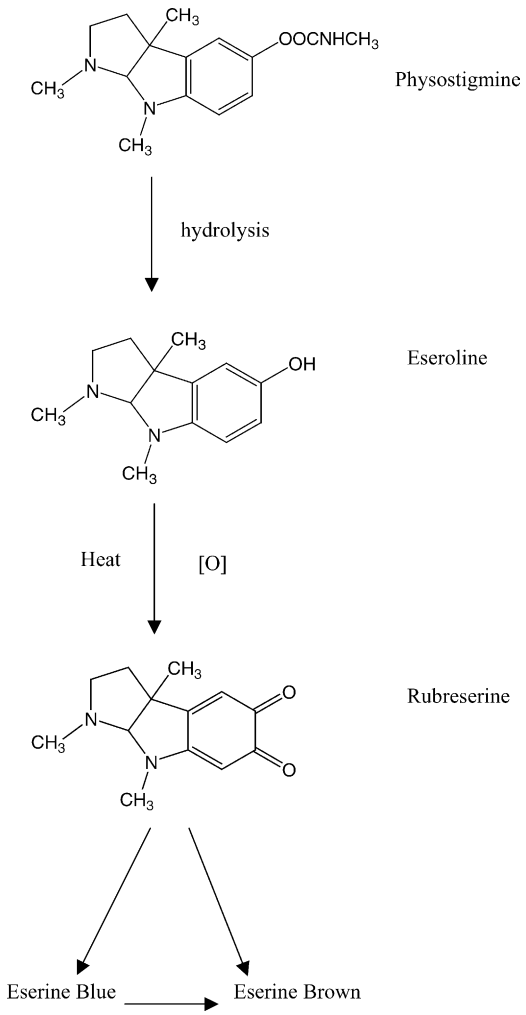

Fig. 1. Decomposition pathways of physostigmine.

2.2. The metabolism of physostigmine

Despite the long history of study of the pharmacologic properties of physostigmine, relatively few pharmacokinetic (PK) studies have been carried out [7]. PK studies with physostigmine have been conducted in several animal species including rat [22–26], guinea pig [27] and dog [28]. Physostigmine and/or its metabolite concentrations can be measured by HPLC [22,23] and by radioimmune assay [29,30]. These studies show that physostigmine has a high clearance and hepatic extraction ratio; and is eliminated almost entirely via several metabolic pathways. Triggle et al. [7] showed that hepatic blood flow is a principal determinant of physostigmine clearance.

Investigation on the route of physostigmine metabolism in the rat has been carried out following intramuscular, intravenous and oral administration [22–26]. It has been shown that the dominant route of metabolism is through the liver. Unni and Somani [26] have reported that about 90% of physostigmine is metabolized by the liver within 2 min of administration. Metabolism is the major route of elimination of physostigmine [22] rather than urinary or biliary excretion [22,31]. The liver plays a particularly important role in the metabolism of physostigmine [26]. The major metabolic products of physostigmine, including eseroline, rubreserine and the condensation products eserine blue and eserine brown are significantly less active inhibitors of AChE than physostigmine itself [21,32,33].

Asthana et al. [34] have shown that plasma concentrations were detectable following 60-min intravenous infusions of 1.5 mg physostigmine in human. There are few PK studies on the metabolites other than physostigmine in human, although they have been identified in animal studies. The lack of PK data on the metabolites other than physostigmine is due to the lack of sensitive determination methods for them in plasma or blood.

3. Separation procedures

3.1. Sample preparation, extraction procedures and removing of coextracted compounds

A critical aspect of physostigmine analysis is the sample extraction step, which requires the isolation of the residue (containing the physostigmine) from a biological material and removing potentially interfering compounds. Fig. 2 provides a general outline of the preparation procedures for the determination of physostigmine.

A potential problem when extracting physostigmine in blood or plasma is the hydrolysis of the compound by plasma esterases after blood samples have been taken. Hydrolysis is affected by time, temperature and pH [40,41]. Whelpton and

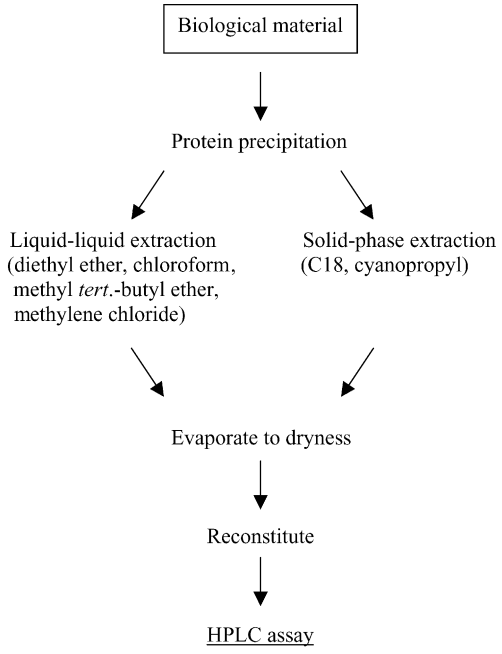

Fig. 2. Preparation procedures for the determination of physostigmine and/or its degradation products in biological material.

Moore reported that physostigmine concentrations declined from an initial value of 5 ng/ml, with apparent first-order kinetics [41]. At 37 °C, the apparent first-order half-time was approximately 15 min. At room temperature (22 °C), 52% physostigmine was lost in 1 h. Even at 4 °C, the loss was not negligible. Physostigmine contains two nitrogen atoms with pK_a values of 1.8 and 7.9 [40]. If the pH of the aqueous medium is high, too much physostigmine may be hydrolyzed during the extraction procedure. Nevertheless, solvent extraction of physostigmine has always been carried out from alkaline aqueous solutions.

Ammonium hydroxide has been used for the precipitation of plasma proteins in many studies [40–42]. Whelpton used 1 ml of 1 M ammonium hydroxide in 3 ml of plasma to extract physostigmine. The pH of the solution via this extraction method was pH 10, which is approximately two units greater than the higher pK_a value of the nitrogen atom in physostigmine [40]. Assuming hydrolysis in dilute solution to be pseudo-first order, an estimate of the decomposition during extraction can be made using the data of Christenson [40]. The decomposition at 25 °C and pH 10 is approximately 5%, whereas at pH 11 it is 38%/h and by pH 12, less than 5% of the original concentration would be present after 1 h. Whelpton [40] was able to complete the extractions in less than 1 h. Once extracted, the residue can be stored overnight at 4 °C without noticeable losses.

Alkaline precipitation of plasma proteins is preferred because acid treatment yielded an extraneous peak that co-eluted with physostigmine [42]. Commonly used extraction solvents in liquid–liquid extraction (LLE) schemes for physostigmine and/or its metabolites, includes diethyl ether [40–42,45], methyl *tert*-butyl ether [20,43,46], chloroform [47,48] and methylene chloride [44]. There is little to distinguish these solvents in terms of their extraction power. Several authors [40–42,45] that have used diethyl ether extraction method to isolate physostigmine from plasma have reported that recoveries range from 63 to 93%. Somani and Khalique [47] and Lau et al. [48] reported recovery ranges from 65 to 99%, using chloroform. Brodie et al. [43], reported a recovery >90% for physostigmine after alkaline precipitation of plasma, extraction with methyl *tert*-butyl ether, and re-extraction from the ether layer into hydrochloric acid. Therefore, alkaline precipitation apparently does not produce a loss of physostigmine recovery through hydrolysis to the metabolites.

However, decomposition of physostigmine can be prevented by adding neostigmine or pyridostigmine to the samples before extraction, since physostigmine hydrolysis is enzymic [40,41,43,44]. Neostigmine or pyridostigmine was chosen for its similarity to physostigmine. But being a quaternary ammonium compound, its extraction into diethyl ether of benzene is negligible [20,41]. Samples of control human plasma stabilized with pyridostigmine bromide (50 μg/ml) and containing physostigmine at concentrations of 1 and 0.2 ng/ml have shown no appreciable loss of physostigmine content after 9 months storage at ca. −20 °C prior to thawing and analysis [20]. Non-enzymatic hydrolysis was not considered important for the few hours that plasma were stored at 4 °C as at 25 °C and pH 7.8 the rate of decomposition is <0.1%/h [40].

Besides plasma, another common biological fluid from which physostigmine is extracted from, is cerebrospinal fluid (CSF). Successful extraction of physostigmine from plasma and CSF using diethyl ether has been reported by Knapp et al. [45]. Although CSF physostigmine was extracted in the same manner, it was unnecessary to deproteinize the sample because of their extremely low content of protein [45]. In contrast, Unni et al. [42] used diethyl ether for plasma; and chloroform for CSF. The extraction of physostigmine from CSF was different from the plasma extraction procedure because contaminant peaks co-eluted with physostigmine [42].

LLE is the most common method for isolating physostigmine and its metabolites from biological specimens. However, the LLE clean-up procedure is labor intensive and time consuming and some involve extraction of physostigmine into an organic phase and then back-extraction into an acidified aqueous phase [43,44,46].

Solid-phase extraction (SPE) is another analysis method which has grown in popularity over the last decade. The SPE clean-up procedure was found to yield more reproducible results and cleaner chromatograms than the conventional extraction procedure using chloroform, ether, ethyl acetate and a mixture of cyclohexane–pentanol (4:1, v/v) [49]. Recovery rates are not often discussed; however, those that have been reported are contradicting. Hsieh et al. [49] reported recovery rates of 54.3% for physostigmine, using the C18 SEP PAK cartridge, whereas Zhao et al. [50] claimed that recovery rates are 88.0% for physostigmine and 61.1% for eseroline. In Zhao et al.'s study, a HCX mixed mode extraction column was initially used in order to obtain clean chromatograms. However, the method was not reproducible and the extraction efficiency was less than 40%. The clean-up procedure using the C18 column was found to yield reproducible results and clean chromatograms. In the clean-up procedure, 0.1 ml of acetonitrile was used for denaturing of plasma proteins. Denaturation with acids or more than 0.1 ml of acetonitrile reduced the extraction recoveries of the analytes. In another study conducted by Hurst and Whelpton [51], an alkaline treated cyanopropyl column was selected after a systematic investigation of nine types of phase. Physostigmine was retained on the cyanopropyl columns and eluted into the minimum volume of methanol. The conditions for SPE were optimized using [^{3}H]physostigmine so that the overall recoveries were greater than 90%.

3.2. History: thin layer procedures

Early investigations used either paper chromatography (PC) or thin-layer chromatography (TLC) for the determination of physostigmine. PC consisted of Whatman filter paper as the stationary phase [35,36]. For TLC, the stationary phase varied, including silica gel and alumina layers [37,38]. TLC

was considered a convenient separation method. It is fast, inexpensive, and several samples can be examined at the same time, side by side.

Berg [38] separated physostigmine (R_F = 0.74) from eseroline (R_F = 0.51) and rubreserine (R_F = 0.55) by TLC on silica gel with chloroform–acetone–33% (w/v) dimethylamine in ethanol (5:4:1) as the solvent for development. The physostigmine was eluted with 0.1N sodium hydroxide and the rubreserine formed by hydrolysis and oxidation was determined colorimetrically at 480 nm. Berg attempted to elute the alkaloid with various organic solvents but the recoveries were low [38].

The method of Berg was criticized by Smith who reported that rubreserine reacted with dimethylamine within 10 min to form a yellow product with an R_F (0.73) close to that of physostigmine [39]. Smith separated physostigmine (R_F = 0.61–0.67) from eseroline (R_F = 0.42–0.45) and rubreserine (R_F = 0.33–0.36) by TLC on alumina, with chloroform–acetone (5:4) as the solvent for development. At a high or a low relative humidity, the R_F value for rubreserine was less than 0.1 and "tailing" was appreciable. It was found necessary to store the alumina plates over a saturated solution of sodium bromide (relative humidity = 58%) for 3 days before use. After the separation of physostigmine from its degradation products, Smith determined the alkaloid by direct-reflectance spectrophotometry; the coefficient of variation of 25 spots on five plates was 5.8% [39].

Rogers and Smith developed an elution technique for the determination of physostigmine from thin-layer chromatograms [37]. Physostigmine was separated from its degradation products on alumina, with chloroform–acetone (5:4) as the solvent for development. The alkaloid was eluted with methanolic hydrochloric acid and determined by ultraviolet spectrophotometry. The reproducibility of the elution method appeared to be slightly better than that of the direct-reflectance method [37,39] but the technique of elution was laborious and slow.

3.3. Liquid column chromatography

3.3.1. General aspects

In the last 20 years, the analytical determination of physostigmine and its degradation products from biological samples has been dominated by the use of HPLC, although biological assays have also been used. All two techniques have advantages and disadvantages. Biological techniques, e.g., immunoassay, are among the most sensitive analytical methods, but are limited by the availability of the specific antisera and are subjected to cross-reactivity. HPLC technique, on the other hand, although not as sensitive as biological techniques, enable to simultaneous screening of both physostigmine and degradation products, and other compounds. Table 1 in this review lists representative examples of the methods devised and columns employed in HPLC separations of physostigmine and/or its degradation products. The table also contains the sample preparation and detection methods. These are organized and tabulated according to analytical sample, i.e., pharmaceutical preparation, biological fluids and tissue samples. The table also indicates the compounds (i.e., physostigmine and/or its degradation products) that are detected and separated.

3.3.2. Chromatographic methods

The most often used columns for the determination of physostigmine and its metabolites are composed of a C18/ODS stationary phase with an internal diameter ranging from 3.9 to 4.6 mm. The particle size in these columns is, in most cases, either 5 or 10 μm. One exception is Whelpton and Moore [41], who reported working with column having 3 μm particles. Column lengths ranged from 150 to 300 mm. Most investigators do not explain their reasons for the choice of column. A few exceptions are Brodie et al., who reported the results from testing two different ODS columns, and Lau et al. [48], who chose C8 and C18 columns. Many authors also did not report using a guard column.

Although reversed-phase technique has been used extensively, the use of normal-phase HPLC on silica column (250 mm × 4.6 mm i.d.; particle size, 5 μm) using a 0.01 M sodium dihydrogen phosphate (pH 3)–acetonitrile (85:15, v/v) at 1 ml flow rate for resolving physostigmine and its metabolite has been reported [50]. The bare silica column was used in place of the C18 column as the peaks of the analytes had a narrower base and a better resolution was achieved [50]. Elsayed et al. [44] also suggested that the use of a normal-phase silica column with an aqueous mobile phase produced results which were more reproducible than those obtained with the more widely used C8 and C18 columns. The low pH of the mobile phase (pH 3) also protected physostigmine from degradation that can occur with more alkaline pH [44,50].

In general, the mobile phases that have been employed with reversed-phase and normal-phase HPLC columns have been methanol and/or acetonitrile in combination with acid and/or buffer. The peak shapes of physostigmine and its metabolites were greatly improved by adding a modifier such as acids. Most often octanesulfonic acid was employed; however, acetic acid, formic acid, phosphoric acid, heptanesulphonic acid and low pH buffer have also been used. Typically, an acid is added to the aqueous phase, although some report adding a small amount of acid to the organic solvents as well [46]. Walter et al. [46] described their mobile phase as consisting of phosphate acid and acetonitrile (75:25, pH 4.0) containing heptanesulphonic acid. In most methods, the mobile phases contain phosphate or acetate buffers (Table 1). In one early investigation, the authors [52] found that the use of sodium dodecyl sulphate (SDS) and $NaClO_4$ as the modifier of the mobile phase (pH 3.0) greatly improved peak shape and reproducibility of retention time of physostigmine in brain tissue samples.

Run times for the determination of physostigmine vary from 5 to 15 min. Some methods have been developed to include its degradation products, thereby increasing the analysis time. Flow rates range from 0.15 to 1.5 ml/min, the most

Table 1
LC procedures for the determination of physostigmine and/or its degradation products

Compound[a]	Sample	Work-up	Stationary phase	Mobile phase	Detection mode	Validation data	References
I and II	Eye-drops		μBondapak C18 (300 mm × 3.9 mm, 10 μm)	40% Methanol with 5 mM heptanesulphonic acid (pH 3.6)	UV, 235	LOD:3 ng/ml (I); 1 ng/ml (II)	[19]
I, II and III	Injection		μBondapak C18 (250 mm × 5.0 mm)	Acetonitrile–0.1 M ammonium acetate (1:1, pH 6.0)	UV, 305		[5]
I, II and III	Plasma (human)	LLE	Spherisorb (250 mm × 4.5 mm, 5 μm)	Methanol–1 M ammonium nitrate (9:1, pH 8.6)	UV, 254; ECD, 0.8 V	LIN: 0.5–20 ng/ml (I); REC: 93% (I); LOD: 0.5 ng/ml (I)	[40]
I	Brain (cat)	LLE	LiChrosorb RP-8 (125 mm × 3 mm, 5 μm)	5 mM NaH_2PO_4, 0.3% (w/v) SDS and 0.1 M $NaClO_4$ in methanol–water (1:1, pH 3.0)	UV, 245	LIN: 100–5000 ng/ml; REC: 90%; LOD: 20 ng/ml	[52]
I	Plasma and urine (human)	LLE	Spherisorb (150 mm × 4.6 mm, 3 μm)	Methanol–0.1 M ammonium nitrate (9:1, pH 8.9)	ECD, 0.4 V	LOD: 0.1 ng/ml	[41]
I and III	Plasma (human)	LLE	Spherisorb 5ODS1 (125 mm × 4.9 mm, 5 μm); guard column: ODS	Acetonitrile–10 mM sodium acetate (95:5)	FL, 254/346	LIN: 0.1–3 ng/ml (I); REC: 90% (I); LOD: 0.1 ng/ml (I)	[43]
I	Plasma and CSF (human)	LLE	Spherisorb (150 mm × 4.6 mm, 5 μm); guard column: silica	Methanol–10 mM sodium acetate (10:1, pH 4.6)	ECD, 0.95 V	LIN: 0.5–40 ng/ml; REC: 60% (plasma); REC: 78% (CSF); LOD: 0.5 ng/ml	[42]
I	Plasma (guinea pig)	LLE	μBondapak C18 (300 mm × 3.9 mm, 10 μm); guard column: C18	Aqueous buffer (0.5 mM 1-octanesulfonic acid–5 mM monobasic sodium phosphate–1% acetic acid)–methanol (60:40, pH 3.1)	RFD	LIN: 0.1–5 ng/ml; LOD: 0.05 ng/ml	[57]
I and III	Plasma and brain (rat)	LLE	μBondapak C18 (300 mm × 3.9 mm, 10 μm)	Aqueous buffer (5 mM 1-octanesulfonic acid–5 mM monobasic sodium phosphate–1% acetic acid)–methanol (60:40, pH 3.1)	UV, 245	I; LIN: 50–500 ng/ml or g; REC: 99–104% (plasma); REC: 96–107% (brain)	[47]
I	Plasma and CSF (human)	LLE	Spherisphorb (100 mm × 4.5 mm, 5 μm)	Methanol–acetonitrile–0.1 M ammonium nitrate (pH 8.9)–water (450:450:80:20)	ECD, 0.8 V	LIN: 75–2000 pg/ml; REC: 66–92% (plasma); REC: 83–92% (CSF); LOD: 25 pg/ml	[45]
I	Skin (human)	LLE	Spherisorb ODS-I (150 mm × 4.6 mm, 5 μm); guard column: ODS	10 mM Octanesulfonic acid and 1% acetic acid in a mixture of acetonitrile–water (52:48, pH 3.5)	UV, 254	LIN: 5–250 μg/g; REC: 65%; LOD: 5 μg/g	[48]

I and III	Serum (human)	SPE	Diol column	Aqueous phosphate buffer (pH 4.0)–methanol (80:20)	FL, 243/418	LIN: 2–100 ng/ml (I); LOD: 2 ng/ml (I); LIN: 5–100 ng/ml (III); LOD: 5 ng/ml (III)	[53]
I	Plasma (human)	SPE	μBondapak C18; guard column: RP-18	5 mM Monobasic sodium phosphate (pH 5.7)–methanol (50:50)	UV, 254	LIN: 0.1–10 μg/ml; REC: 54%; LOD: 50 ng/ml	[49]
I	Plasma (human)	LLE	Altex Ultrasphere-Si (250 mm × 4.6 mm, 5 μm); guard column: silica	Acetonitrile–phosphate buffer (20:80, pH 3.0) containing 10 mM sodium dihydrogen phosphate and 2.5 mM TMA	FL, 240/360	LIN: 0.5–25 ng/ml; REC: 93%; LOD: 0.1 ng/ml	[44]
I	Plasma (human)	LLE	Nucleosil 100 C18	0.02% Phosphoric acid–acetonitrile (75:25, pH 4.0) containing 2.5 mg/ml heptanesulphonic acid	FL, 255/345	LIN: 25–1000 pg/ml; REC: 94%; LOQ: 25 pg/ml	[46]
I	Plasma (human)	SPE	Applied Biosystem narrow-bore column	10 mM Formic acid–acetonitrile–50 mM Tris buffer (52:27:21)	FL, 250/345	LOD: 55 pg/ml	[34]
I and III	Plasma (rat)	SPE	Alltech Ultrasphere-Si (250 mm × 4.6 mm, 5 μm)	Acetonitrile–10 mM sodium dihydrogen phosphate (15:85, pH 3.0)	DAD	LIN: 0.1–2.5 μg/ml; REC: 88% (I); LOD: 10 ng/ml (I); REC: 61% (III); LOD: 25 ng/ml (III)	[50]
I, II and III	Microsomal incubation (mouse)		Biophase octyl (250 mm × 4.6 mm, 5 μm)	Acetonitrile–0.1 M phosphate buffer (40:60, pH 3.0) containing 0.5% (w/v) SDS	ECD, 0.8 V		[16]

[a] I: physostigmine; II: rubreserine; III: eseroline.

common being 1 ml/min. Many investigations have been done to study the effects of mobile phase composition, various sulfonic acids, column temperature and pH on the chromatographic properties of the physostigmine and eseroline peaks. The study revealed that varying the composition of the mobile phase could change the retention times of analytes. Increasing the organic component of the solvent system retarded the retention times [50]. Relative retention time and resolution of physostigmine and its metabolite eseroline peaks decreased with reduction in chain length of sulfonic acids [47]. Higher temperatures (>40 °C) or lower pH values (<4.0) significantly reduced the retention time and improved the shape of the physostigmine peak [49]. However, most authors select ambient temperature to maximize the stability of physostigmine.

3.3.3. Detection

Physostigmine and its degradation products show comparable UV absorbance and their λ_{max} range from 242 to 246 nm. This is consistent with their chemical structures, differing only by the absence or presence of methyl groups on the side chain (Fig. 1). Detection by UV techniques for HPLC investigations is a common method for monitoring wavelengths from 235 to 254 nm. The conventional wavelength of 254 nm is normally used for monitoring. This is perhaps due to convention based on the strong energy line from earlier lamps, because it is not the λ_{max}. A HPLC with photodiode-array detection (PAD) method was developed to detect and quantify physostigmine and its metabolite eseroline in plasma. The signal was monitored at a wavelength of 246 nm. The assay was found to be linear and has been validated over the concentration range of 0.1–2.5 μg/ml from 0.1 ml of plasma. Limits of detection of the assay were 10 ng/ml for physostigmine and 25 ng/ml for eseroline [50].

Other less common means of detection, coupled to LC, are ECD, FL and RFD. Using a dual glassy-carbon working electrode and a silver–silver chloride reference electrode, Whelpton [40], Isaksson and Kissinger [16] and Unni et al. [42] employed EC for determination of physostigmine and its metabolites in biological samples. Electrochemical behavior provided the additional information for the identification of physostigmine and its metabolites not previously identified with HPLC–UV [16]. The electrochemical methods were sensitive and gave a detection limit of 0.5 ng/ml for physostigmine [40,42].

Whelpton's LC–ECD method has been modified further to give approximately a five-fold improvement in sensitivity. Whelpton and Moore used a dual-electrode electrochemical detector in the differential mode utilizing the following three outputs: channel 1, oxidation at +0.7 V; channel 2, reduction at −0.2 V; channel 3, the sum of the signals from channels 1 and 2 [41]. Using 2 ml of plasma or blood, the LOD was 0.1 ng/ml. The sensitivity was increased by taking a 4-ml sample for assay and using the sum of the oxidation and reduction signals [41]. Knapp et al. described their method (using a modification of Whelpton and Moore's detection technique) combining HPLC with ECD using dual electrodes in the redox instead of differential mode [45]. Knapp's technique combined reduction (−0.3 V) in the first electrode followed by oxidation (+0.42 V) in the second one. The redox mode provided for enhanced detector selectivity since only compounds that were oxidized between −0.3 and +0.42 V were detected at detector 2 [45]. This methodology was developed to enable them to carry out PK studies on reasonably small plasma sample volumes and to monitor plasma physostigmine levels in a geriatric population during clinical trials. They achieved a limit of quantitation (LOQ) of 75 pg/ml by using 1–4 ml sample volumes [45].

Quinn and Stewart [53] developed a HPLC post-column fluorescent ion pair extraction system using a new fluorescent ion pair reagent, sodium alpha-(3,4-dimethoxyphenyl) cinnamonitrile-2′-sulfonate (DPS), for the analysis of physostigmine and eseroline. The post-column extraction system consisted of a three-dimensional knitted teflon mixing coil and a membrane phase separator which was modified from an original literature design [53]. Physostigmine and its metabolite eseroline were used as model cations. A solid-phase extraction procedure using octadecylsilane columns was developed to extract the compounds and neostigmine bromide (internal standard) from human serum. The compounds were chromatographed on a diol column using a 80:20 aqueous phosphate buffer pH 4 absolute methanol mobile phase at a flow rate of 1 ml/min. Methylene chloride was used as the on-line extraction solvent for the DPS ion pairs formed. Fluorescence of the extracted ion pairs was measured using an excitation of 243 nm and an emission cut-off filter at 418 nm. Linearity was in the 2–100 and 5–100 ng/ml ranges for physostigmine and eseroline, respectively. The LOD were 2 and 5 ng/ml, respectively. Precision of the method was found to be in the 1.5–3% range and percentage error in the 1.5–7% range for both compounds.

Brodie et al. [43] described an alternative HPLC procedure for the measurement of physostigmine in blood samples incorporating a fluorimetric detector which utilizes the powerful natural fluorescence of physostigmine. The fluorescence detector operated at an excitation wavelength of 254 nm (slit width 10 nm) and emission wavelength of 346 nm (slit width 20 nm). The described analytical procedure for the measurement of physostigmine has been validated in terms of precision, accuracy, specificity and linearity over the concentration range 0.1–3.0 ng/ml. For a sample volume of 2 ml plasma or whole blood, the LOD was 0.1 ng/ml. In the method reported by Elsayed et al. [44], the excitation wavelength was 240 nm, and the emission wavelength was 360 nm for chromatographic run. The LOD for a sample volume of 1 ml plasma was 0.1 ng/ml.

To enhance sensitivity and specificity Lukey et al. [27] developed a radiometric HPLC method for the determination of [^{3}H] physostigmine in plasma samples from guinea pigs, using a Radiomatic Instruments Flo-One-B radioactive flow detector (RFD). The RFD with a 2.5-ml sample chamber was coupled to the μBondapak C18 separation column.

The mobile phase flowed through the column at a flow rate of 1.5 ml/min. Prior to reaching the detector's sample compartment, the column effluent was mixed with Ultrafluor scintillation fluid in a 4:1 (v/v) ratio, the resulting mixture achieving a flow rate of 5.5 ml/min through the Flo-One's cell. The method used flow-through scintillation counting, thus providing decreased labor and increased speed. The mobile phase in this method consisted of an aqueous buffer (0.5 mM 1-octanesulfonic acid–5 mM monobasic sodium phosphate–1% acetic acid) mixed with methanol in a 60:40 ratio (v/v, pH 3.1). The authors reported very high sensitivity (LOD = 0.05 ng/ml) monitoring with this HPLC–RFD technique. However, due to unavailability of radioactive eseroline, the observed separation required ultraviolet detection [27].

3.4. Electromigration methods

Capillary electrophoresis (CE) has already been established as a very powerful tool for analyzing drug substance material and formulations, and has proven to be an attractive alternative to conventional separation techniques such as HPLC and GC [54,55].

A new capillary zone electrophoresis (CZE) method for the rapid determination of physostigmine in pharmaceutical preparation has been developed [56]. An untreated fused-silica capillary tube (75 μm i.d., 44 cm total length, 36.5 cm length to the detector) was employed with detection at 200 for physostigmine. The optimal separation conditions were: 50 mM boric acid–HCl buffer (pH 3.25) with 30 mM $NaClO_4$, electrokinetic injection for 5 s at −5 kV, temperature 25 °C, and separation voltage 15 kV. Different modes of injection were tested in order to obtain the highest sensitivity and best selectivity for the determination. Improvement in the limit of detection (LOD) was obtained when electrokinetic injection (EK) was used instead of hydrodynamic injection (HI), because the positive ions migrate faster than neutral and anionic compounds. EK injection generates cation stacking at the beginning of the capillary. As a consequence of this preconcentration, an increase in sensitivity was attained (LOD: 70 ppb). The reproducibility of the migration time (<1% R.S.D.) was observed for the analyte studied. The method can be used for the monitoring of possible main degradation products in tablets of military antidote formulations [56].

4. Biological assays

Biological techniques are rarely used for the analysis of physostigmine in biological samples. A radioimmunoassays (RIA) was employed by Meyer et al. [29] for the identification and quantification of physostigmine in plasma. Antiphysostigmine antibodies were produced in rabbits using a physostigmine analog, 1,3-dimethyl-3-[2-[*N*-methyl-*N*-(7-carboxyheptanoyl)]aminoethyl]-5-(*N*-methyl-carbamoyloxy)-2,3-dihydroindole hydrochloride, conjugated to keyhole limpet hemocyanin. These antibodies were used to develop a RIA ranging from 0.2 to 15.0 ng/ml of physostigmine in a 0.1-ml plasma sample. A typical standard curve gave an R^2 value of 0.992. This assay measured physostigmine in plasma with good sensitivity and great through-put. In addition, only small volumes (100 μl) of the plasma samples are required. Precision represented by within and among day coefficients of variance was less than 20% for 1.0–50.0 ng/ml and less than 22% for 0.2 ng/ml. Accuracy for the 1.0–50.0 ng/ml range varied less than 15% and was 22% for 0.2 ng/ml [29].

A similar approach, with slightly higher sensitivity, was described by Miller and Verma [30]. In their method, antibodies were produced in rabbits immunized with physostigmine conjugated to bovine serum albumin (BSA) by two different methods: a diazo immunogen coupled to BSA with carbodiimide and a Mannich coupled immunogen. Both immunogens produced antibodies that could be used to develop sensitive and specific RIA for physostigmine. The limit of detection of the RIA was 100 pg of physostigmine in plasma or tissue homogenates (1.0 ng/ml), without the need for an extraction procedure. The interassay and intraassay coefficients of variation were less than 13% in the RIA. No appreciable binding was observed between the antibodies and physostigmine metabolites or other drugs commonly used with physostigmine. The specificity of the RIA was further validated by measuring physostigmine concentrations in biological samples in tandem with high-pressure liquid chromatography. The RIA was used to study the time course of plasma concentrations and tissue distribution of physostigmine in rats [30].

5. Conclusions

Analytical methods to determine physostigmine and its degradation products have been reviewed. HPLC has proven to be an efficient technique for the separation of physostigmine and its metabolites and has been applied successfully for the quantification of these compounds in various biological matrices. LLE is the most common method for isolating physostigmine and its metabolites from biological specimens, however the LLE clean-up procedure is labor intensive and time consuming. The analysis methods published over the last decade have used SPE. A recently reported CE assay has the potential to become the reference method for plasma physostigmine analysis. Other methods to measure plasma physostigmine concentrations include RIA. RIA has been shown to be capable of studying the time course of plasma concentrations and tissue distribution of physostigmine in rats and may become the method of choice in PK studies. Instability of physostigmine in whole blood is a pre-analytical factor that should be accounted for in plasma physostigmine analysis. The decomposition of physostigmine can be prevented by

adding neostigmine or pyridostigmine to the samples before extraction.

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