Determination of pyridostigmine bromide and its metabolites in biological samples

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ABSTRACT Pyridostigmine bromide (PB) is a quartenary ammonium compound that inhibits the hydrolysis of acetylcholine by competitive reversible binding to acetylcholinesterase. PB is used for the symptomatic treatment of myasthenia gravis and has been applied as a prophylaxis against nerve agents. Many studies on PB have involved the reliance on techniques that extract and quantify PB in biological samples. This article presents an overview of the currently applied methodologies for the determination of PB and its metabolites in various biological samples. Articles published from January 1975 to the July 2005 were taken into consideration for the discussion of the metabolism and analytical method of PB. HPLC and GC methods have been used and discussed in most of the references cited in this review. Other methods such as RIA and CE that have been recently reported are also mentioned in this article. Basic information about the type of sample used for analysis, sample preparation, chromatographic column, mobile phase, detection mode and validation data are summarized in a table.

INTRODUCTION

Pyridostigmine bromide (PB) (3-dimethylaminocarbonyloxy-*N*-methylpyridinum bromide), like the structurally-similar, physostigmine, belongs to a class of neuroactive compounds called carbamates (1). It is a powerful and reversible acetylcholinesterase (AChE) inhibitor, which effectively increases the concentration of acetylcholine at the sites of cholinergic transmission (2).

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PB is one of the main drugs currently used to treat myasthenia gravis (3), a progressive neuromuscular disease characterized by an apparent insufficient stimulation of peripheral muscarinic receptors. PB competes with acetylcholine (ACh) for binding to AChE, and like ACh, is hydrolyzed by the enzyme. However, hydrolysis of PB proceeds much more slowly than that of ACh, resulting in effective inhibition of ACh hydrolysis and enhancement of muscarinic stimulation (2).

In military medicine, PB is used as a prophylactic agent against intoxication with irreversible organophosphorus AChE inhibitors, such as the nerve agents sarin and soman (4). The protective property of PB is due to its ability to shield the active site of the AChE from attack and subsequent irreversible inhibition by the nerve agents. Its carbamate functional group binds to the active site of AChE in a similar manner to the binding of organophosphorous AChE inhibitors to AChE (5). The reversible carbamylation of the active site of AChE ensures that the active site is protected from attack by organophosphorous nerve agents which bind 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. The ability to prevent irreversible binding of organophosphorous agents to AChE would make PB useful as a pre-treatment to minimize the effect of nerve gas poisoning if used in conjunction with the standard treatment of atropine and pralidoximine chloride (2-PAM) (6, 7).

The lipophobicity and charge of PB, a quaternary ammonium compound, inhibit its passage across the blood–brain barrier, thus limiting the central nervous system effects (8). There is little evidence of adverse effects of PB treatment in humans (9 – 11). PB undergoes hydrolysis by cholinesterases. It is also metabolized by microsomal enzymes in the liver. The main metabolite of PB is 3-hydroxy- *N*-methylpyridinum (3-OH NMP) to which few reference of a biological action has been made (12). To help understand the full consequences of the biological activity of PB and its metabolites, various analytical techniques, mainly high-performance liquid chromatography (HPLC) and gas chromatography (GC), have been utilised for their measurement in biological samples. In this review, we will concentrate on the identification and quantification of PB and its metabolites by HPLC, GC and other methods.

METHODS

Choice of references

A structured literature search was performed to identify articles in which PB and chromatographic and electrophoretic methods were mentioned. Keywords were used to identify relevant articles from the period of January 1975 to July 2005 in the NLM PubMed Medline database. Reference sections of articles identified by this method were sourced to identify additional articles. Those articles that presented original evidence pertaining to the use of chromatographic and electrophoretic methods to identify and quantify PB and its metabolites were included in this review; those that did not were rejected.

Definitions and terms used

Standard abbreviations used in the review are approved by the Journal. Abbreviations used are included in the list of non-standard abbreviations (Table 1).

Table 1: List of non-standard abbreviations

ACh	Acetylcholine	LOQ	Limit of quantitation
AChE	Acetylcholinesterase	MS	Mass spectrometer
BSA	Bovine serum albumin	NLM	National Library of Medicine
CBA	Carboxylic acid	NPD	Nitrogen-phosphorus detection
CE	Capillary electrophoresis	ODS	Octadecylsilanized silica gel
CV	Coefficients of variance	3-OH NMP	3-Hydroxy-N-methylpyridinum
CZE	Capillary zone electrophoresis	2-PAM	Pralidoximine chloride
ECD	Electron-capture detection	PB	Pyridostigmine bromide
EK	Electrokinetic injection	PI	Pyridostigmine iodide
ESI	Electrospray ionization	PK	Pharmacokinetics
FID	Flame ionization detection	REC	Recovery
GC	Gas chromatography	RIA	Radioimmunoassays
HPLC	High-performance liquid chromatography	RP	Reverse-phase
HI	Hydrodynamic injection	RSD	Relative standard deviation
IS	Internal standard	SDS	Sodium lauryl sulfate
LC	Liquid chromatography	SPE	Solid-phase extraction
LIN	Linearity	TMA^+Cl^-	Tetramethylammonium chloride
LLE	Liquid-liquid extraction	UV	Ultraviolet
LOD	Limit of detection		

DEGRADATION PRODUCTS OF PB

Stability of PB in aqueous solution

The stability studies conducted in aqueous solution showed that PB was stable in acid medium (pH 1.0) at both 25°C and 70°C for 3 hours (13). However, PB is hydrolysed enzymatically (12) and is unstable in aqueous medium at high pH, particularly at pH > 8.5 (13, 14). Even at 25°C, PB is extremely unstable in alkaline solution (pH 11) (13). During hydrolysis the PB absorption spectra indicate a 1:1 transformation by the appearance of isobestic points at 260 and 287 nm. The absorbance of PB at its λmax (269 nm) decreases with a concomitant appearance of absorption maxima at 252 and 322 nm (13). Under alkaline conditions, hydrolysis of PB at the ester linkage can be anticipated.

This would lead to the formation of 3-OH NMP, a reported major metabolite of PB (Fig. 1) (15, 16). It is therefore advisable to avoid alkaline conditions in the assay of PB (13).

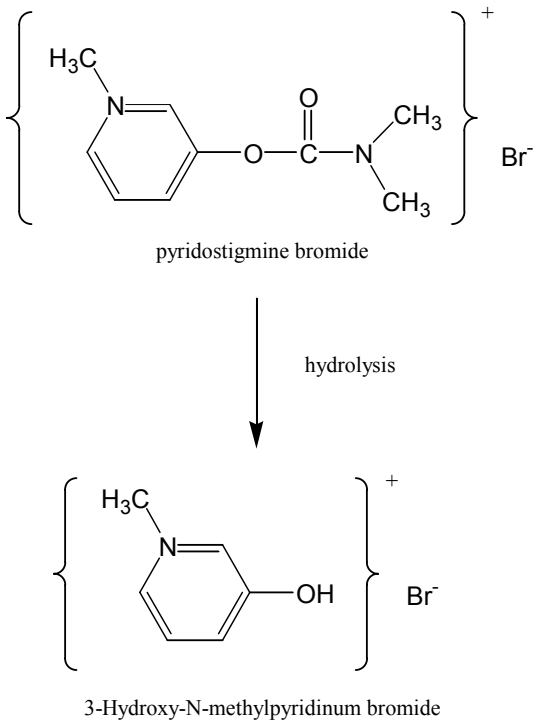

Figure 1: Decomposition pathway of pyridostigmine bromide

The metabolism of PB

Pharmacokinetic (PK) studies with PB have been conducted in rats (14, 16 – 19), dogs (20 – 22) and humans (23 – 25). Investigation on the route of PB metabolism has been carried out following intramuscular (14, 18, 19, 23), intravenous (16, 17, 20, 22) and oral administration (22, 24 – 26). PB and/or its metabolite concentrations can be measured by radioisotopic studies (15 – 17, 23), GC (27), HPLC (13, 14, 26) and by radioimmune assay (RIA) (18, 19). These studies show that PB has a high clearance and hepatic extraction ratio; and is rapidly removed from the circulation. The liver plays a particularly important role in the metabolism of PB (16).

Due to the presence of plasma cholinesterases, the PB hydrolysis in the blood is considerable (12). The main PB metabolite is the hydrolysis product 3-OH NMP, which is rapidly glucuronidated (15, 23). Other metabolites have also been detected in the plasma and urine and their tentative structures have demonstrated the formation of hydroxyl, quinone and demethylated metabolites and their corresponding glucuronides (15, 23). After intravenous administration of PB in rat, an average of 52% was eliminated unchanged in urine after 4 hours. A further 22% was excreted as 3-OH NMP during this time. The proportion of the dose eliminated in urine as PB and its metabolite was independent of the dose administered (16). Somani et al (23) found that during a 24-hour period after administration in man, PB was excreted in the urine as the unchanged drug and its main metabolite in a ratio of 4:1, in accordance with findings by Nowell et al (28). PB can also be detectable in breast milk. Its concentration in breast milk has been found to be 36 to 113% of that in maternal plasma, which implies a very low dose to the nursing infant (about 0.1% of the dose per kilogram bodyweight taken by the mother) (29).

ANALYTICAL METHODS

Radioisotopic techniques

Early investigations used radioisotopic techniques for the determination of PB and its metabolites (15 – 17, 23). The radioisotopic studies can be done with ^{14}C-labeled PB and considered a sensitive analytical method. However, radioactive compounds are difficult to use in human studies. Because of the difficulty, most PK studies of PB have been limited to the use of radioisotopic techniques in laboratory animals (15 – 17).

The relationship between the concentration of PB in plasma and the facilitation of neuromuscular transmission has been studied in the rat by radioisotopic techniques (30). Plasma samples were assayed for total radioactivity by liquid scintillation spectrometry in 9ml NE 260 liquid scintillation fluid and 1ml toluene/iso-amyl alcohol (5:1, v/v). PB and its metabolite were then separated by paper electrophoresis in borate buffer (0.1 M, pH 9.2) for 2 hours at 300V. Radioactivity on the paper electrophoretograms was identified on a Tracerlab 4π radiochromatogram scanner. The concentration of unchanged [^{14}C]-PB and its metabolite was then determined from the total radioactivity in plasma and the results of paper electrophoresis.

Barber et al also used a similar techniques to study the elimination kinetics of [^{14}C]-pyridostigmine iodide (PI) and [^{14}C-methyl]-3-OH NMP in the rat (16). Samples of urine (50 µl) were analysed for total radioactivity by liquid scintillation counting, using 10ml of a homogenous Triton-X-100 solution {6g butyl P.B.D. [2(4'-t-butylphenyl)-5-(4"-bi-phenylyl)-1, 3, 4-oxadiazole] in 667 ml toluene, 333 ml Triton-X-100 and 75 ml water}. The samples were usually analyzed for PI and its metabolites by electrophoresis in borate buffer (30). The paper electrophoretogram was counted to determine the relative proportions of the unchanged PI and its metabolites. Most urine samples were resolved by ion-exchange chromatography using Amberlite IRC-50 resin. The elute was then assayed for the parent drug and its metabolites by liquid scintillation spectrometry.

In studies of the metabolism and excretion of [^{14}C]-pyridostigmine, Wilson and co-workers (17, 31) have identified unchanged pyridostigmine and its metabolite 3-OH NMP in rat urine. In human studies, they have also isolated the unchanged drug and its major metabolite from the urine of a patient given [^{14}C]-PI by intramuscular injection with the use of the combined techniques of radioisotopic studies, ion-exchange chromatography, electrophoresis and paper chromatography (23). PI and 3-OH NMP thus isolated were characterized by ultraviolet spectra and mass spectra, and estimated by a reverse isotope-dilution method (23). These findings have emphasized the importance of investigation of the metabolism and excretion of pyridostigmine.

Chromatographic techniques

HPLC

In the last 25 years, the analytical determination of PB and its metabolites from biological samples has

been dominated by the use of HPLC, although biological assays have also been used. 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, enables simultaneous screening of both PB and its metabolites. Table 2 in this review lists representative examples of the methods devised and columns employed in HPLC separations of PB and/or its metabolites.

Table 2: LC procedures for the determination of pyridostigmine bromide (PB) and/or its metabolites

Compound*	Sample	Work-up	Stationary Phase	Mobile Phase	Detection Mode	Validation Data	Refs.
I & II	Plasma& urine (rat)	SPE	µBondapak C18 (300mm × 3.9mm, 10µm); guard column: C18	Water (adjusted to pH 3.2 using 1 N acetic acid) – acetonitrile; linear gradient	UV, 280	LIN: 100 – 1000ng/ml REC: in plasma, 77% (I); 79% (II). In urine, 79% (I); 78% (II) LOD: 50ng/ml (I & II)	[26]
I	Plasma (guinea pig)	LLE	SCX poly(2-sulfoethyl aspartamide) column (35mm × 2mm)	70% Acetonitrile – 30% 100 mM ammonium formate	MS, *m/z* 180.8 → 124.0	LIN: 0.1 – 50ng/ml REC: >80% LOQ: 0.1ng/ml	[44]
I	Plasma (human)	SPE	Axiom Silica column (250mm × 4.6mm, 5mm)	Acetonitrile – water (1:1, v/v) with final concentrations of 0.05% tetra-methylammonium chloride and 5 mmol/L dibasic ammonium phosphoric acid (pH 7.2)	UV, 208	LIN: 1.53 – 76.3ng/ml LOQ: 1.53ng/ml	[6]
I & II	Serum (human)	LLE	LiChrosorb RP-8 (150mm × 3.2mm, 10µm); guard column: Perisorb RP-2	0.01M Heptanesulfonic acid sodium and 0.01M sodium dihydrogen phosphate in acetonitrile – water (15:85, v/v, pH 3.0)	UV, 214	LIN: 0 – 1000ng/ml (I) REC: 96.4% (I) LOQ: 5ng/ml (I)	[40]
I	Plasma (human)	SPE	25cm column packed with µBondapak C18 (particle size 10µm)	92.25% Aqueous buffer (10mM sodium dihydrogen phosphate – 10mM sodium butanesulfonate – 2.5mM L-tetramethylammonium chloride, and sufficient 0.5M sulphuric acid to adjust pH to 2,8) – 0.75% acetonitrile – 7% 2-propanol	UV. 254	REC: 73.5% LOD: 1ng/ml	[3]
I, II & III	Plasma & urine (human	SPE	µBondapak C18 (300mm × 3.9mm,	5mM 1-Octanesulfonic acid in a mixture of water – acetonitrile – acetic acid (800ml:200ml:5ml)	UV, 270	LIN: 49 – 4900ng/ml (I) REC:	[14]

	& rat)		10μm)			>90% (I)	
I	Plasma (human)	SPE	CPS Hypersil NC-04 column (250mm × 4mm, 5μm)	Acetonitrile – 0.1% triethylamine in water (7:3, v/v, pH 3.2 with acetic acid)	UV, 272	LIN: 5 – 200ng/ml LOD: 1ng/ml	[51]
I	Plasma (human)	LLE	μBondapak C18 (particle size 10μm)	37.5% Acetonitrile in water containing 1 mM sodium lauryl sulphate and 1% acetic acid (pH 4)	UV, 269	LIN: 20.8 – 104.1ng/ml REC: 64%	[13]
I	Plasma (human)	SPE	Radial-Pak CN column (100mm × 5mm, 10μm);	0.1% triethylamine (pH 3.2 with acetic acid) in water – acetonitrile (50:50, v/v)	UV, 270	LIN: 10 – 200ng/g REC: >84% LOD: 2ng/g	[34]
I	Plasma (human)	SPE	Altex Ultra-sphere Octyl column (250mm × 4.6mm, 5μm);	Acetonitrile – water (30:70), 0.1% sodium lauryl sulphate (wt/v), 0.1% H_3PO_4 (v/v), and 2.5mM tetramethylammonium chloride	UV, 208	LIN: 0 – 68.3ng/ml REC: > 73% LOD: 1.37ng/ml	[35]
I & II	Plasma& urine (rat)	SPE	μBondapak C18 (300mm × 3.9mm, 10μm); Supelco guard column	Methanol, acetonitrile, water (adjusted to pH 3.2 using 1 N acetic acid) (10: 40: 50)	UV, 280	LIN: 100 – 1000ng/ml REC: in plasma, 71% (I); 78% (II). In urine, 74% (I); 78% (II) LOD: 50ng/ml (I & II)	[52]
I	Plasma (human)	SPE	Cation exchange column	44% of acetonitrile in sodium sulphate anhydrous buffer (pH 4.7)	UV	LIN: 5 – 100ng/ml REC: 80% LOQ: 5ng/ml	[53]

**I: pyridostigmine bromide; II: N-methyl-3-hydroxypyridinium; III: dimethylcarbamyl ester of 3-hydroxypyridine.*

1. Extraction

A critical aspect of PB analysis is the sample extraction step, which requires the isolation of the residue (containing the PB and its metabolites) from a biological material and removing potentially interfering compounds. A potential problem when extracting PB 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. Pohlmann and Cohan first stated that serum had to be cooled below 12°C within 5 to 6 hours, to prevent significant decreases in PB levels, probably because of the cholinesterase action (32). Aquilonius et al incubated PB in plasma and blood for 6 hours at 37°C and found a loss of 26% and 50%, respectively, while no loss occurred in a buffer at pH 7.4 (33). Thus PB seems to be hydrolyzed by red blood cell AChE as well as by plasma cholinesterase (3). Because the latter enzyme has an optimal pH between 8 and 9,

hydrolysis will be enhanced when the plasma pH rises as a consequence of CO_2 loss with storage (3). Decomposition of PB can be prevented by adding neostigmine or physostigmine to the samples before extraction since PB hydrolysis is enzymic. Neostigmine or physostigmine was chosen for its similarity to PB (34 – 37).

For the pre-treatment of biological fluid, solid-phase extraction (SPE) was applied to isolate PB and its metabolites from the sample matrices. Octadecyl (C_{18})-bonded silica has been SPE most widely employed adsorbent in the cartridge. C_{18} SEP PAK was used to extract PB and 3-OH NMP from rat plasma and urine, being sufficiently effective in cleaning up the samples (26). Abu-Qare et al. (26) reported that recovery rates are 77% for PB and 79% for 3-OH NMP, using the C18 SEP PAK cartridge, whereas Ellin et al (14) claimed recovery rates of >90% for PB. In the later study, Ellin et al studied the recovery of PB from both plasma and urine after their passage through C_{18} SEP PAK and Corasil II (14). The cartridges were washed with water (5 ml) and methanol (5 ml) and PB and its metabolites were eluted with 2ml of 0.1N acetic acid in methanol. They found that C_{18} SEP PAK cartridge provided higher recovery (more than 90%) then Corasil II cartridge (less than 30%). Other SPE techniques using Bond Elut C_2, C_8 and CBA (carboxylic acid) cartridges were reported for the analysis of PB in human biological fluid (6, 38, 39). Successful extraction of PB from plasma using Bond Elut C_2 has been reported by Yturralde et al (39). Since PB and the internal standard (IS), edrophonium chloride, do not elute from the Bond Elut C2 with acetonitrile alone, undesired endogenous substances were washed from the Bond Elut with water (2ml) and acetonitrile (2ml). The Bond Elut was then washed with 1ml of 95% acetonitrile containing 0.1% sodium lauryl sulfate (SDS) and 0.05% tetramethylammonium chloride (TMA^+Cl^-) and subsequently, the PB and IS were eluted with 3ml of 95% acetonitrile containing 0.1% SDS and 0.05% TMA^+Cl^-. Recoveries ranged from 73 to 87% (39). In Matsunada's study (38), he found ion-exchange cartridges such as Bond Elut CBA to be superior to silica-based cartridges for extraction of PB for HPLC studies. The plasma was treated with trichloroacetic acid, applied to Bond Elut CBA, and PB was eluted with 1 ml of 1M hydrocholoric acid in methanol solution. Recoveries were high (95%) and consistent (38).

Liquid-liquid extraction (LLE) is another method for isolating PB and 3-OH NMP from biological samples. Acid precipitation of plasma proteins is preferred because PB is unstable in alkaline solution (13). Commonly used extraction solvents in LLE schemes include dichloromethane and methylene chloride (13, 40). These solvents varied to a great extent, in terms of their extraction power. Yakatan and Tien reported recovery ranges from 64%, using methylene chloride (13). De Ruyter and Cronnelly (40), reported a recovery >90% for PB after precipitation of plasma with 0.1M picric acid in 0.1M sodium hydroxide (pH adjusted to 7), extraction with water-saturated dichloromethane, and re-extraction from the dichloromethane layer into 1mM tetrabutylammonium hydrogen sulfate ($TBA^+HSO_4^-$). Therefore, precipitation with picric acid in sodium hydroxide solution at pH 7 apparently does not produce a loss of PB recovery through hydrolysis to the metabolites (40). Iodide ion (27) and dipicrylamine (41) have been reported as ion-pairing reagents for the extraction of PB. Extraction recoveries with iodide were only 30% compared with the near-quantitative recoveries with dipicrylamine. The use of picrate anion to extract a variety of quaternary ammonium compounds is well-documented (40). Due to its availability and utility over a broad pH range, De Ruyter and Cronnelly examined picrate anion as a counter-ion for biological extractions (40). To remove the picrate anion, which gives an interfering peak on the liquid chromatographic tracings, and to obtain cleaner extracts, a back-extraction step with 1mM $TBA^+HSO_4^-$ was employed. The TBA^+ helps to partition the analytes into the aqueous phase and keeps the picrate anion in the organic phase (40). De Ruyter and Cronnelly indicated that improved extraction procedure gave better efficiencies and greater reproducibility (40).

2. Chromatographic conditions

Columns for the determination of PB and its metabolites are almost exclusively reverse-phase (RP), ranging from 150 to 300 mm in length and usually with an internal diameter ranging from 3.2 to 4.6 mm. The particle size in these columns is, in most cases, either 5 or 10 µm. Most investigators do not explain their reasons for the choice of column. One exception is De Ruyter and Cronnelly (40), who reported the results from testing four different columns: LiChrosorb RP-8 5 µm, LiChrosorb RP-18 10 µm, Varian MCH C_{18} 10 µm and Altex Ultrasphere Octyl 5 µm. In the studies, the Altex Ultrasphere Octyl column proved to be very stable and also had very good height equivalent to a theoretical plate values for the samples (40).

Elution systems are usually binary, with an aqueous acidified polar solvent and/or buffer such as aqueous acetic acid, sulphuric acid, phosphoric acid, heptanesulphonic acid or low pH buffer (solvent A) and a less polar organic solvent such as methanol or acetonitrile, possibly acidified (solvent B). Ellin et al (14) described their mobile phase as consisting of acetic acid, acetonitrile and water (5ml:200ml:800ml) containing l-octanesulfonic acid. Their RP-HPLC method with μBondapak C_{18} has been used for the determination of PB and its metabolites in rat urine and blood samples (14). In most methods, the mobile phases contain phosphate or acetate buffers (Table 2). In one early investigation, the authors (35) found that the use of SDS and TMA^+Cl^- as the modifier of the mobile phase greatly improved peak shape and reproducibility of retention time of PB in plasma samples. Runs are generally 15 minutes maximum. An exception is found in the 30-min run used for the separation of PB and IS (edrophonium chloride) by ion-pair HPLC (35). Flow rates range from 0.8 to 2.5 ml/min, the most common being 1 ml/min. Thermostatically controlled columns are normally kept at ambient temperature. Injections generally range from 5 to 200 μl. Some investigations have been done to study the effects of mobile phase composition on the chromatographic properties of the PB peak (35, 40). The study revealed that PB could not be eluted from the Varian MCH reverse-phase column (monomeric C_{18}) with acetonitrile-water mixtures unless TMA^+Cl^- was added to the mobile phase. These observations might be explained by the irreversible adsorption of the drug to residual silanol groups and deactivation of adsorption sites by TMA^+ (40). A similar observation was reported in the chromatography of curare alkaloids (42). The capacity ratios of PB could be regulated by altering the concentration of TMA^+ in the mobile phase; and increasing the concentration of TMA^+ decreased the retention volume (40). The addition of TMA^+ also had a pronounced effect on the peak shape. It reduced dramatically the tailing of the peaks obtained on the MCH C_{18} and RP-8 columns (40).

3. Detection

PB absorbs in the ultraviolet (UV) region (λ_{max} = 269 nm) (13). Detection by UV techniques for HPLC investigations is a common method for monitoring wavelengths from 208 to 280 nm. The conventional wavelength of 254nm is normally used for monitoring (3). This is possibly due to equipment limitations rather than due to λ_{max}. RP-HPLC methods with μBondapak C_{18} have been developed to detect and quantify PB and its metabolites in both plasma and urine at 270 nm (14), and at 280 nm (26). The assay was found to be linear and has been validated over the concentration range of 0.1 to 1.0μg/ml from a 0.5 ml plasma and a 1.0 ml urine samples. Limits of detection (LOD) of the assay were 50ng/ml for PB and 3-OH NMP (26). Another less common mean of detection, coupled to LC, is the mass spectrometer (MS). Using a thermospray interface between a HPLC system and MS, Malcolm et al (43), developed an assay for the determination of pyridostigmine in human plasma. Plasma was loaded onto an AASP reversed-phase cartridge, injected onto a HPLC system connected to a Finnigan 4500 mass spectrometer via a thermospray interface and the molecular ion monitored. The precision and accuracy at the limit of quantitation was less than ± 5% and 95 – 105%, respectively. The thermospray LC-MS method was sensitive and gave a detection limit of 1.0 ng/ml for pyridostigmine on a routine basis (43). Recently, Needham et al (44) have described a sensitive and highly specific method for the determination of PB in plasma samples from guinea pigs, using HPLC-MS in electrospray ionization (ESI) mode. An advantage of this strong cation-exchange HPLC-MS-MS method was the enhancement of the ESI-MS signal, which provided good retention and good peak shape of PB with a mobile phase of 70% acetonitrile. The high concentration of acetonitrile improved the desolvation process in the HPLC-ESI-MS-MS method and thus produced detection limits five times better than previous HPLC-MS-MS methods (44). In addition, the use of 70% acetonitrile in the mobile phase allowed the direct injection of the supernatant, which is not possible in typical reversed-phase methods without evaporation and reconstitution. The assay was linear from the range of 0.1 to 50 ng/ml using only 25 μl of sample. The precision and accuracy of the assay was better than 9.1 and 113%, respectively (44). There is no doubt that LC-MS will progress further, with more highly-sensitive and specific methods employing LC-MS being developed in the future.

GC

GC is another major chromatographic technique employed for the analysis of PB in biological samples. Early works were typically performed with flame ionization detection (FID), nitrogen-phosphorus detection (NPD) and electron-capture detection (ECD) (27, 45, 46). Amongst the

detection methods employed, the most commonly used is MS (27, 33, 47), which has a sufficient sensitivity for the determination of PB at lower concentrations in biological samples. In the methods reported, volumes of plasma or serum required for adequate detection are 3-ml or less. A LLE technique is used in the publications summarized in Table 3. PB may be analyzed directly by GC.

Table 3: GC procedures for the determination of pyridostigmine bromide

Sample	Work-up	Column	Detection Mode	Validation Data	Refs.
Plasma (human)	LLE	Diatomite CQ (100 – 120 mesh) coated with 3% (w/w) OV-17, 2m × ¼ in. glass	NPD FID MS: *m/z* 72, 166	LIN: 50 – 1000ng/ml (NPD) REC: 88.7% (NPD) LOD: 5ng/ml (NPD)	[27]
Plasma (human)	LLE	Glass column (2.7m × 0.2cm) filled with 10% OV-17 on Gas Chrom Q	MS: *m/z* 167	LIN: 5 – 100ng/ml REC: >80% LOD: 5ng/ml	[33]
Serum (human)	LLE	Chromosorb 101 (6ft × 2mm)	ECD	LIN: 10^{-9} – 10^{-4}M	[45]
Plasma (human)	LLE	10% OV-17 on Chromosorb W-AW (100 – 120 mesh)	NPD	LIN: 5 – 100ng/ml	[46]
Plasma (human)	LLE	5% OV-1 on Chromosorb 750 (100 – 120 mesh), 6ft × 3mm, glass	MS: *m/z* 167.1	LOD: 2ng/ml	[47]

Chan et al (27) developed a sensitive GC method that measured underivatised PB in human plasma. The procedure involved preliminary ion-pair extraction of the drug into dichloromethane, followed by concentration and analysis of the ion-pair complex using a GC system fitted with a NPD. Using the peak area ratio technique, neostigmine bromide was used as the internal standard for the quantitation of PB in plasma. The analytes were separated in a Diatomite CQ (100 – 120 mesh) column (2 m × ¼ in) coated with 3% (w/w) OV-17. The method depends on the thermal dequaternisation of the quaternary amines, and can be used to detect 5 ng/ml in a 3-ml plasma sample. Accurate measurement can be made at levels of 50-1000 ng/ml. This assay procedure had been applied to the determination of the plasma concentration of PB after single administration of intravenous doses in aneasthetised patients. Davison et al (45) also established a GC method for the measurement of the plasma levels of PB. The assay involved a preliminary ion-pair extraction of the drug and the internal marker from plasma using potassium-iodide glycine buffer. The extract was analysed by a GC system (10% OV-17 on chromosorb W-AW, 100-120 mesh) linked to a NPD. The calibration graph of PB was linear and reproducible over the range 5 ng to 100 ng per ml in 3 ml plasma samples. This assay procedure has been used to monitor the plasma levels of PB (2.7 to 18.6 ng per ml) of a myasthenic patient over a period of twelve hours with repeated dosing of PB (60 mg).

Cohan et al (46) investigated the PK of pyridostigmine in human subjects. A GC-ECD method for the analysis of PB in serum was developed. The principle of the method used is to convert the pyridostigmine to its iodide salt, which is then thermally degraded in the injection port of the GC to release methyl iodide, the compound measured by ECD (46). Serum was extracted four times with chloroform, and the volume of the extract was carefully reduced before analysis by GC. The GC contained a 6 ft column with 2 mm internal diameter, which was packed with Chromosorb 101. The portion of the glass column within the injection port was packed with copper wool and maintained at 320°C. The column temperature was maintained at 150°C. Measurement was made by ECD using a nickel 63 source at 250°C.

Sorensen et al (47) introduced a GC-MS method for the determination of PB without derivatisation in plasma. PB was quantitated by GC-MS-selected ion monitoring in the positive chemical ionization mode. PB and the added internal standard d_6-pyridostigmine were extracted with picric acid from plasma into methylene chloride as an ion pair. The extraction was preceded by a washing with ethyl acetate. A 6 ft × 3 mm Chromosorb 750 glass column coated with 5% OV-1 was used for the separation. PB and d_6-pyridostigmine were monitored at m/z 167.1 and m/z 173.1, respectively. LOD ranged from 2 to 5 ng/ml, depending on the condition of the ion source.

Capillary electrophoresis

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 (48, 49). A new capillary zone electrophoresis (CZE) method for the rapid determination of PB in pharmaceutical preparation has been developed (50). 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 270 nm for PB. The optimal separation conditions were: 50 mM boric acid-HCl buffer (pH 3.25) with 30 mM NaClO4, electrokinetic injection for 5 sec 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 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: 60 ppb). The reproducibility of the migration time (< 1% RSD) 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 (50).

Biological techniques

Biological techniques are rarely used for the analysis of PB in biological samples. A RIA was employed by Meyer et al (18) for the identification and quantification of PB in plasma. Antipyridostigmine antibodies were produced in rabbits using a PB analog, 1-(5-carboxypentyl)-3-(N, N-dimethylcarbamyloxy)pyridinium bromide, conjugated to keyhole limpet hemocyanin. These antibodies were used to develop a RIA ranging from 0.5 to 10.0ng/ml of PB in a 0.1-ml plasma sample. A typical standard curve gave an R^2 value of 0.986. This assay measured PB in plasma with better sensitivity and much greater throughput than current state-of-the-art HPLC techniques. In addition, only small volumes (100 μl) of the plasma samples are required. The within- and among-day coefficients of variance (CV) were found to be less than 19% and 29%, respectively. Accuracy for the 1.0 to 50.0 ng/ml range varied less than 20% (18). A similar approach, with slightly lower sensitivity, was described by Miller and Verma (19). In their method, the rabbits immunized with the BSA (bovine serum albumin)-pyridostigmine conjugate and produced specific antibodies in sufficient titre (1:150) for use in the RIA that could reliably detect minute concentrations of PB in plasma and tissue homogenates. The LOD of the RIA was 250 pg of PB in plasma or tissue homogenates (2.5ng/ml), without the need for an extraction procedure. The inter-assay and intra-assay CV were less than 10% in the RIA. No appreciable binding was observed between the antibodies and PB metabolites or other drugs commonly used with PB. The specificity of the RIA was further validated by measuring PB concentrations in biological samples in tandem with HPLC. The RIA was used to study the time course of plasma concentrations and tissue distribution of PB in rats (19).

CONCLUSIONS

A variety of analytical techniques have been described for the analysis of PB in biological samples. These techniques have been reported to be accurate and reproducible and, in the case of HPLC and GC, very specific and sensitive. RP-HPLC methods have been well represented and still offer the best means for the determination of PB and its metabolites. Both SPE and LLE are the common methods for isolating PB and its metabolites from biological specimens. A recently reported CE assay has the potential to become the reference method for plasma PB analysis. Other methods to measure plasma PB concentrations include RIA. RIA has been shown to be capable of studying the time course of plasma concentrations and tissue distribution of PB in rats and may become the method of choice in PK studies.

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