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RESEARCH ARTICLE

PHARMACOLOGICAL ACTIVITY AND ANALYTICAL METHODS FOR DETERMINATION OF FAMPRIDINE – A DRUG FOR MULTIPLE SCLEROSIS TREATMENT

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ABSTRACT

Background: Multiple sclerosis is a chronic autoimmune disease of the central nervous system, characterized by inflammation, demyelinization and axonal injury. The current therapeutic strategies include: disease-modifying, immunomodulatory and immunosuppressive agents (Mitoxantrone). Immunomodulatory drugs are Interferon β, Glatiramer acetate, Leflunomide, Teriflunomide, Fingolimod, Laquinimod (with dual properties of immunomodulation and neuroprotection). For the treatment of relapsing-remitting and secondary progressive multiple sclerosis, new trend is the application of a specific therapeutic strategy with monoclonal antibodies: Alemtuzumab, Daclizumab, Natalizumab and Rituximab. **Objective:** The purpose of the study is the summarizing of the data for pharmacological activity and analytical methods for determination of a drug for treatment of multiple sclerosis – Fampridine. **Methods:** The method of literature survey of data for pharmacological action of Fampridine and analytical methods has been applied. **Results:** For pharmacological activity, literature survey has been shown, that 4-aminopyridine (Ampyra, Dalfampridine, Fampridine, Pymadine), as a selective voltage-dependent potassium channel blocker in the neuronal membrane, prolongs the depolarization phase of the action potential, improves axon potential propagation, increase neurotransmitter release at the neuromuscular junction. Fampridine has been used clinically for multiple sclerosis and Lambert-Eaton myasthenic syndrome. The combination Nivalin P: Galantamine hydrobromide/Pymadine is promising for Alzheimer's disease, due to the synergistic effect of cholinergic potentiation components: Galantamine hydrobromide as an acetylcholinesterase inhibitor and Pymadine as a stimulant of presynaptic release and synthesis of acetylcholine. For the analysis of related substances in 4-aminopyridine substance have been developed gradient HPLC method with UV-detection and capillary electrophoresis. For determination of 4-aminopyridine in capsules, serum and urine have been presented HPLC methods with UV-detection. For analysis of 4-aminopyridine in plasma capillary electrophoresis, spectrofluorimetry, gas chromatography with electron capture detector and HPLC with UV-detection have been described. **Conclusion:** The main pharmacological application of Fampridine is for treatment of multiple sclerosis and the combination Nivalin P: Galantamine hydrobromide/Pymadine is promising for Alzheimer's disease. As the most often applied methods for analysis of 4-aminopyridine in dosage forms and biological samples have been presented HPLC methods with UV-detection, gas chromatography and capillary electrophoresis.

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INTRODUCTION

Multiple sclerosis is a chronic autoimmune disease of the central nervous system, characterized by inflammation, demyelization, axonal injury, motor, visual, speech and memory disturbances (Goldenberg, 2012). The current therapeutic strategies for multiple sclerosis (Gajofatto *et al.,* 2017) are based on the use of disease-modifying, immunomodulatory and immunosuppressive agents (Tsivgoulis *et al.,* 2015).

Mitoxantrone is an intravenously administered immunosuppressant, inhibits T-cell, B-cell and macrophage proliferation and is indicated for reducing of neurological disability and relapse frequency in patients with secondary progressive, progressive relapsing or worsening relapsing-remitting multiple sclerosis (Fox, 2006). As an immunomodulatory agents have been applied Interferon β (Madsen, 2017), Glatiramer acetate (McKeage, 2015), Leflunomide (Aly, 2017), Teriflunomide – active metabolite of Leflunomide (Chan *et al.,* 2016), Fingolimod (Gajofatto *et al*., 2015), Laquinimod (with dual properties of

immunomodulation and neuroprotection) (Hainke *et al*., 2016). The development of monoclonal antibodies is highly specific therapeutic strategy for the treatment of multiple sclerosis. For active relapsing-remitting multiple sclerosis has been introduced Alemtuzumab (Hartung *et al*., 2015), Daclizumab (Shirley, 2017) and Rituximab (He *et al*., 2013). Natalizumab have been used for treatment of secondary progressive multiple sclerosis (Sellebjerg *et al*., 2016).

Pharmacokinetic parameters of 4-aminopyridine: Orally administered 4-aminopyridine (Ampyra, Dalfampridine, Fampridine, Pymadine) is rapidly and completely absorbed from the gastrointestinal tract with relative bioavailability: 95 %. Food reduces approximately 2 % - 7 % of absorption. Fampridine is a lipid-soluble, crosses the blood-brain barrier and bound plasma proteins fraction is between 3 % - 7 %. 5 % of 4-aminopyridine is metabolised under hydroxylation to 3-hydroxy-4-aminopyridine in human liver microsomes by cytochrome P_{450} 2E1 (CYP 2E1) and further is conjugated to the 3-hydroxy-4-aminopyridine sulfate (Fampyra, 2011; Fampyra, 2016). 4-aminopyridine-N-oxide is obtained by oxidation (Parthasarathi *et al*., 2011). Drug and non-active metabolites are eliminated primarily by the kidneys as unchanged by renal excretion, within 24 hours. Faecal excretion accounts for less than 1 %. Drug must not be administered to patients with mild, moderate and severe renal impairment (Fampyra, 2016). On Table 1**.** are presented data for the pharmacokinetic parameters of 4-aminopyridine.

Toxicity of 4-aminopyridine: For 4-aminopyridine LD_{50} data for toxicity after per oral (p.o.), subcutaneous (s.c.) and intraperitoneal (i.p.) application on animals are summarized in Table 2. According to the Hodge-Sterner rating, 4-aminopyridine (LD_{50} = 200 mg/kg b.w. p.o. in rats) is classified as medium to toxic (LD_{50} = 51 mg – 500 mg/kg b.w. p.o.) and very toxic according to the Gosselin scale: 51 mg – 500 mg/kg b.w. p.o).

Pharmacological effects and administration of 4-aminopyridine: In healthy nerves, signals travel over myelin, which is a protective layer that surrounds nerves (Fig. 1.). Multiple sclerosis breaks down myelin, which affects the nerve's ability to conduct signals. The potassium (K^+) channels are located primarily in the paranodal and internodal membrane of the axon, where they are not significantly activated by the passage of an action potential, because the myelin sheath acts as an electrical shield. In demyelinizated axons the internodal membrane and it's ion channels become exposed to larger electrical transients during the action potential. Leakage of ion current through the K^+ channel contribute to action potential conduction block, leading to symptoms as walking difficulties. Electrophysiologic studies of demyelinizated axons show that augmented potassium currents increase extracellular potassium ion concentration, which decreases action potential duration and amplitude and cause conduction failure (Fig. 1.) (Fampyra, 2017). 4-aminopyridine is a selective voltage-dependent potassium potassium channel blockers in the neuronal membrane, prolongs the depolarization phase of the action potential, improves axon potential propagation, increasing neurotransmitter release at the neuromuscular junction, the penetration of calcium ions into muscle fibers, and enhancing muscle redundancies (Dunn *et al.,* 2011). 4-aminopyridine improves synaptic and neuromuscular function in patients with spinal cord injury, myasthenia gravis, or multiple sclerosis by directly stimulation of high voltage-activated Ca^{2+} channels in acutely dissociated neurons (Wu *et al*., 2009). 4-aminopyridine has been applied clinically for: 1) multiple sclerosis (McDonald *et al*., 2011; Jensen *et al*., 2014); Alzheimer's disease: enhances the release of acetylcholine and improves cognitive function; 3) Lambert-Eaton myasthenic syndrome. The drug is the most effective in patients with chronic progressive form of multiple sclerosis due to improving gait (Sahraian *et al*., 2011). Fampridine antagonizes the action of non-depolarising neuromuscular blocking agents Tubocurarine, Atracurium, Doxacurium, Pancuronium, Pipecuronium, Vecuronium and removes a neuromuscular block in botulism (Dunn *et al.,* 2011). In Phase 3 clinical trials (Egeberg *et al*., 2012) it was found that administration of Dalfampridine (Ampyra) 10 mg sustained release tab. l. twice daily, significantly improves motor activity in multiple sclerosis patients (Hayes, 2011), with the rate of motion increasing from 25 % (Goodman *et al.,* 2010) to 42.9 % (Pikoulas *et al*., 2012). The maximum dose is 10 mg twice daily, because higher doses increase the risk of side effects (Cornblath *et al*., 2012).

Dalfampridine 10 mg prolonged-release tabl. (Hersh *et al.,* 2012) has been approved for the treatment of multiple sclerosis by the Food and Drug Administration in 2010 (Blight, 2011). Common side effects which may affect up to 1 in 10 people are: anxiety, back pain, constipation, dizziness, difficulty in breathing and sleeping, headache, nasopharyngitis, nausea, palpitations, tremor, vomiting. Uncommon side effects which may affect up to 1 in 100 people are hypersensitivity, tachycardia and trigeminal neuralgia. The combination Nivalin P: Galantamine hydrobromide/Pymadine = 1: 1 is promising due to the synergistic effect of cholinergic potentiation components: Galantamine hydrobromide is an acetylcholinesterase inhibitor and Pymadine is a stimulant of presynaptic release and synthesis of acetylcholine. In investigation of the effect of Nivalin P on isolated skeletal muscle fibers, Pymadine has been shown to block voltage-dependent potassium channels, prolonging the duration of action potential and the release of calcium ions in the sarcoplasm and retards the uptake of calcium ions into the sarcoplasmic reticulum of muscle fibers (Radicheva *et al*., 1999). For memory testing the effects on total latency, conditional, unconditional and inadequate responses of male Wistar rats, after treatment per oral with Nivalin P at doses of 6.6 mg/kg (1/5 LD_{50}), 3.3 mg/kg (1/10 LD_{50}) and 1.65 mg/kg (1/20 LD_{50}), have been conducted using an active bidirectional avoidance method. Nivalin P at a dose of 1.65 mg/kg facilitates the training of rats and improves the possibilities of reducing the number of inadequate responses. These data indicate that Nivalin P in low doses causes an increase in cholinergic activity and that combination therapy may be useful in the treatment of Alzheimer's disease (Markov *et al*., 1994).

Methods for the analysis of 4-aminopyridine: For the analysis of related substances in 4-aminopyridine substance, capillary electrophoresis was used on a Silicagel column, 300 mm effective length, 370 mm total length, temperature 20 ^oC, applied voltage 25 kV, current magnitude 62 mA, pressure 3447.38 Pa, 50 mM solution of phosphate buffer (pH = 2.5) and UV-detection at λ = 210 nm. Modification of this method is the use of a capillary column Silicagel, 600 mm effective length, an applied voltage: 20 kV and 100 mM sodium acetate solution (pH = 5.15) (Sabbah *et al.*, 2001). For the study of the related related 4-aminopyridine-N-oxide, a gradient HPLC

Table 1. Pharmacokinetic parameters of 4-aminopyridine (Fampyra, 2017)

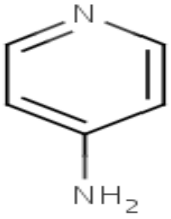
Parameters	4-aminopyridine 
Bioavailability	95 %
Volume of distribution	2.6 l/kg
Bound to plasma proteins	3 % – 7 %
Metabolism	hepatal
Excretion	renal
Renal clearance	370 ml/min.
Terminal elimination half-life 6 hours ($t_{1/2}$)	6 h

Table 2. LD_{50} for 4-aminopydine (Fampyra, 2011)

N:	Animals tested	LD_{50} p.o.[mg/kg b.w.]	LD_{50} i.p.[mg/kg b.w.]	LD_{50} s.c. [mg/kg b.w.]
1.	Rats	200	11.5	18.5
2.	Mice	50	10	5

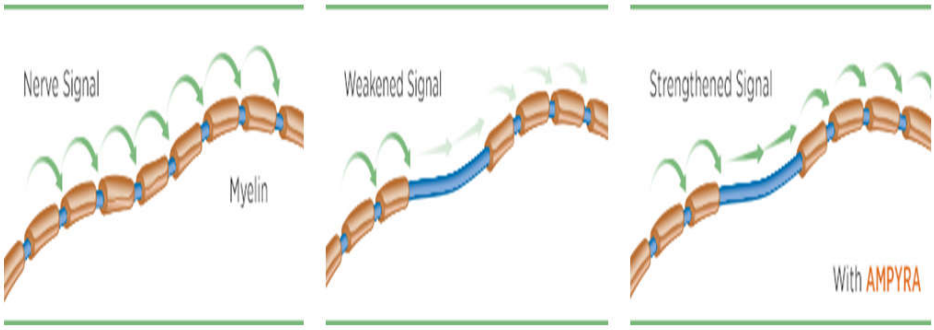


Fig. 1. Signals over myelin in healthy nerves, demyelinizated axons and under the action of 4-aminopyridine

method with RP C_{18} column and UV-detection has been applied (Thomas *et al.,* 2012). Reversed phase high performance liquid chromatographic method for the quantification of related substances in the drug substance and tablet dosage form of Fampridine have been developed by using the following chromatographic system: stationary phase: column Inertsil ODS 3V (150 mm x 4.6 mm x 5 µm particle size), mobile phase A: phosphate buffer pH = 4.0: water solution of potassium dihydrogen orthophosphate, mobile phase B: phosphate buffer pH = 4.0): acetonitrile = 20: 80 v/v, gradient mode with the flow rate of 1.0 ml/min., peaks monitoring at λ = 260 nm (Babu *et al*., 2017). Spectrophotometric methods have been developed for the estimation of Dalfampridine in bulk and its tablet formulation: 1) λ = 262 nm; λ = 274.5 nm in first order derivative spectrophotometry; 2) area under the curve method for zero-order derivative spectrophotometry at 254.2 nm – 269.0 nm; 3) area under the curve method for first-order derivative spectrophotometry at 267.2 nm – 284.2 nm (Vivekkumar *et al*., 2014). For the determination of 4-aminopyridine in capsules HPLC methods with isocratic mode and UV-detection have been developed with the following chromatographic systems: 1) stationary phase: column RP C_{18} Luna octadecylsilane (250 mm x 4.6 mm x 5 µm), mobile phase: acetonitrile: phosphate buffer (25 mM sodium dihydrogen phosphate and 1 mg/ml 1-heptane-sulfonic acid sodium salt) = 15: 85 v/v, flow rate: 1.0 ml/min, UV-detection at λ = 263 nm and internal standard Caffeine citrate (Donnelly, 2004); 2) column Prodigy C_{18} (250 acetonitrile = 165: 35 v v, flow rate: 0.8 ml/min. and UV-detection at λ = 266 nm (Trissel *et al.*, 2002). In plasma 4-aminopyridine have been analyzed by capillary electrophoresis (Namura, 2010), spectrofluorimetry (EL-Fatatry *et al.*, 2013), gas chromatography (Watson, 1981) and HPLC (Hayes *et al.*, 2003). Capillary electrophoresis has been applied for the quantification of 4-aminopyridine in plasma under the following conditions: 1) Silicagel capillary column, 300 mm effective length, 370 mm total length, temperature 20 ^{o}C, applied voltage 25 kV, current magnitude 62 mA, pressure 3447.38 Pa, 50 mM phosphate buffer solution (pH = 2.5) and UV detection at λ = 210 nm (Sabbah *et al.*, 2001); 2) Silicagel capillary column (645 mm x 50 µm), 570 mm effective length, temperature 15 °C, applied voltage 19 kV, 100 mM phosphate buffer (pH = 2.5) and UV-detection at λ = 254 nm (Namura *et al.,* 2010). Spectrofluorimetric methods has been reported for the determination of Dalfampridine in human plasma, based on the reaction between the drug and fluorescamine in borate buffer (pH = 8.5), and fluorescent derivative has been measured at λ = 485 nm using an excitation wavelength at λ = 385 nm (EL-Fatatry *et al.,* 2013). Gas chromatography has been applied for assay in plasma using standard 3-methyl-4-aminopyridine, after extraction of 4-aminopyridine from plasma with dichloromethane and isopropanol, and then derivatization to a pentafluoropropionyl or to a monochlorodifluoro-acetyl derivative, which have been analyzed by an electron capture detector (Watson, 1981). HPLC method with liquid-liquid extraction with ethylacetate from plasma and the following chromatographic system:

stationary phase: RP C_{18} Spherisorb column, mobile phase: acetonitrile: 0.03 mM potassium dihydrogen phosphate, flow rate: 1.0 ml/min., Procainamide internal standard and UV-detection at λ = 263 nm, has been developed (Nattel *et al.,* 2000). HPLC method for determination of 4-aminopyridine in plasma following derivatization reaction with benzoyl-chloride to N-benzoyl-4-aminopyridine on a Ultrasphere column, mobile phase: 12 % aceto-nitrile in 2 g/l ammonium perchlorate, flow rate: 0.1 ml/min., internal standard Procainamide and UV-detection at λ = 265 nm (Pratt *et al.*, 1995) has been reported. For the determination of 4-aminopyridine in serum the following HPLC methods have been described:

- RP C_{18} column, mobile phase: acetonitrile: methanol: ethanol: 1 % ammonium carbonate = 75: 10: 10: 5 v/v and UV-detection at λ = 244 nm (Van Der Horst *et al.,* 1992);
- Nucleosil C_{18} column (150 mm x 3.0 mm x 5 μm), mobile phase: acetonitrile: methanol: ammonium carbonate = 61: 35: 4 v/v, flow rate: 0.8 ml/min. and UV-detection at λ = 245 nm (Uge *et al.,* 1981);
- Bond Elut RP C_{18} column (150 mm x 4.6 mm x 5 μm), mobile phase: 35 % perchloric acid: methanol = 1: 100 v/v, N-propionylprocaineamide standard and UV-detection (Gupta *et al.,* 1996);
- RP C_{18} column, mobile phase: 7.5 % acetonitrile in purified water: tetrabutylammonium iodide: sodium heptanesulfonate, internal standard 2-aminopyridine and UV-detection at λ = 263 nm (Shinohara *et al.,* 1992).

For quantitative analysis of 4-aminopyridine in urine have been introduced an isocratic HPLC methods with UV-detection with the following chromatographic systems:

- stationary phase: Nucleosil C_{18} column (150 mm x 3.0 mm x 5 μm), mobile phase: acetonitrile: methanol: ammonium carbonate = 61: 35: 4 v/v, flow rate: 0.8 ml /min. and λ = 245 nm (Uges *et al*., 1981);
- Bond Elut RP C_{18} column (150 mm x 4.6 mm x 5 μm), 35 % perchloric acid: methanol = 1: 100 v/v, internal standard N-propionylprocainamide and λ = 278 nm (Gupta *et al*., 1996);
- 3) liquid-liquid extraction with methylene chloride from urine, mobile phase: acetonitrile: (15 mM 1-heptanesulfonic acid sodium salt solution, 2 mM tetramethylammonium bromide and 0.01 M sodium dihydrogen phosphate) = 15: 85 v/v, flow rate: 1.0 ml/min. (Casteel *et al*., 1990).

Reverse phase high performance liquid chromatographic method has been developed and validated for the estimation of Dalfampridine in bulk and formulations. Method development has been carried out on stationaty phase: Inertsil C_{18} column (250 x 4.6 mm x 5 μm), achievement of the chromatographic separation using a mobile phase, containing acetonitrile and potassium dihydrogen phosphate buffer (pH = 4) in the ratio of 70: 30 v/v at flow rate of 1.0 ml/min, using UV-detection at λ = 298 nm (retention time = 2.433 min.). For stability study, the drug was exposed to the stress conditions: acid, alkaline, oxidation, thermal by using: 0.1 M HCl, 0.1 M NaOH, 30 % H_2O_2, 100 ºC. The major degradation has been observed at acidic condition (80.11 %), followed by thermal (70.25 %), alkaline (67.12 %) and oxidation (63.42 %) (Dharani *et al.*, 2016). Liquid chromatography-mass spectrometry method has been developed and validated for the simultaneous quantification of Fampridine, Fingolimod and Prednisone in rat plasma using Imipramine as an internal standard. Following protein precipitation, the analysis has been performed on XBridge C_{18} column (150 mm x 4.6 mm x 5 μm), using gradient mobile phase, consisting of 5 mM ammonium formate in water (pH = 9.0) and acetonitrile in a flow gradience program (Suneetha *et al*, 2016).

Conclusion

The main pharmacological application of Fampridine is for treatment of multiple sclerosis and the combination Nivalin P: Galantamine hydrobromide/Pymadine is promising for Alzheimer's disease. As the most often applied methods for analysis of 4-aminopyridine in dosage forms and biological samples have been presented HPLC methods with UV-detection, gas chromatography and capillary electrophoresis.

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