CHARLES UNIVERSITY

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Biochemical Sciences

Anthelmintic and other xenobiotic biotransformation in helminths and its contribution to resistance development

Doctoral thesis

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Deklarace

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracoval samostatně (pod vedením své školitelky a konzultantky). Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

Declaration

I declare that the thesis is my original work that I have written on my own. All literature and other sources used in the thesis are cited in text and listed in the References section. This work was not used to acquire any other or same degree.

Lukáš Prchal

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Abstrakt

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Název disertační práce:

Biotransformace anthelmintik a jiných cizorodých látek u helmintů a její přispění k rozvoji resistence

Parazitičtí helminti jako jsou tasemnice, motolice nebo oblí červi představují hrozbu pro hospodářská, domácí i volně žijící zvířata. Způsobují jim značné zdravotní problémy a chovatelům působí ekonomické ztráty kvůli poklesu produkce a nákladům na léčbu. Léčba veterinárními anthelmintiky je základní metodou boje proti helmintózám. Ovšem dlouhodobé používání anthelmintik vedlo k vývoji lékové rezistence helmintů. Největším problémem je pak rostoucí výskyt multirezistentních kmenů u řady druhů helmintů. Výzkum mechanismů lékové rezistence helmintů se tak stal velmi aktuálním směrem vědeckého výzkumu. Zvýšená biotransformace anthelmintik, urychlení jejich transportu z těl helmintů či kontakt nižších vývojových stádií helmintů s anthelmintiky v životním prostředí jsou jedny z možných mechanismů přispívajících ke snížení účinnosti farmakoterapie a k rozvoji rezistence.

Předložená disertační práce se zaměřuje na studium těchto mechanismů u tří druhů helmintů. Při analýzách léčiv a jejich metabolitů bylo využíváno vysoce účinné kapalinové chromatografie ve spojení s hmotnostní spektrometrií. Při studiích na vlasovce slézové (*Haemonchus contortus*) jsme se zaměřili na rozdíly ve schopnosti metabolizovat a transportovat léčivo flubendazol u citlivých a rezistentních kmenů. Dále jsme zkoumali metabolismus benzimidazolových anthelmintik a aktivity enzymů, které se účastní metabolismu léčiv a jiných xenobiotik, u tasemnice ovčí (*Moniezia expansa*) a u velké jaterní motolice (*Fascioloides magna*). V další části práce byly měřeny hladiny albendazolu a jeho metabolitů v ovčím trusu a jejich vztah k rozvoji helminthorezistence. Dílčím úkolem bylo také zpracování dostupných znalostí o novém aminoacetonitrilovém anthelmintiku monepantelu do souhrnného článku.

Výsledky studií provedených na vlasovkách ukazují, že flubendazol do vlasovek prochází pasivní difúzí a není substrátem žádného transportního proteinu. Vlasovky jsou schopny biotransformovat flubendazol i řadu modelových xenobiotik. Oproti citlivému kmeni vlasovky, kmen multirezistentní má vyšší enzymové aktivity a vytváří více metabolitů, což se může podílet na vzniku rezistence. Ve studiích na tasemnici ovčí a velké motolici jaterní bylo zjištěno, že oba druhy helmintů byly schopny redukovat karbonylovou skupinu u mebendazolu a flubendazolu a oxidovat albendazol na sulfoxid, ale nebyly nalezeny žádné konjugáty těchto léčiv. Tasemnice byla schopná oxidovat sulfoxid dále na sulfon. Tato reakce u motolice chyběla a stejně tak nedocházelo k obdobné oxidaci triklabendazolu dříve popsané u motolice jaterní *Fasciola hepatica*. Farmakokinetická studie albendazolu u ovcí ukázala, že koncentrace albendazolu a jeho aktivního metabolitu v trusu dosahovaly hodnot, které jsou ovocidní i larvicidní pro vlasovky. Preinkubace vajíček a larev vlasovek se subletálními dávkami albendazolu nezvyšovala rezistenci dospělců na albendazol.

Dosažené výsledky přispěly k lepšímu pochopení mechanismů, kterými se helminti chrání před anthelmintiky a dalšími xenobiotiky. Tyto znalosti budou moci být použity pro předcházení vzniku lékových rezistencí helmintů.

Abstract

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Title of Doctoral Thesis:

Anthelmintic and other xenobiotic biotransformation in helminths and its contribution to resistance development

Parasitic helminths such as tapeworms, flukes or nematodes pose a threat for domestic, farm and wild living animals. Helminths cause significant health problems in animals. Moreover, they cause economical losses to farmers due to production decrease and treatment costs. The treatment with veterinary anthelmintics is still a basic method to fight off helminth infections. However, the long-term use of anthelmintics caused an emergence of resistance to anthelmintics. The increasing incidence of multiresistant strains of many helminth species is the greatest problem. Therefore, research of drug resistance mechanisms became an actual field of study. Increased biotransformation of anthelmintics, transport out of parasite bodies and contact of lower developmental stages with anthelmintics in the environment are some of possible mechanisms that lead to decrease of anthelmintic therapy effectivity and resistance development.

The presented thesis focuses on the study of these mechanisms in three helminth species. High performance liquid chromatography coupled with mass spectrometry was used in drug and their metabolite analysis. In a barber's pole worm (*Haemonchus contortus*) study, we focused on differences in metabolism and transport of a drug flubendazole in resistant and susceptible strains. We studied metabolism of benzimidazole anthelmintics and activities of xenobiotic-metabolising enzymes in a sheep tapeworm (*Moniezia expansa*) and a giant liver fluke (*Fascioloides magna*) as well. Another part of the thesis was focused on measurement of albendazol and its metabolites levels in sheep faeces and relationship to helminth resistance development. Summarising the knowledge about novel aminoacetonitrile anthelmintic monepantel to a review article was another partial task.

The results of *H. contortus* studies show that flubendazole gets to *H. contortus* bodies via passive diffusion. Flubendazole appears not to be a substrate of any transport protein. *H. contortus* is able to biotransform flubendazole and many other model xenobiotics. Multiresistant strain creates more metabolites and has higher enzyme activities compared to susceptible one. These differences might participate in resistance development to other drugs. Studies with a sheep tapeworm and a giant liver fluke determine both helminth species are able to reduce carbonyl group of mebendazole or flubendazole and both are able to oxidise albendazole to sulphoxide. However, no conjugates of studied drugs were found. *M. expansa* is able to oxidise albendazole sulphoxide to sulphone. This reaction is not present in the fluke and neither oxidation of triclabendazole documented in liver fluke *Fasciola hepatica* is present. The pharmacokinetic study of albendazole in sheep shows the concentrations of albendazole and its active metabolite in faeces are ovicidal and larvicidal for *H. contortus*. Preincubation of eggs and larvae of *H. contortus* with sublethal doses of albendazole did not increase resistance of adults to albendazole.

Obtained results contribute to better understanding of mechanisms that helminths use to protect themselves against anthelmintics and other xenobiotics. This knowledge could be used to prevent further spread and emergence of resistance.

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1 Introduction

During evolution, many life forms decided to live together with other organisms to mutual benefit. However, sometimes this profit was just one sided. These parasites exist in all kingdoms as well as their hosts. Parasitic helminths are one of a common parasite group consisting of tapeworms, flukes and roundworms. They are one of the most widespread parasite groups and they affect wide range of hosts from molluscs to insects and up to all vertebrae species including human. These parasites are not only of medical concern, but also economical as they decrease yields from farm animals like sheep and cattle.

Anthelmintics of various classes were developed to treat parasitic infections. However, over time, strains resistant to particular anthelmintic emerged especially in veterinary important roundworms. With further use of different classes of anthelmintics, parasites developed resistance to those as well. These multiresistant strains became a threat that limited treatment of affected animals. A few years ago, new classes of anthelmintics were introduced. However, there are concerns about their effectivity in the long run, so mechanisms of resistance and their overcoming became a more intensively studied field. Apart from mutations in target genes, drug metabolism is one of limiting factors as it determines how long the parasite would be affected by the treatment. In mammals, mostly enzymes in liver tissue perform metabolism of foreign compounds. Parasites are trying to protect themselves by metabolising drugs to less effective compounds or by expelling drugs from their bodies by membrane transporters.

Mass spectrometry is a very useful method to study these mechanisms. Its beginning dates to the previous century and gradually it developed into a versatile method for qualitative and quantitative analysis. At first, it was coupled with gas chromatography, but with the development of ion sources working in atmospheric pressure, it started to be coupled with high performance liquid chromatography. Finally, mass spectrometer became the most used detector for high performance liquid chromatography. It enables us to determine mass of unknown metabolites as well as a partial structure of these molecules. A relatively small amount of samples is required compared to methods like nuclear magnetic resonance. Sensitivity of mass spectrometry is an advantage as helminth parasites are small in size and do not produce large amount of metabolites. Apart from qualitative analysis, it gives us an opportunity to quantify found metabolites. With proper sample pre-treatment and HPLC separation mass spectrometry can detect metabolites even in very complex matrices.

All in all, drug metabolism and membrane transport can be an important factor in existence of multiresistant strains of parasites and high-performance liquid chromatography with mass spectrometry gives us excellent opportunity to study these mechanisms.

2 Theoretical part

2.1. Parasitic helminths

Parasitic helminths are usually considered as representatives of platyhelminths from classes Trematoda, Cestoda and Monogenea, and also nematodes from class Nematoda and Acantocephala (Volf and Horák, 2007). Parasitic helminths have various life cycles with development stages either in the environment and/or in up to four host organisms. This work focused on parasites that have mammals as their definitive hosts and there are various invertebrates in a role of intermediate hosts. Diseases caused by parasitic helminths are some of worldwide present-day problems. Parasites that affect humans during their life cycle are threat for populations in developing countries, especially in tropical areas. Parasites affecting animals threaten the welfare of animals and cause significant economic losses in breeding worldwide (Charlier *et al.*, 2014).

2.1.1. Trematodes

Flukes are a class of platyhelminths affecting wide range of intermediate and definitive hosts. Flukes usually have at least one intermediate host and in the majority of cases, the first one is a member of Mollusca phylum. They have bilateral bodies with surface covered with tegument which has various functions; the most important function is protection and apart from that tegument carries organs for attachment to host bodies; in the subclass Digenea those are mainly two suckers whose position and morphology are determining features for parasite (Wilson *et al.*, 2011). Most of the class are hermaphrodites except for Schistosomatidae, where there is a distinct sexual dimorphism with bigger males and small filiform females. Their life cycles have several stages. Miracidia are hatched from eggs and begin to search for the first intermediate host, mostly from Mollusca phylum. There they undergo transformation to sporocysts, rediae and finally to a next infective stage cercariae which leave the mollusc body and either infect another intermediate host where they develop further or they encyst in the environment creating metacercariae (Dixon, 1966; Souza *et al.*, 2002; Volf and Horák, 2007). When ingested by a next host, metacercariae excyst and migrate in a definitive host's body to a final organ, where adult flukes develop.

2.1.1.1. Fasciola hepatica

Even though it is not directly mentioned in this work, a liver fluke (Fig. 1; A) is one of the most studied flukes and has great impact on animal populations. It has a two-host cycle;

with snails *Galba truncatula* being common intermediate hosts in Czech Republic and *Galba truncatula* and *Lymnea* species are hosts worldwide (Rondelaud *et al.*, 2007). The spread of infection is limited by the fact that snail hosts are usually semiaquatic. Definitive hosts are various herbivores or omnivores like sheep (*Ovis aries*), cattle (*Bos taurus*), red deer (*Cervus elaphus*), fallow deer (*Dama dama*), rat (*Rattus norvegicus*) and even humans (Saleha, 1991; Vengušt *et al.*, 2003; Mas-Coma *et al.*, 2005). A liver fluke finishes its development in bile ducts and liver tissue of definitive hosts. It has impact on sheep and cattle heath and their production, therefore it causes significant economic loses (Saleha, 1991; Kaplan, 2001). Its habitat spreads across all continents, however, in Asia there are other dominant liver flukes. It also poses threat to humans, as they can serve as hosts (Mas-Coma *et al.*, 1999, 2005; Wu *et al.*, 2012).

2.1.1.2. Fascioloides magna

A giant liver fluke (Fig. 1; B) is one of the most abundant flukes in wild living animals in the Czech Republic. It is larger than *F. hepatica*, but in many other features they resemble each other (Swales, 1935). It has a two-host cycle and shares hosts with *F. hepatica* and therefore its habitat. However, it becomes an adult in wild living cervids like red, fallow and roe deer (*Capreolus capreolus*). Domestic animals like cattle or sheep are dead end or aberrant hosts but their health and condition can be dramatically affected by flukes migrating in liver tissue (Foreyt and Todd, 1976). Its origin is in North America and it was imported to Italy in the 19th century with game animals. In Europe, it is very common in Central European states like the Czech Republic, Slovakia, Hungary and neighbouring states especially around Danube River; it also spreads with migration of definitive hosts (Majoros and Sztojkov, 1994; Pybus, 2001; Rajković-Janje *et al.*, 2008; Králová-Hromadová *et al.*, 2011; Kasny *et al.*, 2012; Sattmann *et al.*, 2014).

2.1.2. Cestodes

Cestodes are another class of platyhelminth parasites with several subclasses. The biggest and most typical is the subclass Eucestoda. Tapeworms from this subclass have their body segmented. There is a frontal “head” segment called scolex with attachment organs and a “body” segment called strobilus, which is divided to multiple segments that mature gradually (Smyth, 1946; Heyneman, 1996; Auer and Aspöck, 2014). Its surface is covered with tegument, syncytium from multiple cells whose functions are to protect parasite, absorb nutrients from surrounding environment and excrete various compounds (Oaks and Holy, 1994). An absorption is direct from a host’s intestine without the use of a digestive system. Excretion

is performed by protonephridia in each segment. Eucestoda are hermaphrodites with both male and female sexual organs in each segment. Fertilization is usual between two tapeworms, but could be also between two segments of the same individual. The most mature segments, which contain eggs, are on the end of strobilus. When they mature enough eggs are fertilized, released and leave body of the host with faeces (Haag *et al.*, 1999). In the environment larvae hatch from eggs and find host in which they undergo development. Tapeworms have up to two intermediate hosts and mature tapeworms live in intestines of various animals, where they can reach considerable lengths (Haukisalmi *et al.*, 1998; Ito, 2015).

2.1.2.1. Moniezia expansa

The mature tapeworms of this species live in intestines of sheep, goats and cattle, so it is commonly called a sheep tapeworm (Fig. 1; C). It has two-host cycle with oribatid mites living in soil as intermediate hosts and various ruminants as definitive hosts. It has a typical tapeworm body and can spread up to several meters (Stunkard, 1938; Volf and Horák, 2007). It is equipped with inteproglotidal glands which can produce regulatory peptides. Its scolex lacks hooks in its attachment organ so it does not seriously damage host's intestinal wall. *M. expansa* habitat spreads worldwide and this tapeworm is a threat mainly for young calves and lambs, especially when the infestation is massive (Denegri *et al.*, 1998). Apart from these cases there are doubts about its risk (Elliott, 1984, 1986).

2.1.3. Nematodes

In contrast to previous two groups, nematode parasites do not belong among platyhelminths but among roundworms. It could be determined from the name there are distinct differences between these groups. Nematodes bodies are bilaterally symmetric and circular in the cross-section and there is usually considerable sexual dimorphism between male and female worms. They are considered gonochorists with 4 larval stages; however, some members can also use other reproductive strategies like parthenogenesis or hermaphroditism (Blaxter, 1998; Volf and Horák, 2007). Their body surface is covered with a cuticle consisting mostly of collagen with lipids and glycosaminoglycans and with outer layer being glycocalyx (Sood and Kalra, 1977; Johnstone, 1994). These layers have protective and other functions, like excretion or parasite-host interactions. Excretion is provided by systems consisting of small convergent tubules or it is unicellular; both variants end with excretion pores. Nematodes have tubular digestive system with mouth, pharynx and intestine ending in an anal orifice or cloaca. Some nutrients can also be transferred through cuticle and body surface. Their mouths have various organs that allow them to get to and consume their food. Nematodes can be either parasites or

free living in soil or water. Free living *Caenorhabditis elegans* is one of the most studied model organisms and it was the first nematode to have its genome sequenced (Kenyon, 1988; C. elegans Sequencing Consortium, 1998). Considering parasites, many species affecting animals, humans and even plants belong to this group.

2.1.3.1. Haemonchus contortus

Commonly known as "barber's pole worm", due to appearance of female worms where white ovaries and red blood filled digestive tube create a spiral, which looks like a typical sign in front of a barber shop (Fig. 1; D). This parasite has only one definitive host; its three larval stages develop in soil with a larval stage L3 moving atop of grass to be eaten by a ruminant in which it undergoes transformation to a stage L4 and its maturation to an adult stage and then it starts to reproduce (Veglia, 1916; Anderson, 2000). *H. contortus* could also enter hypobiotic state and wait for better conditions. It is dioecious with apparent sexual dimorphism where females are bigger with distinctive ovaries and cloaca endings and males can be distinguished by bursa copulatorix Y shaped dorsal ray. Adults live in abomasum of ruminants, where they suck blood from abomasal wall. One individual can intake up to 0.05 ml of sheep blood per day, causing anaemia, which is life-threatening especially for young lambs and weakened sheep (Rowe *et al.*, 1988). *H. contortus* possess one lancet in its oral cavity to penetrate an abomasal wall, where it causes additional damage, as it leads to permanent changes in wall structure. All in all, *H. contortus* causes serious harm to animals and significant economic loses as well. To determine level of anaemia special FAMACHA (stands for FAffa MAlan CHArt) system was created; it uses changes of coloration to the mucous membranes (Van Wyk and Bath, 2002). *H. contortus* is also one of nematode parasites which developed strains resistant to multiple anthelmintics (van Wyk and Malan, 1988). Moreover *H. contortus* genome has already been sequenced (Laing *et al.*, 2013).

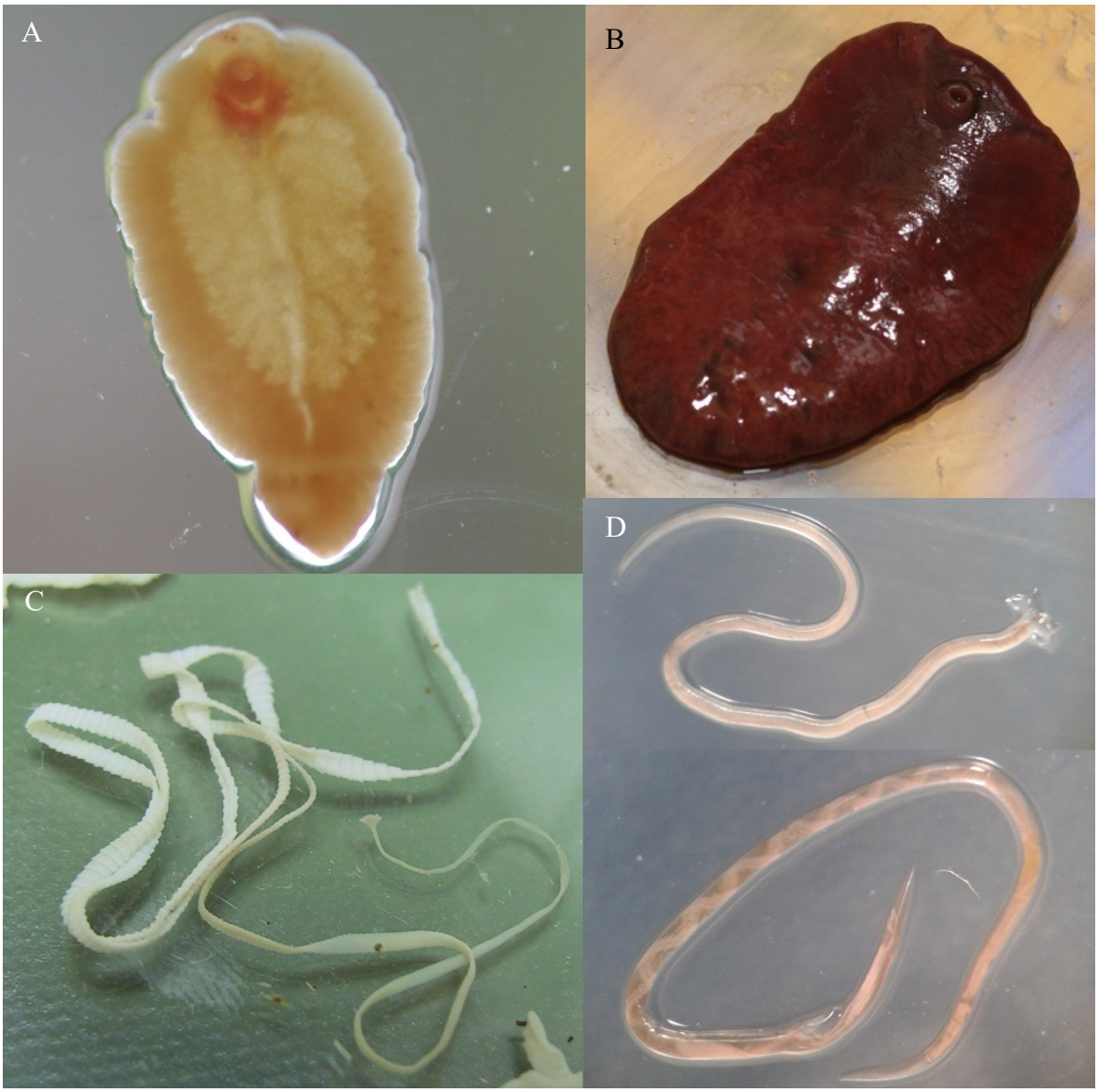

Fig. 1: Helminth parasites A) *Fasciola hepatica*; B) *Fascioloides magna*; C) M*oniezia expansa*; D) *Haemonchus contortus*, top male, bottom female; photographs by Lenka Lecová, Ivan Vokřál and Jiří Lamka

2.2. Animal hosts

Because this work deals with adult parasites from definitive hosts, there is a short notice on them. Intermediate hosts for flukes and tapeworms are left out, because definitive hosts were naturally infected and therefore intermediate hosts could not be identified.

2.2.1. Domestic sheep (*Ovis aries*)

Belonging to phylum Chordata, clade Synapsida, class Mamalia, order Artiodactyla, family Bovidae sheep is one of the most widespread mammals in the world and it is important for agriculture and economy of regions where it is bred. Sheep are quadrupedal ruminants; still,

due to intensive breeding, there are many breeds differing in many aspects of appearance, for example coloration, physical build, presence of horns *etc.* as well as their productivity. They live up to 10-12 years, with maturity reaching in 5-9 months and their production peaks in about 3 to 6 years.

Sheep were domesticated from wild mouflons (*Ovis orientalis* or *Ovis musimon*) during the Neolithic agricultural revolution most probably in the Middle Eastern region (Pedrosa *et al.*, 2005; Tapio *et al.*, 2006; Meadows *et al.*, 2007). Sheep were subjected to long-term breeding and hybridization to develop breeds with best traits (Terrill, 1958; Ryder *et al.*, 1964). Sheep are bred for their meat, milk and wool and breed traits are usually focused only on one of these products. British National Sheep Association lists 65 breeds in the UK (National Sheep Association | NSA) and the website Sheep101 (Schoenian Susan) lists over 200 different breeds.

Belonging among ruminants, sheep have complex digestive system to disintegrate cellulose present in their natural diet consisting of various plants (Nicholson and Sutton, 1969; Mobæk *et al.*, 2012). Their digestive tract consists of 4 compartments of "stomach" preceded by oesophagus and followed by small and large intestine, where digestion and absorption continues. The first parts of stomach are a reticulum and a rumen, which gave name to the whole group; in these two compartments rumination (repeated regurgitation and grinding with teeth) and food fermentation performed by various microbes take place. These two parts are followed by omasum, where water, fatty acids and ions are absorbed and finally by the "true stomach" abomasum which secretes enzymes, hydrochloric acid and performs further grinding of food similarly to human stomach (Gray *et al.*, 1954; McLeay *et al.*, 1973; Francoise Domingue *et al.*, 1991; Freer and Dove, 2002; Stiverson *et al.*, 2011).

Sheep are not usual model or experimental animals, due to their size and time to born or mature however, they were used for cloning research. A sheep named Dolly was the first mammal to be cloned from adult's cells (Campbell *et al.*, 1996, 2005). From the parasitology point of view, sheep are hosts to variety of helminth parasites including roundworms *Haemonchus spp., Teladorsagia spp., Strongyloides spp., Trichostrongylus spp*. parasiting in their gastrointestinal tract; *Dictyocaulus* living in their lungs, flukes *F. hepatica* and *Fasciola gigantica* in their liver and bile ducts and tapeworms like *Moniezia* living in their intestines. There are also many ectoparasites like flies, mosquitoes, botflies, ticks and mites preying on sheep (Schwartz, 1921; Rose, 1973; Elliott, 1986; Zhang *et al.*, 2005; O'Connor *et al.*, 2006).

2.2.2. Wild hosts

Apart from domesticated animals, parasites are a threat for wild living animals as well. In case of game animals, economic losses are similar to those in domestic ones. However, wild animals can spread parasites from an outbreak of infection to new places with suitable conditions (presence of intermediate hosts *etc.*). This could be seen in the case of *F. magna* spread in central Europe (Novobilský *et al.*, 2007; Kasny *et al.*, 2012). In my work, mouflon (*Ovis mousimon*) is mentioned. It is one of wild sheep species (Rezaei *et al.*, 2010) that have many common traits with domesticated forms; however, there are still present traits lost with domestication. All mouflons for example have horns, which consist of keratin and especially in males they are well developed. Besides these common traits wild sheep can share the parasites of domestic ones living in the same area (Meana *et al.*, 1996; Magi *et al.*, 2002).

Apart from that, parasites affect wide range of game animals like fallow deer, red deer white-tailed deer (*Odocoileus virginianus*) and other members of family Cervidae. Cervidae belong among ruminants, they are quite close to Bovidae family (sheep, cattle…); however, there are still vast differences. For example, they have antlers which consist of hard bony substance instead of horns and they shed them annually. There are also distinct differences in parasites and their development e.g. deer and sheep are both hosts for *F. magna*. Still, while in *O. virgianus*, *D. dama* and other definitive hosts *F. magna* would fully mature and starts to produce eggs; in sheep (dead-end host) it does not finish its life cycle and only causes severe damage by migration in sheep liver tissue (Foreyt and Todd, 1976). Besides *F. magna*, there are other trematodes like a lancet fluke (*Dicrocoelium dendriticum*) parasiting in wild animals (Ducháček and Lamka, 2003; Otranto and Traversa, 2003). *Haemonchus placei* and other *Haemonchus* species which are common in cattle can also be found in deer (Lichtenfels *et al.*, 1997). Other gastrointestinal nematodes are common in deer as well (Pacoń, 1994; Santín-Durán *et al.*, 2004; Balicka-Ramisz and Pilarczyk, 2005).

2.3. Treatment of parasitic infections

It is apparent parasites can damage health of their hosts in many ways and affect their welfare and production in economically important species. Therefore, there is constant effort to eradicate parasites and treat their host. It is a sort of endless struggle as parasites develop a resistance to new treatments. There are two main ways to deal with parasites, the most common one is to treat hosts with antiparasitic drugs, the other gives solutions based on

nonpharmaceutical interventions to the environment or breeding strategies. Antiparasitic drugs can be divided in several different ways, by their effectivity, structure, mechanism of action *etc.* There are broad spectrum drugs that are effective against all classes of helminths; yet there are also drugs that have a very narrow spectrum, only one class or even certain parasite. (Martin, 1997).

2.3.1. Benzimidazoles

Benzimidazoles (BZD) are one of the most used classes of anthelmintics. Benzimidazole ring, which gave the name to the group, is always present in their structure (Fig 2; A-D). History of BZD starts with thiabendazole, which was introduced to the market at the beginning of 60' (Cuckler, 1961). Their mechanism of action is based on binding to β-tubulin subunits on the same spot as colchicine, called capping and so it stops tubulin polymerization and formation of new microtubules. It leads to errors in vesicle transport and energy metabolism which finally leads to an energy depletion and a death of a parasite (Lubega and Prichard, 1991; Martin, 1997). Its effect is not immediate, because it takes time to deplete tubulin and energy reserves of a parasite. Due to its selectivity and differences in β-tubulin in parasites and mammals, toxicity of BZD to the definitive hosts is quite low (Dawson *et al.*, 1984; Horton, 2000). However, BZD could have an effect on other invertebrates in the environment, as BZD could affect their condition (Wagil *et al.*, 2015). BZD also have wide spectrum of effectivity affecting many species as well as most of their developmental stages, even eggs; BZD are active against unicellular parasites (Edlind *et al.*, 1990). All in all, these properties makes BZD widely used, but also very prone to resistance development (van Wyk and Malan, 1988; Thomaz-Soccol *et al.*, 2004). The group is represented by albendazole (ABZ), mebendazole (MBZ), flubendazole (FLU), triclabendazole (TCBZ), fenbendazole, and many others.

2.3.1.1. Albendazole

Albendazole (structure Fig. 2; C) was developed about forty years ago and still belongs to BZD used in both human and animals. It is oxidised to sulphoxide, called ricobendazole, which seems to be the true effective compound. Further oxidation to sulphone means loss of its anthelmintic properties. Netobimine is a prodrug which is turned to ABZ (Lanusse and Prichard, 1990; Venkatesan, 1998). ABZ is used against variety of roundworms, tapeworms and flukes as well as gastrointestinal protozoa (Ramalingam *et al.*, 1983; Bauer, 1990; Venkatesan, 1998; Fairweather and Boray, 1999).

2.3.1.2. Mebendazole

Mebendazole (structure Fig. 2; A) is another BZD used mainly against nematode infections in humans, sheep, goats, horses and other animals, but it is effective against other helminth classes as well (Goldsmid, 1974; Hutchison *et al.*, 1975; Lanusse *et al.*, 2013a). In the Czech Republic it is currently the only registered anthelmintic for use in humans except for specific treatment programmes (SÚKL).

2.3.1.3. Flubendazole

Flubendazole (structure Fig. 2; B) is fluorinated derivative of MBZ; it is used mainly as a treatment of nematodal infections in pigs and poultry (Bradley *et al.*, 1983; Willemsen, 2009). It also showed *in vitro* activity as a combination treatment of leukaemia and other types of cancer (Spagnuolo *et al.*, 2010; Hou *et al.*, 2015).

2.3.1.4. Triclabendazole

Triclabendazole (structure Fig. 2; D) is quite outstanding in the benzimidazole group; it has slightly different structure, as well as its anthelmintic spectrum. While other drugs could be called broad-spectrum antinematodal drugs, TCBZ however is effective exclusively against liver flukes (*F. hepatica, F. gigantica, F. magna*). TCBZ shows effectivity against early developmental stages compared to other flukicidal benzimidazole ABZ which is effective mainly in mature flukes. On the other hand ABZ shows activity against fluke eggs which TCBZ does not possess (Coles, 1986; Fairweather, 2005; Alvarez *et al.*, 2009; Lanusse *et al.*, 2013b). A mechanism of action of TCBZ is not fully understood yet. It might be binding to tubulin or uncoupling of oxidative phosphorylation; it also influences protein synthesis (Carr *et al.*, 1993; Stitt and Fairweather, 1994; Fairweather, 2005). Presence of thioether group in the molecule allows its oxidation as in ABZ molecule. However, TCBZ keeps effectivity against flukes even as sulphoxide or sulphone (Stitt and Fairweather, 1994; Halferty *et al.*, 2009). Unfortunately, its extensive use against *F. hepatica* caused some of the parasites to develop resistance to the drug (Brennan *et al.*, 2007).

2.3.2. Salicylanilides

Salicylanilides are another group of anthelmintics assayed in this work. These halogenated compounds are used as preferably antitrematodal drugs. A mechanism of their action is that they uncouple oxidative phosphorylation in mitochondria of parasites. Salicylanilides are very lipophilic compounds and they carry a hydrogen proton which they release and then a proton gradient on a mitochondrial membrane becomes balanced and

a phosphorylation stops. Apart from this mechanism closantel is reported to have an effect on intrategumental pH (Pax and Bennett, 1989; Martin, 1997). This direct disruption causes death of a parasite much faster than with BZD. The effectivity of salicylanilides depends on the drug; the group includes drugs effective on flukes, tapeworms and nematodes; still, one drug has usually effect only on one or sometimes two classes of helminths. They are used to treat parasitic infections in ruminants, except for niclosamide which is also used in cats and dogs. With their lipophilicity, they pass easily through membranes and there is a risk that treated host could be affected by toxic effects. It could manifest as a loss of appetite or damage of sight, paresis and it could be fatal (Swan, 1999). Among the most important members there are closantel and rafoxanide (structures Fig. 2; E and F), then there is oxyclozanide, which slowly becomes obsolete, but it is effective against rumen flukes like *Paramphistomum* or *Calicophoron spp*. and niclosamide which is effective against tapeworms (Pearson and Hewlett, 1985; Paraud *et al.*, 2009).

2.3.2.1. Closantel

This member of the group is one of drugs with a broader spectrum. It is effective against fascioliasis in maturing *F. hepatica* from about the sixth week after infection. Apart from a flukicidal effect, it is effective against *H. contortus* and other nematode parasites. It is also used against larvae of a fly *Oestrus ovis* (Swan, 1999; Lanusse *et al.*, 2013b). From these commonly treated parasites only *H. contortus* was able to develop resistance to closantel (Rolfe *et al.*, 1990).

2.3.2.2. Rafoxanide

While less effective against nematodes and ectoparasites, this salicylanilide drug is effective against younger stages of liver flukes (Swan, 1999). In the Czech Republic it is used in a combination with MBZ (Rafendazol®) to treat wild living ruminants from *F. magna* infection, however a treatment might fail due to reinfection (Kasny *et al.*, 2012).

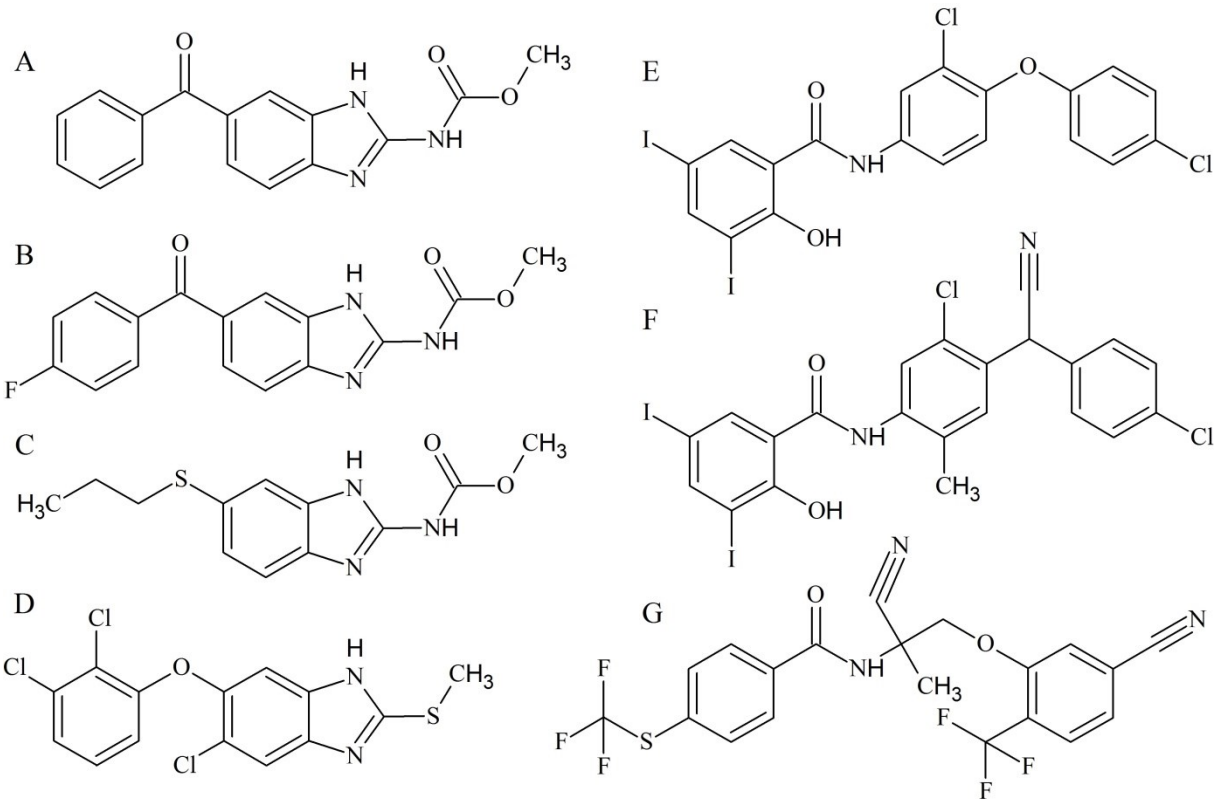

Fig. 2: Structures of anthelmintics A) mebendazole; B) flubendazole; C) albendazole; D) triclabendazole; E) rafoxanide; F) closantel; G) monepantel; structures were made with ChemSketch Freeware software

2.3.3. Macrocyclic lactones

Only a few years ago, this was the most novel class of anthelmintics. Their mechanism of action is based on interaction with glutamate and gamma-aminobutyric acid gated chloride channels and paralysis of various muscle structures, mainly a pharyngeal pump and muscles of wall (Geary *et al.*, 1993; Holden-Dye and Walker, 2014). Apart from the use against parasitic nematodes, there is good effectivity against arthropod ectoparasites (Schröder *et al.*, 1985). These drugs are produced by biosynthesis or semisynthetically. Avermectins are products of *Streptomyces avermitilis,* while mylbemicins are produced by *Streptomyces cyaneogriseus.* The spectrum of treated animals goes from ruminants, pigs, horses to small predators like dogs and cats. However, there are breeds of dogs for example border collies, which show vulnerability to toxic effects of macrocyclic lactones. Apart from these breeds, therapeutic window is quite large and with a correct dosage, it makes macrocyclic lactones safe drugs. Macrocyclic lactones have a highly lipophilic structure that enables them to accumulate in adipose tissues, from where they are slowly released, so withdrawal periods for meat and milk are longer. They can negatively influence non-target species living in soil due to their effectivity on insects and nematodes. The most important member of avermectins is ivermectin, used for variety of

organisms, sometimes combined with drugs to treat cestodes and trematodes to provide complete anthelmintic effect. Moxidectin represents milbemycin group, with similar use as ivermectin, but it appears to have a safer profile (Paul *et al.*, 2000; Ménez *et al.*, 2012; Lanusse *et al.*, 2013c).

2.3.4. Other "old" anthelmintics

Apart from these groups, there are other very important anthelmintics. One of them is imidazothiazole levamisole. It is an agonist of cholinergic receptors; it opens channels and causes spastic paralysis of a parasite. It is effective against many species of nematode parasites and their larvae; yet, it is limited in use by its toxicity and tolerance. There are pyrantel and morantel, members of tetrahydropyrimidine group with the same mode of action. They are safer to use but still limited to be nematocidal only. Praziquantel should be mentioned from anticestodal and antitrematodal drugs. It influences calcium ions penetration to muscles and tegument of parasites, and causes rapid muscle contractions and paralyzes parasites. It has an activity against cestodes and trematodes and is considered safe. It is used in variety of hosts often in combination with antinematodal drugs. There are many other drugs; however, with increasing resistance especially in ruminant gastrointestinal nematodes, need for new drugs with novel mechanisms appeared (Martin, 1997; Lanusse *et al.*, 2013a; b; Holden-Dye and Walker, 2014).

2.3.5. Monepantel

One of the most novel drugs is amino-acetonitrile derivate AAD-1566 later named monepantel (structure Fig. 2; G); it was discovered in the last decade (Kaminsky *et al.*, 2008a; b). It came with a novel target for its mechanism; it has an affinity to nematode-specific nicotinic acetylcholine receptors. With activating those, it opens ion channels and leads to muscle depolarization and paralysis of nematode (Kaminsky *et al.*, 2008a; Rufener *et al.*, 2009). This was an answer to resistant worms like *H. contortus* as it is effective even against adults and an L4 larvae stadium of multi resistant strains of these worms as well as other gastrointestinal nematodes species like *Trichostrongylus spp.* and *Teladorsagia spp.* (Kaminsky *et al.*, 2008a; Epe and Kaminsky, 2013). However, in past years resistant parasites started to appear (details in chapter 2.5). Due to its selectivity to nematode receptor MPTL-1, it also has very pleasant safety profile in hosts and in production animals it has very short withdrawal period (Epe and Kaminsky, 2013). Nowadays it is used to treat only sheep, but in initial studies of amino-acetonitrile derivatives they appeared to have effect on cattle parasites as well; also,

it is promising for treatment resistant nematode strains in horses and other animals (Dadak *et al.*, 2013; Epe and Kaminsky, 2013). It is metabolised mainly to sulphone metabolite, which in contrast to ABZ sulphone keeps its anthelmintic activity. Numerous less abundant monepantel metabolites in both hosts and parasites has been identified (Stuchlíková *et al.*, 2014).

2.3.6. New anthelmintics

Apart from monepantel there are at least two other novel anthelmintics and several potential candidates. Derquantel is a derivative of paraherquamide spiroindole produced by *Penicillium paraherquei* or *P. roqueforti.* It interferes with nicotinic acetylcholine receptors as a competitive antagonist and causes paralysis of worms (Woods *et al.*, 2012; Epe and Kaminsky, 2013; Holden-Dye and Walker, 2014). It is used in combination with abamectin (Startect®) in sheep against similar spectrum of parasites as monepantel. This combination however is toxic for horses and a derquantel precursor paraherquamide is unsuitable for dogs (Woods *et al.*, 2012; Epe and Kaminsky, 2013).

Cyclooctadepsipeptide emodepside is another novel drug that deserves a brief introduction. It is produced by fungus *Mycelia sterilia*. It has two modes of action, one of them is binding to a latrophilin-like receptor and the other is an interference with calcium activated potassium channels; both finally leading to inhibition of a pharyngeal pump, paralysis and death of the parasite (Krücken *et al.*, 2012; Epe and Kaminsky, 2013; Holden-Dye and Walker, 2014). It is used to control nematode parasites like *Toxocara spp*. in small predators e.g. cats and dogs; it is combined with anticestodal praziquantel to enhance antiparasitic spectrum (Epe and Kaminsky, 2013).

2.3.7. Other ways to treat and prevent infections

Besides pharmacological intervention in hosts, there are other ways to deal with infections. Hosts should be in a good shape and with sufficient nutrition which makes them more resilient to infections and decrease final worm counts (Wells, 1999; Getachew *et al.*, 2007). Apart from that, there are ways to improve the environment, for example rotation of pastures. Banks *et al.* (1990) showed *H. contortus* and *Trichostrongylus colubriformis* larvae survive about nine weeks on pastures. There is also a potential to use nematophagous fungi to destroy nematode eggs and free living larvae (Waller and Faedo, 1996; Getachew *et al.*, 2007; de Souza Maia Filho *et al.*, 2017). Prevention might aim to vectors like snails in trematode infections (Berg, 1973; Sunita and Singh, 2011). The experiments with vaccines against various helminth proteins from all helminth classes are conducted: *Taenia spp.*, *Schistosoma*

japonicum, *F. hepatica* and nematodes like *H. contortus*; however, results are still unclear (Lightowlers *et al.*, 2003; McManus and Dalton, 2006; Meier *et al.*, 2016).

2.4. Biotransformation of xenobiotics

Every organism encounters compounds in its environment that can have various effects on it. The compounds could be from various sources; natural, coming from all kingdoms: animals, plants, fungi; or they could be artificial, produced by various sources of human activities. It could be plant alkaloids, flavonoids, pigments, hormones, fungal antibiotics and mycotoxins, animal poisons and excretory products; artificial sources comprise industrial pollutants, some other chemicals from food additives to illegal drugs and of course medicines and their residues. All these compounds have one thing in common they are foreign to organism. They are called xenobiotics (Latin *xeno-* means strange, alien, foreign). Compounds own to the organism are called eubiotics. Xenobiotics can have various effects on organism: toxic, pharmacological *etc.*; it often depends on their concentration in organism. Organism has to deal with the compound or it would accumulate somewhere in its body. It can excrete the compound unchanged or it can transform it via enzymatic systems present in various parts of its body. This process is called biotransformation or metabolism; these terms are commonly interchangeable in this topic (Khojasteh *et al.*, 2011). The scientific discipline dealing with the topic is called xenobiochemistry. Term detoxification could be used in many situations, however it is not universal as some reactions catalysed by these enzymes produce more toxic products, for example highly reactive epoxides (Buhler and Williams, 1988).

In case of drugs that are the main topic of this thesis metabolism is important because it influences bioavailability of drugs in a target compartment and/or in a parasite. Drugs can enter a body in a form of a prodrug and then they can be activated via metabolic change; example of this is netobimine as a precursor for ABZ. Mentioned deactivation of drugs is more important. Drugs lose their physical as well as pharmacological properties. Lipophilicity of drugs is influenced and therefore distribution in tissues and elimination from body is changed. Xenobiotic metabolizing enzymes are present in most tissues, even though they are not equally distributed. Liver is the most important organ, followed by intestinal tissues, kidneys, lungs, brain tissue and others. Microbes present in the gastrointestinal tract are also important in this process, especially in ruminants, where they function as important metabolisers. In plants biotransformed xenobiotics do not leave their body, but are accumulated in vacuoles or in cell walls. Plants have slightly different enzymatic capability as well. (Lanusse *et al.*, 1992; Correia, 2011; Bártíková *et al.*, 2015).

Enzymes that perform biotransformation of xenobiotics usually have lower affinity to substrates; however, they are more universal and able to metabolize wide variety of them. Enzymes can also metabolize endogenous substrates apart from xenobiotics, often physiologically related compound, for example carbonyl-reducing enzymes that metabolises steroids (Lundová *et al.*, 2016). Metabolism of certain drug can influence metabolism of other drugs in many ways like blocking the enzyme due to higher affinity, depleting cofactor needed for reaction or overexpression and inhibition of an enzyme (Correia, 2011).

Biotransformation and transport of drugs can play a significant role in resistance development, like in *F. hepatica* resistance to TCBZ due to transport and metabolism, or expression of β-lactamase in bacteria to cleave bonds in penicillin antibiotics, or transport proteins that protect tumour cells against treatment (Livermore, 1995; Alvarez *et al.*, 2005; Merino *et al.*, 2006).

Biotransformation is usually divided into two phases; in phase I oxidation, reduction and hydrolysis take place and in phase II drugs or their metabolites are conjugated with various endogenous substrates. Phase I reactions usually add or modify functional groups of drugs, often enhancing their hydrophilicity. These metabolites can be either excreted or they can undergo another metabolic reaction of phase I or II. In the phase II the drug or its metabolite is conjugated with endogenous substrates: sugars like glucose, amino acids as glycine, acidic glucuronates, sulphates and others. Transport was sometimes placed as the phase III; however, it is much more common to exclude it from biotransformation so it stands on its own.

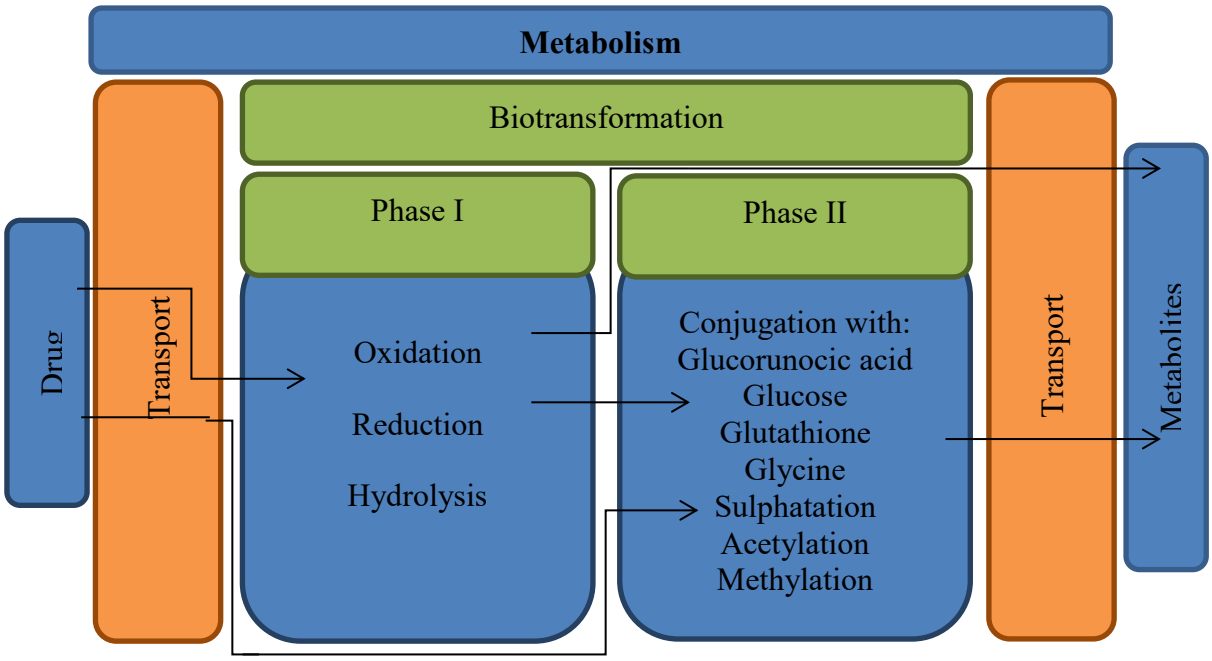

Fig. 3: Drug metabolism diagram (Correia, 2011; Khojasteh *et al.*, 2011; Skálová *et al.*, 2011)

2.4.1. Phase I of biotransformation

2.4.1.1. Oxidation enzymes

Oxidation is fairly common reaction in human and other mammals. Dehydrogenations, hydroxylations, epoxidation, O-, N-, S- dealkylations, deamination, desulphuration, oxidative dehalogenations, N- and S- oxidation all belong among oxidative reactions.

Cytochrome P450 system

Cytochromes P450 (CYP) are members of a group of enzymes that contains hem group with an atom of iron in the centre. Wavelengths of 450 nm is the absorption maximum of reduced iron bound with carbon monoxide. Iron is vital in transferring electrons and thus enabling an enzyme its metabolic function. Enzymes are dependent on reduced nicotinamide adenine dinucleotide phosphate (NADPH) and CYP reductase which regenerate Fe^{3+} to Fe^{2+}. Enzymes are located on the cytosol side of an endoplasmic reticulum (ER) and therefore could be found in microsomal fractions. There are many types of CYP and they are located dominantly in liver and then in intestines, lungs, brain, kidney and other organs and tissues like placenta, heart, skin *etc.* In humans the most important isoforms are CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 (Khojasteh *et al.*, 2011).

Flavin monooxygenases (FMOs)

FMOs are the second most important group of oxidases. They also use NADPH as a cofactor however their reactions are performed by the flavin part of flavin adenine dinucleotide and its hydroxyperoxyflavin form. They are localised on ER in metabolically

important tissues like liver, kidney, lungs and others. There are different isoforms and their localisation can also vary among species. FMOs perform similar reactions as many CYPs, but there are significant differences in their properties and possibility of inhibition or inducibility.

Apart from these two families, there are other groups of oxidation enzymes like monoamine oxidases, which are important targets for many drugs affecting nervous system; alcohol and aldehyde dehydrogenases which protect body from excess of alcohol intake; group of molybdenum hydroxylases consisting of xanthine oxidases and dehydrogenases and aldehyde oxidases (Correia, 2011; Khojasteh *et al.*, 2011; Skálová *et al.*, 2011). Also, enzymes that protect cells from oxidative stress can have enzymatic activity to xenobiotics e.g. peroxidase, and catalase (Calabrese and Canada, 1989; Hollenberg, 1992).

2.4.1.2. Reductases

Even though reduction is often considered less important in xenobiotic metabolism compared to oxidation, reductases are still present and important for correct function of organism. In contrast to microsomes-bound oxidases, reductases are more often soluble and free in cytosol, but still there are several transmembrane members of this group. Apart from typical metabolic organs like liver or intestines, reduction is also performed by a microbiome in a gastrointestinal tract. Reductases perform reduction of various groups e.g. aldehydes and ketones, azo- and nitroreductions, sulphoreductions, reductive dehalogenations.

Aldo-keto reductases (AKR)

It is a very important superfamily of reducing enzymes. AKRs are localised in liver cytosol and reduce keto- and aldehyde- groups with nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate as cofactors. Among their substrates, there are endogenous compounds of steroid nature, various lipids and drugs like dolasetron, doxorubicin, oracin *etc.* (Khojasteh *et al.*, 2011; Skálová *et al.*, 2011).

Short chain dehydrogenases/reductases (SDR)

SDRs form the biggest superfamily of enzymes with up to 47 000 members and about 50 human families. While similar to AKR in many functions and cofactors, they have different structure. They are expressed in different parts of body and organ system depending on their isoform. Some of the important members are carbonyl reductases 1 and 3 metabolising quinone compounds like menadione, anthracycline drugs or warfarin which is a drug with a very narrow therapeutic window. Besides, 11β-hydroxysteroid dehydrogenase is a member of this group;

unlike others it is bound to membrane of microsomes. It has an activity towards steroid hormones like cortisone and similar drugs (Skálová *et al.*, 2011).

Medium chain dehydrogenases

This is also a big and heterogeneous group of enzymes, metabolising many endogenous compounds, also alcohols and they are part of an oxidative phosphorylation chain. One or more Zn^{2+} ions are often present in the molecule. (Hedlund *et al.*, 2010; Skálová *et al.*, 2011).

NADPH quinone oxidoreductases 1 and 2 are important metabolisers of quinone-containing structures in drugs or their metabolites to less toxic, non-radical products. Thioredoxin reductases are another group of quinone reducing enzymes. Some drugs can be reduced by CYP systems as well.

2.4.1.3. Hydrolytic enzymes

There are many enzymes with a function to cleave ester, amide and epoxide bonds in various molecules with addition of water molecule. They are localised mainly in liver tissue; still, there are important members in a nervous system and they are present in microbes in a gastrointestinal tract often cleaving metabolites of phase II and enabling parent molecules to recirculate. Also, β-lactamase in penicillin-resistant bacteria is a member of this group. Among important members there are peptidases, acetylcholine and pseudocholinesterases, paraoxonases, epoxide hydrolases, and carboxylesterases.

2.4.2. Phase II of biotransformation

In phase II a parent molecule or its phase I metabolite are conjugated with endogenous compound usually creating more polar and soluble metabolite which is easier to excrete from body. The enzymes are called transferases. Conjugation is a means to metabolise and excrete some endogenous compounds like bile acids and lipids and therefore they can compete with xenobiotics for cofactors. Conjugation with glucuronic acid, glutathione, sulphate, acetyl, methyl and conjugation with amino acids are the most common reactions in humans. In plants, there are also common glycosyltransferases, which conjugate substrates with variety of saccharides.

2.4.2.1. Uridine diphosphate glucuronosyltransferases (UGT)

These enzymes use uridine diphosphate glucuronic acid to bind to nucleophilic parts of a substrate molecule (alcoholic, phenolic, carboxyl, amine, thiol and other groups). They are localised mainly in lumen of ER in liver and then in other systems (kidneys, brain, intestines),

UGTs conjugate bile acids and other endogenous substrates as well as drugs. Most important isoforms are UGT1A1, 1A4 and 2B7. Glucuronic conjugates are prone to hydrolysis with β-glucuronidases in intestines, enabling enterohepatic circulation (Khojasteh *et al.*, 2011; Skálová *et al.*, 2011).

2.4.2.2. Uridine diphosphate glycosyltransferases

They are often considered plant analogues of UGTs mentioned above; however, they are also present in helminths. They share localisation on lumen of ER, but instead of activated glucuronic acid they transfer uridine diphosphate activated sugars. Most common one is glucose, however there could be other sugars like xylose or rhamnose, multiple sugars are sometimes bound to one substrate (Ross *et al.*, 2001; Matoušková *et al.*, 2016).

2.4.2.3. Glutathione S-transferases (GST)

GSTs are important enzymes performing range of functions in organism. They transfer reduced glutathione to eu- and xenobiotic substrates. They are dominantly soluble and present in cytosol, but there are isoforms bound to mitochondria, peroxisomes or ER. They conjugate steroids and bile acids, prostaglandins and leukotrienes and huge amount of xenobiotics and drugs, mainly electrophilic compounds. They play a role in prostaglandin synthesis and enable cell communication (Handy *et al.*, 2009; Skálová *et al.*, 2011). They are also considered vaccine candidates against parasites (Morrison *et al.*, 1996).

2.4.2.4. Sulphotransferases (SULT)

These cytosolic enzymes work by transferring a sulphone group donated by 3'-phosphoadenosine-5'-phosphosulfate to substrate. Three families of SULTs are important in human biotransformation. SULT1 conjugates phenols, SULT2 focuses on alcohols and hydroxysteroids and SULT3 processes amines; no SULT conjugates carboxylic acids.

2.4.2.5. Methyltransferases

Compared to other phase II enzyme groups, methyltransferases do not bring hydrophilic structure to a substrate molecule. Methyl transferred to the molecule comes from S-adenosyl methionine. They can form O-methylated compounds from phenols, S-methylated ones from thiols and N-methylated from amines and heterocycles. They are present in both cytosolic and microsomal fractions. An important member of transferases is, for example, catechol O-methyl transferase metabolising catecholamine neurotransmitters and similar compounds (Skálová *et al.*, 2011).

2.4.3. Transport proteins

Biotransformation enzymes as well as many potential targets for xenobiotics are located inside a cell; therefore, xenobiotics have to permeate through a cytoplasmic membrane to have an effect or to be metabolised. Lipophilic molecules with adequate size are able to permeate through membranes passively without any means of active transport into or out of cells. Apart from size it depends on their charge and as mentioned above their lipophilicity, which could be expressed as Log P, partition coefficient between solvents: octanol/water (Liu *et al.*, 2011).

For the rest of the molecules there are systems to facilitate their permeation. These transport proteins are localized in a membrane of a cell or organelle. Most of them transport preferably eubiotic compounds; however, there are several types that transport xenobiotics as well; they are usually called drug transporters. They can facilitate uptake of compounds into cells or excrete them out of cells.

There are two big classes: adenosine triphosphate (ATP) binding cassette (ABC) transporters and solute carrier (SLC) transporters. ABC transporters have ATP binding sites, use ATP hydrolysis as an energy source and are mostly considered active transporters. The group contains transporters like massively studied P-glycoprotein (P-gp), multidrug resistance protein or breast cancer resistant protein; these are more often considered efflux transporters as they transport xenobiotics out of a cell.

In contrast to ABC, SLCs does not contain ATP binding sites and they facilitate substrate permeation using energy which is generated by electrochemical potential difference in substrates in syn- or antiport, or by ion gradient on membrane. Members of this group are influx transporters and important members among them are organic cation transporters, organic anion transporting polypeptides, organic anion transporters and oligopeptide transporters (Khojasteh *et al.*, 2011; Skálová *et al.*, 2011).

2.4.3.1. P-glycoprotein

Known also as ABCB1, P-gp is localised on an apical side of epithelial cells in intestines, liver, kidneys, brain and placenta. Six transmembrane domains create a channel through membrane with two ATP binding sites to power efflux of substrates out of cells. Its expression in brain-blood barrier and placenta shows its role of important protective protein of these highly sensitive organs. P-gp is one of proteins that allow cancer cells to survive therapy. Its substrates are dominantly lipophilic compounds such as digoxin, antihistaminic cetirizine, several antibiotics or anthelmintic ivermectin which cause that P-gp deficient dogs are

susceptible to toxicity of this anthelmintic (Edwards, 2003). There are also xenobiotics or drugs that inhibit this efflux transporter for example verapamil and cyclosporine. These are often used for inhibition tests in assays (Yasui-Furukori *et al.*, 2005; Rautio *et al.*, 2006; Skálová *et al.*, 2011).

2.4.4. Helminths and anthelmintic biotransformations

Biotransformation reactions take place in all living organisms from bacteria to humans, therefore it is performed by parasites as well as hosts and many anthelmintics are metabolised by these organisms. This work is focused on these interactions, thus here is a brief introduction to metabolism in helminths.

2.4.4.1. Oxidations in helminths

CYPs are much less investigated in helminths compared to their hosts. Some CYPs were proven in genomes of several helminths like *C. elegans, H. contortus* and fluke *Opisthorchis felineus* (Laing *et al.*, 2015; Pakharukova *et al.*, 2015; Matoušková *et al.*, 2016). Presence of CYPs also depend on a developmental stage of parasite as free-living larvae stages of *H. contortus* have more CYPs than adults living in hosts (Laing *et al.*, 2015). In many cases activity was proven towards one of CYP substrates especially their monooxygenase activity (Saeed *et al.*, 2002; Cvilink *et al.*, 2009a).

In addition to CYPs there are peroxidases present in parasites, which could contribute to oxidative metabolism of xenobiotics (Kotze, 1999). Aside from those, FMOs or antioxidant enzymes can contribute to the formation of oxidised metabolites (Cvilink *et al.*, 2009a; Brophy *et al.*, 2012).

Considering anthelmintics metabolism, oxidative products of ABZ and TCBZ are very important. Sulphoxidation significantly change their pharmacological effectivity from very potent ABZ sulphoxide to harmless ABZ sulphone. TCBZ undergoes sulphoxidation in *F. hepatica,* and it might be performed by CYP-like enzyme there (Mottier *et al.*, 2004; Cvilink *et al.*, 2009a; Devine *et al.*, 2010). Stuchlíková *et al.* (2014) also described S-oxidised metabolites of novel drug monepantel in both sheep and *H. contortus*; this information is quite interesting as sulphone was formed only in multiresistant White River strain.

2.4.4.2. Reduction and hydrolysis in helminths

Reductases and hydrolases seem to be more common and active in helminth metabolism than oxidases. In *H. contortus, D. dendriticum* and *H. diminuta* there were found reduction

activities against several anthelmintics and model substrates (Cvilink *et al.*, 2008a; Skálová *et al.*, 2010; Bártíková *et al.*, 2012). Also, several AKR proteins were found in various parasites as *F. hepatica* or *S. mansoni* (Brophy *et al.*, 2012). Hydrolysis of esters and amides in xenobiotics is also quite common in helminths (Cvilink *et al.*, 2009a). Anthelmintics FLU and MBZ are drugs that have their carbonyl group reduced by the variety of parasites and their hosts. ABZ sulphoxide was reverted to ABZ in *M. expansa* (Cvilink *et al.*, 2009a).

2.4.4.3. Phase II biotransformation in helminths

There is not much information about phase II xenobiotic metabolising enzymes in helminths. Activities towards specific substrates were proven and activities towards eubiotic substrates were found as well. Conjugates with glucose were formed, which is rather surprising as UDP-glucosyl transferase should be present in plants or bacteria (Cvilink *et al.*, 2009a). In *ex vivo* experiments *H. contortus* formed N-glucosylated metabolite of FLU and O-glucosylated metabolite of reduced FLU and also another metabolite of ABZ (Cvilink *et al.*, 2008b). Moreover, methylation of previously reduced FLU was observed in rat tapeworm *H. diminuta* (Bártíková *et al.*, 2012). Nevertheless there is one exception and which is GST; this enzyme was studied very deeply in various helminths, showing high activities with model substrates (usually 1-chloro-2,4-dinitrobenzene) (Brophy *et al.*, 1989, 1990b, 2012). Several classes of GST were identified (Matoušková *et al.*, 2016). GST was also considered to be one of the vaccine candidates as mentioned in previous chapters. Several eubiotic and xenobiotic substrates were found as well as affinity to anthelmintics; however, the conjugate of an anthelmintic with glutathione is yet to be found (Douch and Buchanan, 1978; Brophy *et al.*, 1990a, 2012; Brophy and Barrett, 1990).

2.4.4.4. Transport in helminths

Helminths need to excrete toxic compounds out of their bodies as any other organisms. ABC-transporters sequences were found in their genomes (Brophy *et al.*, 2012; Lespine *et al.*, 2012). The most studied transporters are P-gp and multidrug resistance associated protein, as they are connected to multidrug resistance in cancer cells and can be considered to influence resistance to multiple anthelmintics in parasites. Substrates exported by these transporters are for example ivermectin in roundworms and TCBZ in *F. hepatica* (Devine *et al.*, 2012; Ardelli, 2013). As anthelmintics are often very lipophilic compounds their entry into a parasite is usually passive through a membrane (Alvarez *et al.*, 2007).

2.5. Anthelmintic resistance

With widespread use of anthelmintics, parasites that are not affected by them started to appear. This was not a serious problem due to rapid development of newer and newer classes of anthelmintics in the past. Nevertheless, when multiresistant strains of especially roundworms emerged, this anthelmintic resistance started to be of concern. Anthelmintic resistance occurs when parasites treated with anthelmintics are no longer sensitive to it and this condition is inherited in next generations. In case of side resistance, the parasite is not affected by drugs of entire class of anthelmintics (Sangster, 1999).

Resistance starts with mutation in a gene of a small number of parasites which makes the drug inefficient. However, with repeated drenching with now-not-so-efficient drug and continuous reinfection with surviving parasites these genes become evolutional advantage and are further selected by drenching. The process is finished by creation of resistant strain of a parasite. Once resistance is established in a population, it was never observed to revert. Clinical resistance occurs when reduction of worm burden after treatment is lower than 95 %. Resistance is developing in parasites of the majority of domestic animals with most concerns focused on small ruminants like sheep and goats. Still, other animals like cattle and horses are also affected. The highest number of resistant isolates comes from the group of gastrointestinal roundworms parasitizing these animals. Nowadays resistance in liver flukes is becoming a problem too.

Several factors contribute to resistance development; they can be combined in different ways and can influence an emergence or a spread of resistance. It can be a survival of eggs and larvae in the environment or hypobiotic stages, underdosing of anthelmintics and a frequency of treatments (Jackson and Coop, 2000). Anthelmintic resistance can be detected by several tests; classical are faecal egg count reduction test, egg hatch test or microagar larval development test. However, today more and more tests are based on molecular biology findings, either polymerase chain reaction or pyrosequencing (Coles *et al.*, 2006; Beech *et al.*, 2011).

There are several different mechanisms for anthelmintic resistance. One of the most studied is *H. contortus* resistance to BZD, which is caused by change in isoforms 1 and 2 of β-tubulin molecules. Changes are present in a codon 200 in an isotype 1 and in a codon 167 in an isotype 2 that replace phenylalanine with tyrosine. One or both of these mutations have been found in other helminths resistant to BZD (Wolstenholme *et al.*, 2004). In *F. hepatica* resistance

to TCBZ, ABC transporters and increased metabolism to sulphone are probably to be blamed (Robinson *et al.*, 2004; Devine *et al.*, 2012; Scarcella *et al.*, 2013).

The resistance to macrocyclic lactones is a result of increased expression of P-gp and/or differences in glutamate and gamma amino acid gated chloride channels (Whittaker *et al.*, 2016). Loss or decrease of receptor numbers which are targets for drugs is a probable cause for levamisole and other drugs interacting with nicotinic receptors for acetylcholine channels (Wolstenholme *et al.*, 2004; Whittaker *et al.*, 2016).

Today, the resistance problem is caused by long-term stagnation in novel drug development. Organophosphates came in 1950', BZD and levamisole in 60', salicylanilides in early 70' and finally in 80' macrocyclic lactones. Then there was a period when after parasites lost susceptibility to one drug it was replaced with another and parasites gradually acquired more and more resistances, this was leading to using pasture management strategies, treatment plans and other non-pharmacological methods to control worm populations in hosts. *H. contortus* is one of parasites that developed multiresistant strains in sheep. It happened in areas heavily focused on sheep breeding such as South Africa or Australia (van Wyk and Malan, 1988; Gill *et al.*, 1991; Le Jambre *et al.*, 1995). In this work, multiresistant White River *H. contortus* strain was used in several experiments. Its origin lies in the Transvaal area in South Africa. There are many other helminth species especially among nematodes that developed resistance to at least one anthelmintic group appearing in all regions of the world (Sangster, 1999; Sargison *et al.*, 2010; Traversa and von Samson-Himmelstjerna, 2016).

In new millennium new drugs with effectivity against these resistant strains were invented and brought to practice like monepantel and derquantel (Kaminsky *et al.*, 2008a; Epe and Kaminsky, 2013). However, while it took almost 20 years for parasites to develop resistance to macrocyclic lactones, there were reports of monepantel resistant *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* in 2013 and about resistant *H. contortus* in 2014 (Scott *et al.*, 2013; Van den Brom *et al.*, 2015). Mechanisms of resistance to monepantel as well as some other drugs remain mystery and needs to be thoroughly studied to fully understand it and prevent further spread.

2.6. High performance liquid chromatography

High performance liquid chromatography (HPLC) is an analytical method that separates compounds in dependence on their distribution and interactions with stationary and mobile phases. Compared to classical "low pressure" liquid chromatography, HPLC operates in high

pressure up to 50 MPa (Meyer, 2013). Systems that operate with higher pressure are called ultra-high performance liquid chromatography (UHPLC) systems. Higher pressure allows the use of columns with smaller particles that improves separation resolution and allows greater flows of mobile phases.

In HPLC techniques mobile phase is liquid and stationary phase is usually solid (it can be also liquid bonded to some sort of sorbent). Properties of both the stationary phase as well as the mobile phase can dramatically influence separation. A present-day HPLC system consists usually of a degasser, pumps, an autosampler, a column oven and a detector (usually UV-detector, fluorescence-detector or mass spectrometer).

When dissolved sample is injected to a system, it is driven to a chromatographic column and there it interacts with stationary phase according to its size, functional groups, charge and other properties accordingly to the chosen column. Compounds become trapped and start to accumulate in different parts of column and then they are eluted in short time from column to detector. Results come in a form of a chromatogram where time is on the horizontal axis and intensity of observed value is on the vertical one; eluted compounds are visible as peaks. Example of chromatogram is in Fig. 4 below.

In our research, HPLC is used to: 1) separate compounds before mass spectrometry analysis; 2) get information about presence and behaviour of parent drugs and their metabolites in samples.

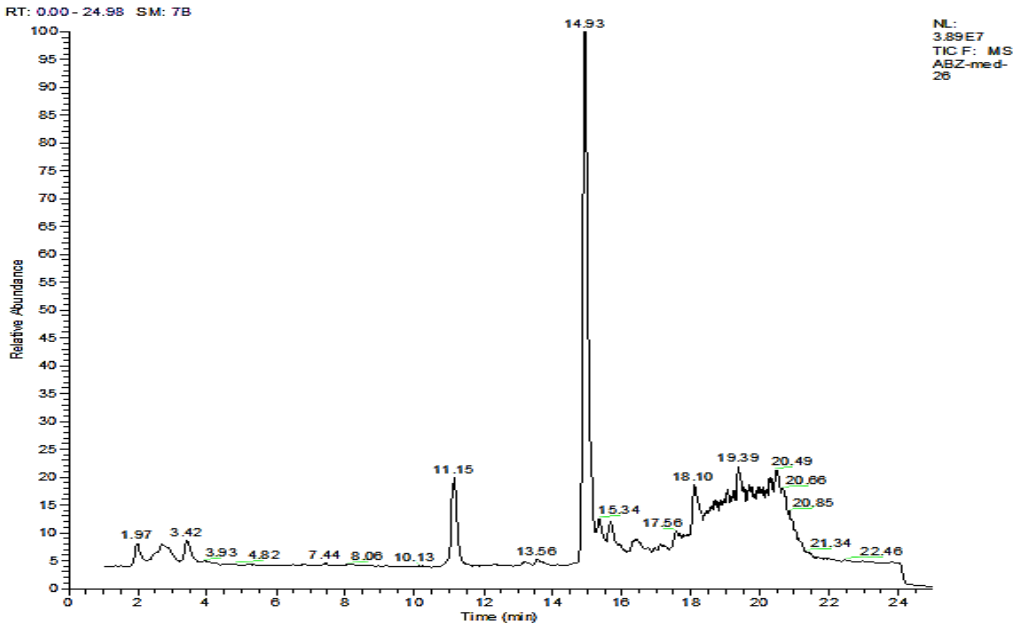

Fig. 4: HPLC-MS chromatogram from analysis of *F. magna* ABZ metabolism; peak in 14.93 min represents ABZ, peak in 11.15 min represents ABZ sulphoxide metabolite

2.6.1. Mobile phase

Mobile phase could be almost any liquid, which does not interfere with parts of the system. Composition of mobile phase during analysis can be constant (isocratic elution), or variable (gradient elution). Selection of mobile phase is dependent on used column (e.g. for reverse phase columns used in this work, mixtures of organic solvents and aqueous solutions are used). Solvents should be fully miscible.

Buffers are usually used as an aqueous part of mobile phase to ensure pH stability during analysis; however, they must not interfere with signal detection. Buffers should be chosen carefully for gradient elution to prevent precipitation or recrystallization inside the system and its clogging. In case of coupling HPLC with mass spectrometry, buffers need to be volatile and should not contain inorganic ions or for example quarter ammonium salts that tend to contaminate mass spectrometer and are hard to wash out.

Organic solvents are used as nonpolar parts of mobile phase. In reverse phase chromatography these are alcohols, acetonitrile or for example tetrahydrofuran. For normal phase chromatography, aliphatic hydrocarbons are used as less polar solvent and hydrocarbons with polar groups (e.g. propanol) are more polar ones (Meyer, 2013; Nováková and Douša, 2013).

Mobile phases need to be filtered before use to prevent undissolved particles or impurities to clog capillaries or the column. Mobile phases should also be degassed before use to get rid of dissolved gases and to save work of degasser in the instrument (Meyer, 2013).

2.6.2. HPLC column and stationary phase

The column is an essential part of the system. Basically it has two main parts; a body which is usually stainless steel hollow tube filled with whatever stationary phase and column terminators, which hold stationary phase inside and enable to connect column to capillaries by ferules (Nováková and Douša, 2013). Apart from steel, there are other materials as glass or plastic used on inner walls of column. Diameter of column determines the use of the column. Columns with a diameter greater than 5 mm are usually used for preparative or semi preparative chromatography, columns with diameter between 2-5 mm are typically used for analytical HPLC and columns with 2.1 mm and smaller diameters are mainly used in UHPLC analysis. In terminators, there are frits enclosing stationary phase in the column body and preventing large impurities entering and plugging the column.

Frits or guard columns are usually installed at the front of column to protect it. They catch impurities and can be easily replaced once they become dirty. Guard columns usually contain the same stationary phase as main column. Disadvantage of using guard columns and frits could be increase of dead volume of the system.

Column efficiency is characterised by a number of theoretical plates, where higher number means less peak broadening. To compare different columns Height Equivalent to a Theoretical Plate is used. It is calculated as a ratio of column length and number of theoretical plates. Another quantity to determine efficiency of the column is peak symmetry, as ideal peaks are those that are symmetrical without tailing or fronting (Nováková and Douša, 2013). Resolution of chromatographic separation is a quantity to determine two neighbouring peaks one from another. Resolution of 1 means it is visible there are two different peaks; resolution 1.5 means that they are fully separated; though, when one peak is much smaller than the other higher resolution might be needed (Meyer, 2013).

Various stationary phases and stationary phase forms are used today based on an intended use. Porous particles of various materials are the most common form today Their size ranges from 1.8 to 10 μm with pore size given in nm or angstroms (0.1 nm=1Å). There are other possibilities in use as well e.g. small nonporous particles used in biopolymer chromatography, porous layer beads, perfusive particles made of cross-linked styrene-divinylbenzene with two pore types coated in active stationary phase. Monolithic column is interesting stationary phase. The stationary phase is made from one piece of silica or organic polymer in a form of porous rod filling a column body (Meyer, 2013). Recently, porous shell particles were developed; they combine nonporous core coated with porous silica, they appear to have improved abilities for many HPLC analyses (Nováková and Douša, 2013; Hayes *et al.*, 2014).

The material is very important for stationary phase. Silica is the basis of most stationary phases. This inorganic compound is used either alone in normal phase or size exclusion chromatography or because of its many silanol groups as a matrix where other functional groups can be chemically bonded. pH limit for silica based columns is between 1 and 8. The silica can be modified using many functional groups of various hydrophobicity and other properties. Bonds can be formed by esterification (obsolete, unstable in water), reaction with thionyl chloride (enabling Si-N bonds) or reaction with mono-/dichlorsilane, creating Si-O-Si-C bonds, which are the most stable and the most widespread. The reaction might also lead to

polymerization and creation of polysiloxanes, which provide cover to silica core and improve stability.

Remaining free silanols can be endcapped by trimethylchlorosilane, to reduce hydrophilicity and interference. The most used alkyl modifier is octadecylsilanole (ODS or C_{18}), apart from this one there are other lengths of alkylsilanoles like C_8, C_{30}. Other stationary phases can have bonded phenyl, nitrophenyl, pentafluorophenyl (PFP), which enable interaction of π electrons and in case of PFP also interactions with fluorine in analytes. More polar groups are represented by aminopropyl, cyanopropyl and diols. Silica also enables to bond phases for ion exchange or chiral analysis (Meyer, 2013; Nováková and Douša, 2013).

From non-silica based stationary phases. there are polymers as mentioned styrene-divinylbenzene resins, which have useable pH range from 1 to 13 and permit creation of alkylated or ion exchanging phases. Similarly, methacrylate gels and agarose can be used. Among inorganic stationary phases, there are alumina (aluminium oxide), titanium (titanium oxide), zirconia (zirconium oxide) and porous graphite carbon or controlled-pore glass (Meyer, 2013).

2.6.3. Modes of chromatography

Chromatography can be performed in several modes which differ in principles of separation and therefore the arrangement of the chromatographic system like used column and mobile phases. Very common modes are normal and reversed phase chromatography and then hydrophilic interaction chromatography (HILIC), size exclusion and ion exchange chromatography are also popular.

Many other methods are available. There can be interactions caused by affinity of bound bio-specific ligand and substrate in sample (affinity chromatography), creation of ion pairs and their interaction with nonpolar stationary phase, partition of chiral compounds *etc*. (Nováková and Douša, 2013). Idea of green chromatography came with environmental concern of last decades. This approach focuses on ecology and sustainability with reducing solvents and other environmentally unfriendly compounds, decrease of energy consumption *etc*. (Płotka *et al.*, 2013).

2.6.3.1. Normal phase chromatography

It is based on adsorption on polar stationary phase with mobile phase being less polar organic solvents and their mixtures. Interactions can be performed by dipole-induced dipole, dipole-dipole, hydrogen bonding or π bonding (Meyer, 2013).

Using normal phase, more polar compounds are highly absorbed compared to nonpolar or less polar. The absorption can be also influenced by steric protection or isomerism. However, normal phases are currently on the descent, they are used mainly in chromatography of lipophilic compounds or division of isomers (Nováková and Douša, 2013).

2.6.3.2. Reversed phase chromatography

As could be determined from the name of the technique the phases are functionally reversed to normal phase chromatography. Stationary phase is nonpolar and mobile phase is based on polar liquids often aqueous, with addition of well miscible organic solvent (acetonitrile, methanol, and acetone).

Reversed phase chromatography is the most widespread and the most popular technique today and C_{18} stationary phase can be considered as the most popular stationary phase in reversed phase chromatography. Water is very weak eluent for any compound that is less polar. These less polar compounds are therefore retained on nonpolar phase which water can not interfere with and are slowly eluted. Organic additives decrease time to elute compounds from columns.

Apart from organic additives, other additives are added to mobile phase to improve deposition or elution of compounds of specific properties (acidic, basic). Retention can be changed with changing length of side chain or by using other modified phases (Meyer, 2013; Nováková and Douša, 2013). All research performed in my work used reverse phase chromatography using C_{18} and PFP columns.

2.6.3.3. Hydrophilic interaction chromatography

HILIC have things in common with both previously mentioned modes of chromatography. It uses polar stationary phases as normal phase chromatography (however columns need to be manufactured for HILIC), but as eluent there are mobile phases with large portion of acetonitrile and water. It is best suitable for polar compounds.

2.6.3.4. Ion exchange chromatography

This chromatography uses electrostatic interaction of ionised compounds with ion exchangers, which are part of stationary phase or bound to it. The compound in flow is exchanged for another ion or interacts with charged ion exchanger previously bound to stationary phase and afterwards it is eluted using change in pH or an ion power of mobile phase. Density of ion exchanger groups determines maximum ion exchange capacity of phase. Ion exchangers are divided according to polarity and ion power: strong anionic exchangers

(quaternary amine), weak anionic exchangers (diethylamine), weak cationic exchangers (carboxymethyl) and strong cationic exchangers (sulfonic acid). This method is used in analysis of ionic or easily charged compounds, especially amino acids (Meyer, 2013; Nováková and Douša, 2013).

2.6.3.5. Size exclusion chromatography

It is known also as gel chromatography, because gel is used as stationary phase. The sample is divided according to size of present molecules. While bigger molecules bypass the particles, smaller molecules enter pores in particles and diffuse through the gel. The gel should be inert to prevent any other interactions with sample or mobile phase that could distort results. The most common gels are made of methacrylate, acrylamide, styrene-divinylbenzene, agarose, and dextran or modified silica. They are used to study large molecules like biopolymers to purify them or to determine their molecular weight (Nováková and Douša, 2013).

2.6.4. Detectors used in connection with HPLC

After compounds in sample are separated and eluted from column, their presence in mobile phase has to be determined. This process is performed by a detector. Most of detectors are based on measuring various physical properties of flowing mobile phase. Some of them can be considered universal detectors (e.g. UV); some of them measure specific properties of analysed compounds.

Ideal detector should be universal, sensitive, give stable response, linear, reliable and easy to use, independent on changes in temperature or mobile phase, not destructive and should give qualitative information about detected compounds. However, no detector can do all this and therefore it is necessary to choose one or combination the most suited for intended samples (Nováková and Douša, 2013).

2.6.4.1. Spectrophotometric detector

This was the most popular detector to use with HPLC for a long time, losing only to mass spectrometry nowadays. Its principle is based on absorption of light of specific wavelength by compounds passing through the cell of the detector. This absorption is caused by different functional groups and structures in molecule like carbonyls, aromatic circles, conjugated bonds and others (Meyer, 2013). Detector working range of wavelengths is from 190 to 800 nm (from ultraviolet to visible spectra – therefore called UV/VIS). There can be instruments with one or more fixed wavelengths or they can be adjusted in the certain range (photodiode-array detectors) (Nováková and Douša, 2013). Two lamps can be used for emitting

light; a deuterium lamp for UV and lower visible wavelengths and a tungsten halogen lamp for visible spectra up to 850 nm. In this work UV/VIS detector was placed before other more sensitive detectors (fluorescence detector or mass spectrometer) (Nováková and Douša, 2013).

2.6.4.2. Refractometric detectors

These detectors are based on detecting any changes in refractive index compared to pure mobile phase. They are universal, but about 1000 times less sensitive compared to UV/VIS detectors (Nováková and Douša, 2013).

2.6.4.3. Fluorescence detectors

This detector is based on detecting light that is emitted by compound after excitation by light of certain wavelength, produced by mercury-vapour lamp. This transition is usually specific for each compound; however as with UV/VIS, certain structures or functional groups must be present in detected compounds. Compared to UV/VIS this method is much more sensitive (Meyer, 2013; Nováková and Douša, 2013).

2.6.4.4. Electrochemical detectors

Principle of these detectors is change of current while oxidation or reduction occurs on electrodes. Usually the measurement is with positive potential as it could analyse many organic compounds via oxidation and is not heavily influenced by dissolved oxygen or metallic ions like reductive measurement. In amperometric detection only a small portion of the analyte is consumed by oxidation or reduction, while in coulometric detection all analyte in cell is oxidised/reduced. Sensitivity is similar to the fluorimetric detector, but it suffers from a limited life of electrodes and their contamination (Meyer, 2013; Nováková and Douša, 2013).

2.6.4.5. Aerosol based detectors

These detectors are based on nebulising and then drying solvents with particles of studied analyte. It is detected by one of three ways: light scattering, ionisation or nucleation technology where analyte particles are condensed with water vapours and then analysed via light scattering. These detectors are universal, but prone to mobile phase composition and purity.

Other detectors work with measuring conductivity, chemiluminescence, infrared absorption or radioactivity. HPLC can be also coupled with mass spectrometry or nuclear magnetic resonance (Nováková and Douša, 2013).

2.7. Mass spectrometry

Mass spectrometry (MS) is an advanced analytical method which allows to determine the mass of positively or negatively charged molecules (based on polarity mode). Results are produced as a mass-to-charge ratio (*m/z*), which in case the molecule has only one charge is same as its molecular weight.

MS allows both, qualitative and quantitative analysis and can provide important information about analysed compounds' structure. Information about the structure is very helpful especially in metabolite analysis.

MS history runs back to 1950' when it was used coupled with gas chromatography. Connection was simple as both techniques use gas phase. HPLC works with liquid phase and therefore it took longer to develop interphase to connect these two techniques. Nowadays HPLC/MS become most utilised detection method for HPLC analysis (Nováková and Douša, 2013).

MS can be today coupled with various separation techniques. Apart from HPLC and gas chromatography it is capillary electrophoresis and ion mobility or their combinations. It can also work for direct analysis without separation, with simply injecting the sample directly into a mass spectrometer, or with various offline methods (Hoffmann and Stroobant, 2007; Kanu *et al.*, 2008).

Mass spectrometers consist of an ion source where ions suitable for MS analysis are produced, at least one mass analyser where ions are separated and graded according to desired parameters and a detector which register ions incoming from analyser. The most common type of detector today is an electron multiplier where particle hits conversion dynode of opposite polarity and causes an emission of secondary particles, which again causes another emissions and therefore amplify the signal and finally ends with detector which digitalises the signal. (Hoffmann and Stroobant, 2007)

2.7.1. Ion source

The first step in mass spectrometry is to turn simple molecules to charged ions in gas phase. In coupling gas chromatography and mass spectrometry, there are two main approaches: Electron ionization where electrons give their energy to molecule of sample and chemical ionization where donor of energy is charged molecule of reagent gas. Both are called hard ionization techniques as they produce significant fragmentation of parent compounds. To

simplify and speed up identification process huge fragment spectra libraries are available nowadays. These hard ionization techniques need to be performed in vacuum.

On the contrary to previous techniques, soft ionisation techniques also exist. These techniques work in atmospheric pressure, allow transfer of liquid phase to gas phase, are used in HPLC-MS connection and keep molecules mostly unfragmented. As HPLC-MS coupling was dominantly used in my research I focus on those.

2.7.1.1. Electrospray ionisation

Electrospray ionisation (ESI) is based on applying high potential to liquids and spraying them in a form of aerosol from a tip of a capillary. With evaporation of solvent (can be eased by stream of neutral gas) droplets carrying charges are shrinking and breaking (Coulombic explosion) until they transfer charge to the molecule of the sample in a droplet, leading to the stream of ions that are directed into a mass analyser via ion optics. In case of large molecules (5-10 thousand Da), there is usually only one molecule per droplet. Moreover, especially large molecules that have more ionisable groups can be charged multiple times influencing *m/z* measured (Hoffmann and Stroobant, 2007). It also possess capability to work in very small volumes for rare samples and ionisation of specific hydrophilic samples as nanoelectrospray (Schmidt *et al.*, 2003). ESI can be used for ionic compounds, neutral and polar compounds that can be protonated or deprotonated and for nonpolar compounds that are oxidised or reduced on the tip of ESI needle. In the ESI source adducts of parent molecules with ions present in solvent (mobile phase) can be formed. In positive mode, mostly Na^+ and K^+ adducts can be observed. Adducts with carboxylic acids are typical for negative ion mode (Ardrey, 2003).

2.7.1.2. Atmospheric pressure chemical ionisation

Atmospheric pressure chemical ionisation (APCI) is another common method in HPLC-MS. It resembles the chemical ionisation used in gas chromatography. A stream of mobile phase is nebulised in nebuliser by a stream of nebulising gas (usually nitrogen) and then the fog is traveling through a tempered chamber where desiccation occurs. After desiccation flying molecules are subjected to corona discharge, which make them charged ions desired for MS. Particles from mobile phase have a role of ionising gas as they are primary charged and then they pass their charge to the molecules of dissolved samples (Hoffmann and Stroobant, 2007). APCI is suitable for polar and nonpolar compounds. While it is softer than electron or chemical ionisation it is not very suitable for large molecules above 2000 Da. Also, thermolabile compounds should be handled with care. Compared to ESI, it copes better with buffers and

additives in mobile phase. As well as in ESI, so APCI can create adducts in both polarities (Ardrey, 2003).

2.7.1.3. Atmospheric pressure photoionisation

Atmospheric pressure photoionisation (APPI) is a method very similar to the previous APCI, though instead of creating ions using a corona discharge it has gas (usually krypton) discharge lamp, which emits UV photons. Photons react with dopant molecules emitting electrons and creating highly reactive positive radicals. Radicals then ionise solvent molecules by transferring protons that are finally passed on analysed compound. If the ionisation energy of studied compound is lower than that of dopant it can be ionised directly (Hoffmann and Stroobant, 2007; Lee and Zhu, 2011). Due to its dependency on dopants it is more sensitive to composition of mobile phase than APCI; however, it enables analysis for especially nonpolar compounds that are hard to ionise with two previous methods. It can work in both positive as well as negative mode (Kauppila *et al.*, 2004; Hoffmann and Stroobant, 2007).

2.7.1.4. Other ionisation techniques

There are more ionisation techniques used in MS. Many are based on laser desorption ionisation (MALDI - Matrix assisted laser desorption ionisations) or can analyse surface of sample like DESI (desorption ESI) or DART (Direct analysis in real time). Others use plasma or fast atom bombardment.

2.7.2. Types of mass analysers

After ions are created and directed through ion optics, they are analysed in mass analyser. Analysis is performed in high vacuum to prevent the influence of air composition. Variety of mass analysers differing in accuracy, robustness, regularity of service and other parameters are used.

There are several main characteristics that describe mass analyser. One of those is mass range limit which determines range of *m/z* over which mass analyser detects. Analysis speed is another characteristic; it determines how fast analyser can scan a range of *m/z*. Transmission describes a portion of ions that are enabled to reach a detector. Mass accuracy shows difference between observed *m/z* and theoretical *m/z.* And finally there is resolution which tells us how precisely mass analyser can distinguish two compounds with small *m/z* difference (Hoffmann and Stroobant, 2007). Usually analysers working with more than two decimals resolution are considered high resolution analysers. These can determine accurate mass of molecule which is a sum of accurate masses of all atoms in the molecule. Compared to nominal mass which is in

unit Da (Dalton) accurate mass is accurate to thousandth of Da. Mass defect calculation is enabled by determination of accurate mass. It is difference between nominal mass and exact mass, based on elemental composition. It is unique for every molecule and it is usually in mDa (Holčapek *et al.*, 2008; Lee and Zhu, 2011).

2.7.2.1. Quadrupole analysers

Quadrupole analyser consists of four rods of a circular or hyperbolic cross-section. Two opposite rods are connected to direct current while others have radiofrequency current brought to them. Each ion with particular *m/z* have a stable trajectory through analyser to a detector. Changing currents will enable to separate ions in the sample (Ardrey, 2003). Quadrupole analysers are considered low resolution analysers. They can be used in various configurations. Probably most used configuration is triple quadrupole where first and third quadrupoles are separation analysers and central one is used as collision cell. It can be coupled with other analysers like time of flight (TOF) detectors as well.

2.7.2.2. Ion trap analysers

There are two main types of ion trap analysers. 3D ion trap and 2D ion trap. 3D ion trap, sometimes considered as 3D quadrupole, has ring electrode and two cap electrodes. These cap electrodes enable to keep ions "trapped" inside the ion trap. Change of potential on cap electrodes release trapped ions to a detector.

Linear ion trap is called 2D trap and resembles a classical quadrupole, however direct current is sent on ends of cell to prevent escape of ions. Compared to 3D ion trap there is more space inside and efficiency of ion trapping is better (Lee and Zhu, 2011).

Both ion trap types are considered low resolution and both are capable to perform tandem spectrometry. In addition, they can be combined with other mass analysers such as time of flight or orbital ion trap.

2.7.2.3. Time of flight detectors

Time of flight (TOF) detector is based on a principle that if ions are dosed into analyser in the same time with same energy then their speed of passage to detector is equivalent to their *m/z* with lighter ions traveling faster than heavier. Its ability to determine small differences in *m/z* is based on a length of an analyser tube. An ion mirror called reflectron is placed to the end of tube to increase the length. It effectively doubles the distance along which ions can separate. Modern machines can have more reflectrons and length can be prolonged even further. This

gives TOF detectors ability to work in high resolution (Ardrey, 2003; Hoffmann and Stroobant, 2007).

2.7.2.4. Orbitrap

In the meantime most novel type of mass analyser, described in late 90' by Makarov (2000). It consists of two electrodes, inner and outer, creating an orbital cell. In the trap ions oscillate in cyclotron and magnetron motions. Generated current is measured by detection plates and then transformed to mass spectra by Fourier transformation. Orbitrap achieves high resolution and mass accuracy with much reduced dimensions (Hoffmann and Stroobant, 2007; Lee and Zhu, 2011). Another detector operating with Fourier transformation is ion cyclotron resonance (ICR).

2.7.3. Tandem mass spectrometry

The ionisation techniques mentioned above are considered soft as they produce no, or very low amount of parent molecule fragments. Fragmentation of a parent molecule can help to identify the molecule and to determine structures of unknown compounds. Fragmentation can occur in-source (e.g. ESI and APCI sources), or it can be performed in analyser as tandem mass spectrometry (Ardrey, 2003). In tandem mass spectrometry (abbreviated MS-MS or MS^2) ions are fragmented by increasing their energy due to collision with inert collision gas, commonly noble gas like helium, argon, xenon; this is called collision induced dissociation (CID). MS-MS in triple quadrupoles can be described as follows: the first quadrupole isolates precursor ion (called parent ion) which undergoes CID in the second quadrupole and its fragments or parent ions are analysed in the third quadrupole. This approach when there are more analysers to perform the task is called tandem MS in place.

Other approach is tandem MS in time. In this technique ions are trapped, fragmented and analysed in the analyser (2D/3D ion traps and ICR). These machines also enable to isolate product ions and let them undergo fragmentation again leading to multiple stage tandem mass spectrometry (MS^n) analysis (Hoffmann, 1996; Hoffmann and Stroobant, 2007). Apart from CID, fragmentation can be induced by other means like collision with surface or laser beams.

2.7.4. Types of MS scans

Mass spectrometers can work in several modes or scans in dependence of analyser type (Nováková and Douša, 2013).

1) Basic scan: Spectrometer measure all ions in a chosen range of *m/z* (total ion current-TIC)
2) Selected ion monitoring (SIM): in this mode spectrometer only measure signals of ion/ions of chosen *m/z* in time
3) Product ion scan: scan of all ions resulting from fragmentation of an ion with chosen *m/z*.
4) Precursor ion scan: scan of all ions could provide a fragment of specific *m/z*
5) Neutral loss scan: scan of all precursor ions that provide certain neutral fragment; e.g. difference 44 mass units means CO_2 loss and is typical for carboxylic acids or loss of 18 means loss of water in alcohols (Hoffmann and Stroobant, 2007; Nováková and Douša, 2013)
6) Single reaction monitoring (SRM): in this case spectrometer analyses one particular fragmentation pair of precursor-product ions. It can be helpful for quantitative analysis using MS. Selectivity can be enhanced by observing several reactions of one parent ion (multi reaction monitoring - MRM).

Spectrometers can perform one or more of these tasks simultaneously depending on the construction of spectrometers. Reactions 3-6 are available only for machines capable of at least MS^2. Neutral loss can be measured only with spectrometer performing MS-MS in space (Hoffmann, 1996).

2.7.5. HPLC-MS in xenobiotic metabolism study

MS is a technique of high importance in identification of drug metabolites and other compounds from the environment. Basically, it provides molecular weight of studied compounds and limited amount of information about their structure. In case of metabolism, MS can help to identify structural changes in molecule that occur during metabolic pathways.

HPLC-MS connection increase amount of information about studied compounds (UV spectra, lipophilicity, etc.). HPLC separation also allows study compounds in very complex matrices (blood, urine, faeces, body tissues, etc.). As mentioned above, drug metabolising enzymes influence lipophilicity of parent compound, which leads to change in retention time during HPLC analysis. Metabolism can even change UV and fluorescence properties. Positional isomers and enantiomers cannot be simply recognized using conventional columns and HPLC-MS systems. Special chiral columns or ion mobility are needed for their separation.

Coupling HPLC to nuclear magnetic resonance add information about studied compounds (Want *et al.*, 2005; Holčapek *et al.*, 2008).

High resolution mass analysers (TOF, Orbitraps and ICR) are ideal for metabolite detection as they provide us with knowledge of elemental composition via accurate mass measurement and mass defect detection. It can determine right molecular formula; however, often it is not self-sufficient, but can narrow the search (Holčapek *et al.*, 2008; Lee and Zhu, 2011). High resolution mass analysers provide us with information about isotope composition (Hoffmann and Stroobant, 2007).

When a molecule is added to or removed from the structure mass defect is introduced to the molecule, for example oxidation introduce mass defect of -5 mDa, glucuronidation +32 mDa. For most of biotransformation reactions mass defect is up to 70 mDa of parent molecule, therefore it enables to filter out masses from the matrix that do not suit it. There are examples of elemental mass defects in Table 1 and examples of mass defects in common metabolic reactions in Table 2 (Holčapek *et al.*, 2008; Lee and Zhu, 2011).

Table 1: Mass defects for chosen elements (Holčapek *et al.*, 2008)

Nominal atomic mass (Da)	Element	Mass defect (mDa)
1	H	+7.825
12	C	0
14	N	+3.074
16	O	-5.085
32	S	-27.929

Table 2: Examples of metabolic reactions and their MS spectra changes (Holčapek *et al.*, 2008; Lee and Zhu, 2011)

Nominal mass shift (Δ Da)	Elemental composition change	Reaction	Exact mass shift (mDa)
-44	$-CO_2$	Decarboxylation	+10.2
-14	$-CH_2$	Demethylation	-15.7
-2	$-H_2$	Alcohol oxidation	-15.7
+2	$+H_2$	Aldehyde/Ketone reduction	+15.7
+14	$+CH_2$	Methylation	+15.7
+16	+O	Hydroxylation/S-oxidation	-5.1
+176	$+C_6H_8O_6$	Glucuronidation	+32.1
+162	$+C_6H_{10}O_6$	Glucosylation	+52.8

Other types of mass analysers are suitable to different tasks. Ion traps are used in structure identification due to possibility of MS^n spectres. Triple quadrupoles are very useful in quantification of metabolites via single/multiple reaction monitoring. Hybrid machines as quadrupole-TOF can be used to observe fragmentations in high resolutions (Holčapek *et al.*, 2008).

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4 List of abbreviations

4-PCA	4-pyridincarboxaldehyde
ABC	ATP binding cassette
ABZ	Albendazole
AKR	Aldo-keto reductases
APCI	Atmospheric pressure chemical ionisation
APPI	Atmospheric pressure photoionisation
ATP	Adenosine triphosphate
BR	Benzimidazole resistant strain of *H. contortus*
BZD	Benzimidazole
C_{18}	Octadecylsilanole stationary phase
CID	Collision induced dissociation
CYP	Cytochrome P450
DART	Direct analysis in real time
DESI	Desorption ESI
ER	Endoplasmic reticulum
ESI	Electrospray ionisation
FAMACHA	Faffa Malan Chart
FLU	Flubendazole
FMOs	Flavin monooxygenases
GST	Glutathione S-transferase
HILIC	Hydrophilic interaction chromatography
HPLC	High-performance liquid chromatography
HPLC-MS	High-performance liquid chromatography coupled with mass spectrometry

ICR	Ion cyclotron resonance
ISE	Susceptible strain of *H. contortus*
ISE-S	Ivermectin resistant strain of *H. contortus*
MALDI	Matrix assisted lased desorption ionisations
MBZ	Mebendazole
MS	Mass spectrometry
MS-MS or MS^2	One stage tandem mass spectrometry
MS^n	Multiple stage tandem mass spectrometry
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
PFP	Pentafluorophenyl
P-gp	P-glycoprotein
SDR	Short chain dehydrogenases/reductases
SIM	Selected ion monitoring
SLC	Solute carrier
SRM	Single reaction monitoring
SULT	Sulphotransferases
TCBZ	Triclabendazole
TIC	Total ion current
TOF	Time of flight
UDP	Uridine diphosphate
UGT	Uridine diphosphate glucuronosyltransferase
UHPLC	Ultra-high performance liquid chromatography
UV	Ultraviolet
UV/VIS	Ultraviolet to visible spectra
WR	White River strain of *H. contortus* (multiresistant)

5 Aims

The main aim of this work was to broaden the knowledge about metabolism and behaviour of anthelmintics in parasites and their hosts using high performance liquid chromatography with mass spectrometric detection. In addition, the connection of these mechanisms to anthelmintic resistance development was studied.

Partial aims of this work:

1. Study of biotransformation and transport of anthelmintic drug flubendazole in susceptible and resistant strains of a parasitic roundworm *Haemonchus contortus*
2. Collection and evaluation of current information about monepantel, the novel anthelmintic drug
3. Determination of activities of xenobiotic metabolising enzymes and biotransformation of anthelmintic drugs in a sheep tapeworm (*Moniezia expansa*) and a giant liver fluke (*Fascioloides magna*)
4. Analysis of albendazole and its metabolites in faeces from treated sheep and analysis of effects of these compounds on lower development stages of *H. contortus* and on white mustard seeds germination

6 Experimental part: results and discussion

6.1. Analysis of flubendazole transport in barber's pole worm (*Haemonchus contortus*) susceptible and resistant strains.

Bártíková, H., Vokřál, I., Kubíček, V., Szotáková, B., **Prchal, L.**, Lamka, J., Várady, M. and Skálová, L. (2012). Import and efflux of flubendazole in *Haemonchus contortus* strains susceptible and resistant to anthelmintics. *Veterinary Parasitology* 187, 473–9. doi:10.1016/j.vetpar.2012.02.008. IF_{2012}=2.381

In this work, we tried to determine transport of flubendazole into and out of bodies of *H. contortus* in an *ex vivo* study. To examine various types of transport, transport of FLU between dead and living nematodes was compared and effect of verapamil, inhibitor of main efflux ABCB1 transporter (P-glycoprotein), on FLU accumulation in nematodes was assayed as well, because the role of ABC transporters is suspected in resistance to other anthelmintics. All these parameters were cross-examined among strains which were either susceptible to all anthelmintics (ISE) or resistant to benzimidazoles (BR) or to ivermectin (ISE-S) or multi-resistant (WR); these worms were obtained from experimentally infected sheep. After incubations, worms were homogenised, FLU and metabolites were extracted and analysed by HPLC with UV/VIS detection.

The results showed FLU is able to enter *H. contortus* adults directly due to high lipophilicity of FLU, which was also measured during the experiment ($\log P = 3.05$). The amounts of flubendazole were steadily growing in both living and dead nematodes with increasing time. In efflux studies, the results were similar. Release of flubendazole from worms was constant in time and independent on the state of parasite. The inhibition of P-gp with verapamil did not have any effect on amounts of FLU found in worms and its participation on FLU efflux can be excluded. When the strains were compared to each other very similar results were found out, therefore the transport was not affected by any enzyme or transporter expressed in resistant strains.

All in all, this study proved passive diffusion is main and probably the only way how flubendazole can enter or exit body of *H. contortus* as it is independent on inhibition, presence of body functions and a strain of the worm.

6.2. Reduction of xenobiotics and biotransformation of flubendazole in barber's pole worm (*Haemonchus contortus*)

Vokřál, I., Bártíková, H., **Prchal, L.**, Stuchlíková, L., Skálová, L., Szotáková, B., Lamka, J., Várady, M. and Kubíček, V. (2012). The metabolism of flubendazole and the activities of selected biotransformation enzymes in *Haemonchus contortus* strains susceptible and resistant to anthelmintics. *Parasitology* 139, 1309–16. doi:10.1017/S0031182012000595. IF_{2012}=2.355

This study was focused on biotransformation of flubendazole in *H. contortus* strains susceptible or resistant to anthelmintics. The study consists of several parts. Worms were collected from experimentally infected sheep. An *ex vivo* study was used to determine which metabolites are produced. Worms were cultivated for 24 h in media with flubendazole and then their homogenate and medium was extracted and analysed by HPLC-MS analysis. Part of worms was homogenised and subcellular fractions were prepared. With obtained subcellular fractions *in vitro* formation of reduced flubendazole and its glucosylation were studied. *In vitro* specific activities for model substrates (naloxone, metyrapone, menadione, 4-pyridinecarboxaldehyde - 4-PCA) were assayed in a cytosol-like fraction and specific activity of UDP-glucosyltransferase towards p-nitrophenol was assayed in a microsome-like fraction. Activities were compared among strains of *H. contortus* susceptible (ISE) and resistant to anthelmintics (BR, WR, ISE-S).

It was found by HPLC-MS analysis that *ex vivo H. contortus* produces four different metabolites – phase I: reduced flubendazole, and phase II: reduced glucosylated flubendazole and two glucosylated flubendazole metabolites. Using tandem mass spectrometry, it was determined reduced flubendazole is glucosylated on newly formed hydroxyl group while flubendazole is glucosylated on both nitrogens in the benzimidazole structure. All metabolites were present in all strains and in both, worm homogenate and medium. Resistant strains were more active in metabolising than ISE strain.

During *in vitro* experiments, it was found that formation of reduced flubendazole is mostly inhibited by menadione and partly by 4-PCA, however carbonyl-reducing activity towards menadione was not present in any strain. Cytosolic activities towards 4-PCA and metyrapone were present in all strains, but activity towards naloxone was present only in ivermectin resistant WR and ISE-S strains. Also, *in vitro* glucosylation of p-nitrophenol was found only in resistant strains. Glucosylation of reduced flubendazole *in vitro* was found in all strains with resistant ones forming more glucosylated metabolites.

The metabolic pathways in resistant strains of *H. contortus* are more active compared to susceptible one and could protect helminths from anthelmintic therapy. Resistant strains are fully capable of reducing flubendazole and forming phase II glucosides.

6.3. Insight and brief summary of information on novel drug monepantel

Lecová, L., Stuchlíková, L., **Prchal, L.** and Skálová, L. (2014). Monepantel: the most studied new anthelmintic drug of recent years. *Parasitology* 141, 1686–1698. doi:10.1017/S0031182014001401. IF_{2014}=2.560

This review is focused on monepantel, one of novel drugs against parasitic nematodes, which was thoroughly studied in last years. It briefly describes origin of the drug and its mechanism of action in comparison to other anthelmintics with similar effects. Next section is focused on its toxicity, which is found quite low, and pharmacokinetic studies performed in various animals. Further part is focused on various metabolites produced in sheep as well as in roundworm *H. contortus*. It concludes most of the drug is metabolised via sulphoxidation and in sheep it is conjugated with various endogenous substrates. Next section deals with efficacy of monepantel on spectrum of helminths and their life stages; also, the possible use of monepantel as anticancer drug with rather low toxicity is discussed. Influence of monepantel on nontarget organisms like various insects living on the pastures or even in sheep dung is mentioned as well. Last section discusses rising resistance to monepantel in Australia and New Zealand and problems of resistance and its prevention as a whole.

6.4. Study of biotransformation enzymes and biotransformation of benzimidazole anthelmintics in sheep tapeworm (*Moniezia expansa*)

Prchal, L., Bártíková, H., Bečanová, A., Jirásko, R., Vokřál, I., Stuchlíková, L., Skálová, L., Kubíček, V., Lamka, J., Trejtnar, F. and Szotáková, B. (2015). Biotransformation of anthelmintics and the activity of drug-metabolizing enzymes in the tapeworm *Moniezia expansa*. *Parasitology* 142, 648–659. doi:10.1017/S0031182014001711. IF_{2015}=3.031

The study was focused on drug metabolism in a sheep tapeworm *Moniezia expansa* and its comparison with previous results of a rat tapeworm *Hymenolepis diminuta*. The study was performed both *ex vivo* and *in vitro*; tapeworms were obtained from naturally infected sheep. *Ex vivo* meant cultivation of tapeworms with BZD anthelmintics (albendazole, mebendazole, flubendazole) and subsequent search for metabolites by LC-MS. *In vitro,* specific enzyme activities towards range of substrates were determined using subcellular fractions obtained by centrifugation from tapeworm homogenate; cytosol-like, microsomes-like and mitochondria-like fractions. *In vitro* incubations with albendazole, flubendazole and mebendazole were done as well. Kinetic parameters were assayed for production of reduced metabolites of mebendazole and flubendazole.

In the *ex vivo* experiments *M. expansa* showed ability to form only oxidised or reduced metabolites such as sulphoxide and sulphone of albendazole and carbonyl reduced mebendazole and flubendazole. No conjugates as glycosides of albendazole or methylations of flubendazole were detected compared to previous works with parasitic helminths *H. contortus* and *H. diminuta*. All metabolites were proven in media and tapeworm homogenate except for albendazole sulphone, which was found only inside the worm; it has to be effluxed in much slower rate or not at all and it is stored inside a worm's body. In *in vitro* studies reduction of anthelmintics was performed as expected in a cytosol-like fraction, while oxidation of albendazole to sulphoxide was present in all fractions. Comparison of kinetic parameters of *M. expansa* with *H. diminuta* studied previously showed sheep tapeworm's enzymes have greater affinity (lower K_M value) towards mebendazole and are able to metabolise more flubendazole (higher V_{max} value). The specific enzyme activities of different helminths are summarized in Table 3; data for H. *diminuta*, *H. contortus* and *D. dendriticum* are from previous works (Cvilink *et al.*, 2008a; b, 2009b, Bártíková *et al.*, 2010, 2012; Vokřál *et al.*, 2013).

The table is taken from the article and moreover includes data for a fluke *Fascioloides magna*, which is going to be discussed below.

Table 3: Xenobiotic metabolising enzymes and metabolites of anthelmintics in helminth parasites

Enzyme/parent drug	*M. expansa*	*H. diminuta*	*H. contortus*	*D. dendriticum*	*F. magna*
Peroxidase	Cyt, Mic, Mit	Cyt, Mic, Mit	Cyt, Mic, Mit	Cyt, Mic, Mit	Cyt, Mic
Flavin monooxygenase	-	-	n.d.	n.d.	Mic
Catalase	Cyt, Mit	Cyt, Mic, Mit	Cyt, Mic, Mit	Cyt, Mic, Mit	Cyt, Mic
Superoxide dismutase	Cyt, Mic, Mit	Cyt, Mic, Mit	Cyt, Mic, Mit	Cyt, Mic, Mit	-
Glutathione reductase	Cyt, Mit	-	-	Cyt, Mic	-
Glutathione peroxidase	-	-	-	-	Cyt, Mic
Thioredoxin reductase	Cyt, Mic, Mit	-	-	Cyt, Mic	-
Acenaphthenol dehydrogenase	Cyt, Mic, Mit	Cyt	Cyt	Cyt	Cyt, Mic
Menadione reductase	Cyt, Mic, Mit	Cyt	-	Cyt	Cyt, Mic
4-PCA reductase	Cyt	Cyt, Mic,	Cyt	Cyt	Cyt
Daunorubicine reductase	Cyt	Cyt	Cyt, Mic, Mit	-	-
Metyrapone reductase	n.d.	n.d.	Cyt	Cyt	Cyt
Oracin reductase	-	Cyt	Mit, Cyt	-	-
UDP-glucosyl transferase	Mic, Mit	Mit	Mic	-	Mit
UDP-glucuronosyl transferase	Cyt, Mic, Mit	n.d.	Cyt, Mic, Mit	-	Mit
Glutathione S-transferase	Cyt, Mit	Cyt	Cyt	Cyt	Cyt
Albendazole sulphoxide	Cyt, Mic, Mit, Ex	n.d.	Mic, Mit, Ex	Mic, Mit, Ex	Cyt, Mic, Mit, Ex
Albendazole sulphone	Ex	n.d.	Mic, Mit	Mit	n.d.
Albendazole glucoside	n.d.	n.d.	Ex	-	n.d.
Reduced mebendazole	Cyt, Ex	Cyt, Ex	Cyt	Cyt, Mic, Mit	Cyt, Ex
Reduced flubendazole	Cyt, Ex	Cyt, Ex	Cyt, Ex	Cyt, Mic, Mit	-
Flubendazole glucoside	n.d.	n.d.	Ex	-	-
Methylated reduced flubendazole	n.d.	Ex	n.d.	-	-

Cyt, cytosol-like fraction; Mit, mitochondria-like fraction; Mic, microsomes-like fraction; Ex, *ex vivo*; n.d., not detected; -, not tested

In *M. expansa*, several carbonyl-reducing enzymes and antioxidant enzymes (protection against oxidative stress) are active. Compared to *H. diminuta,* UDP-glucosyl transferase and

UDP-glucuronosyl transferase activities were determined; however, compared to *H. contortus,* no glucosides of anthelmintics were found.

Taken together, a sheep tapeworm has available several ways how to lower the danger of anthelmintic treatment and is able to produce basic metabolites of benzimidazole anthelmintics.

6.5. Study of biotransformation enzymes and biotransformation of anthelmintics in giant liver fluke (*Fascioloides magna*)

Prchal, L., Vokřál, I., Kašný, M., Rejšková, L., Zajíčková, M., Lamka, J., Skálová, L., Lecová, L. and Szotáková, B. (2016). Metabolism of drugs and other xenobiotics in giant liver fluke (*Fascioloides magna*). *Xenobiotica* **46**, 132–140. doi:10.3109/00498254.2015.1060370. IF_{2015}=1.723

This study is focused on metabolism of a giant liver fluke (*Fascioloides magna*). The samples were obtained from naturally infected fallow deer. An *ex vivo* study was preceded with preincubation period to let flukes get rid of excess blood, which could influence extractions and analysis. Flukes were incubated with BZD albendazole, mebendazole and triclabendazole and salicylanilides closantel and rafoxanide, because these compounds are usually constituents of medicines used against *F. magna*. Closantel and rafoxanide were used in lower concentrations compared to benzimidazole drugs, because of their toxicity for flukes. *In vitro* biotransformation study was performed with BZD and specific activities of xenobiotic metabolising enzymes were determined in microsomes-like and cytosol-like subcellular fractions. A mitochondria-like subcellular fraction was prepared as well; however, due to impurities probably from remaining digested blood it was unusable in enzyme activity studies and was only used in *in vitro* incubations. Compared to previous work HPLC with UV and fluorimetric detection was replaced with UHPLC-MS-MS analysis.

The obtained results of *ex vivo* incubations were disappointment, as almost none metabolites were found. It is known that *Fasciola hepatica* oxidise triclabendazole up to sulphone, in *F. magna* none metabolite was found; also, albendazole was oxidised only to sulphoxide. Mebendazole was reduced on its carbonyl group like it was in previous studies in various helminths. There were no metabolites of closantel and rafoxanide found in flukes or media. *In vitro* it was confirmed that reduced mebendazole is produced in cytosol-like fraction and kinetic parameters for mebendazole reduction were determined showing similar affinity as in *M. expansa* but with much lower maximal velocity of reaction. Albendazole was *in vitro* metabolised mostly in a microsomal fraction compared to a mitochondrial one in *M. expansa*. Enzyme activities of *F. magna* from *in vitro* enzyme assays can be seen in Table 3. Moreover, activities of sulfotransferase and quinone oxidoreductase were found in cytosol. Flavin

monooxygenase activity was also found; still, as it was told it did not contribute to oxidation of anthelmintics very much.

In conclusion, it was found *F. magna* have enzymes capable of performing biotransformation, however only a few metabolites were actually formed. This finding could be promising for future therapy as triclabendazole resistance is often associated with biotransformation and transport.

6.6. Analysis of albendazole and its oxidised metabolites levels in sheep faeces and influence on free living stages of barber's pole worm (*Haemonchus contortus*) and germination of white mustard (*Sinapis alba*)

Prchal, L., Podlipná, R., Lamka, J., Dědková, T., Skálová, L., Vokřál, I., Lecová, L., Vaněk, T. and Szotáková, B. (2016). Albendazole in environment: faecal concentrations in lambs and impact on lower development stages of helminths and seed germination. *Environmental science and pollution research international* **23**, 13015–22. doi:10.1007/s11356-016-6472-0. IF_{2015}=2.76

Veterinary anthelmintics represent risk to the environment as they may impact non-target organisms including plants. This study was focused on passage of albendazole through sheep gastrointestinal tract to faeces, where *H. contortus* eggs are located and where larvae start their development. This work represents sort of a shift to anthelmintic-plant relations which is nowadays studied in our research group. As we could not find faecal concentrations after administration of albendazole to sheep in available scientific literature this study was proposed and realized. Lambs were treated with 10 mg per kg of body weight with albendazole and then the faeces were collected in 1.5 h interval for the first 12 h and then each 6 h until 72 h post administration. Samples of faeces were then processed and analysed via UHPLC with tandem mass spectrometry analysis looking for parent drug and its sulphoxide and sulphone. Meanwhile a test for hatching of susceptible ISE strain of *H. contortus* and a test for larvae development were carried out in presence of albendazole and its sulphoxide in concentrations found in faeces. Also, a test of influencing germination of *Sinapis alba* seeds after exposure to albendazole and its sulphoxide in different concentrations was done.

We were surprised how much albendazole passed to the faeces; its concentration rose sharply till 12^{th} hour post administration and then slowly decreased. The course of albendazole sulphoxide was very similar to the parent drug, however sulphone concentrations started to rise slower and peaked in about 42 h post administration. Hatching and larval development tests proved there is very narrow window where worms or eggs are in contact with sublethal doses of anthelmintics that are critical for resistance development. It may vary in partially resistant strains and also influences on the pasture can broaden the window. On the other hand, germination of white mustard was not affected by even highest tested concentrations.

This study gave us information about pharmacokinetics of albendazole in sheep after oral administration and about influence that it can have on lower soil-living developmental stages of parasites and plants in the environment.

6.7. Author's participation to the works presented in thesis

Bártíková, H., Vokřál, I., Kubíček, V., Szotáková, B., **Prchal, L.**, Lamka, J., Várady, M. and Skálová, L. (2012). Import and efflux of flubendazole in *Haemonchus contortus* strains susceptible and resistant to anthelmintics. *Veterinary Parasitology* **187**, 473–9. doi:10.1016/j.vetpar.2012.02.008. IF_{2012}=2.381

Cultivation of H. contortus with flubendazole.

Vokřál, I., Bártíková, H., **Prchal, L.**, Stuchlíková, L., Skálová, L., Szotáková, B., Lamka, J., Várady, M. and Kubíček, V. (2012). The metabolism of flubendazole and the activities of selected biotransformation enzymes in *Haemonchus contortus* strains susceptible and resistant to anthelmintics. *Parasitology* **139**, 1309–16. doi:10.1017/S0031182012000595. IF_{2012}=2.355

Participation on in vitro experiments with reduction of flubendazole in susceptible and resistant H. contortus. Cultivation, extraction and analysis of samples.

Lecová, L., Stuchlíková, L., **Prchal, L.** and Skálová, L. (2014). Monepantel: the most studied new anthelmintic drug of recent years. *Parasitology* **141**, 1686–1698. doi:10.1017/S0031182014001401. IF_{2014}=2.560

Contribution to the publication elaboration

Prchal, L., Bártíková, H., Bečanová, A., Jirásko, R., Vokřál, I., Stuchlíková, L., Skálová, L., Kubíček, V., Lamka, J., Trejtnar, F. and Szotáková, B. (2015). Biotransformation of anthelmintics and the activity of drug-metabolizing enzymes in the tapeworm *Moniezia expansa*. *Parasitology* **142**, 648–659. doi:10.1017/S0031182014001711. IF_{2015}=3.031

Extraction and preparation of ex vivo samples.

Contribution to analysis of results

Contribution to the publication elaboration

Prchal, L., Vokřál, I., Kašný, M., Rejšková, L., Zajíčková, M., Lamka, J., Skálová, L., Lecová, L. and Szotáková, B. (2016). Metabolism of drugs and other xenobiotics in giant liver fluke (*Fascioloides magna*). *Xenobiotica* **46**, 132–140. doi:10.3109/00498254.2015.1060370. IF_{2015}=1.723

Contribution to material collection

Ex vivo experiments

HPLC and MS analysis

Publication elaboration

Prchal, L., Podlipná, R., Lamka, J., Dědková, T., Skálová, L., Vokřál, I., Lecová, L., Vaněk, T. and Szotáková, B. (2016). Albendazole in environment: faecal concentrations in lambs and impact on lower development stages of helminths and seed germination. *Environmental science and pollution research international* **23**, 13015–22. doi:10.1007/s11356-016-6472-0. IF_{2015}=2.76

Preparation of samples

UHPLC-MS analysis

Albendazole kinetics elaboration

Contribution to publication elaboration

7 Summary

With a commercial use of anthelmintics, resistance of helminths to drugs started to appear and became a great problem. Drug metabolism and transport is one of suspected mechanisms of drug resistance development. In my thesis, I focused on broadening the knowledge about metabolism of anthelmintic drugs and other xenobiotics in parasitic helminths of different classes and in their hosts. The relationship between anthelmintics metabolism and resistance development was studied. Several methods for UHPLC-MS analysis were introduced for these studies.

In *Haemonchus contortus,* reduction and glycosylation of flubendazole were described. Differences in these metabolic activities were found between susceptible and anthelmintic resistant strains of the parasite; resistant strains having higher activities and forming more metabolites than susceptible ones. Flubendazole enters and leaves body of *H. contortus* solely by passive diffusion and participation of efflux transporter P-glycoprotein was excluded.

The recent information about novel anthelmintic drug monepantel was summarised in the review article.

In *Moniezia expansa*, only metabolites of anthelmintics of biotransformation phase I were found. Several enzyme activities of biotransformation and antioxidant enzymes were assayed as well. The obtained results indicate *M. expansa* is able to deactivate anthelmintics, and thus protects itself against their action. *Fascioloides manga* showed very low metabolic activities especially in oxidations compared to other helminths previously studied. Very interesting was inability of *F. magna* to deactivate triclabendazole and albendazole. Only reduced metabolite of mebendazole was found in this parasite. *F. magna* possesses lower xenobiotic-metabolizing system compared to other helminths.

Pharmacokinetic study of albendazole in sheep faeces showed there is only a low risk for transforming a susceptible strain of *H. contortus* to a resistant one when recommended doses of anthelmintic are used. On the other hand, concentrations of albendazole in faeces are so high they might affect non-target soil invertebrates.

8 Appendices

8.1. Publications included in thesis

I. Bártíková, H., Vokřál, I., Kubíček, V., Szotáková, B., **Prchal, L.,** Lamka, J., Várady, M. and Skálová, L. (2012). Import and efflux of flubendazole in *Haemonchus contortus* strains susceptible and resistant to anthelmintics. *Veterinary Parasitology* **187**, 473–9. doi:10.1016/j.vetpar.2012.02.008. IF_{2012}=2.381

II. Vokřál, I., Bártíková, H., **Prchal, L.,** Stuchlíková, L., Skálová, L., Szotáková, B., Lamka, J., Várady, M. and Kubíček, V. (2012). The metabolism of flubendazole and the activities of selected biotransformation enzymes in *Haemonchus contortus* strains susceptible and resistant to anthelmintics. *Parasitology* **139**, 1309–16. doi:10.1017/S0031182012000595. IF_{2012}=2.355

III. Lecová, L., Stuchlíková, L., **Prchal, L.** and Skálová, L. (2014). Monepantel: the most studied new anthelmintic drug of recent years. *Parasitology* **141**, 1686–1698. doi:10.1017/S0031182014001401. IF_{2014}=2.560

IV. **Prchal, L.,** Bártíková, H., Bečanová, A., Jirásko, R., Vokřál, I., Stuchlíková, L., Skálová, L., Kubíček, V., Lamka, J., Trejtnar, F. and Szotáková, B. (2015). Biotransformation of anthelmintics and the activity of drug-metabolizing enzymes in the tapeworm *Moniezia expansa*. *Parasitology* **142**, 648–659. doi:10.1017/S0031182014001711. IF_{2015}=3.031

V. **Prchal, L.,** Vokřál, I., Kašný, M., Rejšková, L., Zajíčková, M., Lamka, J., Skálová, L., Lecová, L. and Szotáková, B. (2016). Metabolism of drugs and other xenobiotics in giant liver fluke (*Fascioloides magna*). *Xenobiotica* **46**, 132–140. doi:10.3109/00498254.2015.1060370. IF_{2015}=1.723

VI. **Prchal, L.,** Podlipná, R., Lamka, J., Dědková, T., Skálová, L., Vokřál, I., Lecová, L., Vaněk, T. and Szotáková, B. (2016). Albendazole in environment: faecal concentrations in lambs and impact on lower development stages of helminths and seed germination. *Environmental science and pollution research international* **23**, 13015–22. doi:10.1007/s11356-016-6472-0. IF_{2015}=2.76

8.1.1. Publication No. 1

I. Bártíková, H., Vokřál, I., Kubíček, V., Szotáková, B., **Prchal, L.,** Lamka, J., Várady, M. and Skálová, L. (2012). Import and efflux of flubendazole in *Haemonchus contortus* strains susceptible and resistant to anthelmintics. *Veterinary Parasitology* **187**, 473–9. doi:10.1016/j.vetpar.2012.02.008. IF_{2012}=2.381

8.1.2. Publication No. 2

II.	Vokřál, I., Bártíková, H., **Prchal, L.,** Stuchlíková, L., Skálová, L., Szotáková, B., Lamka, J., Várady, M. and Kubíček, V. (2012). The metabolism of flubendazole and the activities of selected biotransformation enzymes in *Haemonchus contortus* strains susceptible and resistant to anthelmintics. *Parasitology* **139**, 1309–16. doi:10.1017/S0031182012000595. IF_{2012}=2.355

8.1.3. Publication No. 3

III.	Lecová, L., Stuchlíková, L., **Prchal, L.** and Skálová, L. (2014). Monepantel: the most studied new anthelmintic drug of recent years. *Parasitology* **141**, 1686–1698. doi:10.1017/S0031182014001401. IF_{2014}=2.560

8.1.4. Publication No. 4

IV. **Prchal, L.,** Bártíková, H., Bečanová, A., Jirásko, R., Vokřál, I., Stuchlíková, L., Skálová, L., Kubíček, V., Lamka, J., Trejtnar, F. and Szotáková, B. (2015). Biotransformation of anthelmintics and the activity of drug-metabolizing enzymes in the tapeworm *Moniezia expansa*. *Parasitology* **142**, 648–659. doi:10.1017/S0031182014001711. IF_{2015}=3.031

8.1.5. Publication No. 5

V. **Prchal, L.,** Vokřál, I., Kašný, M., Rejšková, L., Zajíčková, M., Lamka, J., Skálová, L., Lecová, L. and Szotáková, B. (2016). Metabolism of drugs and other xenobiotics in giant liver fluke (*Fascioloides magna*). *Xenobiotica* **46**, 132–140. doi:10.3109/00498254.2015.1060370. IF_{2015}=1.723

8.1.6. Publication No. 6

VI. **Prchal, L.,** Podlipná, R., Lamka, J., Dědková, T., Skálová, L., Vokřál, I., Lecová, L., Vaněk, T. and Szotáková, B. (2016). Albendazole in environment: faecal concentrations in lambs and impact on lower development stages of helminths and seed germination. *Environmental science and pollution research international* **23**, 13015–22. doi:10.1007/s11356-016-6472-0. IF_{2015}=2.76

8.2. Other author's publications

Lecová, L., Růžičková, M., Laing, R., Vogel, H., Szotáková, B., **Prchal, L.**, Lamka, J., Vokřál, I., Skálová, L. and Matoušková, P. (2015). Reliable reference gene selection for quantitative real time PCR in *Haemonchus contortus*. *Molecular and Biochemical Parasitology* **201**, 123–127. doi:10.1016/j.molbiopara.2015.08.001.

8.3. List of author's presentations

Poster presentations

20th Helminthological Days 2013, Štědronín u Orlíku, May 2013

Prchal L., Bártíková H., Jirásko R., Vokřál I., Skálová L., Szotáková B., Benzimidazole drugs metabolism in the tapeworm *Moniezia expansa*. 20th Helminthological Days 2013 Programme & Abstracts p. 44

V4 Parasitological Meeting, Stará Lesná, May 2014

Prchal L., Rejšková L., Lamka J., Vokřál I., Zajíčková M., Szotáková B., Giant liver fluke: Common anthelmintic metabolism. Book of Abstracts p. 109

XVI. Interdisciplinary meeting of young biologists, biochemists and chemists, Milovy, May 2016

Lukáš Prchal, Russel Morphew, Peter Brophy: Glutathion S-transferáza u *motolice Calicophoron daubneyi* a její interakce s oxyklozanidem. (poster in Czech)

XII. Czech and Slovak parasitological days, Ledeč nad Sázavou, May 2016

Prchal L., Válková K., Vokřál I., Stuart R., Szotáková B.: Metabolism and transport of xenobiotics in liver fluke *Fasciola hepatica*. Book of abstracts, p. 96

Oral Presentations

Student Scientific conference of Faculty of Pharmacy, Hradec Králové, April 2012

Prchal L., Bártíková H., Szotáková B.: Biotransformation of flubendazole in susceptible and resistant strains of *Haemonchus Contortus*

4th Postgraduate and 2nd Postdoc conference of Faculty of Pharmacy, Hradec Králové, January 2014

Prchal L., Rejšková L., Szotáková B.: Metabolism of anthelmintic drugs in giant liver fluke, Book of abstracts - PhD-posdok konference FaF 2014 p. 159

6th Postgraduate & 4th Postdoctoral conference Faculty of Pharmacy UK, Hradec Králové, February 2016

Prchal L., Brophy P., Morphew R., Szotakova B.: Glutathione S-transferase in rumen fluke Calicophoron daubneyi, Book of abstracts p. 73-74