REPORT

An *ex vivo* up-take of levamisole molecules by cestode (*Monezia expensa*) of goat (*Capra hirsa*) and its detection through RP-HPLC

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Abstract: Detection of various molecules of drugs remained a prime issue especially in tissues of animals, humans and in their target parasites. The cestode/tapeworms pose a dilemma because of their weird body composition and uptake pattern of nutrients and medicines especially through absorption by tegument. We selected levamisole; thought to be potent antiparasitic/ ani-cestodal drug. The uptake of levamisole (LEV) through cestodeal tissues is studied through HPCL in this paper. High performance liquid chromatography technique has been utilized to know the uptake of levamisole in tissues of cestodes of Goat (*Monezia expensa*) in small ruminants. The drug was exposed to *M. expensa* by *in vitro* till its death or a parasite ceases its movement. The tissue/ part of proglattids of the *M. expensa* were homogenized with some modifications and levamisole extraction was performed with liquid phase extraction method. The evaporation of solvent was done and the residual cestodal tissues were cleaned by solid phase. After the solid phase extraction method, the recovery of drug, detection and quantification of levamisole from cestodal tissues was determined through Reverse Phase Column High Performance Liquid Chromatography (RP-HPLC). Levamisole (LEV) molecules assay was obtained on a C18 reverse-phase (20um, 6mm x 150mm) column at flow rate of 1ml/min using acetonitrile and ammonium acetate as mobile phase and UV detection was done at 254nm. The development of method of Levamisole (LEV) detection from cestodal tissues by HPLC *in vitro* samples has been demonstrated first time in Pakistan, which can provide the solution of parasitic control and provide in sight in to the uptake of anti cestodal drugs either against human or livestock parasites.

Keywords: RP-HPLC, Levamisole, Cestodes, *Ex vivo*, Tegument

INTRODUCTION

Levamisole is an anthelmintic and immunomodulator (Siwicki *et al*., 1989) belonging to imidazothiazole synthetic derivatives (Kamal *et al*., 2013) that are under trial as anti-cancer (Kamal *et al*., 2013). It was first discovered in 1966 and then to now used as veterinary dewormer for livestock (Ploeger *et al*., 1989, Miller, 1980). The levamisole has been reported to immobilize the *Caenorhabditis elegans (C. elegans)* by temporarily suppressing muscular activity by blocking the function of nicotinic acetylcholine receptors (nAChR), (Rieckher *et al*., 2011) for molecular study. Very less is known about its action on Cestodes/ tape worms either *in vivo* or *in vitro* studies in goat (*Capra hirsa*) (Elliot, 1986, Arundel, 1986). A lot of work has been done on the levamisole detection through HPLC from plasma (Vandamme *et al*., 1995), through meat samples (de Bukanski *et al*., 1991) and through intestinal fluids (Marriner *et al*., 1980) of livestock but little literature is available on the detection of levamisole from the tap worm tissues (Crosby, 1991). The concentration levels of the drug or metabolites has been measured in plasma, meat, GIT fluids and in milk (Chirollo, 2010) but cannot achieved from tissues of *Monezia expensa* tape worms/ platyhelminths commonly found in the intestine of free ranging goats. However the quantitative concentration of levamisole metabolites either *in vivo* or *in vitro* in target tapeworm/ platyhelminths tissue/ material after the routine administration of dewormer is required to know the exact amount of drug absorbed by the target parasite through paranchyma or tegument and to correlate information on pharmacokinetics of uptake of drug by tegument of parasite *in vitro* and same will help in *in vivo* in host. The same will provide the comprehensive informations on the uptake pattern of drug by the platyhelminths for the treatment purpose and *ex vivo* diffusion of Levamisole molecules in to tapeworms of livestock for the greater understanding of absorption process by the targeted parasites. These all factors will lead to better understanding of clinical efficacy of the levamisole in animals. The aim of the present work was to validate the quantification method for the detection of Levamisole molecules from tissues of *Monezia expensa* exposed with drug (Levamisole) *in vitro* through HPLC.

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MATERIALS AND METHODS

Design of the experiment

Collection of the Cestode (Monezia expensa)

The *M. expensa* specimens were collected from the small intestines of goats brought to the local abattoir of Multan, Pakistan. The live parasites were rinsed with normal saline for the removal of intestinal contents and placed in normal saline solution. The live Cestodes/ platyhelminths were brought to the laboratory of Parasitology, Department of Pathobiology, Faculty of Veterinary Sciences, Bahauddin Zakariya Univeristy Multan, Pakistan. The standard drug was purchased from Sigma-Aldrich as (−)-Tetramisole hydrochloride, (*S*)-(−)-6-Phenyl-2,3,5,6-tetrahydroimidazo[2,1-*b*] thiazolehydrochloride, L (−)-2,3,5,6-Tetrahydro-6 phenylimidazo [2,1-*b*] thiazole hydrochloride, having empirical formula $C_{11}H_{12}N_2S \cdot HCl$ (Kaya *et al*., 2011). Levamisole hydrochloride and was constituted up to 1.5 % in distilled water (Kaya *et al*., 2011) as most of veterinary formulations claim for the efficacy.

Ex vivo essay of M. expensa

The tapeworms (*M. expensa*) were placed in two groups in patri plates containing 1.5% Levamisole Hydrochloride solution and normal saline (control group). The live *M. expensa* were placed in the plates and time was noted for the effectiveness (Lethal Dose, LD_{50}) of the drug on the cestode. As the targeted parasite ceased its activity in any group first it was known as LD of that group. The parasite ceased in 5 seconds in Levamisole HCL group while remained live in normal saline (pH 7.4)for 30 minutes and so at 37°C.

Sample preparation

Cestode (*M. expensa*) obtained from small intestine of small ruminants. After *Ex vivo* incubation of *M. expensa* for 10 minutes to Levamisole HCL was removed from the petriplates and rinsed with distal water for the removal of extra drug residues on the tegument. The parasitic material was blotted on filter paper and made it ready for HPLC analysis for the determination of concentration of Levamisole in the tissues of cestode. Shortly after the incubatory phase the preparatory phase for the extraction was initiated.

Extraction of levamisole from the cestodal tissues

The extraction of the drug was done with certain modification (Mottier *et al*., 2003) About 1gm of the tissue of cestode was weighed and was homogenized in pistol and mortar. The homogenate was mixed with 3 ml of methanol and shaken on vortex shaker/ mixer (Mylab, LV-6, Seoulin Biosciences, Korea) over 5minutes to extract the drug analyte(s) present in tissue and then centrifuged by temperature controlled centrifuge machine (Mikro 200R, Hettich, Germany) at 2500 rpm for 10 minutes at 10°C. The collected methanol phase was concentrated to dryness in a vacuum container/ desiccator. The dry matter residue obtained was dissolved in 1ml methanol/ water (50/50 v/v). The molecules were extracted with certain modifications by using disposable C_{18} sterile syringe filter (Corning PES 0.20um, Corning incorporated, Corning, NY 14831, Part No. 431229) previously conditioned with HPLC grade 0.5 ml methanol followed by 0.5ml deionized water. Sample was injected into PES filter and washed with 0.5 ml HPLC grade water followed by 3ml/ 300ul methanol (mobile phase). 50 ul of the sample was analyzed by HPLC to measure the concentration of the drug to be assayed. The blank un-spiked drug was also done by injecting through PES 0.20 ul filters to get the spikes for known concentration of the drug.

Chemicals and equipments

Chemicals

Pure reference standards of levamisole were used for the validation as purchased from Sigma-Aldrich as (−)-Tetramisole hydrochloride, (*S*)-(−)-6-Phenyl-2,3,5,6-tetrahydroimidazo [2,1-*b*] thiazolehydrochloride,L(−)-2,3,5,6-Tetrahydro-6 phenylimidazo [2,1-*b*] thiazole hydrochloride, having empirical formula $C_{11}H_{12}N_2S{\cdot}HCL$ prepared in HPLC grade distilled water at final concentration of 1.5% as claimed by various manufacturers. The solutions were stored at 4°C till further use. All the solvents like acetonitrile and methanol for mobile phase or during extraction phase and drug validation phase were HPLC grade and water was double distilled and deionized using water purification system at Institute of Chemical Sciences.

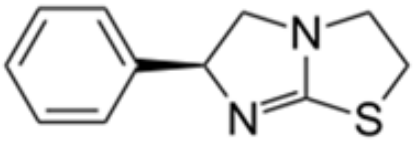

Fig. 1: Chemical structure of Levamisole molecules to be assayed by HPLC in the study

Equipment and chromatographic conditions

Chromatography was done on Shimadzu HPLC equipment (Shimadzu Corporation, Koyoto, Japan) with LC-10AT LC solvent pump, CTO-10A Column oven at 30C, CDD-6A Conductivity Detector, CBM-102 Communication Bus Module, an ultraviolet visible spectrophotometer detector (UV) (SPD-10AT), an automatic sample injector (SIL-10A) with 20ul loop. Data and chromatogram collected was analysed by manipulating the software Real Time Analysis Classic GC 10. For the analysis of levamisole by HPLC octadecylsilyl Shim-Pack CLC-ODS reverse phase column was employed. The length of the separation column was 6.0mm I.D. x 150mm L. with 5ul particle size. Elution from stationary phase was done at flow rate of 1ml/min using the HPLC grade acetonitrile and ammonium acetate buffer (0.025M, pH 6.6) as mobile

phase. The elution gradient linearity of acetonitrile and ammonium acetate buffer was set and maintained at 50:50 from 3 minutes to 15 minutes. The detection of levamisole molecules was done at wave length of 254nm.

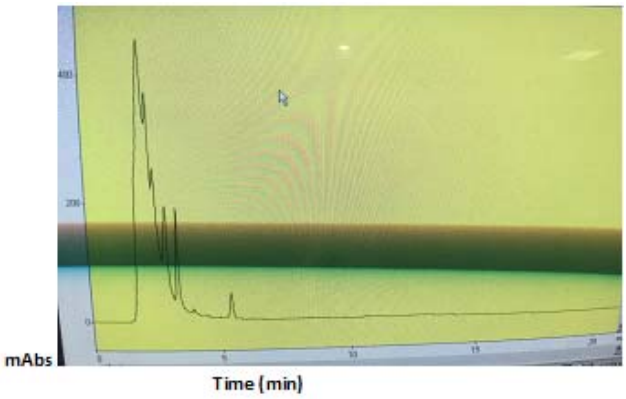

Fig. 1: Chromatogram showing the surge of levamisole molecules in cestodeal tissues

Calibration standards

The stock solution for Levamisole as tetramisole was prepared in HPLC grade distilled water at the concentration of 1.5% and stored at 4°C in refrigerator.

Validation of methods

Recovery of absolute analyte of levamisole

Recovery of absolute analyte of levamisole was assayed at 1.5% concentration. The coefficient of variant (CV) of drug recovery method was calculated statistically (Sari *et al*., 2014).

Linearity of standard curves

Linearity calibration curves provide the evidence that system is working/ performing properly through the concentration range of interest (Sistla *et al*., 2005, Snyder, 1997). The data for linearity was analysed statistically by using ANOVA and regression methods if differed from a straight line (Lavagnini and Magno, 2007).

Internal standard (IS)

The internal Standards (IS) of levamisole was obtained from the maximum peaks of the drug (Stubbings and Bigwood, 2009, Woestenboghs *et al*., 1981, Cherlet *et al*., 2000).

Precision and accuracy

Precision (inter-day and intra-day) was determined by evaluation of parasitic material and expressed in %CV while accuracy is defined as closeness between the experimentally measured and true value (Woestenboghs *et al*., 1981, Schewelor *et al*., 2004, Shabir, 2003, Menditto *et al*., 2007) and was expressed in relative error (%RE). The accuracy was measured by differences between observed result and calculated result.

Limit of detection (LOD) and limit of quantification (LOQ)

The minimum/ smallest concentration to be detected is called LOD (Armbuster *et al*., 1994). The LOQ was calculated as the lowest drug concentration on standard curve (SC) that could be quantified with not exceeding 20% precision and 20% accuracy of normal (Snyder, 1997, Mc Whinney *et al*., 2010).

RESULTS

HPLC is now widely used to estimate the quantity of drug in serum, milk, meat and body fluids in animals(Martos *et al*., 2010) but here in this work the aim was to estimate the drug molecules in the tissues of *Monezia expensa* that is a common small intestinal platyhelminths or Cestodes of goat (Hendrix and Robinson, 2014). In proglottids of *Monezia expensa* the molecules of levamisole were never reported by HPLC or any other technique except in other Cestodes (Crosby, 1991). This method was validated first time for the detection of Levamisole in tegument/ parenchyma of *Monezia expensa* through reverse-phase high performance liquid chromatography (RPHPLC). The C18 high performance column ODS reverse-phase were used as stationary phase that are commonly used and generalized in purpose for high speed liquid chromatography. The analyte was separated by RPHPLC with a gradient of ammonium acetate (pH 6.6) and acetonitrile. The total run time for the method was 20 minutes and the method employed for sample extraction was simple and highly efficient fig. 1. The representative chromatograms obtained are shown in fig. 2, with drug and fig. 3, the spike of levamisole as IS. The ultra violet (UV) detector used was set at 254 nm while other workers used to take chromatogram on 292nm (Mottier *et al*., 2003). *Ex vivo* incubation of levamisole was done for 5 seconds to 30 minutes that is very less time for incubation and exposure of the drug to the parasite while other workers exposed/ incubated the parasites for over 90 minutes (Lavagnini and Magno, 2007). In this work it is worth mentioning that a very less time drug exposure up to 5 seconds can be sufficient for the absorption into the tegument or parenchyma of the cestodal proglottids either complete or part of it. The *M. expensa* blank was free of interferences in the time region. The mean absolute recovery of drug was assessed at its concentration of 1.5%. The levamisole molecules have high affinity for lipid and protein present inside the body of Cestodes or nematodes.

DISCUSSION

The pharmacokinetics of the drug may include the affinity of drug-cell receptors present on the parasite, metabolism and up take in body of the host, absorption time and peak serum levels for the maximum concentration in the body tissues/ fluids of the host (Alvarez *et al*., 2007). The

Table 1: The Highest peak report of levamisole molecules in correlation with time in cestodeal tissues

S. No.	Time	Area	Height	Concentration
1.	1.407	8418	693	1.9466
2.	2.111	11128	1348	2.5731
3.	2.379	73288	7364	16.9466
4.	2.600	104407	6235	24.1424
5.	3.113	100036	5502	23.1319
6.	3.366	15453	1683	3.5732
7.	3.581	11614	1062	2.6856
8.	3.733	9406	813	2.1715
9.	4.028	6981	644	1.6143
10.	4.266	7983	649	1.8458
11.	4.423	15701	1050	3.6306
12.	4.808	6300	500	1.4569
13.	4.983	6605	414	1.5272
14.	5.308	2378	320	0.5500
15.	5.450	3505	321	0.8105
16.	5.668	9849	499	2.2775
17.	6.358	2068	114	0.4781
18.	7.842	5135	450	1.1874
19.	9.077	28151	426	6.5095
20.	17.817	4055	157	0.9377
Total		432461	30244	100.0000

Table 2: Highest concentration of levamisole molecules in cestodeal tissues in correlation to time

S. No	Time	Area	Height	Concentration
1.	1.948	631207	45679	5.2223
2.	2.888	1171360	131669	9.6912
3.	2.952	1225208	135285	10.1367
4.	3.426	8446011	937702	69.8777
5.	4.429	546675	17112	4.5229
6.	10.118	33315	2686	0.2756
7.	12.256	33078	2074	0.2737
	Total	12086852	1272207	100.000

penetration concentration available in the body of host available for cestode to make certain actions to control the parasites present in the body of the host because some time the body fat makes less availability of the drug for their actions. The drug optimal efficacy of drug depends on the entry and accumulation of the specific drug in the parenchyma/tegument or tissues of the targeted parasites. Major physicochemical determinant for the drug like levamisole to reach its therapeutic level in the target parasite is the determination of its affinity like lipophlic or lipoproteophilic or neucleic acid lipophilic affinity. Understanding the processes that regulate drug transfer into helminth parasites is an important aspect in improving the control of parasites in human and veterinary medicine and controlling the drug resistance problem reported commonly. The optimal drug concentration was obtained with equal ratio of methanol and water (50:50 v/v). The drug recovery from very minute portion of cestode that was exposed for incubation for 5 seconds to 30 minutes proved that RPHPLC chromatogram method was accurate, reliable and reproducible method for levamisole molecules detection in platyhelminths specially *Monezia expensa* in goat as chromatogram showing in table 1. This method could be a best example for the trans-tegumental-diffusion of the drug and its recovery by RPHPLC as shown in table 1 &2. The results here demonstrates that the nature penetration of drug into *M. expensa* like platyhelminths can help in better understanding of the affinity and penetration capability of the levamisole drug (Wang, 2013) where most of the mysteries are to solved in the veterinary sciences drug discovery and drug delivery and up take mechanisms specially in the field of parasitology.

CONCLUSION

This is the ever first report of recovery of levamisole from goat GIT platyhelminth like *M. expensa* through fast drug recovery method RPHPLC. The method is simple, accurate and reproducible for the recovery of levamisole

from parasitic parenchyma/ tegument. It has been gleaned that the method could deliver the beneficial informations regarding the pharmacokinetics of the drug and better understanding the dilemma of drug resistance mostly reported in anthelmintics by the parasites. This method can measure the lowest concentration of the drug up taken by the tegument for a very meager exposure by *Ex vivo*. The informations obtained could be beneficial to solve the future problems to be addressed in human and veterinary parasitology.

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