Original Article

Development of Fast, Precise and Selective RP-HPLC Methods for Identification of Possible Degradation Products of Ivermectin in Isolated Rat Hepatocytes and in Different pH Media

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ABSTRACT

Aim: A new stability indicating RP-HPLC based methods were developed to identify the possible degradation products of Ivermectin in isolated rat hepatocytes and in different pH media using a C_{18} RP column. Complete validation, including linearity, accuracy, recovery, precision, robustness, stability, and peak purity, was performed. **Materials and Methods:** HPLC system of UltiMateDionex 3000 DAD with LiChrosorb C_{18} Column were used in this study. During the investigation, for the removal of interfering fraction of isolated rat hepatocytes a purification procedure, consisting of protein precipitation followed by double filtration was developed. **Results:** Method I was applied effectively and the results indicated two major hepatic metabolites of Ivermectin. Method II was developed to monitor the chemical stability in close to physiological conditions. The appearance of a new peak and the proportional decrease in the sample concentration indicate a liability in buffer with pH12. **Conclusion:** The obtained results demonstrated that the degradation products of Ivermectin in hepatic cells and in alkali media are different.

Key words: Ivermectin, *in vitro,* Metabolism, Chemical stability, RP-HPLC.

INTRODUCTION

Ivermectin is a semi-synthetic macrocyclic lactone, which consists of 22, 23-dihydroavermectin B_{1a} (80%) and 22, 23-dihydroavermectin B_{1b} (20%). Avermection B_{1a} has been utilized for the semi-synthetic route of Ivermectin and even though the effect of the product was close to that of Avermectin B_{1a}, the toxicological data have shown significant difference between both molecules.[1]

Ivermectin as a broad-spectrum drug possesses numerous effects which are used for treating onchocerciasis, strongyloidiasis, enterobiasis, ascariasis, filariasis, trichuriasis and scabies, in humans.[2-5] It has proven its activity in the treatment of a number of viral infections such as Dengue virus, West Nile virus, Newcastle virus.[6-8] In the last year, a number of studies are performed on its antiviral effect against SARS –CoV-2 and its use in both treatment, and prevention.[9,10]

The evaluation of the chemical liability is an important step in the monitoring of the pharmacokinetics behavior of biologically active molecule pointed to facilitation in the search for the most appropriate dosage form.[11] Therefore, the quality guidelines of ICH include the conduct of stability studies prior to and during the development of novel drugs, and application of pharmacologically active substances based on their exposure to various conditions as air, light, elevated temperature and humidity.[12]

Frequently some *in vitro* studies have been applied as a simulated monitoring mechanism to characterize the drug metabolism.

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Thus for identification of the pharmacological and toxicological responses, rat hepatocytes are often utilized.[13]

Several studies of Ivermectin have proven that it is extensively metabolized by cytochrome P450 enzymes in human liver. The structures of 10 metabolites in human liver microsomes have been determined by ^{1}H-NMR and LC/MS,[14] with more than 13 metabolites identified using human liver microsomes, human recombinant enzymes, hepatocytes and blood samples. The data revealed that the major pathways of IVM-B_{1a} metabolism in humans include methyl-hydroxylation, O-demethylation and oxidation reactions.[15]

The aim of this study is to develop reliable, sensitive and rapid RP-HPLC methods for investigation of possible metabolism of Ivermectin in isolated rat hepatocytes and identification of its chemical stability under different pH conditions.

MATERIALS AND METHODS

Investigation of possible metabolites in isolated rat hepatocytes

Animals

Male Wistar rats (body weight, 200–250 g) were used. Rats were housed in plexiglass cages (3 per cage) in a 12/12 light/dark cycle, temperature 20 ± 2°C. Food and water were provided *ad libitum*. Animals were purchased from the National Breeding Centre, Sofia, Bulgaria. All performed procedures were approved by the Institutional Animal Care Committee and were in accordance with European Union Guidelines for animal experimentation.

Isolation of hepatocytes

Rats were anesthetized with sodium pentobarbital (0.2 mL/100 g). *In situ* liver perfusion and cell isolation were performed as described by Fau,[16] with modifications. After portal catheterization, the liver was perfused with 100 mL HEPES buffer (pH = 7.85), containing 10 mM HEPES, 142 mMNaCl, 7 mMKCl, 5 mM glucose + 0.6 mM EDTA (pH = 7.85), followed by 200 ml HEPES buffer (pH = 7.85), without any addition and finally 200 ml HEPES buffer containing collagenase type IV (50 mg/200 mL) and 7 mM $CaCl_2$ (pH = 7.85). The liver was excised, minced into small pieces and hepatocytes were dispersed in 60 mL Krebs-Ringer-bicarbonate (KRB) buffer (pH = 7.35), containing 1.2 mM KH_2PO_4, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5 mMKCl 5 mM $NaHCO_3$, 4.5 mM glucose and 1% bovine serum albumin. After filtration, the hepatocytes were centrifuged at 500 g for 1 min and washed 3 times with KRB buffer. Cells were counted under the microscope and the viability was assessed by Trypan blue exclusion (0.05%).[16] Initial viability averaged 89%. Cells were diluted with a KRB, to make a suspension of about 3×10^6 hepatocytes/mL. Incubations were carried out in 25 mL Erlenmeyer flasks. Each flask contained 3 mL of the cell suspension (i.e. 9 x 10^6 hepatocytes). Incubations were performed in a 5% CO_2 + 95% O_2 atmosphere.

Incubation of Ivermectin in isolated rat hepatocytes

The hepatocytes were incubated at KRB buffer with 10 μM Ivermectin for 2 hr.

Sample handling before RP-HPLC analysis

Several diverse methods were applied in order to achieve the optimal separation from the accompanying biological impurities of the extracted analyte. Initially, the volume of the used methanol was altered ranging from 400μL to 1 mL. Additionally, the time and the revolutions needed for the final precipitation were modified.

For the current analysis, the optimal protocol was obtained after precipitation with 1 mL HPLC grade methanol and centrifugation for 15 min at 14 000 rpm. The supernatant solution was double filtered through PVDF sterile syringe filters (firstly through 0,47 μm, and through 0.22 μm).

Identification of chemical stability in close to physiological conditions

Buffer preparation

Preparation of buffers pH 1.2

Measured amounts of 6.57 g of KCl (dissolved in water free from CO_2) and 119.0 mL of hydrochloric acid (0.1 mol/L) were dissolved in 1000 mL distilled water.[17]

Preparation of buffer pH 7.4

Measured amounts of 2.38 g of sodium hydrogen phosphate dihydrate, 0.19 g of potassium dihydrogen phosphate dihydrate and 8.0 g NaCl were dissolved in 1000 ml distilled water.[17]

Preparation of buffer pH 12

Buffer was prepared by mixing accurate volumes of the primary salt solutions- 100 mL 0.05 M disodium hydrogen phosphate and 53.8 mL 0.1 M sodium hydroxide ad 200 mL distilled water. Each buffer was filtered through a membrane filter (0.47 μm) using a Millipore glass filter holder.

Sample preparation

For the indicated stability evaluations 10mg of Ivermectin were dissolved in 10 mL methanol (gradient grade, ≥99.8%) (Concentration in stock 1 mg/mL).

Thereafter, 5 mL of the stock solution was pipetted out in a 10 mL flask and the corresponding buffer of pH 1.2, pH 7.4 and pH 12.0 was added up to the mark in order to acquire the discussed pH metrics.

Analytical procedures

The HPLC system consists of UltiMateDionex 3000 SD pump connected to UltiMateDionex DAD 3000 detector. HPLC separations were performed on a LiChrosorb C_{18} Column (5 µm, 15 x 0.46 cm), conditioned at 25°C in a column oven.

The applied mobile phase for investigation of possible metabolites consists of acetonitrile: distilled water: methanol in ratio 5/35/60(v/v/v).

The mobile phase for chemical stability evaluation consists of the same solvents in a ratio 20/60/20 (v/v/v). For both methods the flow rate was set to 0.8 mL/min with injection volume of 10 µL.

RESULTS AND DISCUSSION

Methods development

The methods were developed and optimized by evaluating changes in chromatographic conditions including mobile phase contents, flow rate, temperature and wavelength of the detector.

The applied RP-HPLC C_{18} column was selected after a detailed literature survey. A number of mobile phases were used, where in order to achieve good separation for both analytical procedures was determined that two different methods are necessary.

In the search for good separation and suitable peak forms, we examined all foresaid buffers. Series of trials were performed, which demonstrated that introducing a buffer with different pH values in the mobile phase leads to poor separation of the chromatographic peaks (Figure 1).

The necessity of alteration in the ratio of applied solvents for chemical stability evaluation was based on poor separation achieved with the mobile phase used in the experiment for evaluation of possible metabolic transformations of Ivermectin incubated with isolated rat hepatocytes, as visible on Figure 2.

Based on the performed experiments as most appropriate for both methods were determined to be the conditions presented in Table 1.

Validation

The developed RP-HPLC methods were validated according to the following parameters: specificity, linearity, precision and accuracy.

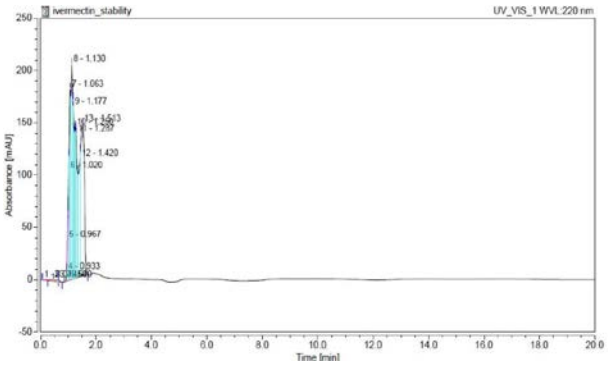

Figure 1: Poor separation using buffer included in the mobile phase: CH_3CN: Buffer pH 3: CH_3OH (5:35:60).

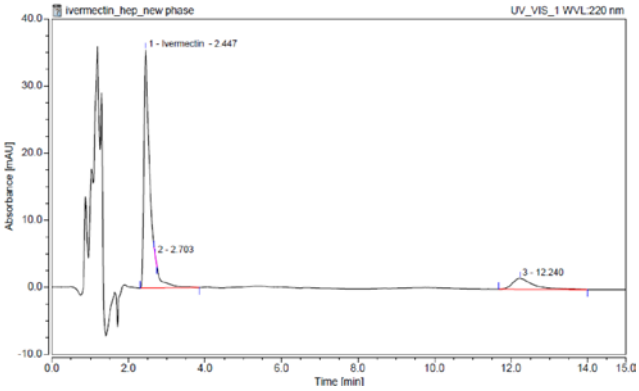

Figure 2: Poor separation in chemical stability evaluation using mobile phase: CH_3CN: H_2O: CH_3OH (20:60:20).

Table 1: Chromatographic parameters selected as most appropriate for the chosen analytical procedures.

Parameter	Method I	Method II
Column temperature	25°C	25°C
Flow	0.8 ml/min	0.8 ml/min
Inject volume	10 µl	10 µl
Run time	15 min	20 min
Wavelength	220 nm	220 nm

Specificity

No significant interfering peaks (peak area >0.1%) were observed during the analysis of the compounds and the mixtures in blank solution.

Precision

The method's precision was evaluated by analyzing six samples of Ivermectin at 100% level. The Relative standard deviations (RSD) were 0.2478% (Method I) and 0.0367% (Method II) ($n = 6$), thus meeting the criteria of RSD ≤ 2%. These results demonstrate that the methods are precise for determination of Ivermectin.

Linearity

In this study, the linearity of Ivermectin was evaluated from 50% to 250% of the nominal concentration - 500µg/mL. Linear regression was used to process the calibration data. The correlation coefficient of linearity of the both methods was 0.9989, which indicated good correlation between the peak areas and the range of the concentrations analyzed. The methods are proven to be linear within the investigated range (Figure 3).

Accuracy

The accuracy of an analytical procedure expresses the closeness of results obtained by that method to the true value. The percentage recovery and RSD were calculated for all active ingredients; all the results are within limits. Acceptable accuracy was within the range of 99.98% to 100.4% recovery and not more than 2.0% RSD, as demonstrated in Table 2.

The validated methods were used to evaluate the biotransformation and the chemical stability of Ivermectin under the above defined conditions.

Investigation of possible metabolites in isolated rat hepatocytes

According to the literary data, an appropriate method for purification of the analyzed sample consists of the utilization of C_{18} cartridges. The latter sample treatment protocol has been described by Meier and Blaschke in which paper the Praziquantel metabolites in isolated rat hepatocytes have been investigated.[18] Another procedure includes purification based on pressurized liquid extraction. Hansen *et al.* applied this method to determine to Toltrazuril and its two metabolites.[19]

Based on this information we developed a purification procedure, consisting of standard protein precipitation. For the removal of interfering fraction of samples, four different pretreatment clean-up combinations of conditions were applied (Table 3). As observed in the first three cases, after centrifugation the processed solutions remained opalescent and not appropriate for chromatographic analysis. In an attempt to improve the quality of the solution the method was optimized by changing the revolutions per minute and the duration of centrifugation (Table 3).

The purified by the above developed procedure samples were chromatographed under the conditions following the developed Method I. This method demonstrated that the analyzed compound has gone throughhepatocytic metabolism, as visible on Figure 4.

As demonstarted in Figure 4A, only one peak corresponding to the retention time of Ivermectin, at a concentration of 500 µg/mL, is observed. However, after being incubated for 15 min in isolated rat hepatocytes, the concentration of Ivermectin dropped to

Table 2: Evaluation of the accuracy of the developed methods.

Compound	Spiked level (µg/mL)	Replicate number	Recovery (µg/mL) Method I	Recovery (µg/mL) Method II	% Recovery Method I	% Recovery Method II	% RSD Method I	% RSD Method II
Ivermectin	250	1	249.74	249,97	99.9	99,9	0,069	0,108
		2	250.00	250,22	100.	100,1		
		3	249.61	249,68	99.9	99,9		
	500	1	500,08	499,94	100.0	99,9	0,069	0,027
		2	499,40	500,03	99.9	100,0		
		3	499,59	499,76	99.9	99,9		
	750	1	748,75	750,91	99.8	100,1	0,214	0,124
		2	751.75	749,28	100.2	99,9		
		3	749.25	749,30	99.9	99,9		

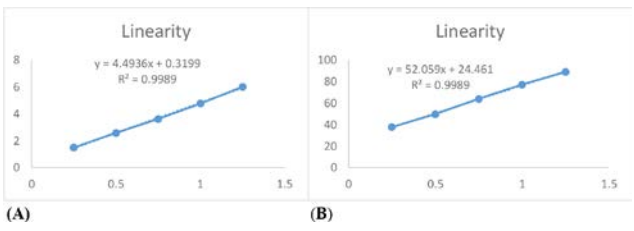

Figure 3: Linearity of the RP-HPLC Method I (A) and Method II (B).

Table 3: Different pretreatment clean-up combination.

Sample (µL)	Methanol (µL)	Speed (rpm)	Time (min)	Result
200	200	8000	10	opalescence
200	400	9000	12	opalescence
400	1000	10000	15	opalescence
400	1000	14000	15	lack of opalescence

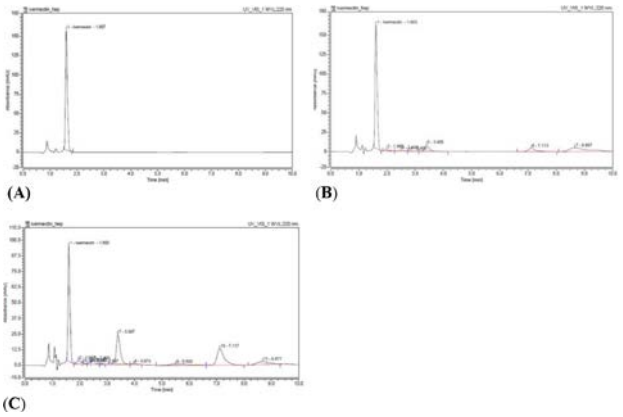

Figure 4: Chromatograms demonstratingthe hepatocytic metabolism of Ivermectin at 0th min (A), at 15th min (B) and at 30th min (C).

489.6 µg/mL and several additional peaks were detected (Figure 4B). In the 30th min mark, the presented on Figure 4 chromatogram C, the area of the new identified peaks was found to increase, while the area of the peak corresponing to the analyzed Ivermectin was observed to be reduced to 292 µg/mL.These observations led to the conclusion that the tested compound is subjected to metabolic degradation.

The developed RP-HPLC method identified several new peaks, where the areas of two of them substantially increased during the incubation which corresponds to the expected results pointing presence of metabolites. The chromatographic behavior of these two major peaks designated that in isolated rat hepatocytes the major metabolic pathways are methyl-hydroxylation and O- demethylation, which is in agreement with the previously published data.[20]

Evaluation of chemical stability in close to physiological conditions

On Figures 5 and 6 the chromatograms of the samples incubated with buffers 1.2 and 7.4, respectively are presented. The absence of new peaks with a retention time different from that of the studied molecule reveals that the structure is stable under these conditions.

In addition, the chemical stability in strong alkali media of pH 12.0 and temperature of 37°C was also investigated. The appearance of a new peak and the proportional decrease in the sample concentration indicate a structural change (Figure 7) in the analyzed Ivermectin.

The obtained results demonstrate that the degradation products of Ivermectin in hepatic cells and in alkali media are different. These observations led to the necessity of identification of chemical instability products which will be our next aim.

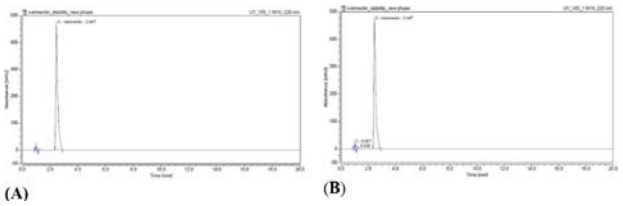

Figure 5: Chromatograms indicating the behavior of Ivermectin in the presence of buffer with pH 1.2 and at 37°C at 0th min (A) and at 210th min (B).

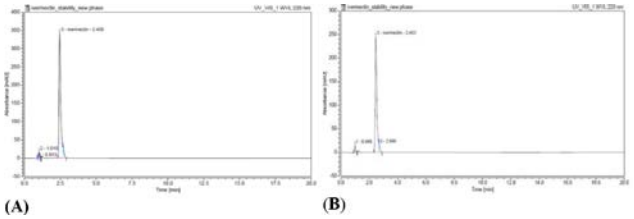

Figure 6: Chromatograms indicating the behavior of Ivermectin in the presence of buffer with pH 7.4 and at 37°C at 0th min (A) and at 210th min (B).

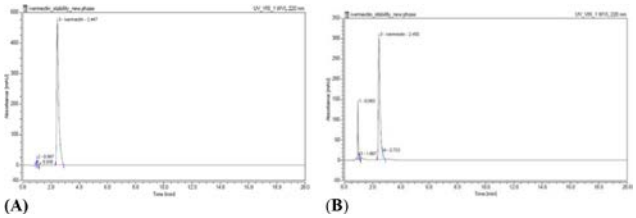

Figure 7: Chromatograms indicating the behavior of Ivermectin in the presence of buffer with pH 12 and at 37°C at 0th min (A) and at 30th min (B).

CONCLUSION

In the current study two precise, specific, accurate and stability indicating RP- HPLC methods were developed and validated. The applied Method I was successfully used for monitoring the biotransformation of Ivermectin in hepatic cells, while the developed Method II indicated that the tested molecule is sensitive in aqueous media with pH 12 and temperature of 37°C and is stable under the other two evaluated pH values. The analysis showed that the major hepatic metabolites are obtained through methyl-hydroxylation and O- demethylation.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

^{1}H-NMR: Proton nuclear magnetic resonance; **HPLC:** High performance liquid chromatography; **ICH:** International Council for Harmonisation; **IVM:** Ivermectin; **LC/MS:** Liquid chromatography–mass spectrometry; **SAR:** Severe acute respiratory; **CoV2:** Syndrome coronavirus 2.

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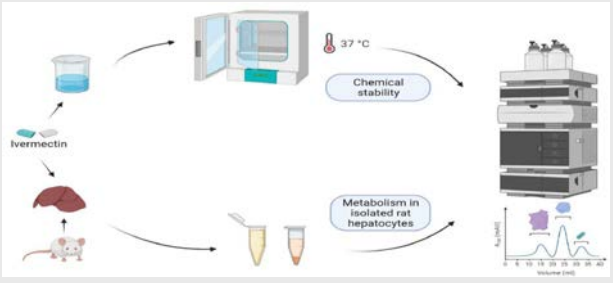
PICTORIAL ABSTRACT



SUMMARY

Ivermectin is a semi-synthetic macrocyclic lactone with a broad anti-parasitic spectrum. In recent years, anti-viral activity was also demonstrated *in vitro*. Thus, it was in our interest to develop fast, precise and selective RP-HPLC methods for identification of possible degradation products of Ivermectin in isolated rat hepatocytes and in different pH media. Our investigation showed the appearance of a new peak which indicates a liability in buffer pH12. In addition, the obtained results demonstrated that the degradation products of Ivermectin in hepatic cells and in alkali media are different.

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