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RESEARCH ARTICLE

Determination of isohydrafural and methyluracil in ear drops by high performance liquid chromatography

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Objective: The aim of current study was the development and validation of a reversed-phase high-pressure liquid chromatography (RP-HPLC) method for the quantitative determination of two active ingredients, isohydrafural (IHF) and methyluracil (MU) in fixed dose combination ear drops. **Methods**: An efficient separation of the two compounds was achieved on a Teknokroma C1 5µm (150*4,6mm) column, with a methanol: water 60:40 (V: V) mobile phase, at 300C temperature and 0.6 mL/min flow-rate. The total analysis time was 5.5 min. **Result**: The verified validation parameters were: linearity, selectivity, specificity, precision (repeatability and reproducibility), robustness, limit of detection and quantification. **Conclusions**: Good separation and lack of interference from other chromatographic peaks, rapidanalysis times were obtained. The newly developed chromatographic method can be applied for the analysis of fixed dose combination ear drops with isohydrafural and methyluracil.

Keywords: high pressure liquid chromatography, method validation, ear drops, isohydrafural, methyluracil

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Introduction

The modifications of the chemical structure of nitrofurans are carried out in the direction of obtaining less toxic compounds, with higher efficacy and a wider spectrum of action, but also with better bioavailability [1-9]. Isohydrafural (Figure 1), an autochthonous substance, is a good example in this respect. Within the State University of Moldova, the hydrazone derivative of 5-nitrofuran - isohydrafural (IHF) was synthesized, whose antibacterial action was investigated in the Intrahospital Infections Laboratory of „*Nicolae Testemițanu*" SUMPh. The compound exhibits pronounced antibacterial action (bactericidal and bacteriostatic), surpassing its analogues in terms of potency and safety [10]. IHF (5-nitro-2-furan aldehyde isonicotinoylhydrazone) was obtained by the condensation of 5-nitrofuran aldehyde with isonicotinic acid.

The substance possesses significant antibacterial properties against both aerobic and anaerobic bacteria, 2 to 7 times higher than nitrofural. The bactericidal activity in concentrations between 1.25 - 5.0 µg/mL on all strains of the genus *Staphylococcus* that have been investigated, makes this remedy one of real value. Also, IHF is less toxic (LD50 = 990 mg/kg) than nitrofural (LD50 = 166.7 mg/kg). Another major advantage is its effectiveness when applied topically [10].

A rather important role in the treatment of auricular infection is the stimulation of reparative processes in the epithelization phase for the formation of new tissue [11, 12].

Methyluracil (MU) (Figure 2) has been used successfully as a tissue repair stimulator in the form of ointment and suppositories. MU has several advantageous properties, which determine its wound healing effects: anti-inflammatory, anabolic and immunostimulatory [13-16]. This substance has not previously been used in ototopic pharmaceutical forms.

For the first time, a formulation of ear drops containing IHF and MU as active principles, based on PEG 400 and propylene glycol, was developed; nipagin and acetate buffer was added to ensure antimicrobial and optimal pH stability. The compatibility of active substances and excipients has been demonstrated [17]. *In vivo* studies on rats have established that IHF and MU do not exhibit ototoxicity [18].

The quantitative analysis of IHF and MU in pure substances and in monocomponent forms can be performed by chemical (neutralization in anhydrous medium, iodometric) and physico-chemical (UV-VIS spectrophotometric, HPLC) methods [19, 20].

The aim of the present study was the development and validation of a reversed-phase high-performance liquid

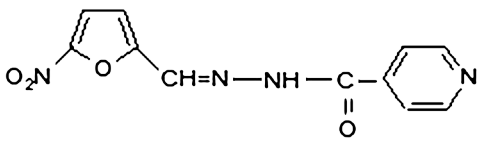

Fig. 1. Structural formula of IHF

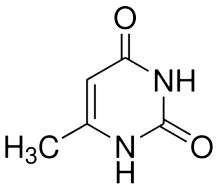

Fig. 2. Structural formula of MU

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chromatography (RP-HPLC) for the quantitative determination of the two active pharmaceutical substances (IHF, MU) in ear drops.

Methods

Active substances: MU (pharmaceutical secondary standard, USP,PhEur), was purchased from Sigma–Aldrich (HPLC standard GmbH, Germany). IHF was obtained and standardized in the Laboratory of the Department of Organic Chemistry of the State University of the Republic of Moldova. PEG 400 and propylene glycol were purchased from Sigma–Aldrich (Fisher Chemical, Belgium). Three experimental series (01, 02, 03) of ear drops containing IHF and MU were prepared in the Laboratory of Drug Development, Analysis, Standardization and Control of the „*Nicolae Testemițanu*" SUMF Scientific Center of Medicines, with concentration of IHF 0.05 g/100 g and MU 2.00 g/100 g.

Chemicals: Acetic acid, sodium acetate, hydrochloric acid, sodium hydroxide, analytical grade 30% aqueous hydrogen peroxide (Riedel-de-Haen, Reag. Ph. Eur., Germany),HPLC grade methanol were purchased from Honeywell (Riedel-de-Haen, for HPLC, gradient grade, ≥99.9%, France).

Apparatus: A HPLC system Shimadzu-20A with UV-VIS detection (Kyoto, Japan) was used for method development. The active ingredients (IHF and MU) were separated on a column Teknokroma C1 5µm (150x4,6mm) coloumn.

Chromatographic separation conditions: To prepare the mobile phase, methanol and purified water were mixed in a ratio of 60:40 (v/v); the mixture was filtered through a Millipore XF 5423050 capron filter (0.45 µm) and subjected to degassing. The temperature of the chromatographic column was 30°C; injection volume 20 µl; mobile phase flow 0.6 mL/min; isocratic elution was used. IHF detection was performed at 360 nm , while MU detection took place at 244 nm. The retention time was 4.3 min for IHF and 3.5 min for MU.

Method development and validation

Preparation of IHF standard solution: Appropriate amount of IHF (5 mg) was weighed and transferred into a 25 mL volumetric flask, then dissolved and diluted with a mixture of methanol:water (60:40) mobile phase (by heating and shaking on the ultrasonic bath) to 25 mL. A volum of 0.5 mL of this solution was diluted with the same solvent to 10 mL to obtain a solution of 10 µg/mL IHF.

Preparation of MU standard solution: Appropriate amount of MU (200 mg) was weighed and transferred into a 25 mL volumetric flask, then dissolved and diluted with a mixture methanol:water (60:40) mobile phase to 25 mL. A volum of 0.5 mL of this solution was diluted with the same solvent to 10 mL to obtain a solution of 400 µg/mL.

Preparation of pharmaceutical sample solution: 1.0 ml of the ear drops solution was transferred to a 50 mL volumetric flask and diluted with the mobile phase methanol:water (60:40).

Preparation of the blank solution: All excipients without the active substances were mixed in the quantities provided for the preparation of the pharmaceutical form. 1.0 mL of the blank mixture was transferred to a 50 mL volumetric flask and diluted with methanol:water (60:40) mobile phase.

Chromatographic system control: After stabilizing the chromatographic column with the mobile phase for 10 min, 20 µl of standard solutions were injected until retention times of 4.3 min for IHF and 3.5 min for MU were obtained after two consecutive injections; 3 measurements for each standard solution were repeated. Column performance was verified, based on IHF and MU peaks, a lower limit of 2000 theoretical plates was set; the peak asymmetry coefficient, calculated at ½ of the peak height, did not exceed 1.5; the relative standard deviation (RSD) of the peak areas of the determined substances did not exceed 2.0%. [21].

Forced degradation studies

Degradation studies were performed to verify whether the proposed method is capable of separating analytes from chemically related substances formed upon degradation. The pharmaceutical form was subjected to various accelerated stress conditions: UV light irradiation, temperature, acid hydrolysis, basic hydrolysis and oxidative stress [22].

- Sample preparation for acid hydrolysis: 3.0 mL of pharmaceutical form was mixed with 3.0 mL 1 M hydrochloric acid solution, stired for 3 hours on a water bath at 70^0C.
- Sample preparation for basic hydrolysis: 3.0 mL of pharmaceutical form was mixed with 3.0 mL 1 M sodium hydroxide solution, stirred for 3 hours on a water bath at 70^0C.
- Preparation of sample for oxidative stress: 3.0 mL of pharmaceutical form was mixed with 3.0 mL of 5% hydrogen peroxide solution, stirred for 6 hours at 25^0C.
- Sample preparation for thermal stress: pharmaceutical form was placed in a thermostat at 60°C for 1 month.
- Sample preparation for exposure to light: 5.0 mL of pharmaceutical form was exposed to UV light (254 nm) for 7 days.

After exposure to various stressl factors, 0.5 mL samples were diluted with the mobile phase to a volume of 25 mL (concentrations of 10 µg/mL IHF and 400 µg/mL MU were obtained), filtered through membrane filters of 0.22 µm and subjected to chromatography by the proposed method.

Quantitative determination

For quantitative determination, 20 µl of sample and standard solutions were injected in five replicates, using the selected chromatographic conditions. The average values for IHF and MU peak areas in the chromatograms of the sample and the standard solutions were determined. The chromatograms of the standard solutions, the sample solu-

tion with concentrations of 10 µg/mL IHF and 400 µg/mL MU and for the blank solution are shown in Figure 3 and Figure 4.

The content of IHF and MU in the pharmaceutical form was calculated based on the results of standards.

Validation of the method

The method was validated according to the ICH guidelines [23].

Linearity. The linearity of the chromatographic method was evaluated (in the range of 8.0–12.0 µg/mL for IHF and 320.0 – 480.0 µg/mL for MU) using five concentration levels. Regression analysis was applied to determine the linearity (Table I). Five levels of concentrations were prepared for each of the three series of IHF and MU standard solutions. All determinations were repeated three times, and calibration curves were constructed [21, 23].

Sensibility The limits of detection (LOD) and quantification (LOQ) of IHF and MU were determined by measuring the signal-to-noise (S/N) ratio with RSD values (n = 3) lower than 10%, [21, 23, 24].

Accuracy. To determine accuracy, standard addition (sample enrichment) method was used. Solutions were prepared with concentrations of 80%, 100% and 120% level (8.0 µg/mL, 10 µg/mL, 12.0 µg/mL for IHF and 320 µg/mL, 400 µg/mL, 480 µg/mL for MU), and analyzed in triplicate and the recovery was calculated. The RSD values were evaluated for each concentration level (Table II) [21, 23].

Precision. In order to determine the precision of the method, the repeatability was evaluated for 6 samples, on the same day, (Table III) and intermediate precision, was determined performing 6 determinations in 2 different days, under the same analytical conditions.(Table IV) [21, 23].

Robustness. When determining the robustness, small variations were made in the chromatographic conditions: the flow rate of the mobile phase ± 0.1 mL/min, co-ratio of methanol and water in the mobile phase ± 2% and column temperature by ± 5°C (Table V) [21, 23].

Table I. Regression analysis in of the linearity study of HPLC determination method assay of IHF and MU from ear drops

Statistical parameters	IHF	MU
Level of significance, α	0,05	0,05
Number of determinations	30	30
R2	0.99987	0.99988
R	0.99993	0.99994
Standard deviation, s	318255.9	4420162.5
Slope, a	201269.6	70017.5
Intercept, b	-318216.4	235891.1

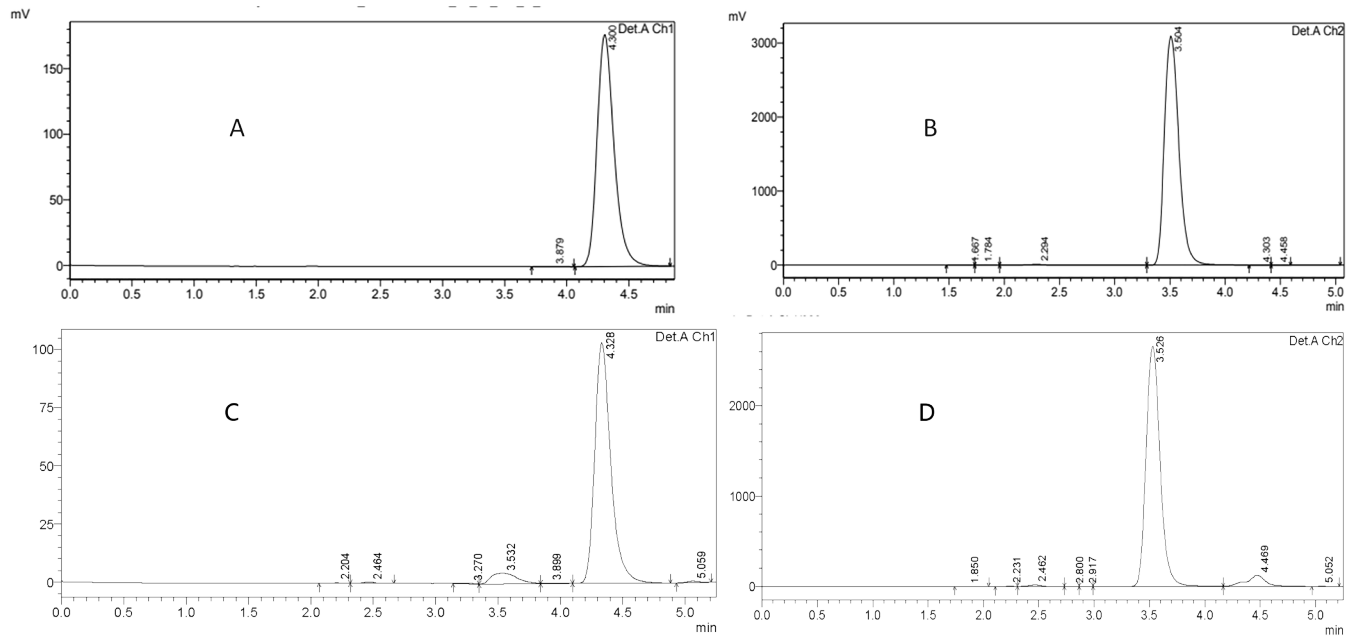


Fig. 3. Chromatograms of IHF (A, 360 nm) and MU (B, 240 nm) standard solutions and sample solutions at 360 nm (IHF, C) and 240 nm (MU, D)

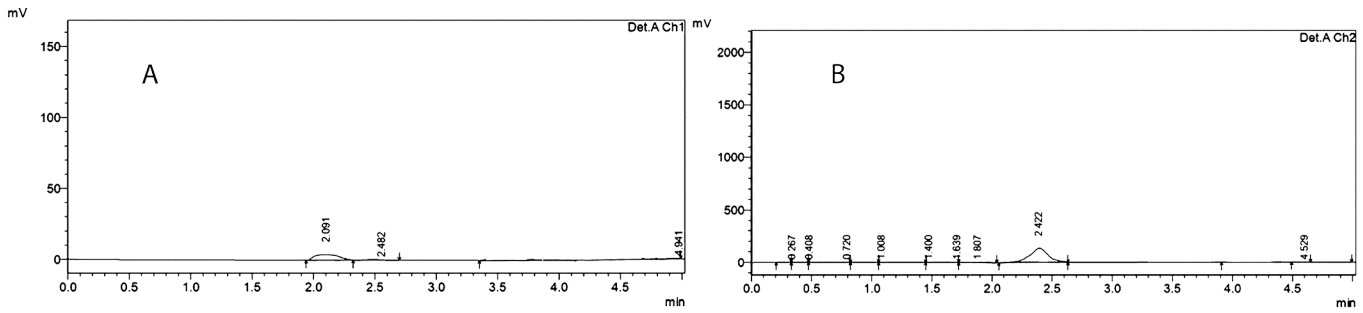


Fig. 4. Chromatograms of the blank solution at 360 nm (A) and 240 nm (B)

Table II. Accuracy determination of HPLC determination method assay of IHF and MU from ear drops

	Concentration levels, %	Determination number	Theoretical concentration of spiked sample, µg/mL	Peak area	Concentration in spiked sample, µg/mL	% Recovery	Average, % RSD
IHF	80	1	8	1287464.11	7.98	99.33	100.11
		2	8	1289905.64	7.99	99.67	
		3	8	1299085.86	8.04	101.33	1,07
	100	1	10	1695451.00	10.00	100.00	100.00
		2	10	1695449.01	10.00	100.00	
		3	10	1695459.41	10.00	100.00	0.000
	120	1	12	2095255.76	11.99	99.86	99.29
		2	12	2090355.37	11.97	99.57	
		3	12	2074029.30	11.89	98.43	0.57
MU	80	1	320	22678499.61	319.71	99.76	98.55
		2	320	22573079.80	318.20	98.50	
		3	320	22480531.64	316.88	97.40	0.80
	100	1	400	28249278.01	399.43	99.72	99.72
		2	400	28250149.12	399.44	99.72	
		3	400	28250098.37	399.44	99.72	0.00
	120	1	480	33919956.31	480.57	100.20	99.55
		2	480	33641474.81	476.59	98.78	
		3	480	33814742.41	479.07	99.67	0.72

Note: RSD – relative standard deviation

Table III. Repeteability determination of HPLC determination method assay of IHF and MU from ear drops

No.	Retention time, min		Peak area		Assay, %	
	IHF	MU	IHF	MU	IHF	MU
1	4.353	3.527	1522219	28260214	0.05394	2.00420
2	4.353	3.526	1512397	28359871	0.05359	2.01127
3	4.345	3.521	1520117	28099658	0.05387	1.99282
4	4.338	3.513	1510048	28162578	0.05351	1.99728
5	4.342	3.518	1521877	28352178	0.05393	2.01073
6	4.347	3.525	1534892	28642587	0.05439	2.03132
Average	4.346	3.522	1520258.33	28312847.70	0.05390	2.00790
RSD, %	0.138	0.154	0.578	0.676	0.578	0.676

Table IV. Intermediate precision determination of HPLC determination method assay of IHF and MU from ear drops

No.	Day 1						Day 2					
	Retention time, min		Peak area		Assay, %		Retention time, min		Peak area		Assay, %	
	IHF	MU	IHF	MU	IHF	MU	IHF	MU	IHF	MU	IHF	MU
1	4.353	3.527	1522219	28260214	0.05394	2.00420	4.356	3.528	1521289	28258971	0.05391	2.00412
2	4.353	3.526	1512397	28359871	0.05359	2.01127	4.353	3.526	1551578	28365214	0.05498	2.01165
3	4.345	3.521	1520117	28099658	0.05387	1.99282	4.344	3.523	1520001	28095641	0.05386	1.99253
4	4.338	3.513	1510048	28162578	0.05351	1.99728	4.337	3.511	1525697	28150018	0.05406	1.99639
5	4.342	3.518	1521877	28352178	0.05393	2.01073	4.342	3.517	1523149	28363578	0.05397	2.01154
6	4.347	3.525	1534892	28642587	0.05439	2.03132	4.347	3.525	1527741	28636398	0.05414	2.03088
Average	4.346	3.522	1520258	28312847	0.05390	2.00790	4.346	3.522	1528242	28311636	0.0542	2.0079
RSD %	0.138	0.154	0.578	0.676	0.578	0.676	0.138	0.154	0.771	0.682	0.771	0.682

Table V. Robustness determination of HPLC determination method assay of IHF and MU from ear drops

Variable parameters		Retention time, min		Peak area		Assay, %	
		IHF	MU	IHF	MU	IHF	MU
Mobile phase flow 0.5 ml/min		4.352	3.528	1524852	28260559	0.05403	1.93766
Mobile phase flow 0.7 ml/min		4.402	3.587	1527310	28354859	0.05412	1.94412
Methanol: purified water 58.8:41.2		4.345	3.522	1520148	28082949	0.05387	1.92548
Methanol: purified water 61.2:38.8		4.337	3.514	1545991	28158859	0.05478	1.93068
Column temperature 250 C		4.34	3.517	1524811	28378227	0.05403	1.94572
Column temperature 350 C		4.348	3.524	1548709	28846585	0.05488	1.97784
The average value	$\bar{x}$	4.354	3.532	1.53E+06	2.83E+07	0.05429	1.94358
Standard deviation	S2	5.82E-04	7.51E-04	1.48E+08	7.26E+10	1.86E-07	3.41E-04
	S	0.024	0.027	12166.964	269523.310	0.00043	0.01848
Relative standard deviation	RSD	0.554	0.776	0.794	0.951	0.794	0.951

Specificity. The specificity of the method was performed by analyzing the chromatograms of the analyzed samples and the blank solution at both wavelengths (Figures 3 and 4). The excipients of the pharmaceutical form did not interfere with the determination.

Statistical analysis. Statistical analysis was carried out by using the Statistical Package for the Social Sciences (IBM SPSS Statistics) 10.5 software [21].

Results

Method Development and Optimization.

A good separation of IHF and MU peaks was observed when using methanol and water as mobile phase in ratio of 60:40 and at a flow rate of 0.6 ml/min. The total running time was 5.5 minutes. The optimum temperature of the chromatographic column was 30^0C. Under optimised conditions, the retention times were 4.305±0.016 min for MU and 3.500±0.006 min for IHF, respectively. The developed method allowed the simultaneous determination of the two analytes in a single test (Figures 3 and 4).

Linearity. The results of the linearity study showed a linear relationship over the concentration range of 8-12 µg/mL for IHF and 320-480 µg/mL for MU. The evaluation of the residuals following the regression analysis demonstrated the accuracy of the obtained results. A normal distribution of the residuals was found (the probability value for the Shapiro-Wilk test is higher than 0.05). The results of the regression analysis are presented in Table I.

Calibration parameters indicate good linearity: correlation coefficients (R) and coefficient of determination (R^2) were higher than 0.9999 for both substances. After determining of the statistical significance of the regression coefficients, it was found that in the calibration curves for IHF and MU, the intercept does not significantly differ from zero ($p > 0.05$).

Sensibility. Limit of detection and quantification (LOD and LOQ) were 0.55 µg/mL for IHF, 8.38 µg/mL for MU; and 1.66 µg/mL for IHF and 25.39 µg/mL for MU respectively.

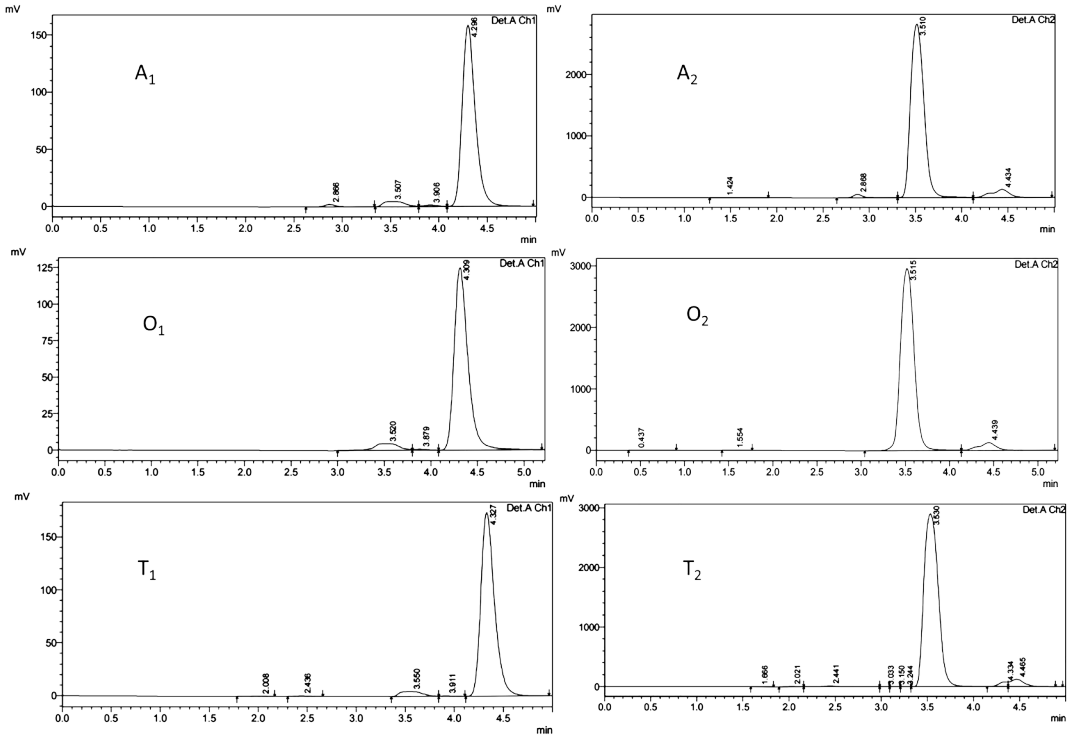
Specificity. As can be seen in Figures 3 and 4, there are no peaks corresponding to the retention times of the active substances in the chromatogram of the blank solution.

Accuracy. As indicated in Table II, RSD values were between 0.00 – 1.07. The recovery of the substance of 98.55% - 100.11%, indicates the accuracy of the method, the accepted limits beeing between 98.0% - 102.0%.

Precision. The precision evaluation showed an acceptable concordance between the results. RSD values, calculated for retention times, peak areas and analyte concentrations of less than 1% confirm the precision of the method (Table III and Table IV).

Robustness. After making some minor changes in the chromatographic conditions (co-ratio of solvents in the mobile phase, flow rate and column temperature) a good separation of the components was achieved, the calculated RSD values are within the limits and do not exceed 1.0%, which demonstrate the robustness of the method (Table V).

Forced degradation studies. Forced degradation studies showed that the investigated pharmaceutical form is resistant to heat (storage at 60^0C for 1 month), to oxidative stress (5% H_2O_2, stirring for 6 hours at 25^0C) and to acid hydrolysis (HCl 1M, 3 hours at 70^0C) (Figure 5).



Note: $*_{1=}$ 360 nm, IHF; $*_{2=}$ 244

Fig. 5. Chromatograms of sample exposed to acid hydrolytic stress conditions (A1 and A2), oxidation (O1 and O2), temperature (T1 and T2)

The chromatograms obtained after exposure to these stress conditions were identical to the chromatogram of the sample without exposure (Figure 3 C,D). Basic hydrolytic (1M NaOH, 3 hours at 70^{0}C) triggered IHF degradation, while MU was stable. Also, IHF showed degradation upon exposure to UV light (UV exposure 254 nm for 7 days). On the chromatograms of the sample solutions, after exposure to the mentioned stress conditions, additional well-defined peaks appeared at 360 nm; no degradation products were observed at 244 nm (Figure 6). The homogeneity of the IHF peak was also assessed. Peak purity values were over 98.61% for IHF at 360 nm.

Method application. The analysis (identification and dosage) of IHF and MU in ear drops (laboratory experimental series) was performed applying the validated HPLC method. The retention times of IHF and MU on the chromatograms of the sample solution were identical to those on the chromatograms of the standards. The content of active substances in the analyzed samples was calculated based on the calibration curves. The obtained results showed RSD values less than 1%, satisfactory accuracy and precision (Table VI).

Discussions

An HPLC method for the simultaneous quantification of two active substances (IHF and MU) in a fixed dose ear drop combination for the treatment of otitis was developed for the first time. In the method validation process, it was found that all parameters were within the limits accepted by the ICH guidelines, establishing a good linearity, and sensitivity. The results obtained confirmed the specificity of the method, precision, accuracy and robustness, RSD values of less than 2% are within acceptable limits. Forced degradation studies confirmed the method's capability to distinguish the active pharmaceutical ingredients from degradation products under various stress conditions. The pharmaceutical form exhibited resistance to heat, oxidative stress, and acid hydrolysis, with IHF showing instability under basic hydrolytic stress and UV light exposure.

Conclusions

The study successfully developed and validated a RP-HPLC method for the simultaneous quantification of two active pharmaceutical ingredients, IHF and MU, in ear drops. The method demonstrated good linearity, sensitivity, specificity, precision, accuracy, and robustness, meeting the criteria set by the ICH guidelines. The method allowed for the analysis of both active ingredients in a single test, providing a practical and efficient approach for quality control in pharmaceutical formulation.

The applicability of the developed method was demonstrated through its successful application in analyzing laboratory experimental series of ear drops, providing accurate and precise quantification of IHF and MU. The results support the method's potential integration into the quality specifications for combined ear drops containing IHF and MU.

Author contribution

LU: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Validation; Visualization; Writing – original draft; Writing – review & editing.

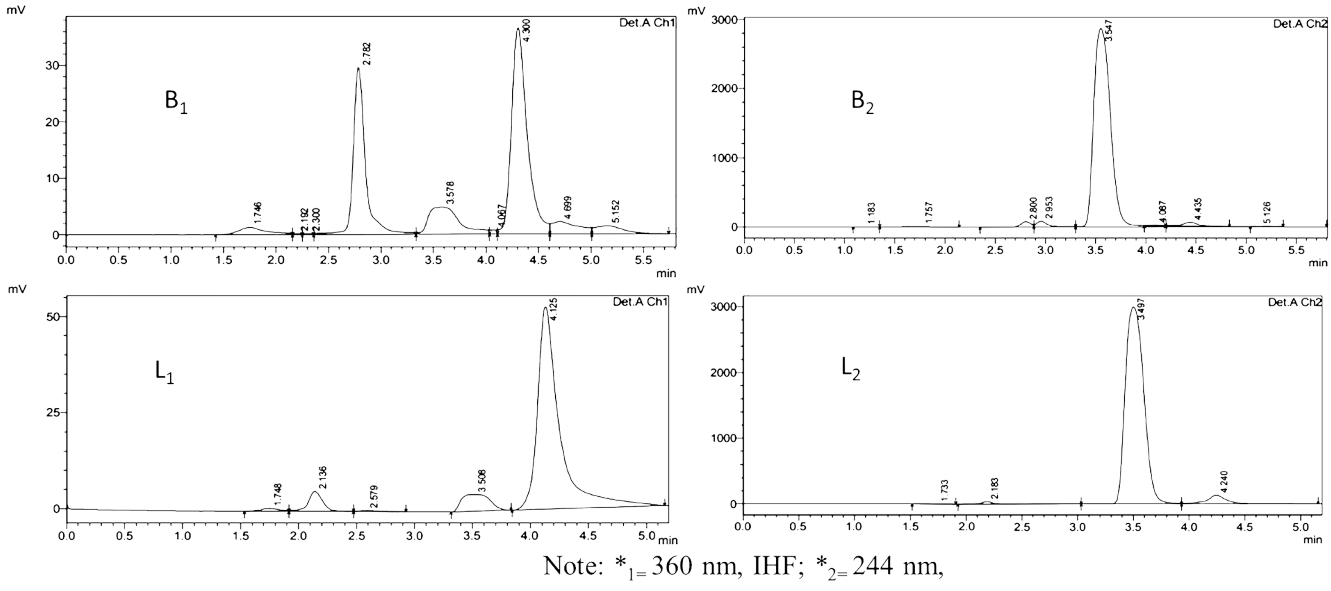


Fig. 6. Chromatograms of sample exposed to basic hydrolytic stress conditions (B1 and B2), light (L1 and L2)

Table VI. Determination of IHF and MU in ear drops (experimental series) by the developed and validated HPLC method

Test substance	Declared content, g	Determined content (n=6, α=0.05) (g per 100 g of ear drops)	Relative standard deviation (RSD, %)
IHF	0.05	0.0539±0.0003	0.578
MU	2.00	2.0079±0.0142	0.676

Conflict of interest

None to declare.

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