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GREENING OF THE METHOD FOR SIMULTANEOUS DETERMINING THE ENISAMIUM IODIDE AND TILORONE DIHYDROCHLORIDE USING GC-FID ASSAY

Anastasiia Belikova, Ludmila Sidorenko, Liudas Ivanauskas, Vasyl Chornyi, Anna Kononenko, Alla Koval, Victoriya Georgiyants

Pharmaceutical companies in Ukraine aspire to develop their innovative medicinal products and successfully introduce them to the global market. However, along with the prospects of increased usage of these pharmaceuticals, there arises a challenge of heightened waste production, making them a part of the over twenty million tons of PPCPs produced annually. Consequently, one of the tasks in producing new pharmaceuticals is the development of methodologies and approaches not only for quality control but also for their determination in the environment matrices.

The aim. *Develop and validate GC-FID chromatographic method for the simultaneous determination of Enisamium iodide and Tilorone dihydrochloride, evaluate their applicability, and compare their "greenness" with the previously developed HPLC method.*

Materials and methods. *The determination of the Tilorone dihydrochloride and Enisamium iodide was carried out by gas chromatography with a flame ionization detector using the Rxi-5 ms (30 m long, 0.25 mm outer diameter and 0.25 μm liquid stationary phase thickness)*

Results. *Chromatographic GC-FID methods have been developed for the simultaneous determination of Enisamium iodide and Tilorone dihydrochloride. Optimal sample preparation conditions were established, and a validation process was conducted. A comparison with the previously developed HPLC method was made regarding "greenness."*

Conclusions. *The developed GC-FID methodology is accurate and more environmentally friendly compared to the previously established methods. It can be recommended to determine Enisamium iodide and Tilorone dihydrochloride in the environmental matrices. It is considered environmentally friendly based on the overall GREENness (AGREE) scale, scoring 0.73 (>0.70), which demonstrates the environmentally favourable nature of the proposed analytical approach*

Keywords: *Enisamium iodide, Tilorone dihydrochloride, GC-FID, method development, validation, "green" analytical analysis, pharmaceutical wastes*

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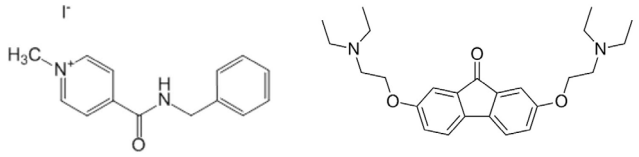
1. Introduction

The Ukrainian pharmaceutical industry is rightfully considered one of the most advanced in Europe. This recognition is not solely due to the many manufacturers capable of supplying citizens with generic drugs. Ukrainian pharmaceutical companies aspire to develop their innovative medicinal products and successfully introduce them to the global market. Examples of such drugs include Enisamium Iodide (Amizon, Farmak) and Tilorone dihydrochloride (Amixin, Interchim).

Enisamium iodide (Fig. 1), developed at the Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine, has undergone a full cycle of experimental and clinical studies and has been approved by the Pharmacological Committee of the Ministry of Health of Ukraine for use as an antiviral, anti-inflammatory, and antipyretic agent [1–3]. Tilorone dihydrochloride is an oral synthetic inducer of endogenous α-, β-, and γ-interferons produced by immune competent cells, which is a 2,7-bis [2-(diethylamino) ethoxy]-fluoren-9 dihydrochloride [4, 5] (Fig. 1). In the mid-1970s, tilorone was synthesized in Ukraine and was named Amixin [6]. Its high immunomodulatory activity has been established [5].

These drugs are already registered in various countries [6–8] and are used for viral diseases, including during the coronavirus pandemic, where their potential use as an alternative therapy has been investigated [3, 9]. European scientists also study their effectiveness against other viruses [10, 11]. Along with the prospect of increased use of these drugs, there arises the issue of increasing their waste production, and they become a part of the over twenty million tons of PPCPs produced annually [12, 13]. Therefore, one of the tasks in the development of new pharmaceuticals is the creation of methods and approaches not only for quality control but also for

their determination in the environment (environmental matrices). Chromatographic methods are usually used for this purpose [14, 15].



Enisamium Iodide (Amizon) Tilorone dihydrochloride (Amixin)

Fig. 1. Chemical formulas of Enisamium Iodide and Tilorone dihydrochloride

HPLC is usedfor the quality control of Enisamium Iodide dosage forms [16, 17]. In this methodology, a Zorbax Eclipse XDB – C18 column was used. The mobile phase consisted of water and a buffer solution of acetonitrile at a pH of 2.5, with a flow rate of 0.5 mL/min, and detection was carried out at a wavelength of 225 nm. The presented HPLC methodology for the quantitative determination of Enisamium Iodide can be developed to investigate the dissolution profiles of Enisamium Iodide tablets. For the analysis of Tilorone dihydrochloride, reverse-phase high-performance liquid chromatography (HPLC) with a photodiode array detector is also employed. A Diasorb 130-C16T column (150×4 mm, 7 μm) is used in this methodology, with the mobile phase consisting of chloroform and a flow rate of 1 mL/min and detection at a wavelength of 225 nm. This method has been utilized to determine Tilorone dihydrochloride in blood serum [18, 19].

Previously, we developed a method for simultaneously determining these substances [20]. The methodology for the determination of Enisamium iodide and Tilorone dihydrochloride in a single sample, as well as their mixture with antibiotics (ceftriaxone, tetracycline, ampicillin, and levofloxacin), was developed using reverse-phase HPLC with a photodiode array detector. We used a SunFire C18 column with a mobile phase consisting of a buffer solution of sodium perchlorate (pH=2.5) and acetonitrile, flowing at a rate of 0.8 mL/min. The analytes were identified at 205 and 265 nm. This method can be used to determine Enisamium iodide and Tilorone dihydrochloride, as well as their presence in a mixture with antibiotics in the environmental matrices. The methodology proposed is characterized by excellent substance separation and suitability for application in "eco-pharmacy." However, it does have certain drawbacks concerning its "greenness", such as the usage of a substantial amount of solvents, making the methodology less environmentally friendly and incurring significant costs. It is known [21] that gas chromatography typically assists in addressing the "greenness" issue of the methodology, where feasible, through component separation.

The aim of this study is to develop and validate GC-FID chromatographic method for the simultaneous determination of Enisamium iodide and Tilorone dihydrochloride, assess the feasibility of its application, and compare it in terms of "greenness" with the previously developed HPLC methodology.

2. Planning (methodology) of the research

The main stages of the study are:

– to optimize the conditions for using GC-FID for the simultaneous determination of Enisamium iodide and Tilorone dihydrochloride;

– validate chromatographic method GC-FID;

– to evaluate the applicability of GC-FID method;

– to compare in terms of «greenness» with the previously developed HPLC method using the AGREE scale.

3. Materials and methods

3. 1. Reagents, solvents

APIs and Standard samples of Enisamium iodide manufactured by JSC Farmak batch 07–16; of tilorone hydrochloride manufactured by Interchim, Ukraine No. 2922197000. Acetonitrile (99.9 %) was purchased from Sigma–Aldrich Co., UK. The GC-equipment was run with helium (purity 5.0) as the carrier gas was purchased from Gazchema (Lithuania).

3. 2. Equipment

GC/FID method analyses were performed using a GC-2010 Plus Shimadzu with flame ionization detector and autosampler AOC-20i+s (Shimadzu Technologies, Kyoto, Japan). The separation of analytes was carried out on a with Rxi-5 ms (Restek Corporation, Bellefonte, PA, USA, capillary column (30 m long, 0.25 mm outer diameter and 0.25 μm liquid stationary phase thickness). A robotic autosampler and a split/splitless injection port were used.

3. 3. Methods (chromatographic conditions, preparation of standards and samples)

Chromatographic conditions GC/FID method.

Injection port temperature was kept at 250 °C until the end of the analysis. The separation of analytes was carried out on a with Rxi-5 ms (Restek Corporation, Bellefonte, PA, USA, capillary column (30 m long, 0.25 mm outer diameter and 0.25 μm liquid stationary phase thickness) with a liquid stationary phase) 5 % diphenyl and 95 % polydimethylsiloxane) with helium at a purity of 99.999 % as the carrier gas in a constant flow of 1.49 mL/min. The oven temperature was programmed at 75 °C for 5 min, then increased to 290 °C at 10 °C/min and increased to 320 °C at 20 °C/min and kept for 10 min. The total time was 41 min. Injection volume was 1.0 μl, injection mode was split (split ratio 10), carrier gas – helium.

Preparation of sample and standard solutions.

A portion of Tilorone dihydrochloride and Enisamium iodide 30.0 mg of the each substance were dissolved in 10 mL of acetonitrile, the volume of the solution is adjusted to 100.0 mL with the same solvent and mixed. The subsequent solution was transferred to 200 μL insert placed autosampler vials, and 1 μL aliquot was injected into GC-MS system for analysis. The comparison of chromatographic responses was used to evaluate the extraction efficiency.

GC/FID Method Validation.

The GC/FID method validation was carried out in accordance with the International Conference on

Harmonisation (ICH) guidelines for analytical methods in the quality control of medicinal products [22]. The method verification was conducted to assess linearity and calibration curve ranges. Regarding linearity, a mixture of the standard solution was prepared as follows. The exact volume of the standard solution (3.3.2) was transferred to a volumetric flask (200 µL). The method's precision was assessed by calculating repeatability. The precision of the method was verified by repeating the procedure with standard solution mixtures six times. Upper and lower limits were determined. An aliquot of each sample was then introduced and quantitatively determined. The precision of the chromatographic system was checked by examining the %RSD of retention times and peak areas. Six injections were made daily for three consecutive days.

Statistical Analysis.

The results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test with software package Prism v. 5.04 (Graph Pad Software Inc., La Jolla, CA, USA). We estimated the average of measurement (*AVG*), sample standard deviation (*Sx*), a standard deviation of mean (*SD*), the coefficient of variation (*CV*), and the statistical significance of results (*p*). The value $p<0.05$ was taken as statistically significant. The correlation and regression analysis were performed for evaluation of impact of clover pollen on the content of fatty acids in the samples.

The environmental friendliness of the developed GC-FID was evaluated using the AGREE Calculator [23, 24].

4. Results

4. 1. Chromatographic conditions

The selected conditions for GC-FID analysis provide good separation of compounds compared to the chromatogram of the solvent (Fig. 2).

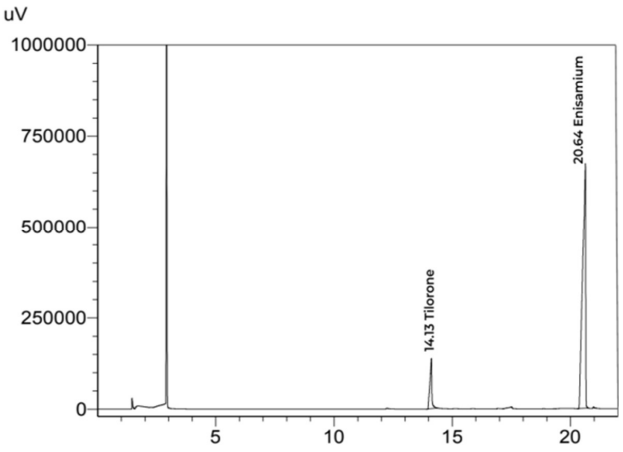


Fig. 2. Chromatogram of test solution by GC-FID method proposed

4. 2. Method Validation

The developed methods were validated in terms of specificity, linearity, precision, stability, and accuracy, besides the Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated for both methods.

Linearity, LOD, LOQ.

LOD and LOQ were determined based on the standard deviation (*SD*) of y-intercept of the regression line (*s*) and the slope of the calibration curve (*S*) as $LOD=3.3\times(s/S)$ and $LOQ=10\times(s/S)$. The study of linearity was carried out by the analysis of a series of solvents with different concentrations of Tilorone dihydrochloride and Enisamium iodide. The results of linearity studied with the calculated LOD and LOQ are shown in Table 1.

Table 1

Obtained results from studying linearity, LOD and LOQ

Method	Calibration curve	Correlation coefficient r^2 (n=3)	Linear range (µg/mL)	RSD (%)	LOD (µg/mL)	LOQ (µg/mL)
Enisamium iodide	$y=-24968.5075+$ $+55185810.9453x$	0.9987	0.12–16	1.29	0.047	0.110
Tilorone dihydrochloride	$y=-35058.9008+$ $+381686954.4267x$	0.9999	0.12–16	2.65	0.035	0.095

Precision.

The study has been carried out within two days by different analysts. Test solution with 100 % concentration was analyzed. The results of the RSD deviation of assay determination, also the errors of the method, are shown in Table 2.

Table 2

Results of precision study for HPLC and GC-FID methods

Method	Concentration (µg/mL)	Intra-Day (n=6)			Inter-Day (n=12)		
		Found± ±S.D., %	RSD, %	Error, %	Found± ±S.D., %	RSD, %	Error, %
Tilorone dihydrochloride	16	100.12± ±0.24	0.52	0.45	100.25± ±0.34	0.63	0.63
Enisamium iodide	16	100.1± ±0.20	0.55	0.48	100.5± ±0.18	0.50	0.46

Accuracy.

To assess accuracy, recovery assays were conducted by spiking sample solutions with known quantities of the reference compound. The added analyte quantities represented 50 %, 100 %, and 200 % of Tilorone dihydrochloride and Enisamium iodide in the samples. The results are presented in Table 3.

Table 3

Accuracy of the developed method

Reference Value (%)	Amount measured (%)		Relative standard deviation (%)		Recovery (%)	
	Tilorone dihydrochloride	Enisamium iodide	Tilorone dihydrochloride	Enisamium iodide	Tilorone dihydrochloride	Enisamium iodide
50 %	50.08	49.91	0.13	0.06	100.16	99.82
100 %	100.21	99.80	0.07	0.11	100.21	99.80
200 %	196.70	199.83	0.21	0.15	98.35	99.92

The stability of Tilorone dihydrochloride and Enisamium iodide was carried out within 24 h for a

standard solution. It was established that stored solutions were stable for up to 24 h in the case of the GC-FID methods. Hence, peak deviations of substance were 0.395 % and 0.387 %, respectively.

4. 3. Eco-scale calculation

All developed chromatographic methods for the analysis of Tilorone dihydrochloride and Enisamium iodide were estimated for their greenness using the analytical Eco-scale to choose the method with the least environmental impact. Furthermore, different conditions of pre-treatment for each sample were taken into account. The results of the HPLC method were taken from a previously developed methodology for the determination of Tilorone dihydrochloride and Enisamium iodide [20].

Table 4

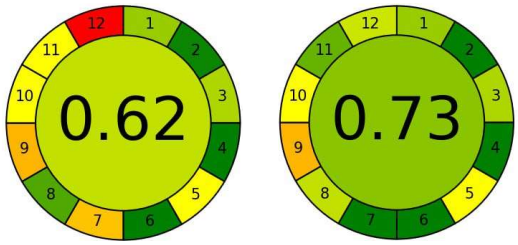
Calculation of penalty points for the HPLC and GC-FID detector analysis in the analysis of one sample for Tilorone dihydrochloride and Enisamium iodide [20].

Reagents	Penalty points (HPLC)	Penalty points (GC-FID)
Acetonitrile (mL)	4	0
Water (mL)	–	–
Instruments		
HPLC/ GC-MS	1	3
Waste	8	4
Occupational hazard	3	–
Sampler	–	1
Transport	–	1
Sample preparation solutions		
Water 50 mL	0	–
Acetonitrile (mL)	–	0
Total penalty points:	16	9
Analytical Eco-Scale total score:	78	35

5. Discussion

Chromatography analysis involves a variety of techniques as well as pre-treatment of samples. In addition, it is generally acceptable to use multiple determination methods when assessing a sample. Analytical techniques are typically selected based on factors such as accuracy, precision, cost, and potential environmental and health effects. We developed the GC-FID method with the aim of improving its environmental friendliness when compared to the previously established HPLC method. Initially, we anticipated the need for derivatization, as was the case with thiotriazoline [25], but we managed to avoid derivatization in that instance. Consequently, we sought to design a GC-FID method without derivatization to enhance its ecological profile. Experimental investigations revealed that the mixture of target compounds dissolved most effectively in chloroform and acetonitrile. From a green chemistry perspective, acetonitrile was chosen as the sample preparation solvent. The chromatographic conditions used in this analysis were based on previous successful trials [25], which resulted in excellent separation and peak symmetry for Tilorone dihydrochloride and Enisamium iodide, as shown in Fig. 2. The validation studies confirmed the suitability of the methodology for the defined objectives. In the case of all the analyzed substances, the linear regression coefficients (R^2) exceeded 0.99, demonstrating excellent linearity of the calibration curves. Furthermore, the methodology can be employed for quality control and waste detection beyond the limit of detection. This aligns with the recommendations of the European Chemicals Agency (ECHA) [26] (allowable substance concentrations ranging from 0.1 mg/L to 0.5 mg/L). Consequently, these preliminary results indicate that the developed methodology can be used for the determination of Tilorone dihydrochloride and Enisamium iodide in the environmental matrices. The results demonstrated the good precision of the method, with RSDs for the repeatability and intermediate precision below 6,4 % for the determination of the majority of the substances (Table 2). The developed method is correct since the requirements for the error criterion are ≤6.4 %. Recoveries were obtained in the range of 98.13–100.12, depicting that the proposed methods are accurate for the determination of Tilorone dihydrochloride and Enisamium iodide. All procedures showed satisfactory results in the data and could be recommended for the analysis of Tilorone dihydrochloride and Enisamium iodide in their mixtures and is possible for waste analysis.

Due to the results obtained, HPLC and GC-FID (Table 4) showed enough different Eco-scale values. Thus, the HPLC for Tilorone dihydrochloride and Enisamium iodide has 16 penalty points, respectively, compared to the GC-FID detector has 9, respectively. Conversion into eco-scale points results in 78 points for HPLC and 35 points for GC-FID (Table 4). We used the "AGREE methodology" [18] as it is the only one that encompasses all 12 GAC principles. The calculations were carried out using the "AGREE Calculator."



a *b*

Fig. 3. Analytical GREEnness (AGREE) scale for the greener: *a* –HPLC; *b* – GC-FID method

Fig. 3 depicts the AGREE results and assessment for each individual GAC principle. The overall AGREE score for the developed analytical approach (Fig. 3, *b*) for GC-FID was 0.73, which, in comparison to HPLC with a score of 0.62 (Fig. 3, *a*), is greener. From this, we can conclude that the proposed analytical approach for the analysis of Tilorone dihydrochloride and Enisamium iodide is more environmentally friendly and aligns with GAC principles.

Study limitations. The proposed methods for the quantification of Enisamium iodide and Tilorone dihydrochloride have not been studied to determine API data in the presence of other drugs and in environmental samples.

The prospects for further research lie in the development of various methods for the analysis of samples taken from the most important medium (soil, wastewater).

6. Conclusions

In the present study, we developed and validated a GC-FID method for the simultaneous determination of Enisamium iodide and Tilorone dihydrochloride. This method eliminates the derivatization step, minimizing time and solvent usage compared to the previously established HPLC method. The methodology was validated, and limits of detection (LOD) and quantification (LOQ) were determined. According to the obtained results, the developed GC-FID method can be employed for routine monitoring of Enisamium iodide and Tilorone dihydrochloride in various environmental samples. Compared to the previously developed HPLC method, the GC-FID method proves to be more environmentally friendly. Its assessment using the analytical GREENness (AGREE) scale resulted in a score of 0.73 (>0.70), surpassing HPLC's score of 0.62, demonstrating the environmentally favourable nature of the proposed analytical approach. According to the obtained results, the GC-FID method was recognized as the most suitable for analysis, as it has a smaller environmental footprint compared to the HPLC method, requires lower operational costs than GC-MS, and demonstrates satisfactory accuracy.

Conflict of interests

The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this article.

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Data availability

Data will be made available on reasonable request.

Use of artificial intelligence

The authors confirm that they did not use artificial intelligence technologies when creating the current work.

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