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Development of an HPLC Method for the Determination of Levothyroxine Applied to Four Different Preparations

Anum Sattar[1]*, Hina Rehman[2], Safila Naveed[1], Samreen Aziz[3], Asma Eraj[1]

[1] Department of Pharmacy Practice, Jinnah University for Women, Karachi, Pakistan

[2] Department of Pharmacy Practice, Institute of Pharmaceutical Sciences, Jinnah Sindh Medical University, Karachi, Pakistan

[3] Department of Pharmacy, Iqra University North Campus, Karachi, Pakistan

Abstract: HPLC is an important research tool that can identify, extract and measure the substance, its numerous components and drug-related additives that may occur during either the manufacture or storage of the medication. Understanding the chemistry of the drug ingredient is required for this, which makes it easier to create analytical methods. To find the most effective way to conduct the procedure, many chromatographic parameters were investigated and tested. It is essential to identify a suitable mobile phase, column, column temperature, wavelength, and gradient to achieve the desired suitability and durability of the medicine in addition to its degradants and contaminants. This study aims to develop simply and rapidly the HPLC method for the determination of levothyroxine and its different brands. Levothyroxine is the drug of choice for hypothyroidism. In this study, a high liquid chromatography method was developed for the quantification of different brands of levothyroxine. The chromatographic study was analyzed at an ambient temperature of 28°C with isocratic elution. The mobile phase of the chromatograph was water and methanol Me OH (45:55) (1:1V/V) or 0.01 M phosphate buffer, the solution with adjusted pH=3. The flow rate of the pump was set at 1.5ml/min, the volume of the solution was 20 µl injected into the HPLC column, and the wavelength at 225 nm. The accuracy (99.9% recovery), precision (99.9 to 100% recovery), linearity (≥ 0.99), and retention time (16.8 min) were observed for all brands of levothyroxine. In this study, we have evaluated the HPLC method for the quantification of levothyroxine and its different brands. Levothyroxine is considered as a controversial drug throughout the globe. In the recent past, we have found no study that proved clinical and biological interchangeability between levothyroxine brands. Our study is its self-unique in its case. There is no such publication proving that generic drugs are equivalent.

Keywords: levothyroxine, HPLC, analysis, different brands.

开发用于测定四种不同制剂中左旋甲状腺素的高效液相色谱方法

摘要：高效液相色谱是一种重要的研究工具，可以识别、提取和测量药物制造或储存过程中可能出现的物质、其众多成分和药物相关添加剂。为此需要了解药物成分的化学性质，这使得创建分析方法变得更加容易。为了找到执行该程序的最有效方法，研究和测试了许多色谱参数。必须确定合适的流动相、色谱柱、色谱柱温度、波长和梯度，以实现药物及其降解物和污染物所需的适用性和耐久性。本研究旨在建立简便、快速测定左旋甲状腺素及其不同品牌的高效液相色谱法。左旋甲状腺素是甲状腺功能减退症的首选药物。在本研究中，开发了一种高效液相色谱法，用于定量不同品牌的左旋甲状腺素。色谱研究在 28°C 的环境温度下进行等度洗脱分析。色谱流动相为水和甲醇我哦(45:55)(1:1 电压/电压)或 0.01 米磷酸盐

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About the authors: Anum Sattar, Department of Pharmacy Practice, Jinnah University for Women, Karachi, Pakistan; Hina Rehman, Department of Pharmacy Practice, Institute of Pharmaceutical Sciences, Jinnah Sindh Medical University, Karachi, Pakistan; Safila Naveed, Department of Pharmacy Practice, Jinnah University for Women, Karachi, Pakistan; Samreen Aziz, Department of Pharmacy, Iqra University North Campus, Karachi, Pakistan; Asma Eraj, Department of Pharmacy Practice, Jinnah University for Women, Karachi, Pakistan

Corresponding author Anum Sattar, anumsattar2018@gmail.com

缓冲液，溶液调酸碱度=3。泵的流速设定为 1.5 毫升/分钟，溶液体积为 20 微升注入高效液相色谱柱，波长为 225 纳米。对所有品牌的左旋甲状腺素的准确度（99.9%回收率）、精密度（99.9 至 100%回收率）、线性（≥ 0.99）和保留时间（16.8 分钟）进行了观察。在本研究中，我们评估了用于左旋甲状腺素及其不同品牌定量的高效液相色谱方法。左旋甲状腺素在全球范围内被认为是一种有争议的药物。在最近的过去，我们没有发现任何研究证明左旋甲状腺素品牌之间的临床和生物学互换性。我们的研究在其个案中独树一帜。没有这样的出版物证明仿制药是等效的。

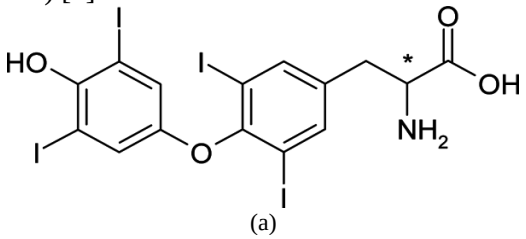
关键词：左旋甲状腺素，高效液相色谱，分析，不同品牌。

1. Introduction

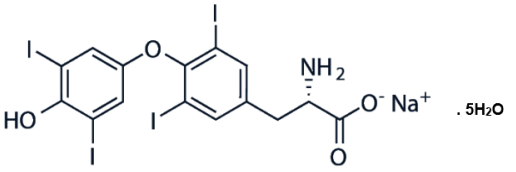
Levothyroxine is indicated for treating hypothyroidism. Hypothyroidism is occurring when the thyroid gland does not produce enough hormone [1]. T3 and T4 hormones of the thyroid gland play a significant part in the release of metabolic hormones, synthesis, and storage. Thyroid hormones play an important part in metabolism and cardiovascular effects. T3 and T4 are essential in the regulation of numerous metabolic methods and are important for typical growth and enlargement [3]. Thyroid hormones control gene transcription by crossing the cell nucleus that binds to DNA-bound thyroid receptors [4].

Hypothyroidism affects globally up to 5 % of the European population and 0.3-3.7% in the USA. The prevalence of subclinical hypothyroidism in China and India is about 16%, and 10% [5]. The prevalence of hypothyroidism is 4.1% in Pakistan [6]. TSH level is monitored in patients who were diagnosed with hypothyroidism; about 99% population is diagnosed with primary hypothyroidism. Hypothyroidism is treated with thyroid-stimulating hormone replacement; only one drug is available that is levothyroxine.

Levothyroxine is an oral drug that is used for the treatment of hypothyroidism[7]-[8]. Levothyroxine sodium (L-3, 5, 3, 5-tetraiodothyronine sodium salt) pentahydrate is a salt of the levo- isomer of thyroxine (Figure 1) [9].



(a)



(b)

Fig. 1 Structure of (a) thyroxine and (b) levothyroxine sodium pentahydrate

Levothyroxine is a crystalline powder that is white to pale water and alcohols are marginally soluble but insoluble in acetone, ether, and chloroform [9]. Levothyroxine is also known as L-Thyroxin and is a synthesized form of the thyroid hormone thyroxine [4]. The adult dose of levothyroxine is 1.7 μg/kg, or 100–125 mcg [10]. Levothyroxine has a narrow therapeutic index, so its dose fluctuation produces adverse effects [11].

Side effects include angina, increased heart rate, increased pulse rate, myocardial infarction, breath shortness, arrhythmias, myocardial infarction, anxiety, tiredness, headache, hotness intolerance, sleeplessness, rash, hair loss, goiter, weight loss, menstrual abnormalities, stomach cramps [12]-[15]. In the past many studies on the determination and dissolution of levothyroxine by applying HPLC and its different types [16]-[21]. In 2000 analysis of thyroid hormones T3 or T4 in human fluids developed the HPLC method [22].

The research was conducted in 2004 to investigate levothyroxine and its degradation using HPLC to inductively coupled plasma mass spectrometry [23]. Newly worked on the extent of levothyroxine in the extended-acting delivery system by using high-performance liquid chromatography The use of bovine serum albumin as a levothyroxine stabilizer has been validated and evaluated [24]. HPLC is the core product of the current pharmaceutical industry [22]. High-performance liquid chromatography is a methodology of investigation [25]. An essential investigative methodology is pragmatic in all phases of drug detection, progress, and manufacturing [26].

HPLC is used in different procedures of selection to verify the highest pureness of new substances, in artificial objects, new preparations, procedures, and the reassurance of the ultimate medication [27]. The HPLC technique aims to isolate and measure the primary medication, and any impurities of the reactions [28]. HPLC efficiency is one of the strong investigative chemistry instruments. It is capable to isolate, recognize or measure mixtures, which are existing in

any analytes that can be dissolved in a specimen or a fluid. A reliable form of analysis is an HP generally used for both measurable and qualitative purposes. Medicine substance review and usage for medicine determination, the constancy of substance [29].

This study aims to develop the HPLC method for the different brands of levothyroxine.

2. Method

2.1. Instrumentation

Shimadzu model no SPD-204 High-performance liquid chromatography design comprises an ultraviolet detector with fluctuating wavelengths and allied with the pump Nawaid scientific trader model no Rocker 300. Column C18 (5 mm, 250 x 4.6 mm) was used for the resolution of constituents for injecting drugs into the HPLC auto-injector used with a volume of 20 µl. For statistics computation and acquisition, HPLC was linked with the PC.

2.2. Materials and Chemical Reagents

Different brands of levothyroxine were purchased from the National Medical Centre pharmacy. These brands include Thyroxine, Thyronorm, Synthroid, and Eltroxin in the strength of 50 µg to 100 µg. All these drugs have more than one year of expiry. For HPLC, different HPLC grade reagents were used, including methanol, water, and 0.01M phosphate buffer. For all of these and the preparation of the mobile phase, deionized filtered water was used. A drug solution of levothyroxine was used as the mobile phase and was freshly prepared every day. Prepared different dilutions of drug concentration solutions like (2.5 ml, 5 ml, 6.25 ml, 10 ml, 12.5 ml, 25 ml, and 100 ml). All these solutions were used for the HPLC validation method.

2.3. Solution Preparation

First, we checked the solubility of levothyroxine. The solubility of levothyroxine in water and methanol, then we prepared different concentration solutions of methanol and water for HPLC. The different concentrations of methanol and water were methanol 90: water 10, methanol 80: water 20, methanol 70: water 30 methanol 60: water 40 methanol 50: water 50.

2.4. Sample Preparation in Different Levothyroxine Brands

Taken suitable quantity of levothyroxine of different four brands and a volume of mobile phase methanol and water and mixed well to achieve 100 ug/ml concentration. And made its serial dilutions for calibration. The sample solution is prepared fresh daily, and the stock solution can be stored for a long time.

2.5. Assay Preparation

Assay was prepared by taking twenty tablets of four different brands of levothyroxine (thyroxine, thyronorm, Synthroid, Eltroxin) and weighing to achieve the average weight, then crushing or powdering twenty tablets of four brands of levothyroxine (thyroxine, thyronorm, Synthroid, Eltroxin) and pouring into a volumetric flask. Initially, the drug was dissolved into 50 ml of mobile phase, then the volume was increased to the mark 100 ml. After obtaining concentrations of the drug, serial dilutions (2.5 ml, 5 ml, 6.25 ml, 10 ml, 12.5 ml, 25 ml, 100 ml) of each drug were made with the mobile phase Me OH (45:55) (1:1V/V) or 0.01 M phosphate buffer. Before using, these solutions were filtered through a disposable filter and then 0.45 µm were injected.

2.6. Wavelength

Wavelengths of all drugs are allocated between 200 to 400 nm in the UV-spectrum. Levothyroxine and it's all its brands showed its absorbance at 225 nm wavelength.

2.7. Chromatographic Settings

The chromatographic study was analyzed at an ambient temperature of 28°C with isocratic elution. The mobile phase of the chromatograph was water and methanol Me OH (45:55) (1:1V/V) or 0.01 M phosphate buffer, the solution with adjusted pH 3. The flow rate of the pump was set at 1.5 ml /min, the volume of solution 20 ul was injected into the HPLC column, and the wavelength was at 225 nm.

2.8. Method Development

The isocratic elution method was applied for selecting the mobile phase for isolating Levothyroxine from different brands. The composition, flow rate, and pH of the mobile phase were varied to optimize the analytical procedure. The retention time of levothyroxine was 16.8 min.

2.9. Validation Protocol

Method validation comprises numerous specifications that are accuracy, linearity precision and limitations of detection, and limitations of quantifications. Strategies for the validation of these parameters are described in ICH recommendations [30]. These guidelines are used for the completion and study of linearity, accuracy precision, and LOD and LOQ technique [31].

The techniques of system suitability used various parameters such as peak symmetry, resolution, retention time, mass distribution ratio, and theoretical plates of the column. All parameters ranged within the recommendations of the international conference on harmonization.

Three multiple concentrations (80, 100, 120) were used to estimate the accuracy with the familiar aggregates of the drug sample of levothyroxine in different brands. For the percent recovery, we injected the three injections of each solution into the high-

performance liquid chromatography and evaluated the percent recovery.

For estimating the linearity of different brands of levothyroxine, we prepared various concentrations of solutions that are 6.25, 12.5, 25, and 50, 100 μgmL^{-1}. Concentrations were assembled for the standard curve. We attained a linear graph by plotting the concentration vs. (AUC). Under all circumstances, the correlation coefficient is magnificent if it is ≥ 0.99.

The precision of different brands of levothyroxine was evaluated using six replicate concentrations for each drug introduced into the HPLC on 2 non-sequential days in all circumstances of percent recovery. Percent recovery was evaluated.

Although, according to recommendations LOD and LOQ calculations via equations, in chromatography methods experimentally determined LOD and LOQ values based on the signal-to-noise ratio (S/N) are more significant and representative.

3. Results

This research aimed to establish a speedy, precise, consistent high-liquid chromatography procedure for the quantification and comparative assessment of different brands of levothyroxine. High-pressure liquid chromatography is also known as high-performance liquid chromatography. It is a significant method of investigation. HPLC is the core product of the current pharmaceutical industry. An essential investigative methodology is pragmatic in all phases of drug detection, progress, and manufacturing. Recent studies High liquid chromatography method development is used frequently for the discovery of drugs because of its accuracy and significant role in routine analysis. High liquid chromatography needs a bulk of solvents and exclusive and complex apparatus and requires distinctive procedures. The chromatographic assays were analyzed at an ambient temperature of 28°C with the isocratic elution. The mobile phase of the chromatograph is water, methanol, and 0.01 M phosphate-buffered saline with adjusted pH 3. The flow rate of the pump was set at 1.5 ml/min, the volume of a vial was 20 ul injected into the HPLC column, and the wavelength was 225 nm.

3.1. Method Development

The best resolution of levothyroxine and its different brands was achieved in column C18. It makes available the best, most convenient effective, and reproducible resolutions of constituents. Under the same experimental circumstances, using another category of column provides a resolution time larger than C18. For the checking of system suitability, various concentrations of mobile phase methanol and water were used. Variation in the pH of the mobile phase has a greater impact on the chromatographic parameters. The mobile phase of the chromatograph is water, methanol, and 0.01M phosphate-buffered saline with adjusted pH 3. The composition, flow rate of 1.5 ml/min, and pH of the mobile phase were varied to optimize the analytical procedure. The retention time of levothyroxine is 16.8. In contrast to the other available procedures for determining levothyroxine, HPLC is a more convenient, accurate, and cheap solvent, less time-consuming, and provides the acceptable LOD and LOQ that qualify for levothyroxine pharmacokinetics studies. As compared to other published procedures of levothyroxine, HPLC provides satisfactory separation, fine recovery, and precision of levothyroxine and its brands.

3.2. Method Validation

Method validation comprises numerous specifications that are accuracy, linearity precision and limitations of detection, and limitations of quantifications. Strategies for the validation of these parameters are described in ICH recommendations. These guidelines are used for completing linearity, accuracy precision, and LOD and LOQ technique.

3.3. System Suitability

The techniques of system suitability use various parameters that peak symmetry, retention time, mass distribution ratio, and theoretical plates of the column. All parameters range within the recommendations of the international conference on harmonization. The results of all parameters are demonstrated in Table 1.

Table 1 System suitability of different brands of levothyroxine

Product Names	Tailing Factor	Theoretical Plate	Retention Time
Synthroid	0.92	14713.25	16.86
Thyroxine	0.92	14713.25	16.869
Thyronorm	0.92	14713.25	16.869
Eltroxin	0.92	14713.25	16.869

3.4. Accuracy

Three multiple concentrations (80, 100, 120) were used for estimating the accuracy with the familiar aggregate of the drug sample. For the percent recovery, we made three injections of each solution into the high-performance liquid chromatograph and evaluated the percent recovery. The percent recovery for different brands of levothyroxine is given in Table 2.

Table 2 Percent recovery of different brands of levothyroxine

Product Names	Conc, μgmL^{-1}	%RSD	% Recovery
Eltroxin	80	0.0006	99.91
	100	0.0003	99.92
	120	0.0001	99.93
Synthroid	80	0.0006	99.93
	100	0.0003	99.94
	120	0.0001	99.95
Thyroxine	80	0.0007	99.96
	100	0.0003	99.91
	120	0.0002	99.93
Thyronorm	80	0.0008	99.93
	100	0.0002	99.92
	120	0.0001	99.94

3.5. Linearity

For estimating linearity, we prepared various concentration solutions that are (6.25, 12.5, 25, and 50, 100 µgmL-1). These concentrations were assembled into a standard curve. The correlation coefficient for Eltroxin is 0.99 for Synthroid, 0.98 for Thyroxine 0.99, and Thyronorm 0.98. We attained a linear graph by plotting the concentration vs AUC. Under all circumstances, the correlation coefficient is magnificent if it is ≥ 0.99. The linearity of the four brands of levothyroxine is shown in Table 3 and Figures 2, 3, 4, and 5.

Table 3 Regression equations

Product Names	Regression equation	r^2
Eltroxin	y = 6020x + 1.5833	0.99
Synthroid	y = 6088.2x + 0.2917	0.98
Thyroxine	y = 6097.2x + 0.9167	0.99
Thyronorm	y = 7170x + 1.5833	0.98

Note: r = correlation coefficient

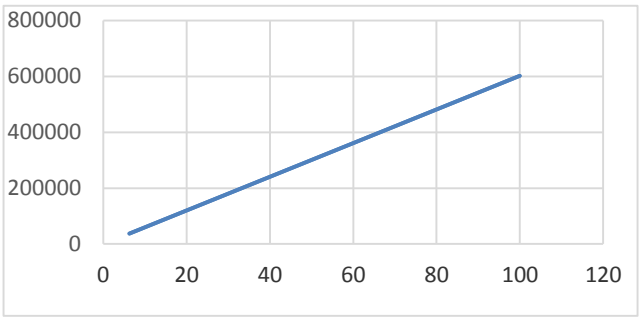


Fig. 2 Linearity of Eltroxin

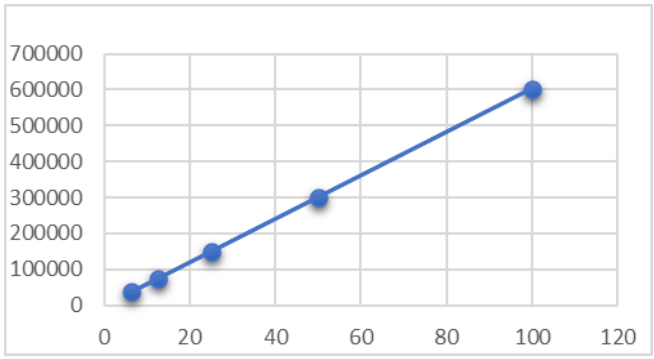


Fig. 3 Linearity of Synthroid

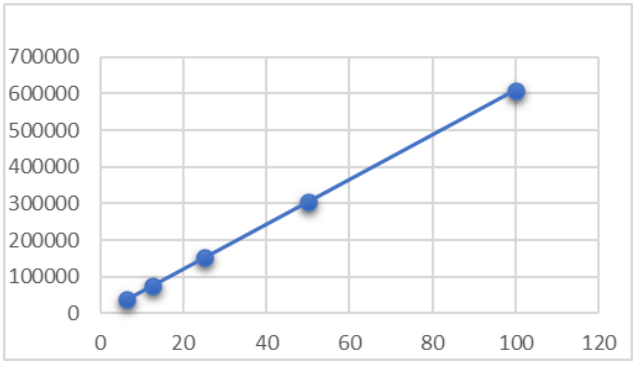


Fig. 4 Linearity of Thyroxine

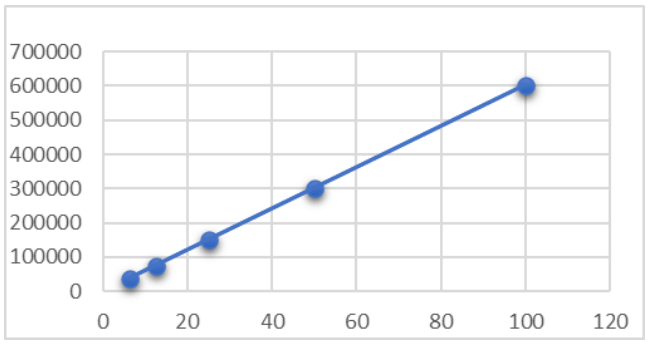


Fig. 5 Linearity of Thyronorm

3.6. Precision

Precision was evaluated using 6 replicate concentrations for each drug introduced into the HPLC on 2 non-sequential days in all circumstances of percent recovery. Percent recovery was evaluated. The precision results for different brands of levothyroxine are demonstrated in Table 4.

Table 4 Inter-day and intraday precision of different brands of levothyroxine

Drugs	Conc. Injected	Inter-day		Intra-day	
	µgmL^{-1}	%RSD	%Recovery	%RSD	%Recovery
Eltroxin	2.5	0.005	99.9	0.011	99.9
	5	0.002	99.9	0.004	100
	10	0.002	99.9	0.004	100
	25	0.0006	99.9	0.002	100
	50	0.0003	99.9	0.0008	100
	100	0.0001	99.9	0.0005	100
Synthroid	2.5	0.005	99.9	0.008	99.9
	5	0.002	99.9	0.005	99.9
	10	0.001	99.9	0.002	100
	25	0.0007	99.9	0.001	100
	50	0.0003	100	0.0003	100
	100	0.0002	100	0.0003	99.9
Thyroxine	2.5	0.008	99.9	0.018	100
	5	0.004	99.9	0.004	99.9
	10	0.001	99.9	0.002	100
	25	0.0004	99.9	0.0013	99.9
	50	0.0004	100	0.0006	100
	100	0.004	100	0.0003	99.9
Thyronorm	2.5	0.004	100	0.008	100
	5	0.002	100	0.004	100
	10	0.001	100	0.063	99.9
	25	0.0006	100	0.001	100
	50	0.0003	100	0.0010	100
	100	0.0001	100	0.0002	100

3.7. Limits of Detection and Quantifications

The LOD and LOQ are evaluated using this formula

$$LOD = \frac{3.3\alpha}{S} \text{ and } LOQ = \frac{10\alpha}{S} \quad (1)$$

where α - standard deviation of the lowest concentration; S - slope of the standard curve.

The LOD and LOQ of the different brands of levothyroxine are shown in Table V.

Table 5 LOQ of different brands of levothyroxine

Concentration µgmL^{-1}	Product Names	LOD µgmL^{-1}	LOQ µgmL^{-1}
6.25	Eltroxin	0.276	0.805
6.25	Synthroid	0.266	0.807
6.25	Thyroxine	0.256	0.806
6.25	Thyronorm	0.266	0.807

4. Discussion

This study was designed to develop a fast, accurate,

and reliable high-liquid chromatography technology for drug measurement. It is a key investigative tool. The current pharmaceutical industry's main product is high-performance liquid chromatography (HPLC). A crucial investigative tool is used in all stages of drug detection, progression, and production. we developed an HPLC method for the determination of levothyroxine applied to four different preparations. Levothyroxine is an oral preparation and is indicated for hypothyroidism. Different brands of levothyroxine were distinguished by the use of methanol mobile phase and chromatographs were obtained for each drug with sharp and symmetrical peaks with good baseline resolution (Figures 6-9).

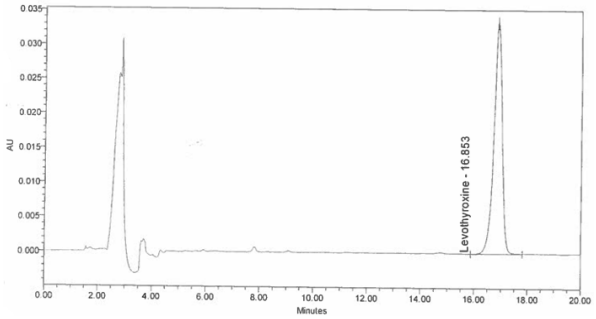


Fig. 6 The chromatograph obtained from Thyronorm

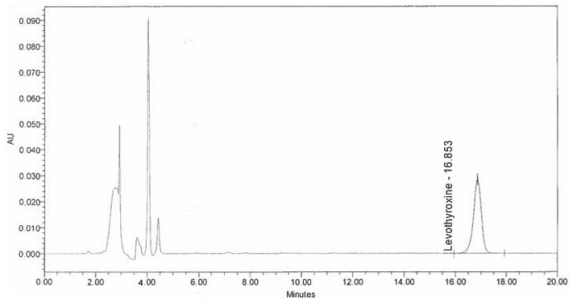


Fig. 7 The chromatograph obtained from Synthroid

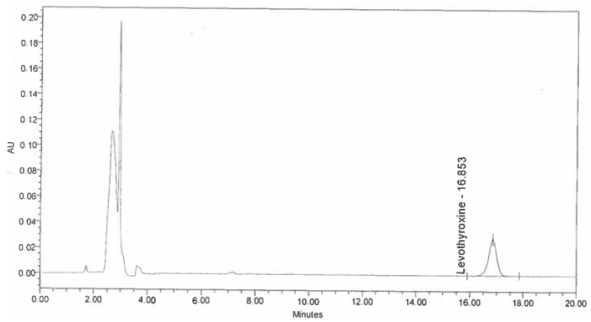


Fig. 8 The chromatograph obtained from Thyroxine

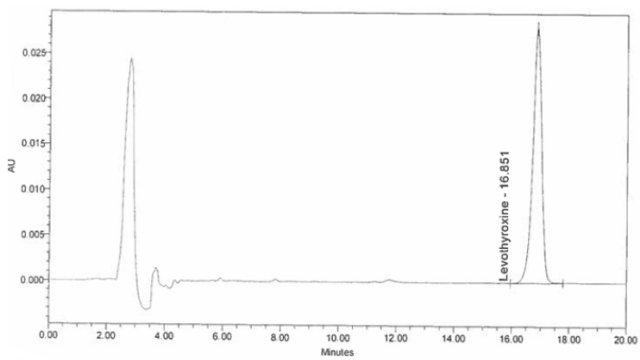


Fig. 9 The chromatograph obtained from Eltroxin

The linearity of all brands was ≥ 0.99. The validation revealed that the precision accuracy and LOD or LOQ were within the range. In the past, numerous studies on high-liquid chromatography method development are used regularly for detecting preparations because of its correctness and substantial role in a routine examination. In the literature review, many studies were performed on the quantification and determination of different drugs by using the HPLC technique. The research was conducted in Karachi for the determination of captopril and diuretics by performing the HPLC method [28]. Another research was conducted on the determination of the antihypertensive drug enalapril and statin by execution of HPLC validation and development method [29].

In 2021, a study was conducted on the development and validation of thyroid-stimulating hormones in vivo studies by HPLC. This study successfully developed the validated method for thyroid-stimulating hormones in vivo studies [32]. Recently another study was performed on the levothyroxine tablet formulation considerations and factors that affect the solid dosage form stability and storage conditions [33].

5. Conclusion

5.1. The Main Finding

In this study, we evaluated the HPLC method for the quantification of levothyroxine. In this method, we need to evaluate the accuracy, precision, linearity of LOD, and LOQ percent assay, of different brands of levothyroxine with ICH recommendations. HPLC is an appropriate and rapid method to evaluate the quantification of levothyroxine in different brands. HPLC technique can be used in the dissolution of different medicine and clinical research of innovative drugs and numerous applications in various fields.

5.2. Implications and Explanation or Findings

The accuracy (%recovery 99.9), precision (%recovery 99.9 to 100), Linearity (≥ 0.99), and retention time is 16.8 min for all brands of levothyroxine.

5.3. Limitations of the Study and Research Perspective

Bioequivalence is the consideration of the limitation of the study if we will look into the bioequivalence that it would be concluded that all brands of levothyroxine are chemically and biologically equivalent.

5.4. Scientific Novelty

Many third-world countries are known to counterfeit medications. Many drugs are essentially crucial and need to be equal in terms of bioequivalence.

Levothyroxine is considered as a controversial drug throughout the globe. In the recent past, we have not

found any study that proved clinical and biological interchangeability between levothyroxine brands. Our study is its self-unique in its case. None of such publications proved that generic drugs are equivalent. In numerous studies, Pakistan shows the strength that the products available in the different communities are equivalent in terms of interchangeability. This study proves that the available community pharmacies have equivalent marketed drugs and can be easily interchangeable with all quality standards and price discretion.

5.5. Recommendations and Future Research

Primary and secondary hypothyroidism requires the daily oral dosage of levothyroxine. Oral levothyroxine administration counters several difficulties in terms of administration and adherence. To overcome these difficulties, levothyroxine sodium-loaded implanted devices can be the future recommended option that may deliver a prolonged medication for a long period.

Pharmaceutical businesses and researchers are becoming more interested in such medication delivery technologies. In the future, we can work on these types of technologies.

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