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RP-HPLC BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF METFORMIN AND LEVOTHYROXINE IN HUMAN PLASMA- DRUG INTERACTION STUDIES

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ABSTRACT: A simple, sensitive, robust and specific high performance liquid chromatographic (HPLC) method was developed and validated for the simultaneous determination metformin and levothyroxine in human plasma. In the current study, the analysis was performed on Phenomenex Luna 5µ C18 100A (250 x 4.6mm, 5 micron) column using acetonitrile: methanol: 0.03M phosphate buffer (pH 2- 3.5) 50:10:40 v/v/v as mobile phase at flow rate 1.0 mL/min. The analytes were monitored with UV-PDA detector at 251nm. In this developed method Metformin and Levothyroxine elutes at a retention time of 2.698 and 5.929 min respectively. The proposed method is having linearity in the concentration range from 5 to 50µg/mL of Metformin and Levothyroxine. The current method was validated with respect to linearity; precision, lowest limit of detection (LOD) and lowest limit of quantification (LOQ), accuracy and recovery according to the USP guidelines. The system consisted of a pump (Shimadzu, prominence, HPLC), with 20µl sample injector, along with a PDA detector at a wavelength of 251nm. Data was compiled using Shimadzu LC Solution software. A good linear relationship over the concentration range of 5-50µg/ml was shown. Validation of the method was carried out as per the USFDA. The method developed was found to be precise, accurate, specific and selective. Statistical analysis shows that the method is reproducible and selective for the estimation of Metformin and Levothyroxine in dosage form.

INTRODUCTION: Metformin, (CPG) 1,1-Dimethylbiguanide hydrochloride **Fig. 1A** a drug used to treat diabetes mellitus (a condition in which the body cannot control the level of sugar in the blood).

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Its mechanism of action is the alteration of the energy metabolism of the cell. Metformin exerts its prevailing, glucose-lowering effect by inhibiting hepatic gluconeogenesis and opposing the action of glucagon.

Metformin is a first-line therapy for the treatment of type 2 diabetes, due to its robust glucose-lowering effects, well-established safety profile, and relatively low cost. While metformin has been shown to have pleotropic effects on glucose metabolism, there is a general consensus that the major glucose-lowering effect in patients with type

2 diabetes is mostly mediated through inhibition of hepatic gluconeogenesis. However, despite decades of research, the mechanism by which metformin inhibits this process is still highly debated. A key reason for these discrepant effects is likely due to the inconsistency in dosage of metformin across studies. Literature survey reveals that few analytical methods have been reported for metformin include RP-HPLC methods [1-4], HPTLC method [5, 6], UV method [7], normal phase HPLC [8], GC method [9], LC-MS method [10], capillary electrophoresis method [11]. Levothyroxine, sodium (2S) – 2 – amino – 3 -[4 - (4 - hydroxy-3, 5-diiodophenoxy)-3,5-diiodophenyl] propanoic acid **Fig. 1B** is synthetically produced form of thyroxine, a major endogenous hormone secreted by the thyroid gland. Also known as L-thyroxine or the brand name product Synthroid, levothyroxine is used primarily to treat hypothyroidism, a condition where the thyroid gland is no longer able to produce sufficient quantities of the thyroid hormones T_4 (tetraiodothyronine or thyroxine) and T_3 (triiodothyronine or Levothyroxine), resulting in diminished down-stream effects of these hormones. Literature survey reveals that few analytical methods have been reported for levothyroxine include has been estimated by colorimetry [12], Spectrophotometric methods [13, 14], LC-MS/MS [15], RP-HPLC [16-21].

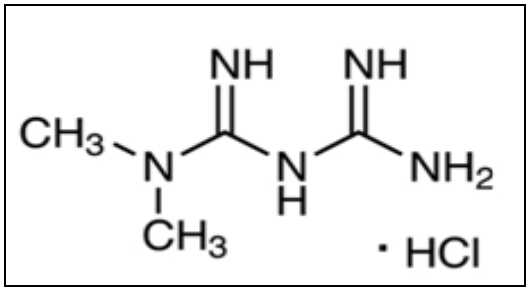

FIG. 1A: STRUCTURE OF METFORMIN HYDROCHLORIDE

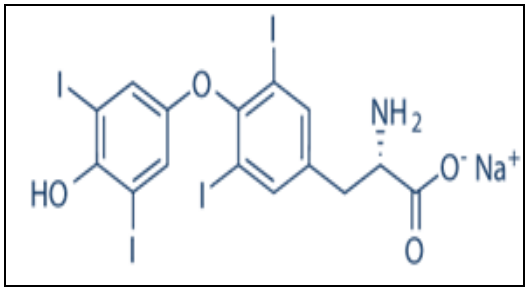

FIG. 1B: STRUCTURE OF LEVOTHYROXINE SODIUM

MATERIALS & METHODS:

Chemical and Reagents: Pure sample of metformin and levothyroxine were received from Wintac Limited, Bangalore. The human plasma was received from JSS Hospital, Mysore, Karnataka, India. All the chemicals and reagents used were of analytical grade only. Milli-Q-water was used throughout the process, methanol, acetonitrile of HPLC grade were procured from Merck Chemical Laboratories, Bangalore, India.

Instrumentation: The present research was carried on HPLC (SHIMADZU) equipped with PDA detector with LC solution software. Separation was attained using Phenomenex C8 column. The mobile phase was a mixture of potassium dihydrogen orthophosphate buffer (pH-3.0) and acetonitrile (40:60 v/v) at flow rate 1.2 mL/min. The contents of mobile phase were filtered before use through membrane filter (0.45 µ). The optimized chromatographic conditions are shown in **Table 1.**

TABLE 1: OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Chromatographic Conditions	
Column	C8 (250 x 4.6 mm. 5 µ) Phenomenex
Flow rate	1.2 mL/min
Run time	10 min
Wavelength	252 nm
Injection Volume	10µL
Detector	PDA Detector
Elution	Isocratic
Mobile Phase	potassium dihydrogen orthophosphate buffer (pH-3.0) and acetonitrile (40:60 v/v)
Column oven temperature	25 ± 5ºC

Preparation of Mobile Phase: Mobile phase is prepared by adding 4.08g potassium dihydrogen orthophosphate in 250ml of Millipore water, dissolve and adjust the pH to 3.0 using ortho phosphoric acid and made up to 1000ml (0.03M) using Millipore water and acetonitrile were used in the ratio of 40: 60 (v/v).

Preparation of Standard Solutions: Stock solution of Metformin and levothyroxine was prepared by dissolving 100 mg of drugs Metformin and levothyroxine in 50 mL of methanol in 100mL volumetric flask dissolved and volume was made up to 100 mL using the methanol to get the standard stock solutions of concentration 1 mg/mL (1000 µg/mL) for both Metformin and levothyroxine. Different working standard solutions were prepared from the above solution.

METHOD DEVELOPMENT:

Selection of Mobile Phase: Different mobile phases were tried in various ratios for selection of solvents of desired polarity. The drugs metformin and levothyroxine were injected with different mobile phases at different ratios and flow rates till a sharp peak, without any interference was obtained. The mobile phase selected with good resolution was phosphate buffer (pH 3) and acetonitrile in the ratio 40:60 (v/v).

Stock and Standard Solution: The stock solution of metformin and levothyroxine were prepared by dissolving 10mg of each separately into methanol and volume was made up to 100ml with same solvent.

From stock solutions (100 µg/ml of each) 5, 10, 20, 30, 40, 50 µg/ml concentration were prepared separately using methanol as solvent. Equal volumes of both concentrations were mixed and used as standard solutions.

Preparation of Calibration Curve: From the stock solution (1000 µg/mL) aliquots of Metformin and levothyroxine were pipette into a series of 10 mL volumetric flask. The final volume was made up to the mark by using HPLC grade methanol. 10µL solution was injected to the column and peak areas were measured and the calibration curve was obtained. Linear correlations were found between peak ratios of Metformin and levothyroxine and are described by regression equation. The Beer's law was obeyed in the concentration range of 5 – 50 µg/mL **Fig. 2.** The regression parameters and system suitability of the method were shown in **Table 2.**

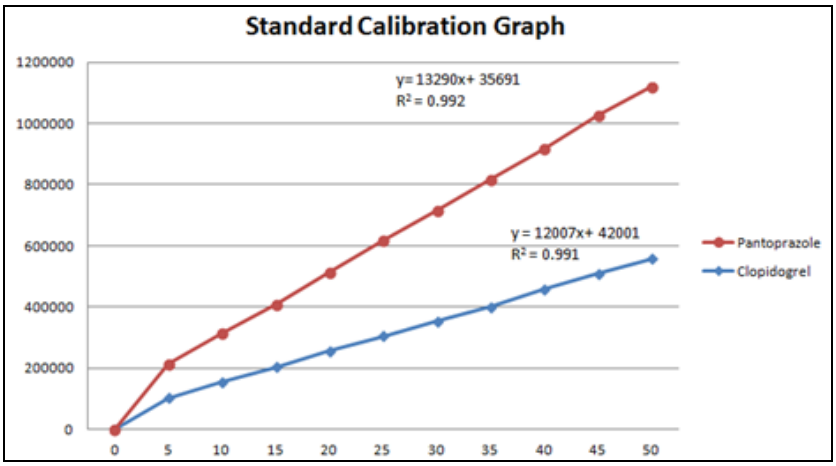

FIG. 2: STANDARD CALIBRATION GRAPH OF METFORMIN AND LEVOTHYROXINE

TABLE 2: THE REGRESSION AND SYSTEM SUITABILITY PARAMETERS OF THE METHOD

Parameter	Metformin	Levothyroxine
Linearity (µg/ml)	5-50	5-50
Regression Equation	12007x + 42001	13290x + 35691
Regression coefficient (R^2)	0.9913	0.9924
Slope	97774	85001
Intercept	458786	583384
Retention Time (RT)	2.698 min	5.929 min
LLOQ (µg/ml)	2.810	6.072
Resolution factor (RS)	6.7	6.7
Capacity Factor (K')	5.2	5.2
Tailing Factor (T)	1.1	1.7
Theoretical Plates	4376.51	7810.79
HETP	81.0	90.0

Determination of Drugs in Plasma (Spiking Method): 0.1 ml of drug from stock solution was added to 0.1 ml of plasma (obtained by centrifuging the blood samples at 10,000 rpm for 10 minutes) in append off tubes and made up to the volume (1.8 ml) with acetonitrile for the precipitation of proteins. It is further centrifuged at 10,000 rpm for 10 minutes. Supernatant fluid is decanted into vial by filtering with syringe filters of 0.45µ size. The obtained chromatograms are shown in **Fig. 3A** and **B**.

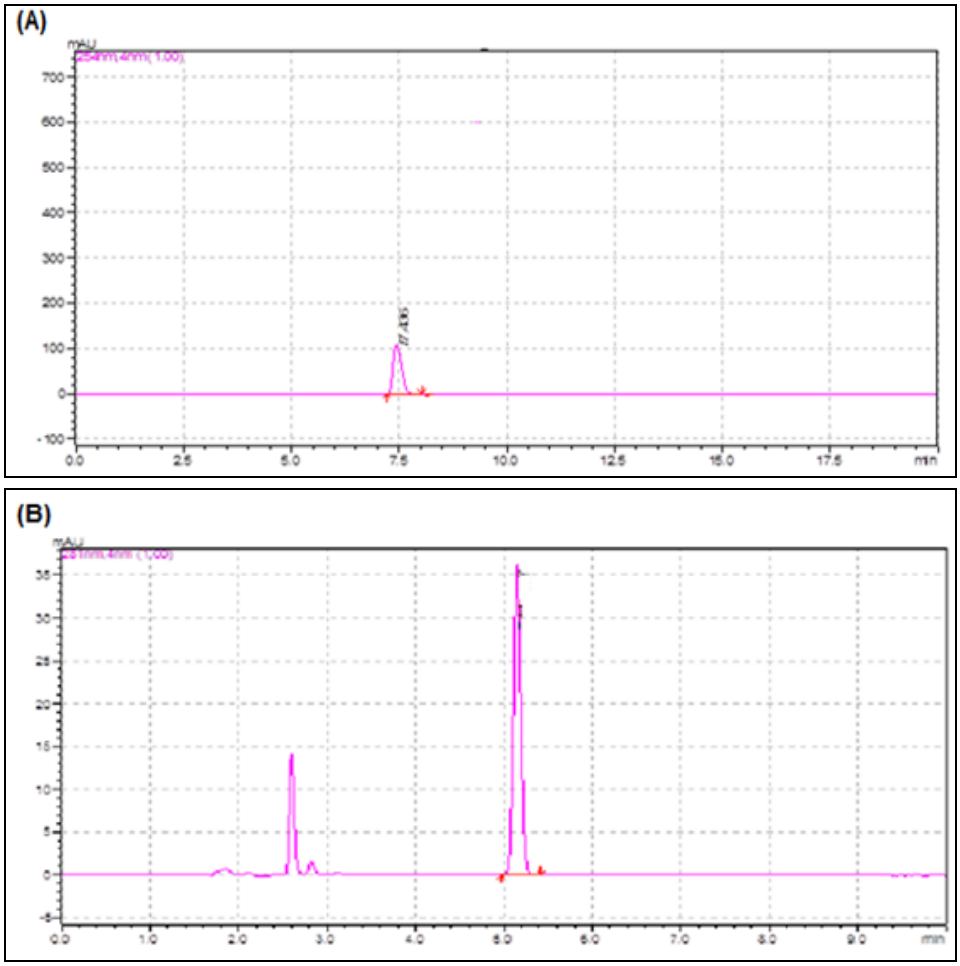

FIG. 3: CHROMATOGRAM OF (A) BLANK, (B) METFORMIN AND LEVOTHYROXINE IN PLASMA

RESULTS & DISCUSSION:

Method Validation: Since, the HPLC method was developed, validation of the method by using various parameters was performed to ensure that the accomplishment of the method meets the requirements of the described bioanalytical applications.

Following parameters were performed for method validation:

1. System suitability
2. Specificity
3. Detection Limit (LOD)
4. Quantification Limit (LOQ)
5. Linearity
6. Precision

Accuracy:

Linearity: From the experimental conditions described above, linear calibration curves of Metformin and levothyroxine were obtained for ten different concentrations level for both. The r^2 for metformin was 0.991 and for levothyroxine was 0.990. Linear correlations were found between peak area of Metformin and levothyroxine concentration and are described by the regression equation. The linearity range for Metformin and levothyroxine is 5-50 µg/ml. Results are specified in **Table 2.**

Specificity: Specificity is the capability to evaluate the analyte distinctly in the presence of expected impurities and degraded products.

20 µl of the blank was injected in duplicate to the UPLC system and chromatographed. 20 µl of Metformin and levothyroxine standard solutions were injected in duplicate to the UPLC system. Standard chromatograms obtained are presented in **Fig. 4 (A, B** and **C)**.

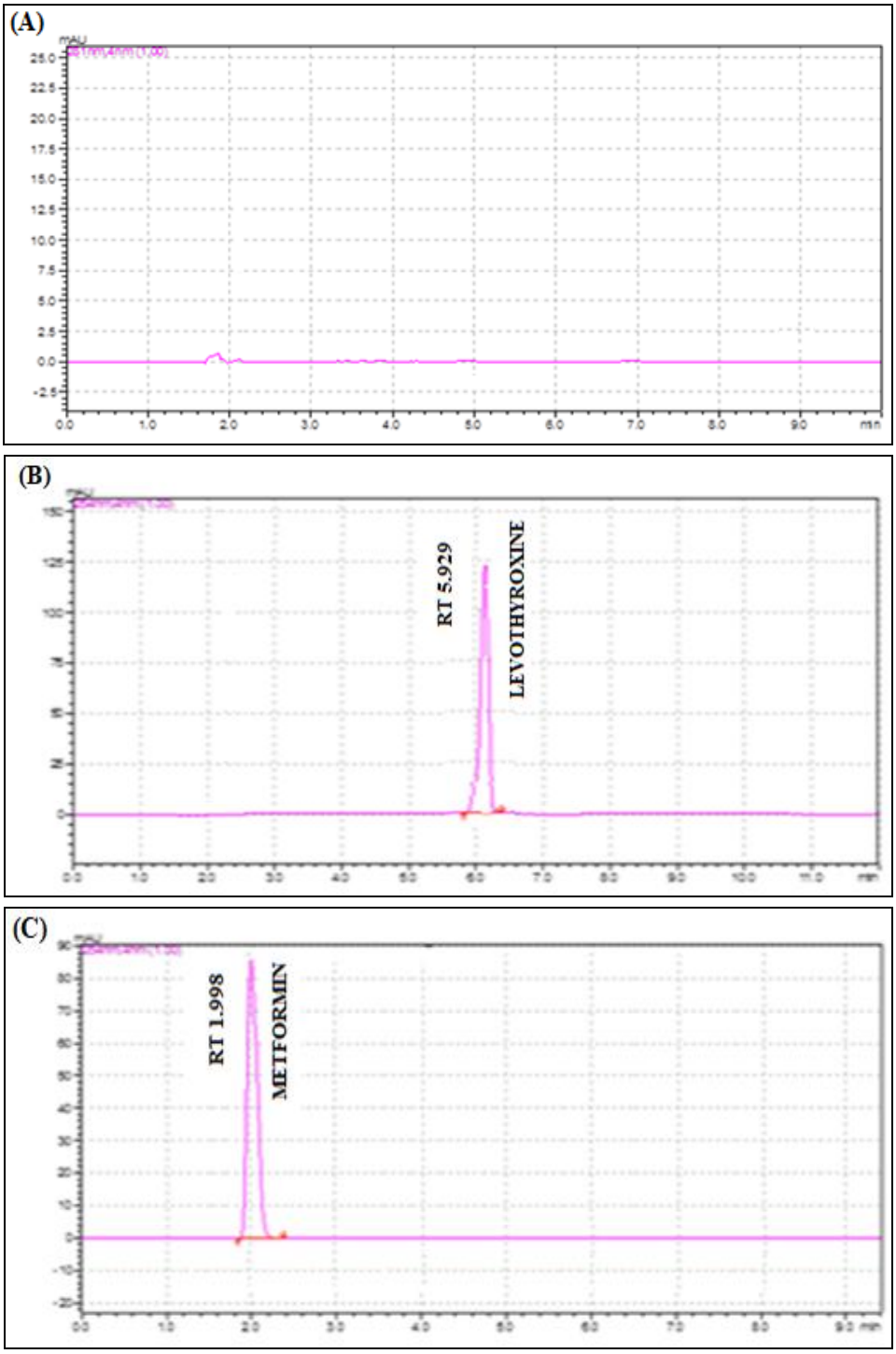

FIG. 4: CHROMATOGRAM OF (A) BLANK, (B) STANDARD SOLUTION OF LEVOTHYROXINE (50 µG/ML), (C) STANDARD SOLUTION OF METFORMIN (50 µG/ML)

Precision and Accuracy: The accuracy of an analytical method is the percentage of relativeness between the conventional true value and the value obtained by that method. Precision and Accuracy were determined by replicate analysis of known content of sample. The mean value should be within 15% of the actual value as per the acceptance criteria. The difference between mean amounts added and recovered (RE, %) serves as a measure of accuracy. The coefficient of variation

(CV, %), as a measure of precision at each concentration, should not exceed 15%. Intra-day and inter-day accuracy and precision were evaluated by analysis of quality-control samples containing metformin at three different concentrations a low concentration (LQC), a concentration near the centre of the calibration plot (MQC) and a concentration near the upper limit of the calibration plot (HQC). Intra-day accuracy and precision were evaluated by analysis of these QC samples prepared and analyzed on the same day (eight samples of each concentration; three replicate injections). Inter-day accuracy and precision were evaluated by analysis of these QC samples prepared and analyzed on five different days (three samples of each concentration; three replicate injections). The intra-day precision and accuracy of the method for metformin and levothyroxine are presented in **Table 3A**. The inter-day precision and accuracy of the method for metformin and levothyroxine are presented in **Table 3B.** All values for accuracy and precision were within the recommended limits.

TABLE 3: INTRADAY AND INTERDAY PRECISION OF METFORMIN AND LEVOTHYROXINE

(A) Intraday Precision

Concentration (µg/ml)		Mean (µg/ml)		%RSD	
		Metformin	Levothyroxine	Metformin	Levothyroxine
Low (n=3)	5	5.11	5.25	0.07	0.06
Medium (n=3)	25	25.5	26.6	0.08	0.06
High (n=3)	50	51.30	50.16	0.06	0.07

(B) Intraday Precision

Concentration (µg/ml)		Mean (µg/ml)		%RSD	
		Metformin	Levothyroxine	Metformin	Levothyroxine
Low (n=3)	5	5.21	5.30	0.06	0.08
Medium (n=3)	25	25.7	25.96	0.07	0.05
High (n=3)	50	51.30	50.35	0.05	0.06

Recovery: Recovery of the method was performed comparing the three quality control (QC) samples at low, medium and high concentrations (5, 25, 50 µg/ml). The recoveries of metformin and levothyroxine were determined by comparing peak area obtained for QC samples that were subjected to the extraction procedure with those obtained from blank plasma extracts that were spiked post extraction to the same nominal concentrations.

Stability Studies: The stability in human plasma over three freeze–thaw cycles and during short-term, long-term, and post-preparative storage was tested by analysis of LQC and HQC samples. The freeze–thaw stability was determined over three freeze–thaw cycles within 3 days. Spiked plasma samples were frozen at -22^{o}C for 24 h and thawed at room temperature in each freeze–thaw cycle. To study short-term stability, the frozen (-22^{o}C) and then thawed plasma samples were kept at room temperature for 6 h before sample preparation. The results obtained from these test samples were compared with those from freshly thawed and processed samples (reference samples). Long-term stability was determined after keeping spiked plasma samples frozen at -22^{o}C for 1 month. For this stability test the samples (test samples) were analyzed and the results were compared with those obtained from freshly prepared and processed samples (reference samples). The stability in stock solutions was studied after storage at 2^{o}C for 1 month. Three freeze thaw cycles of the quality control samples did not seem to affect quantification. Quality-control samples stored in a freezer at -22^{o}C were stable for at least 1 month. Thawing of the frozen samples and keeping them at room temperature for 6 h had no effect on quantification. The stability in stock solutions was confirmed after storage for 29 days at 2^{o}C.

CONCLUSION: The method involves simple and precise method for bioanalytical determination of metformin and levothyroxine in human plasma. This study showed that metformin along with levothyroxine significantly decreased plasma level of metformin. Such a variation would lead to sub therapeutic concentration and a consequent lack of therapeutic efficacy of metformin. This consequence may be expected due to inhibition of enzyme cytochrome P450 2C19 which is

responsible for bioactivation of metformin. In conclusion, present study showed that levothyroxine can alter the pharmacokinetics of metformin to significant levels. Summary of validation parameters data for Metformin and Levothyroxine is presented in **Table 4.**

TABLE 4: SUMMARY OF VALIDATION PARAMETERS DATA FOR METFORMIN AND LEVOTHYROXINE

Parameters		Metformin	Levothyroxine	Acceptance criteria
Retention Time (min)		5.14	2.62	-
LOD (µg/ml)		5	5	-
LLOQ (µg/ml)		6.5	7.2	-
Linearity (µg/ml)		5-50	5-50	-
Accuracy (% Recovery)		96.7-98.2%	96.2-98.4	90 -110%
Precision (%RSD)	System	0.025		
	Method	0.0020		< 2%
	Intermediate precision	0.72		
Specificity		No peak of diluent, excipients and impurities were detected.		No peak should be detected
System Suitability Parameters	N	9772		>2000
	HETP	0.0021		-
	Asymmetry	1		~1
	Resolution	1.115		

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