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Research Article

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Development of an Ionic Liquid-Based Dispersive Liquid-Liquid Micro Extraction Method Combined with RP-HPLC for Determination of Methyclothiazide in Plasma

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ABSTRACT

Methyclothiazide, a diuretic antihypertensive agent is a member of benzothiadiazine class of drug. It helps in prevent your body from absorbing too much salt which can cause fluid retention ,used to treat high blood pressure, fluid retention in people with congestive heart failure, Cirrhosis in liver, kidney problems or oedema caused by using steroids or estrogens. Along with its needful effects, Methyclothiazide causes some unwanted effects among which mostly are of unknown reason. Bio analysis helps to provide information about metabolic fate, pharmacokinetics of drugs and unknown facts of the drug. Hence a new HPLC-UV method has been developed using Inertsil ODS C18 column and Actonitrile and Methanol in 20:80 ratio as stationary phase and mobile phase for determination of Methyclothiazide in plasma using Ionic Liquid DLLME method. Novelty of the proposed method is extraction method used for sample preparation. Use of Ionic liquids as extraction solvent made the method environmentally friendly. Proposed method was accurate, precise, and robust. Matrixed calibrated standards were prepared and the developed method was linear over the range of 20-70 µg/ml. The percentage recovery for Quality controls was between 98%-102%. Hence the proposed method can be used for determination of Methyclothiazide in biological fluid.

Keywords: Bio-analysis; RP-HPLC; DLLME; Ionic Liquid; Methyclothiazide

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INTRODUCTION

Bio analysis has an important role in drug development and bioanalytical method development is one of the bottle necks in drug development. The bio analysis procedure includes sampling, sample preparation, analysis, calibration and data evaluation and reporting [1]. Bio analysis is well established in pharmaceutical companies, clinical, pre-

clinical and toxicological laboratories to support drug discovery and drug development and has become an important discipline in many research areas such as the development of new drugs, forensic analysis, doping control and identification of biomarkers for diagnostic of many diseases. Bio analysis is challenging due to the complexity of the sample matrix [2].

The validation of developed method plays a key role to demonstrate the bioanalytical method performance [3]. High performance liquid chromatography (HPLC) is a powerful technique for bio analysis of drugs. HPLC columns have a wide range of selectivity and therefore HPLC was applied for the separation of many drugs and metabolites in different biological matrices. The main type of detectors used in HPLC is UV-VIS which is cost effective.

Ionic Liquid Based Liquid-Liquid Microextraction

Method is newly adopted by the researchers in which organic solvents with densities less than 1 g/ml were used has reversed this trend [4]. This technique is typically used for environmental applications, therefore only few bioanalytical methods dealt with DLLME. Ionic liquids which are considered as green solvents developed as eco-friendly solvents in various micro extraction techniques such as LLME, SDME, SPME and DLLME. As a result of their unique physicochemical properties, Ionic liquids have aroused increasing interest for their promising role as alternative medium for classical solvent extraction [5-11]. The application of IL based DLLME methods to bio analysis were limited.

Methyclothiazide, a diuretic antihypertensive agent is a member of benzothiadiazine class of drug. It helps in prevent your body from absorbing too much salt which can cause fluid retention ,used to treat high blood pressure, fluid retention in people with congestive heart failure, Cirrhosis in liver, kidney problems or oedema caused by using steroids or estrogens. Very few methods have been reported for determination of Methyclothiazide in plasma by HPLC-UV [12], determination of Methyclothiazide in combination with Amiloride [13] and Deserpidine [14] in tablet dosage form and determination of Anti-hypertensive in plasma by LC-MS/MS where Methyclothiazide is used as internal standard [15]. Hence an attempt has been made to explore a bioanalytical method by HPLC-UV method using emerging Liquid-Liquid Micro extraction method in which Ionic liquid is used as extraction solvent (Figure 1).

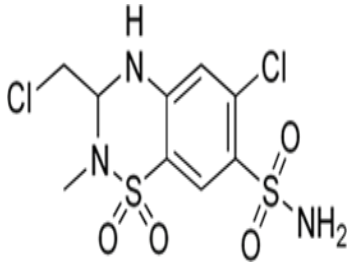

Figure 1. Chemical structure of Methyclothiazide

EXPERIMENTAL SECTION

Instrument Employed

HPLC – WATERS Model NO. 2695 series Compact System Consisting of Inertsil-C_{18} ODS column was used for quantification of recorded chromatograms.

Materials and Reagents

Methyclothiazide reference was obtained as gift sample. Acetonitrile and Methanol of HPLC grade were procured from Sigma Aldrich. Millipore water system was of HPLC grade. BMIMPF6 (>96%) was procured from Fluka, Steinheim.

Preparation of Stock Solutions

A total of 10 mg of Methyclothiazide was weighed accurately and taken in 10 ml of volumetric flask and dissolved in 10 ml of methanol to give 1.0 mg/ml stock solution (stock-A). 2 ml from stock-A was mixed 10 ml with methanol to give 200 µg/ml stock solution. 1 ml solution was vortexed for 1 min and 0.5 ml aliquot was transferred to 1.5 ml micro centrifuge tubes and stored at 20°C until used (stock-B)

Calibration Standards and Quality Controls

Matrix based Calibration standards were prepared by mixing six volumes of Methyclothiazide working solutions in the range of 20-70 µg/ml concentrations of Methyclothiazide. Four quality control samples with concentrations of 20 µg/ml (50%), 30 µg/ml (80%), 40 µg/ml (100%) and 50 µg/ml (120%) µg/ml solutions were prepared by diluting working standard. 1 ml plasma was added to each concentration and vortexed for 1 min. 0.5 ml of aliquots were transferred to 1.5 ml Eppendrof micro centrifuge tubes and stored at -20°C until used.

Extraction of Methyclothiazide from Plasma Using Ionic liquid Based DLLME

The extraction was performed in Eppendorf tubes. 100 µl of plasma spiked with 100 µl of Methyclothiazide working standard was taken in 1.0 ml centrifuge tube. To this, 50 µl Methanol, 50 µl 5%Nacl solution and 50 µl IL were added then vortexed for 60 s and centrifuged at 4500 rpm for 5.0 mins. The IL was found at the bottom of the centrifuge tube well separated from plasma. Chromatogram was obtained and no peak was observed corresponding to protein. It shows that the proteins present in the plasma do not interact with the IL selected in the present investigation.

Instrument Employed

Inertsil C_{18}ODS (4.6 x 250 mm; 5 µm) column and Acetonitrile and Methanol in 20:80 ratio were used as stationary and mobile phases respectively. Optimised chromatographic conditions are listed out in Figure 2 and Table 1.

Table 1. Optimised chromatographic conditions

Column	Inertsil ODS C18(250 x 4.6 mm, 5 µ)
Mobile Phase	Acetonitrile : Methanol (20:80)
Flow rate	1.0 ml/min
Run time	7 mins
working Concentration	40 µg/ml
Column Temperature	25°C
Volume of injection	20 µl
Detection wavelength	240 nm
Retention Time	3.521 mins
Ionic liquid	BMIMPF6
Dispersive solvent	Methanol

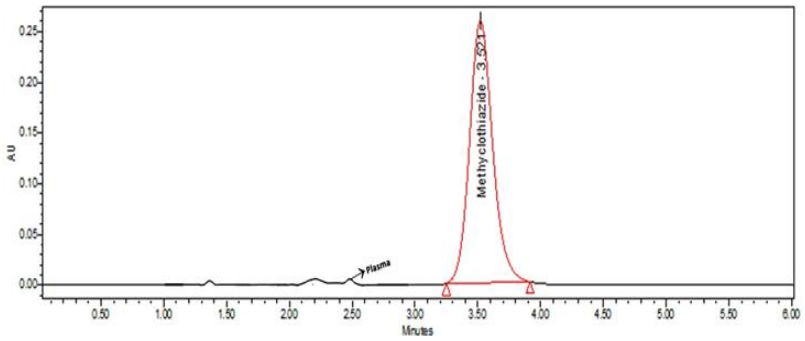

Figure 2. HPLC-UV Chromatogram of Methyclothiazide in rat plasma

METHOD VALIDATION

System Suitability Parameters

Study was done by injecting six replicates of 50% (LLOQ) and 180%(ULOQ) calibration standards solutions of Methyclothiazide into HPLC. USP plate count was 9442. Suitability of proposed method was checked by determining %RSD for retention time was found to be 0.0054. Blank plasma runs were performed to check the interference of matrix. (carry over test). The results are in Table 2.

Table 2. System suitability parameters

Injection	RT	USP Plate count	USP Tailing
1 LLOQ (50%)	3.521	9428	1.106
2	3.523	9443	1.109
3	3.520	9432	1.110
4	3.518	9451	1.107
5	3.519	9460	1.108
6	3.159	9459	1.107
Blank plasma	-	-	-
1 ULOQ (180%)	3.525	9425	1.107
2	3.523	9442	1.108
3	3.520	9423	1.110
4	3.517	9452	1.109
5	3.518	9460	1.108
6	3.156	9445	1.109
Blank plasma	-	-	-
Mean	3.5202	9442	1.107
SD	0.001924	-------	-------
% RSD	0.054643	-------	-------

Selectivity

Selectivity was evaluated using blank samples obtained from six individual slots. Chromatogram of LLOQ concentration analyte was compared with that of Blank plasma to now the possible interference components and no interfering components were observed at the retention times of analyte. Results are in Table 3.

Table 3. Identification and Interference

Sample name	Injections	Run time	RT of Methyclothiazide (mins)
Sample (50%)	1	6 mins	3.524
Slot-1	1	6 mins	-
Slot-2	1	6 mins	-
Slot3	1	6 mins	-
Slot4	1	6 mins	-
Slot-5	1	6 mins	-
Slot-6	1	6 mins	-

Linearity

The calibration standards were prepared and graph was constructed between calibration standards versus peak areas and the linear regression lines were used for the determination of concentration of Methyclothiazide in extraction samples. The method was linear over the range of 20-70 µg/ml. Preparation of linearity solutions and Linearity detector response are tabulated in Figure 3, Tables 4 and 5 respectively.

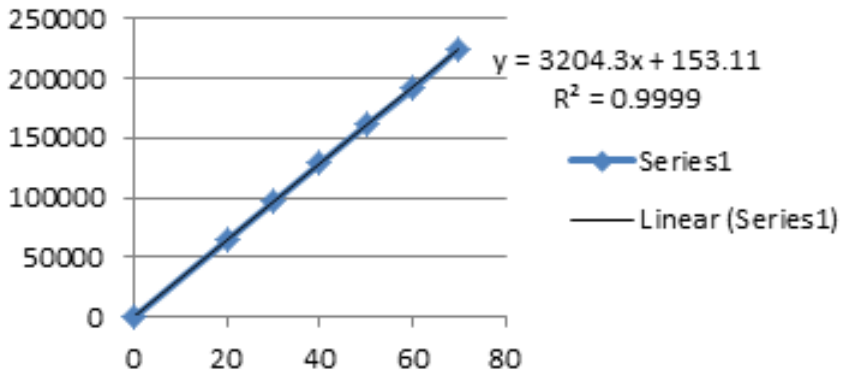

Figure 3. Linearity curve for Methyclothiazide

Table 4. Linearity solutions

Linearity level	Amount of linearity stock solution to be added (ml)	Final dilution with methanol (ml)	Concentration (ppm)
Linearity level-1 50%	1	10	20
Linearity level-2 80%	1.5	10	30
Linearity level-3 100%	2	10	40
Linearity level-4 120%	2.5	10	50

Linearity level-5 150%	3	10	60
Linearity level-6 180%	3.5	10	70

Table 5. Linearity of detector response

Sample name	No of injections	Mean area of Methyclothiazide peak
Blank	1	-
Linearity level-50%	3	64282.5
Linearity level-80%	3	96420.75
Linearity level-100%	3	128565.25
Linearity level-120%	3	160760.30
Linearity level-150%	3	191225.25
Linearity level-180%	3	224988.80

Accuracy and Precision Data

The accuracy and precision data of the analytical method was obtained by replicate processing. Precision was calculated as percentage RSD and accuracy as %Recovery. 80%, 100% and 120% concentrations were selected to determine accuracy and precision of quantification. The %Recovery for Methyclothiazide was between 98%-102% and %RSD was less than 2% which shows that the result obtained was within the limits as per ICH guidelines. The results are in Table 6.

Table 6. Accuracy and Precision data of Methyclothiazide

Analyte Level	Analyte Peak Area	Nominal Concentration (µg/ml)	Actual Concentration (µg/ml)	Individual %Recovery	Mean %Recovery	% RSD
Level 1	64280.03	20	20.08	100.04	100.23	0.22
	64264.22	20	20.07	100.35		
	64272.65	20	20.06	100.30		
Level 2	96421.78	30	30.01	100.03	100.09	0.22
	96420.75	30	30.00	100.00		
	96423.85	30	30.08	100.26		
Level 3	128582.12	40	40.08	100.20	100.20	0.22
	128514.34	40	40.06	100.15		
	128555.54	40	40.07	100.18		
Level 4	160765	50	49.63	99.26	99.27	0.21
	160763	50	49.64	99.28		
	160768	50	49.65	99.26		

Robustness Data

Robustness of the proposed method can be determined by bringing small changes in flow rate of mobile phase. Change in system suitability (tailing factor and retention time) was checked to confirm robustness of the method. Lowest and Highest concentrations in the calibration curve were used to determine the robustness of the method. Results are in Table 7.

Table 7. Robustness Data of Methyclothiazide

Injection sequence for flow rate change of mobile phase by ±10%			
Flow rate change of mobile phase by +10%			
Sample name	**No of injections**	**Retention time**	**Tailing Factor**
LLOQ	1	3.521	1.106
	2	3.523	1.110
ULOQ	1	3.520	1.112
	1	3.518	1.118
	1	3.519	1.117
Mean		3.520	1.112
SD		0.001924	0.0049
%RSD		0.054643	0.4475
Flow rate change of mobile phase by -10%			
LLOQ	1	3.525	1.123
	2	3.528	1.125
ULOQ	1	3.530	1.124
	1	3.528	1.124
	1	3.519	1.123
Mean		3.526	1.123
SD		0.001927	0.0008
%RSD		0.054736	0.0744

LOD and LOQ for Methyclothiazide

Limit of Detection is the lowest concentration of the analyte that can be determined and Limit of Quantification is the lowest that can be quantified with acceptable accuracy and precision were determined according to ICH guidelines (Table 8).

Table 8. LOD and LOQ for Methyclothiazide

Drug	LOD (µg/ml)±SD	LOQ (µg/ml)±SD
Methyclothiazide	0.134 µg/ml	0.406 µg/ml

RESULTS AND DISCUSSION

New Ionic liquid based Dispersive Liquid-Liquid Micro extraction combined with RP-HPLC method was developed for simultaneous estimation of Methyclothiazide in plasma on Inertsil ODS C18 (250 x 4.6 mm, 5 µ) column using mobile phase Acetonitrile:Methanol (20:80) to obtain good resolution of Methyclothiazide with reasonably symmetrical sharp peak, optimum retention time of with 3.521 minutes. Methanol as a Dispersive solvent and BMIMPF6 as Ionic liquid resulted in 97.10% of recovery. 5% Sodium chloride addition increased the efficiency of extraction.

The system was found to be suitable for analysis of Methyclothiazide. %RSD for retention time was 0.054%. Theoretical plates were found to 9442 and tailing factor was 1.108. There was no interference of plasma peak at the retention time drug. Standard curve for Methyclothiazide was linear over the investigational range of (20-70 µg/ml). A co-relation-coefficient of 0.999 shows that the developed HPLC method for Methyclothiazide is linear over the range of 20-70 µg/ml. The developed method for analysis Methyclothiazide was found to be accurate and Precise. The mean percentage recovery was between 98% to102% and %RSD for accuracy samples (80%, 100% and 120%) was found to be less than 2%.

Robustness of developed method was established by changing flow rate of mobile phase by ±2%. However the retention times and peak areas kept slight changing from actual values but %RSD for all the samples was not more than 2%. LOD and LOQ were determined by preparing calibration curve using samples containing analyte in the range of detection and quantification limits. LOD and LOQ were 0.134 µg/ml and 0.406 µg/ml found to be respectively.

CONCLUSION

A new, accurate, precise and robust HPLC-UV method was developed with combination of Ionic Liquid based Dispersive Liquid-Liquid Micro extraction method. It was an excellent Pre concentration method for determination of Methyclothiazide in plasma. Ionic liquids being green solvents make the method environmentally friendly. The proposed method resulted in good resolution within less chromatographic runtime of 6 mins. The developed method exhibited excellent performance in terms of selectivity, sensitivity and %recovery. The results obtained were within the limits as per ICH guidelines. Hence the method can be applied for bioanalysis of Methyclothiazide in plasma at therapeutic levels for Therapeutic drug monitoring.

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