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In Vitro Studies for Nephrotoxicity in Renal Failure Patients by Using Human Plasma Sample and Bioanalytical Method Development and Validation of Torsemide by RP-HPLC Method

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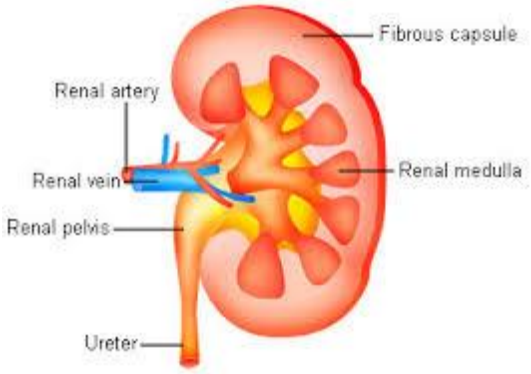
ABSTRACT

A rapid high - performance liquid chromatographic bioanalytical method has been developed and validated for torsemide in human plasma. Torsemide is a pyridine-sulfonyl urea type loop diuretic mainly used in the management of edema associated with conjunctive heart failure. Torsemide was found with symmetrical peak shapes on an analytical column, phenomenex Luna C18 using 60 % acetonitrile with and 40% water as the mobile phase Flow rate was maintained at 1.0 ml/min, detection wave length was 225 nm, column temperature was set as ambient and diluent was mobile phase. Conditions were finalized as optimized method. The retention times of torsemide and spironolactone, the internal standard were 3.014 and 6.754 min respectively. System suitability parameters were studied by injecting the standard five times and results were well under the acceptance criteria. Linearity study was carried out between 5% to 100 % levels, R 2 value was found to be as 0.999. Precision was found to be 0.2 for repeatability and 1.2 for intermediate precision. LOD and LOQ are 0.10µg/ml and 1.00µg/ml respectively. By using above method assay of marketed formulation was carried out 100.01% was present.

INTRODUCTION:

The kidneys lie on the posterior abdominal wall, one on each side of the vertebral column, behind the peritoneum and below the diaphragm. They extend from the level of the 12th thoracic vertebra to the 3rd lumbar vertebra, receiving some protection from the lower rib cage. The right kidney is usually slightly lower than the left, probably because of the considerable space occupied by the liver.



Kidney function tests: 1) Blood pressure.

2) Blood creatinine test.

3) Blood urea test.

4) Urine analysis.

5) Urea clearance test.

6) Creatinine clearance test.

7) Estimated glomerular filtration rate.

8) Blood urea nitrogen test.

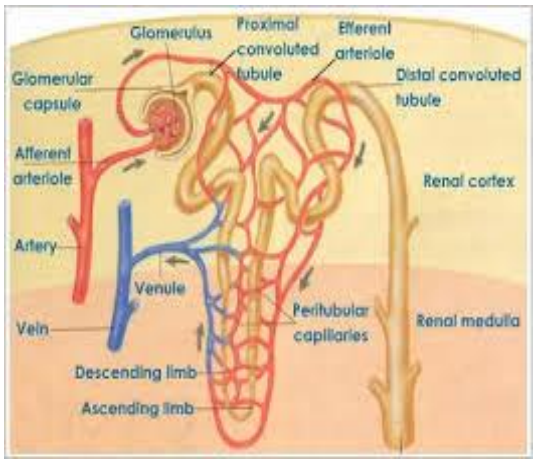
The nephron consists of a tubule closed at one end, the other end opening into a collecting tubule. The closed or blind end is indented to form the cup-shaped *glomerular capsule* (Bowman's capsule) which almost completely encloses a network of arterial capillaries, the *glomerulus.* Continuing from the glomerular capsule the remainder of the nephron is about 3 cm long and is described in three parts:

• The *proximal convoluted tubule*

• The *medullary loop* (Loop of Henle)

• The *distal convoluted tubule*, leading into a *collecting duct.*

The collecting ducts unite, forming larger ducts that empty into the minor calyces.



After entering the kidney at the hilum the renal artery divides into smaller arteries and arterioles. In the cortex, an arteriole, the *afferent arteriole*, enters each glomerulus. The series of blood vessels in the kidney capsule then subdivides into a cluster of capillaries, forming the glomerulus. Between the capillary loops, there are connective tissue phagocytic *mesangial cells*, which are part of the reticuloendothelial system.

A bioanalytical method is a set of procedures involved in the collection, processing, storage, and analysis of a biological matrix for a chemical compound. Bioanalytical method validation (BMV) is the process used to establish that a quantitative analytical method is suitable for biochemical applications. Reassurances as to the quality of the method and its reliability come from adopting a minimum series of validation experiments and obtaining satisfactory results. Characterization of the stability of analytes in biological samples collected during clinical studies together with those critical assay reagents, including analyte stock solutions, is recognized as an important component of biological assay validation. Validation involves documenting, through the use of specific laboratory investigations, that the performance of characteristics of the method is suitable and reliable for the intended analytical applications.

The quality of these studies is directly related to the quality of the underlying Bioanalytical data. It is therefore important that guiding principles for the validation of these analytical

methods be established and disseminated to the pharmaceutical community. Both RP - HPLC and LCMS.

MS can be used for the bioanalysis of drugs in plasma. Each of the instruments has its own merits. The fundamental parameters for this validation includes selectivity, accuracy, precision, linearity, limit of detection, limit of quantification, recovery, robustness, stability and range. The objective of validation of Bioanalytical procedures is to demonstrate that it is suitable for its intended purpose. The most widely accepted guidelines for method validation is the ICH guidelines Q2 (R1), which is used both in pharmaceutical and medical science.

Method Development: Analytical method development is the process of creating a procedure to enable a compound of interest to be identified and quantified in a matrix. A compound can often be measured by several methods and the choice of analytical method involves many considerations, such as chemical properties of the analyte, concentrations levels, sample matrix, cost of the analysis, speed of the analysis, quantitative or qualitative measurement, precision required and necessary equipment. The analytical chain describes the process of method development and includes sampling, sample preparation, separation, detection and evaluation of the results.

Sample collection and preparation: Collected by following methods-

1) Liquid – Liquid extraction: Liquid-Liquid extraction generally involves the extraction of a substance from one liquid phase to another liquid phase. Now a day's traditional LLE has been replaced with advanced and improved techniques like liquid phase microextraction, single drop liquid phase microextraction and supported membrane extraction.

2) Solid Phase Extraction (SPE): Solid phase extraction is selective method for sample preparation where the analyte is bound onto a solid support, interferences are washed off and the analyte is selectively eluted. Solid phase consists of four steps; conditioning, sample loading, washing and elution.

3) Protein Precipitation: Protein precipitation is often used in routine analysis to remove proteins. Precipitation can be induced by the addition of an organic modifier, a salt or by changing the pH which influences the solubility of the proteins. The samples are centrifuged and the supernatant can be injected into the HPLC system or be evaporated to dryness and thereafter dissolved in a suitable solvent.

4) Bioanalytical Method Validation (BMV): The reason for validating a bioanalytical procedure is to demonstrate the performance and reliability of a method and hence the confidence that can be placed on the results. In addition, Shah *et al*. has stated that all Bioanalytical methods must be validated if the results are used to support registration of a new drug or the reformulation of an existing one.

MATERIALS AND METHODS:

MATERIALS

Reagents Used For *In Vitro* Studies-

Reagents	Uses
ERBA wash	solution for cleaning micro flow cells & cuvets (*In vitro* diagnostic use only)
Strips	Used for urine analysis
Glucose – LS reagent	Used for sugar test
Creatinine buffer reagents (Picric acid, NaOH reagent)	Used for creatinine test
Uric acid reagents	Used for urea test

Instruments Used For *In Vitro* Studies-

Instruments	Uses
Bioanalyzer (AGILENT MODEL)	Used for evaluation parameter tests
Sphygmomanometer	Used for blood pressure

Instruments Used For HPLC-

S. No	Name	Model	Manufacturer
1.	pH meter	-	Eutech
2.	Weighing balance	-	Denver
3.	Ultrasonicator	UCA 701	Unichrome
4.	HPLC	LC	Waters 2695- Empower software
5.	Flow rate	1.0 mL/min	--
6.		Isocratic model	--

Reagents and Chemicals-

S. No.	Name	Grade	Manufacturer
1.	Water	HPLC	Rankem
2.	Acetonitrile	HPLC	Merck

Drug Samples- Torsemide & spironolactone was purchased from Indian market manufactured by Cipla Pvt Ltd, Ankleshwar. Commercial pharmaceutical preparation of Torsemide and spironolactone tablets which are claimed to contain 10 mg and 25 mg of were used in analysis.

METHOD

In vitro studies for nephrotoxicity in renal failure patients by using human plasma samples and bioanalytical method development and validation of torsemide by RP-HPLC method for the ratio (40:60 v/v) used as mobile phase and flow rate of 1.0 ml/min. The detection was carried out at 218 nm and ambient column temperature was maintained.

Clinical Study- To diagnose the kidney disease by made evaluation parameters (blood tests) on eleven kidney failure patients by the permission of through proper authorization worked as a project trainee in global multispeciality hospital and observe the kidney failure patients case study, symptoms, treatment to that select one patient and perform evaluation parameters for estimation of kidney disease compare the obtained results with normal values by the help of lab technician in laboratory and collect the blood sample for method development.

EVALUATION PARAMETERS (BLOOD TESTS)	NORMAL VALUES	SELECTED PATIENT REPORTS
Blood pressure	120/80	140/90
Blood creatinine	0.6 -1.4 mg /dl	11.9 mg/dl
Blood urea	10 – 45 mg / dl	60 mg/dl
Urine analysis	Absence of Proteins & blood cells	Presence of Proteins and blood cells
Creatinine clearance test	75 -125 ml / min	115 ml / min
Blood urea nitrogen test	20 – 40 mg / dl	80 mg / dl
Blood sugar test	60 - 160	264 mg/dl

Above the obtained results in the table to confirm the patient was suffering from kidney disease and to that patient collect the blood sample for method development and validation of torsemide.

METHOD DEVELOPMENT

Selection of the wavelength for Simultaneous Estimation:

In setting up the conditions for development of the assay method, the choice of the detection wavelength was based on the scanned absorption spectrum for Torsemide and Spironolactone.

Accurately weighed 10mg of Torsemide and Spironolactone, were transferred to 10 ml volumetric flasks. The compounds were then dissolved in Buffer and ACN in the ratio 50:50v/v and the volumes were made up to the mark with solvent. We get 1mg/ml solution of both Torsemide and Spironolactone. From the above solution pipetted out 1ml into one separate 100ml volumetric flasks and made up to the mark with solvent.

The prepared solution was loaded into the autosampler and the system was set in order to take the auto-injection in HPLC with PDA detector. The Spectrum obtained is as follows:

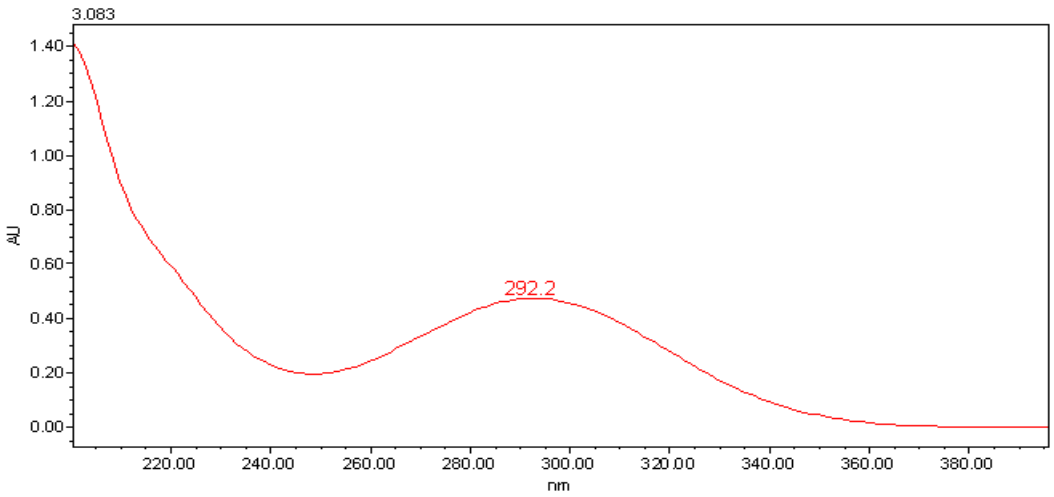


Figure - 1 Spectrum of Torsemide

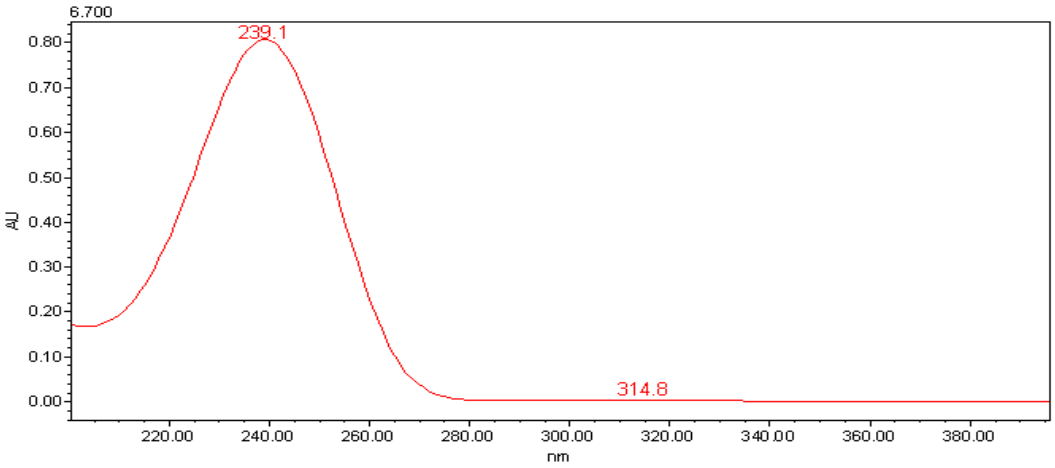


Figure- 2 Spectrum of Spironolactone

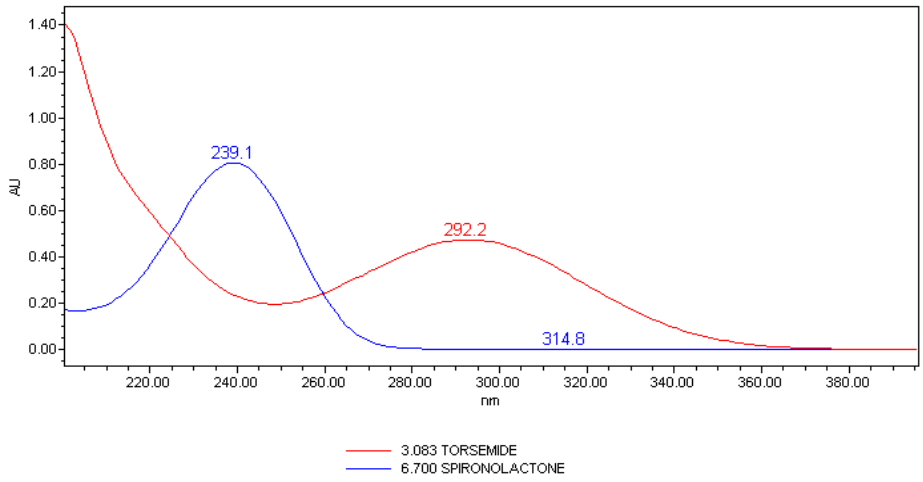


Figure - 3 Spectrum of Overlay

From the above spectra of both drugs, a wavelength was selected at which both the drugs showed maximum absorbance. The wavelength selected was 225 nm.

RESULTS AND DISCUSSION:

In vitro studies for nephrotoxicity in renal failure patients by using human plasma sample and Bioanalytical method Development and Validation of Torsemide by RP-HPLC method.

To perform the *in vitro* studies on renal failure patients by using blood samples to estimate the disease compared with normal values to the obtained results. Select one patient to confirm the disease by made blood tests in laboratory with the help of global multi specialty hospital lab technicians obtained results were compared with normal values.

EVALUATION PARAMETERS (BLOOD TESTS)	NORMAL VALUES	SELECTED PATIENT REPORTS
Blood pressure	120/80	140/90
Blood creatinine	0.6 -1.4 mg /dl	11.9mg/dl
Blood urea	10 – 45 mg / dl	60mg/dl
Urine analysis	Absence of proteins & blood cells	Presence of proteins and blood cells
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Blood sugar test	60 - 160	264 mg/dl

Above the obtained results in the table to confirm the patients was suffering from kidney disease and to that patient collect the blood sample for method development and validation of torsemide.

RP HPLC is commonly used in laboratories for the qualitative and quantitative analysis of drug substances, metabolites. The present review focused on various extraction techniques like liquid-liquid extraction, solid phase extraction and protein precipitation which play important role in sample preparation and detection by RP HPLC and consistent evaluation of the key bioanalytical method validation parameters is discussed: accuracy, precision, sensitivity, selectivity, standard curve, limits of quantification, range, recovery stability *etc*. These validation parameters are described, together with an example of validation methodology applied in the case of chromatographic methods used in bioanalysis. Bioanalytical method development and validation of torsemide was carried out an Luna C18, (250 mm x 4.6 mm, 5 µm) in an isocratic model, using mobile phase composition of Water, and acetonitrile in the ratio (40:60 v/v) with a flow rate 1.0 ml/min. The effluents were monitored at 218 nm. From the results % assay value of Torsemide Extract found to be 99.9% respectively.

Linearity was observed over the concentration range of Torsemide 0-5 µg/ml for Extract. Correlation coefficient was found to be 0.999 which indicates that the concentration had given good linearity.

The % RSD values of Torsemide Extract for System precision and Method precision was found to 0.008 respectively. As the results are within acceptance limit of less than 2%, indicates that the proposed method has good reproducibility. The results are good for both method precision and system precision.

From the results shown in accuracy, it was found that the mean percentage recovery values of pure drug were found to be 99.9% for Torsemide Extract as these results are within the acceptance limit of 98%-102% which indicates that the method was accurate.

The ruggedness of the proposed method was analyzed by two different analysts. The % RSD was found within the limits i.e., should not be more than 2.0. Hence the proposed method has good repeatability.

Table 1- VALIDATION PARAMETERS FOR TORSEMIDE

S. No.	Parameters		Extract
1	Linearity (µg/ml)		0-5
	Correlation Coefficient		0.999
2			
	(i) Method Precision		
	(ii) Intermediate Precision		0.0912
	(iii) System Precision		0.008
3	Accuracy (% of recovery)	50%	0.150
		100%	0.084
		150%	0.111
4	Assay (%)		99.9

CONCLUSION:

- *In vitro* studies performed on renal failure patients to diagnose the disease and compared with the normal values. The obtained results were found to be show the patient was suffering with kidney problem the diseased patient blood sample was collected and performed the bioanalytical method development.

- The bioanalytical method developed is simple and shows good accuracy, precision, assay, linearity, ruggedness within the limits. It can be used for the estimation of biofluids. These paration method developed produce acceptable values of recovery. The chromatogram developed has well resolved peak of torsemide without any interference. The developed method could be applied in pharmacokinetic, bioequivalence, toxicokinetic studies.

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