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NOVEL BIO-ANALYTICAL RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ACECLOFENAC AND DROTAVERINE HYDROCHLORIDE IN HUMAN PLASMA BY DOE APPROACH

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ABSTRACT

A simple, accurate, precise, specific and robust RP-HPLC method is developed for simultaneous estimation of aceclofenac and drotaverine hydrochloride in human plasma by applying DoE approach. Liquid chromatography was performed on a Kromasil C8 column (150 × 4.6 mm, 5µm) and the mobile phase consisted of Acetonitrile: Ammonium acetate buffer pH 3.5 (53:47 v/v) with 1ml/min of flow rate. The effluent was monitored on a UV detector at 230 nm. Out of three types of extraction procedures viz. protein-precipitation, liquid-liquid extraction and solid-phase extraction applied; good recovery was obtained using solid-phase extraction and hence was selected for further extraction. Average recovery of extracted sample from plasma was found to be 71 % and 98 % for aceclofenac and drotaverine hydrochloride respectively. There was a good linear relationship of peak area of both the drugs with the concentration which ranges from 30 – 9000 ng/ml and 50 – 180 ng/ml for aceclofenac and drotaverine hydrochloride respectively. Bio-analytical samples were found to be stable over three freeze-thaw cycles showing good stability of drugs in plasma. The bio-analytical method was validated for selectivity, linearity, accuracy, precision and stability according to USFDA guidelines and can be further applied for therapeutic monitoring of these drugs in patients.

KEYWORDS: Aceclofenac, Drotaverine hydrochloride, Bioanalytical method, RP-HPLC, Human plasma.

1. INTRODUCTION

Aceclofenac (Fig. 1) (ACF), 2- [2- [2- [(2, 6-diclorophenyl) amino] phenyl] acetyl] oxyacetic acid, is a specific cyclooxygenase-2 (COX-2) inhibitor with phenylactetic acid derivative acting as a potent non-steroidal anti-inflammatory and analgesic drug. It is used in the management of osteoarthritis, rheumatoid arthritis, and ankylosing spondylitis.[1] Aceclofenac is official in British Pharmacopeia and Indian Pharmacoepeia.[2] Various UV- Vis spectrophotometric, HPTLC, HPLC and densitometric methods are reported for aceclofenac for its estimation individually or in combination with other drugs.[3–12] Drotaverine hydrochloride (Fig. 2) (DRT), (Z) -1-(3, 4-diethoxybenzylidene) -6, 7- diethoxy -1, 2, 3, 4 tetrahydroisoquinoline is an analogue of papaverine. It acts as an antispasmodic agent by inhibiting phosphodiesterase IV enzyme and is often used to reduce the labor pain.[13] Drotaverine hydrochloride is official in Polish Pharmacopeia.[14,15] Various articles have been published on use of UV spectrophotometry technique for estimation of drotaverine in pharmaceuticals. Membrane selective electrode method, HPTLC, RP-HPLC, cathode adsorptive stripping voltammetry, luminescence, spectrofluorimetry methods are also reported for the estimation of drotaverine in formulations and biological samples.[15–24]

The combination of aceclofenac and drotaverine hydrochloride is used for treating the patients suffering from severe muscular pain and inflammation. The combination can also be used for curing Cholangitis, Cholecystitis, Cholecytolithiasis, Cystitis, Nephrolithiasis, Papilitis, Smooth muscle spasm, Stone formation, Ureterolithiasis, Urolithiasis and Vesical tenesmus.[25]

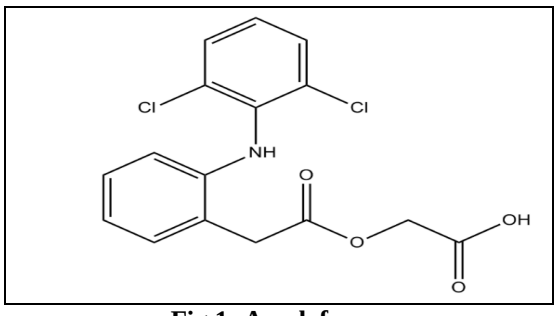

Fig 1: Aceclofenac.

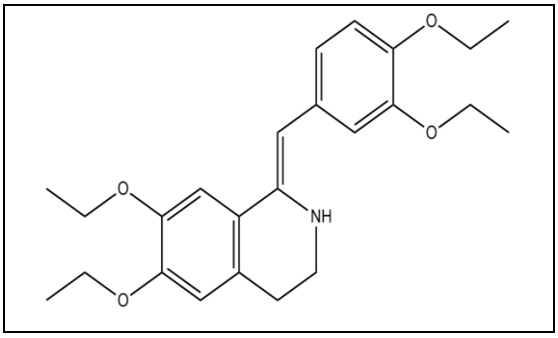

Fig. 2: Drotaverine hydrochloride.

From the literature review, it was revealed that the analytical methods like HPLC, UV are reported for estimation of ACF and DRT in combined dosage form. However, no bio-analytical method is reported for combination of both these drugs in any of the biological fluids. Hence, it was endeavoured to develop and validate bio-analytical method for simultaneous estimation of ACF and DRT in human plasma using RP-HPLC method which can be further used for therapeutic monitoring of these drugs in patients.

Statistical experimental design, called as Design of Experiments (DoE), is the method to carry out and plan experimental processes for extracting out the utmost quantity of informational data with the least analyses steps.[26,27] Due to the higher cost of experimentation, it is difficult for any analyst to examine various factors that can affect the processes; while trial and error method is found to be too tedious for examining their complexity. DoE is the technique that focuses on the vital factors in few numbers and regulates the process to achieve increased productivity and improved quality. Hence, the bio-analytical method was developed and optimized by DoE approach.

2. EXPERIMENTAL WORK

2.1 Chemicals and reagents

Aceclofenac and drotaverine hydrochloride were provided as a gift sample by Piramal Healthcare Pvt. Ltd., Ahmedabad, India and Troikaa Pharmaceuticals, Ahmedabad, India respectively. While, the Internal standard Mefanamic acid was obtained as a gift sample from Nirlife Healthcare Pvt. Ltd., Ahmedabad, India. Orthophosphoric acid was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. While, HPLC grade of Methanol, Acetonitrile, Trichloroacetic acid, Ethyl acetate, n-Hexane, Iso-propyl alcohol were purchased from S. D. Fine Chemicals Ltd., Mumbai, India. Blank human plasma was gifted by Suraktam Blood Bank, Vadodara, India and SPE cartridges were purchased from Orochem India Pvt. Ltd., Mumbai, India.

2.2 Preparation of standard solutions

Ammonium acetate buffer (0.1 M) was prepared by dissolving 0.770 gm of ammonium acetate in 1000 ml of water and adjusting pH to 3.5 with 0.1M *o*-phosphoric acid solution. Standard stock solution of ACF and DRT was prepared by dissolving ACF (10 mg) and DRT (8 mg) in ratio of 100:80 in 10 ml of Methanol and sonicated. From this combined solution of of ACF (1000 µg/ml) and DRT (800 µg/ml), 1 ml of solution was withdrawn and transferred to 10 ml amber coloured volumetric flask and volume was made up with methanol. The standard solution obtained was of concentrations 100 µg/ml and 80 µg/ml of ACF and DRT, respectively. For MFA (Internal standard), 1mg of the drug was dissolved in 10 ml of methanol and sonicated to get a concentration of 100 µg/ml. Further, 1 ml of this solution was pipette out and transferred to 10 ml amber coloured volumetric flask and volume was made up with methanol, to get a concentration of 10µg/ml.

2.3 Instrumentation and Chromatographic conditions

The HPLC method was performed using Jasco HPLC system (LC-2000 Plus series) consisting of a binary pump, column heater, UV- Vis detector. The data collection and analysis were performed using Jasco Borwin version 1.50 software. Chromatographic separation was achieved using reverse-phase KROMASIL C8 (150*4.6mm, 5µm) column with a mobile phase containing a mixture of acetonitrile and ammonium acetate buffer (53:47 v/v) adjusted to a pH of 3.5 with *o*-phosphoric acid. The mobile phase was filtered, degassed and pumped at a flow rate of 1 ml/min The effluent was monitored on a UV detector at 230 nm.

2.4 Optimization of HPLC method by DoE approach

Design Expert Software was used for applying DoE with file version 10.0.1. Quadratic model, Three-level Full Factorial Design with Response Surface Methodology was used for the experiment. Total 9 trial runs were carried out for concentration ratio of 10: 8 ppm of ACF and DRT in a mixture, using mobile phase as the solvent for preparation of the drug solution. Mobile phase ratio and pH were taken as independent factors which were varied to get the trial runs. Resolution, theoretical plates for ACF and theoretical plates for DRT were the responses that were analysed in order to obtain the optimized HPLC conditions.

2.5 Procedure for sample extraction

For protein precipitation, 500µl pre-vortexed blank human plasma was taken in 10 ml micro-centrifuge tube. It was spiked with 50 µl of drug solution and 50 µl of internal standard solution and vortexed for 2 min. Precipitating agent viz. Acetonitrile, Methanol and Trichloroacetic acid were added to the mixture and allowed to precipitate for 8-10 min without shaking it. Upper organic layer was separated out and filtered with syringe filter. It was then evaporated and reconstituted with 2 ml of mobile phase and injected for separation into HPLC system. Optimization of plasma volume and precipitating agent volume was done to get better recovery.

For LLE, pre-vortexed 500 µl blank human plasma was taken in 10 ml micro centrifuge tube. It was spiked with 50 µl drug solution and 50 µl internal standard solution and vortexed for 2 min. To this solution, 3 ml of extracting solvents in different ratios were added. It was then vortexed for 10 min and centrifuged for 10 min at 7000 rpm. Organic layer was separated and then was transferred in 5 ml glass tube. The organic layer was evaporated to dryness in water bath at 50℃. After reconstituting with 2.5 ml mobile phase (ACN: Ammonium Acetate buffer), it was subsequently injected for separation into HPLC system. Different extracting solvents; Hexane, Diethyl ether, Dichloromethane, Ethyl acetate, Iso-propyl alcohol, Methanol in different ratios were tried according to their polarity.

For SPE, conditioning of cartridge was done with 1 ml methanol which was then allowed to pass through the cartridge by starting the extractor. Pre-vortexed 500 µl blank human plasma spiked with 50 µl of drug solution and 50 µl internal standard solution were loaded into the cartridges and allowed to pass through. Cartridges were then washed with 1 ml triple distilled water and dried. To elute out the drugs, 1 ml of mobile phase (ACN:Buffer) was added into the cartridge and taken into an eppendorf. It was then diluted with 1.5 ml of mobile phase and injected into HPLC system for further separation.

2.6 Methodology for validation

The developed bio-analytical method for determination of ACF and DRT in human plasma was validated according as per USFDA guidelines for bioanalytical method validation. The method was validated for selectivity, linearity, accuracy, precision, freeze-thaw stability and bench-top stability.

2.6.1 Selectivity: To test the selectivity, 950 µl of blank human plasma was spiked with 50 µl of ACN: Ammonium acetate buffer (53:47), without analytes and IS. It was then injected directly after extraction. LLOQ sample was prepared by spiking 30 ng/ml of ACF, 50 ng/ml of DRT and 50 ng/ml of IS from their stock solutions, upto 1 ml of blank human plasma. Six replicates of this LLOQ sample were injected after extraction.

2.6.2 Linearity: The linearity was evaluated by analysing standard plots associated with ten concentrations of standard calibration curve and one concentration of LLOQ. Each of these concentrations were prepared by adding 30 µl of ACF, 50 µl of DRT in increasing amounts and 50 µl of IS, separately, to 1 mL of blank plasma and the samples were then extracted by SPE. The final linearity concentrations were: 30, 50, 100, 250, 500, 1000, 2000, 3500, 5000, 7500, 9000 ng/ml for ACF and 50, 75, 90, 115, 120, 130, 140, 150, 160, 170, 180 ng/ml for DRT. A calibration curve was plotted for given concentrations against their peak area.

2.6.3 Accuracy: Accuracy was performed for three concentrations at LQC (LQC should be near to three times the LLOQ concentration), MQC and HQC levels, with five aliquots of each. The final concentrations obtained were 90, 1100 and 7400 ng/ml for ACF and 110, 135 and 165 ng/ml for DRT. 50 µl of IS from standard solution was added to the drug spiked plasma. Extraction was performed by SPE and % CV was calculated.

2.6.4 Precision: The precision was carried out at three different levels. Repeatability was performed by injecting a mixture of LQC, MQC and HQC samples of linearity range: 90, 1100 and 7400 ng/ml for ACF, 110, 135 and 165 ng/ml for DRT with 50 µl of IS standard solution. Five aliquots taken were extracted from plasma by SPE method and % CV was calculated. Intra-day and Inter-day precision was determined by measuring the peak area of ACF and DRT three times within a day (3 hrs, 6 hrs and 9 hrs) and on three different days (1st day, 2nd day and 3rd day), respectively. For intra-day and inter-day precision studies, three QC level samples of concentration 90, 1100 and 7400 ng/ml for ACF and 110, 135 and 165 ng/ml for DRT were taken. 50 µl of IS standard solution was added to the drug spiked plasma, extracted and % CV values calculated.

2.6.5 Stability Studies: For freeze-thaw stability determination, the freezing and melting of stability samples were done for upto three freeze/thaw cycles at regular time interval of 24 hrs, 48 hrs and 72 hrs. %RSD of samples were calculated for LQC (90 ng/ml) and HQC (7400 ng/ml) levels of ACF and LQC (110 ng/ml) and HQC (165 ng/ml) levels of DRT.

Bench top stability was carried out by testing samples placed at one constant place and position at different time interval of 3 hrs, 6 hrs, and 9 hrs. %CV was calculated for LQC (90 ng/ml) and HQC (7400 ng/ml) levels of ACF and LQC (110 ng/ml) and HQC (165 ng/ml) levels of DRT.

3. RESULTS AND DISCUSSION

3.1 Method optimization by DoE approach

An experimental design comprising of mobile phase ratio and pH at three levels led to a total of 9 chromatographic runs. The conditions were evaluated for resolution, theoretical plates of ACF and theoretical plates of DRT. From which it was found that mobile phase ratio (53:47 v/v) with pH 3.5 shows resolution, theoretical plates of ACF and theoretical plates of DRT in acceptable limits. Hence, method optimised by DoE resulted in a robust performance.

Fig. 3 shows the overlay plot and desirability for the applied DoE. From the overlay plot, it has been observed that yellow region gives the favourable conditions for the experiment, while other regions cannot be considered favourable one. And the desirability in that region is 1,

which shows that the region is desirable for experiment to be performed.

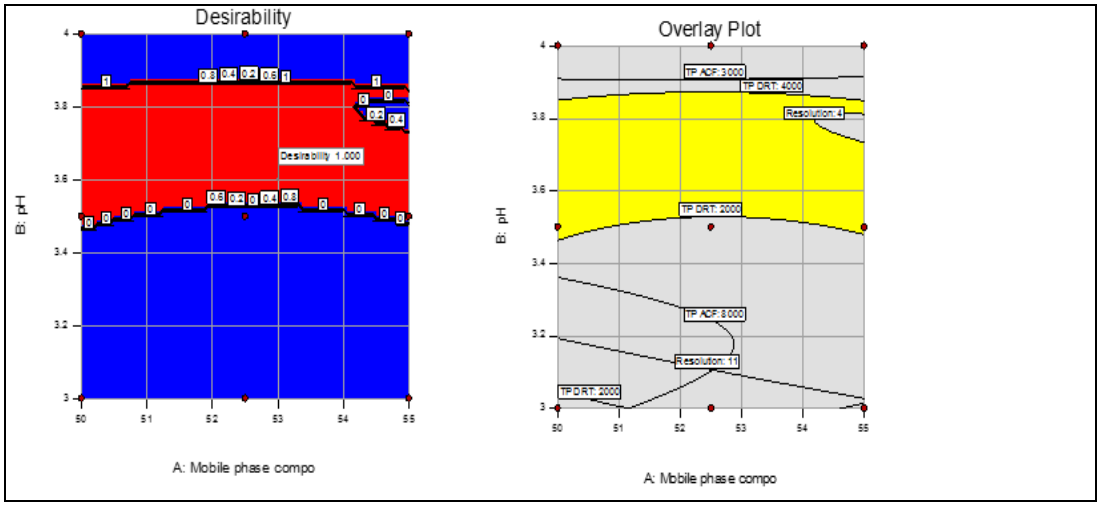

Fig. 3 overlay plot and desirability.

3.2 Sample extraction

Extraction procedure of PP showed recovery of 53% and 22% for ACF and DRT respectively, while LLE showed recovery of 40% and 60% for ACF and DRT respectively. However, SPE showed the best recovery out of the three that of 71% and 98 % for ACF and DRT respectively. Hence, SPE was selected as the optimized method for developing and validating the bio-analytical method for determination of DRT and ACF in plasma. Depending upon the nature and solubility of both the drugs, selection of SPE cartridge was done. ACF is lipophilic while DRT is hydrophilic, so selecting the cartridge was tedious but the recovery of both the drugs were better compared to other extracting methods. Diluted solution of methanol gave less recovery as compared to the pure triple distilled water when used in washing step for SPE. Hence, washing of the cartridge was done by triple distilled water which produced consistent results.

3.3 Method Validation

All method validation parameters performed as per USFDA guidelines are shown in Table 1.

3.3.1 Selectivity

The retention time of the peaks obtained for blank human plasma and for the LLOQ concentration of drugs were compared. No interference was observed between the plasma and the drug peaks as well as that of internal standard.

3.3.2 Linearity

ACF and DRT were found to be linear over the concentration range of 30 - 9000 ng/ml and 50 - 180 ng/ml with an equation of y= 20.745x + 1999.2 and y= 27.558x – 281.57, respectively (Fig. 4). The correlation coefficient obtained were 0.9952 and 0.9926 for ACF and DRT, respectively.

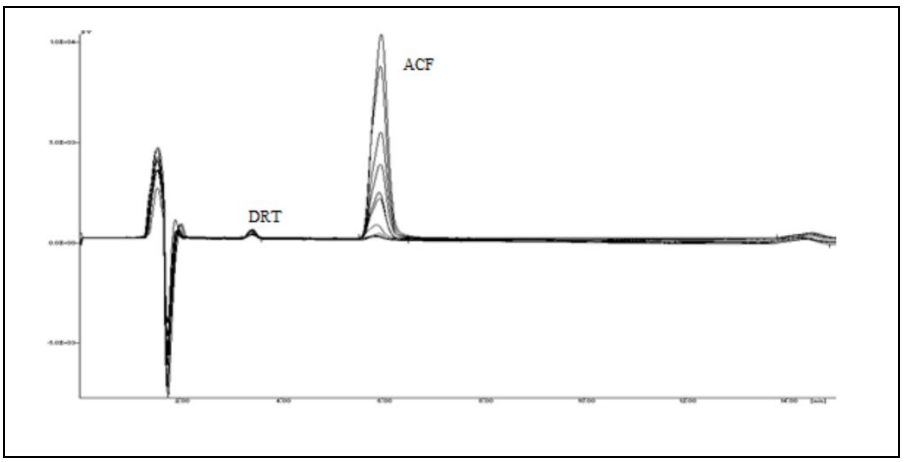

Fig. 4 linearity chromatogram of ACF and DRT.

3.3.3 Accuracy

The % CV for the LQC, MQC and HQC samples of ACF and DRT individually observed were 2.07% and 2.82% respectively indicating the method to be accurate.

3.3.4 Precision

As shown in Table 2, % CV for repeatability and intra-day as well as inter-day precision for all three QC samples were within acceptance criteria indicating a precise method.

3.3.5 Stability

% CV was found to be within the acceptance criteria for both freeze-thaw stability and bench-top stability study. Also, no significant change for ACF and DRT peak areas was observed after exposing the samples to three freeze-thaw cycles.

Table 1: Summary of validation parameters.

Parameter	ACF			DRT		
Selectivity	No interference			No interference		
Linearity	30 – 9000 ng/ml			50 – 180 ng/ml		
Regression equation	y = 20.745x + 1999.2			y = 27.558x - 281.57		
Correlation Coefficient	0.9952			0.9926		
Accuracy	**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
(% CV)	1.18 %	2.07 %	1.06 %	1.82 %	0.93 %	1.23 %
Precision	**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
Repeatability (% CV)	1.34 %	1.44 %	0.71 %	1.36 %	1.04 %	1.02 %
Intraday (% CV)	1.17 %	0.55 %	0.61 %	1.26 %	1.57 %	0.39 %
Interday (% CV)	7.21 %	6.04 %	3.36 %	1.25 %	2.56 %	1.20 %
Freeze Thaw Stability (% CV)	**LQC**		**HQC**	**LQC**		**HQC**
	(1) 1.14 % (2) 0.72 % (3) 2.56 %		(1) 0.72 % (2) 0.20 % (3) 0.45 %	(1) 2.34 % (2) 0.31 % (3) 0.32 %		(1) 1.03 % (2) 0.44 % (3) 0.23 %
Short Term Stability (Bench Top Stability) (% CV)	**LQC**		**HQC**	**LQC**		**HQC**
	(1) 0.59 % (2) 1.05 % (3) 0.34 %		(1) 0.64 % (2) 0.53 % (3) 1.18 %	(1) 2.15 % (2) 1.55 % (3) 1.27 %		(1) 1.22 % (2) 0.27 % (3) 2.78 %

4. CONCLUSION

In summary, the RP-HPLC method optimized by DoE is used for the determination of aceclofenac and drotaverine hydrochloride in human plasma. The biological matrix (plasma) that was used for the extraction method was found to be free from any interference. For the extraction, solvent optimization is done precisely to get the usage of minimum amount of solvent and also to extract maximum amount of drugs from the plasma. After comparing all of the three extraction methods viz. PP, LLE and SPE, SPE was selected as it gave a better recovery. The proposed SPE procedure for both the drugs is efficient, rapid and sensitive for routine analysis while the bioanalytical method meets all the acceptance criteria according to the USFDA guidelines. Hence, it can be used for the quantitative estimation and determination of ACF and DRT in human plasma as well as for its therapeutic monitoring in patients.

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