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Original Article

DEVELOPMENT AND VALIDATION OF A NEW RP HPLC ANALYTICAL METHOD FOR THE DETERMINATION OF ETODOLAC SUCCINIC ACID CO-CRYSTALS IN SPIKED RABBIT PLASMA

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ABSTRACT

Objective: The aim of the study was to develop and validate a novel and sensitive HPLC method for the determination of Etodolac content in Etodolac succinic acid co-crystals in spiked rabbit plasma.

Methods: Chromatographic separation was achieved on an Eclipse C18 column (4.6 mm,100 mm, 3.5 µm spherical particles) using acetonitrile: methanol: acetic acid (100%) (50:49:1) as the mobile phase at a flow rate of 0.8 ml/min and monitored at 278 nm. Tinidazole was used as the internal standard. The run time was 6 min. The method was validated to fulfill International Conference on Harmonisation (ICH) guidelines, which included specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and robustness.

Results: The calibration curve was linear over the concentration range from 2.5 to 15 µg/ml, and the lower limit of detection was 0.3700 µg/ml. and lower limit of quantification was 1.121µg/ml for determination in spiked rabbit plasma. The accuracy and precision of the method were within the acceptable limit of±2% at the lower limit of quantification.

Conclusion: A simple, sensitive, rapid and reproducible RP-HPLC method was developed with short runtime and less flow rate. Statistical analysis of the method proved that this method is suitable for the estimation of Etodolac in Co-crystalin plasma. Hence this method can be employed in the routine assay of the Etodolac Succinic acid co-crystals.

Keywords: Etodolac, Succinic acid, HPLC, Co-crystals

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INTRODUCTION

Etodolac is a nonsteroidal anti-inflammatory drug. Etodolac is widely used for the treatment of osteoarthritis and post-surgery analgesia. The drug is practically water-insoluble, and due to its very poor water solubility, it has only limited bioavailability. By co-crystallization with water-soluble coformer succinic acid, a fivefold increase in aqueous solubility of Etodolac was observed. The co-crystals were prepared by Liquid assisted grinding method and were characterized using analytical methods like FTIR, PXRD and DSC [1, 2]. Literature survey shows that no analytical techniques have been reported so far for the quantification of Etodolac in Etodolac succinic acid co-crystals (ESA) in rabbit plasma. High-pressure liquid chromatography detection (HPLC) is a highly precise method for the determination of drug in plasma.

So, the present study aimed to develop a simple, reliable, rapid, robust, selective, sensitive, and precise HPLC method for the determination of Etodolac succinic acid co-crystals in rabbit plasma. The study was approved by Institutional Animal Ethical Committee (IAEC No.01/04/IAEC/2022/MCT). The assay method was validated using ICH guidelines for linearity, accuracy, precision, specificity, limit of detection (LOD), and limit of quantification (LOQ) for the determination of drug content of the ESA in spiked rabbit plasma.

MATERIALS AND METHODS

Etodolac succinic co-crystals prepared using Etodolac R. S and Succinic acid RS, Tinidazole RS (Sigma Aldrich), Acetonitrile, Methanol and Acetic acid (100%) used were of HPLC grade. (Merck India).

Liquid chromatographic conditions

The HPLC system was Agilent1220 Infinity LC (G4288C) which consisted of Agilent 1220 Infinity LC pump and injector Rheodyne 20 µl sample loop. Separation module fitted with Agilent Eclipse plus C18 column (100 mm *4.6 mm). HPLC system was equipped with a variable UV absorbance detector. Solvent delivery system was used to operate the gradient flow through a symmetry C18 column (4.6-100 mm, 3.5 µm spherical particles). with acetonitrile: methanol: acetic acid (100%) (50:49:1) as a mobile phase (pH 4.6) at a flow rate of 0.8 ml/min and the run time was 6 min. Degassing was achieved via filtration through a 0.45 µm Millipore membrane filter and sonication for 10 min. The injection volume was 20 µl and detection was done at 278 nm. The HPLC system was operated at 25 °C. A standard calibration curve (6 standards ranging from 2.5to 15µg/ml was prepared to determine the unknown concentration of Etodolac in ESA spiked rabbit plasma

Preparation of stock solutions

The standard solution of ESA and Tinidazole R. S were prepared in methanol at a concentration of 1 mg/ml and stored in 50 ml glass bottles in the refrigerator at 4 °C.

Preparation of working standards

Different working standard solutions of ESA (50-300µg/ml) were prepared by diluting of the abovementioned stock solution in acetonitrile: methanol: acetic acid (50:49:1) mixture and were stored at 4 °C.

Sample preparation

Blood samples from the marginal ear vein of 4 healthy rabbits were taken into polypropylene tubes containing EDTA and mixed well. It was centrifuged at 3000rpm for 15 min. The separated plasmas were taken in glass tubes. The plasma samples were pooled and 0.5 ml of plasma was taken into each of 10 ml tubes and were spiked with 0.5 ml of ESA standard solution (corresponding to concentration 50,100,150,200,250,300µg/ml) and 0.5 ml of Internal standard (tinidazole RS) corresponding to concentration 200µg/ml was added to each tubes and 0.5 ml acetonitrile was also added to each tube [3].

After vigorous shaking for 30 s, the mixtures were centrifuged at 3000 rpm for 15 min at room temperature. The organic layers were transferred to a new tube and evaporated. The residue was reconstituted in 10 ml of the mobile phase. (The strength of the drug in final solutions were 2.5 - 15 µg/ml and each containing 10 µg/ml IS). After vortex-mixing for 5 min, 20 µl of the sample was used for HPLC analysis as described below.

Determination of wavelength of detection

In order to fix the wavelength of detection, standard solutions of Etodolac RS and Succinic acid RS and Tinidazole RS were prepared in methanol. Dilutions were made using the mobile phase to get 10µg/ml solutions of Etodolac, Succinic acid and Tinidazole [3].

The solutions were scanned from 200-400 nm using a double-beam UV spectrophotometer.

Preparation of calibration curves

Six spiked plasma samples containing different ESA concentrations corresponding to 2.5 to 15µg/ml were prepared without the addition of IS. Peak area were determined and plotted against concentration. Six spiked plasma samples containing different ESA concentrations corresponding to 2.5 to 15 µg/ml were prepared separately with the addition of IS (10µg/ml) in to each solution. The ratio of peak area of ESA and IS were determined and plotted against concentration.

Validation

The RP-HPLC method for etodolac succinic acid co-crystal assay was validated in term of accuracy, precision, reproducibility, linearity, specificity, LOD, LOQ, and robustness according to ICH Harmonized Tripartite Guidelines [4]. Three standard calibration curves were prepared at different times to evaluate the linearity, precision, accuracy and stability.

Specificity

The specificity of the HPLC method was evaluated to ensure that there was no interference from the other components of the co-crystal. The specificity was studied by performing the assay using succinic acid coformer alone using the same mobile phase and wavelength of detection 278 nm [5, 6].

System suitability

The system suitability was assessed by six replicates of plasma samples spiked with ESA at a concentration of 200µg/ml. (ESA concentration in the sample is 10 µg/ml). The acceptance criterion was±2% for the percent relative standard deviation (% RSD) for the peak area and retention times for ESA

Linearity and range

Linearity is the ability to obtain test results that are directly proportional to the concentration of the analyte. Linearity was determined by three injections of six spiked plasma samples of different ESA concentrations (2.5,5,7.5,10,12.5,15µg/ml). The average of the ratio of peak area of Sample and IS were plotted against concentrations.

Then linearity was evaluated by using the calibration curve to calculate the coefficient of correlation, slope and intercept. In general, a value of correlation coefficient (r2)>0.998 is considered as evidence of an acceptable fit for the data to the regression line.

Accuracy

The accuracy of an analytical method expresses the nearness between the expected value and the experimental value. The studies were performed using the standard addition method at 80%, 100% and 120% of the known concentration of the standard solution to the pre analysed sample. The percent recovery (R%) of the analyte recovered was calculated. In this case, to evaluate the accuracy of the developed method, successive analysis (n = 6) for three different concentrations (5µg/ml, 10µg/ml and 15µg/ml) of standard ESA solution in spiked plasma were performed using the developed method. The data of the experiment were statistically analysed using the formula [% Recovery = (Recovered conc./Injected conc.× 100] to study the recovery and validity of the developed method. The mean recovery should be within 90–110% to be accepted.

Precision

Precision is the degree of agreement among individual tests when the technique is applied repetitively to test multiple replicates in three different occasions. The intraday precision was assessed by analysing the calibration curves of six replicates of plasma samples having different concentrations of ESA within the same day. Similarly, the inter-day precision was determined by analysis of six replicates of samples on three different days. The total precision of the method was expressed as the relative standard deviation (%RSD).

Limit of detection and limit of quantification

LOD is the lowest concentration in a sample that can be detected but not necessarily quantified under the stated experimental conditions. LOQ is the lowest concentration of analyte that can be determined with acceptable precision and accuracy.

These two parameters were calculated using the formula LOD = 3.3 × S D/S and LOQ = 10 × SD/S, where SD = standard deviation of the ratio of peak areas of Sample and IS and S = slope of the calibration curve.

Robustness

The robustness of an analytical procedure is the measure of its ability to remain unaffected by small but deliberate changes in test parameters and provides an indication of its reliability during routine usage. The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. The wave length of detection was changed to 276 nm and analysis was done for replicates of sample concentrations 10 µg/ml of ESA. The assay was repeated by changing the pH of the mobile phase by 0.2 units

Data and statistical analysis

In vitro results were expressed as mean±SD of at least three replicates. The HPLC assay results of plasma samples were calculated using linear regression. The % RSD was calculated for all values. One-way analysis of variance (ANOVA) was used to assess the reproducibility of the assay. The level of confidence was 95%.

Stability studies

To test the short-and long-term stability, three spiked plasma samples, low (5 µg/ml), medium (10µ g/ml) and high (15 µg/ml) concentrations, were stored under different conditions like at 4 ◦ C and room temperature. The assay was done for three samples at 6, 12, and 24 h and for 2 w after preparation. Moreover, the stabilities of stock solutions were tested at room temperature for 6 hours in the daylight. They were considered stable if the variation of the assay was less than 10% of the initial time response [7].

RESULTS

Determination of wavelength of detection

Etodolac RS shows absorption maxima at wavelength 278 nm. While Succinic acid RS did not show any absorption at the entire absorption range of Etodolac, confirming the non-interference of coformer in the detection of API.

Tinidazole also has significant absorption at 278 nm (fig. 1). So, the wavelength of detection was fixed at 278 nm.

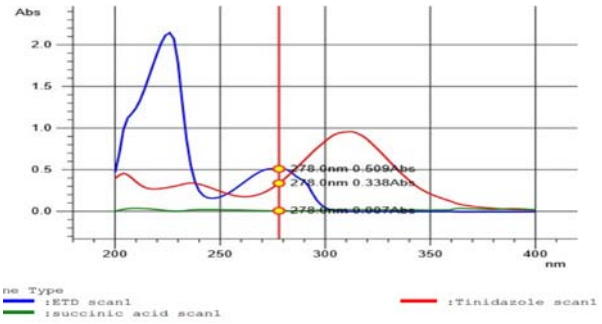

Fig. 1: Overlay wavelength scan of Etodolac, succinic acid and tinidazole

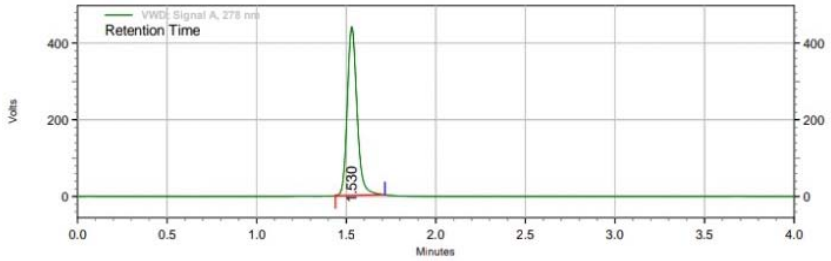

Fig. 2: Representative chromatogram of ESA spiked plasmasample

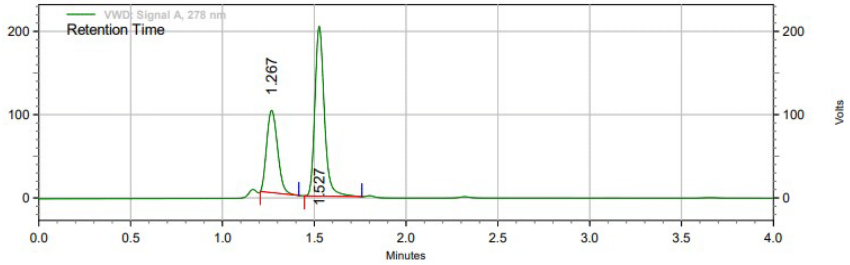

Fig. 3: Representative chromatogram of ESA and IS

Development of HPLC method

The chromatogram (fig. 2) represents ESA10µg/ml and (fig. 3) represent ESA and IS spiked plasma sample.

Validation of HPLC method

Preparation of calibration curve

Calibration curve of ESA in spiked plasma (without IS) was found to be linear in the concentration range 2.5 – 15µg/ml (Fig.4). Correlation coefficient was found to be 0.998. Table 1 shows data of calibration curve of ESA solution. Calibration curve of ratio of peak area of ESA and IS against concentration 2.5- 15µg/ml was also found to be linear with a correlation coefficient 0.9995 (Fig.5).

Limit of detection and limit of quantification

0.370µg/ml was the limit of detection and 1.121µg/ml was the limit of quantification.

Reproducibility

Reproducibility of the method was determined by calculating the linear regression of three calibration plots prepared on three different days [8]. The mean correlation coefficient was≥0.99 with percentage Relative Standard Deviation of the slopes of three lines2.1%. ANOVA of the data indicated no significant difference ($p>0.05$) confirming the reproducibility of the method.

Robustness

It was observed that a small change in the wavelength of detection and pH of mobile phase did not affect the ratio of area under the peak of the chromatogram of ESA and IS [9] (table 2).

Specificity

No peak was found near the chromatogram of the ESA at wavelength 278 nm, confirming high degree of specificity of the method.

System suitability

System suitability was assessed by doing six analysis of ESA at a concentration of 10ug/ml (table 3)

Table 1: Calibration curve data of ESA

S. No.	Concentration (µg/ml)	Peak area
1	2.5	7841269
2	5	15669379
3	7.5	24984648
4	10	34697532
5	12.5	44233251
6	15	54305977

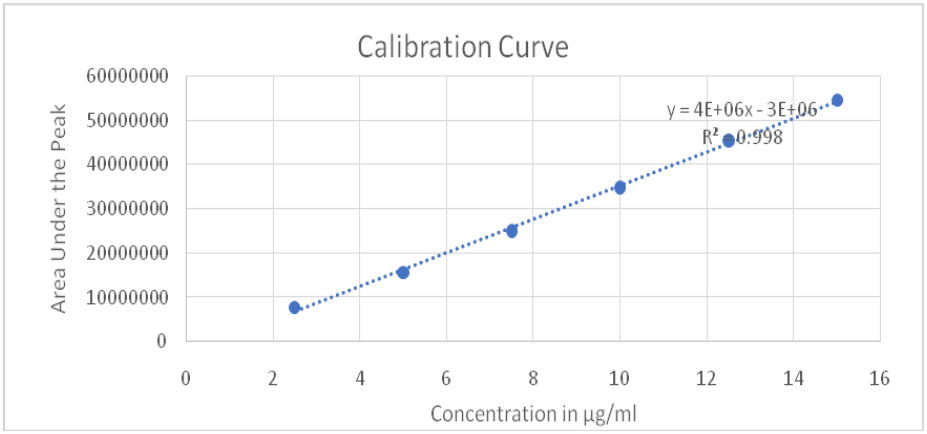

Fig. 4 Calibration Curve of ESA Solution in spiked plasma

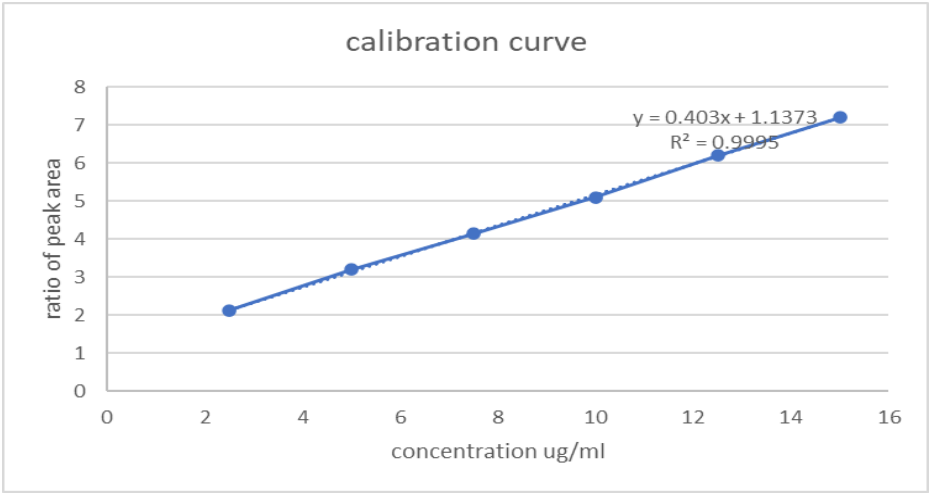

Fig.5 Calibration Curve of ESA and IS in spiked Plasma

Table 2: Robustness of the method

Concentration	factor	level	Retention time of IS	Retention time of ESA	Ratio of peak area
10 µg/ml	pH	4.8	1.267	1.527	5.12
		4.6	1.267	1.530	5.098
10µg/ml	wavelength	278	1.265	1.527	5.43
		276	1.267	1.527	5.32

Table 3: System suitability of HPLC method

Concentration 10ug/ml	Retention time
Mean (n=6)	1.528
SD	0.018
RSD	1.44

Table 4: Accuracy of the HPLC method for determination of Etodolac in ESA in Spikedplasma

ESA	Concentration µg/ml	% of analyte added	% recovered	% RSD
	5	80	103.6	0.75
		100	98.99	0.65
		120	102.35	0.44
	10	80	101.62	0.097
		100	100.63	0.0765
		120	102.85	0.124
	15	80	99.72	1.05
		100	100.64	0.98
		120	98.99	1.8

Accuracy

The percentage recovery was calculated (table 4) and the average recovery was found to be within the standard limit [6].

Interday and intraday precision

Interday (table 5) and intraday (table 6) precision was determined from a replicate analysis of three concentrations of ESA in spiked plasma. % RSD was found to be within the standard limit.

Reproducibility

Reproducibility of the method was calculated by preparing the standard plots on three different days. The mean correlation coefficient was≥0.99 with % RSD of the slopes 2.3. ANOVA of the data indicated no significant difference in the slope, which confirmed the reproducibility of the developed method.

Stability studies

No tendency of degradation of Etodolac at room temperature under daylight for 6 h was observed (table 7). In short term stability study, the solutions were also stable until 24 h at room temperature and also for two weeks at 4 °C [10].

Table 5: Interday precision of Etodolac estimation in spiked plasma

ESA	Concentration µg/ml	Mean ratio of peak Area n=3	SD	% RSD
	5	3.24	0.042453	1.31
	10	5.13	0.060828	1.185
	12.5	6.02	.092378	1.533

Table 6: Intraday precision of etodolac estimation in spiked plasma

ESA	Concentration ug/ml	Mean (n=3) ratio of peak area	SD	% RSD
	5	3.44	0.0576	1.67
	10	5.18	0.0614	1.18
	12.5	5.98	0.0843	1.409

Table 7: Stability of the ESA in spiked plasma

Samples at room temperature after 24 h	Concentration µg/ml	Mean ratio of peak area	SD	% RSD
	5	3.02	0.0076	0.251
	10	5.12	.0980	1.91
	15	7.12	.1436	2.008

DISCUSSION

Many methods were reported for the Estimation of Etodolac in dosage forms [1, 7, 8]. But no method was reported till date for the determination of Etodolac content in Etodolac Succinic acid co-crystal in Rabbit plasma. The reported HPLC method for the determination of Etodolac in bulk were tried for the determination of Etodolac content in ESA co-crystal [10]. But very poor resolution was obtained. Hence the aim of the present study was the development and validation of a simple, reproducible and sensitive HPLC method for the determination of drug content of the co-crystal in rabbit plasma**.**

CONCLUSION

A simple, sensitive, rapid and reproducible RP-HPLC method was developed with short runtime and less flow rate. Statistical analysis of the method proved that this method is suitable for the estimation of Etodolac in Co-crystalin plasma. Hence this method can be employed in the routine assay of the Etodolac Succinic acid co-crystals

FUNDING

Nil

AUTHORS CONTRIBUTIONS

All authors have equal contribution

CONFLICT OF INTERESTS

Declared none

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