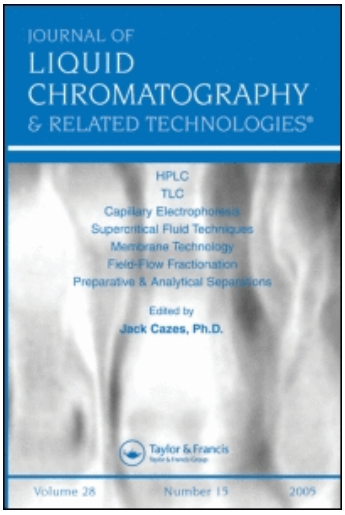

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Drug Dissolution Studies and Determination of Deflazacort in Pharmaceutical Formulations and Human Serum Samples by RP-HPLC

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Drug Dissolution Studies and Determination of Deflazacort in Pharmaceutical Formulations and Human Serum Samples by RP-HPLC[#]

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ABSTRACT

A simple, sensitive, reproducible, and validated RP-HPLC method with UV detection is described for the determination of deflazacort in raw material, pharmaceuticals, human serum samples, and in-vitro drug dissolution studies. The separation was achieved using a C18 (250 × 4.6 mm; 5 μm) column and a mobile phase comprising acetonitrile, methanol, and 0.067 M KH_2PO_4 in the ratio (27 : 20 : 53, v/v/v), adjusted

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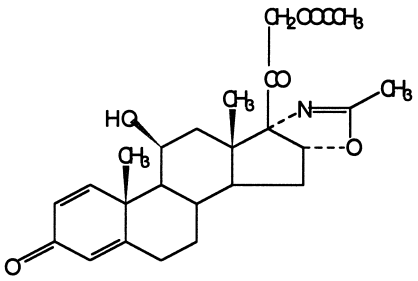
to pH 6.5 with 3 M NaOH. The results of analysis were treated statistically and it has been validated and proven to be rugged. The limit of detection and limit of quantification were found as 2.05 ng mL^{-1} and 6.83 ng mL^{-1} in mobile phase and 4.06 ng mL^{-1} and 13.55 ng mL^{-1} in human serum samples, respectively. The method produced linear response in the concentration ranges 10–30,000 ng mL^{-1} in mobile phase and 25–30,000 ng mL^{-1} in serum samples. The intra- and inter-day assay precision of the method was within 0.92% relative standard deviations in mobile phase and 1.48% relative standard deviations in human serum samples. This method was successfully applied for the determination of the drug in tablet dosage form, human serum, and drug dissolution studies. The results were found to be accurate, reproducible, and free from interference from the excipients or endogenous substance.

Key Words: Deflazacort; HPLC; Dissolution; Pharmaceuticals; Human serum; Determination.

INTRODUCTION

Deflazacort is a glucocorticoid. It is a prednisolone derivative with lower lipid solubility and has been suggested to have lesser effects on bone, carbohydrate, and lipid metabolism than does prednisolone. The corticosteroids are used in physiological doses for replacement therapy in adrenal insufficiency. The corticosteroids are used in pharmacological doses for their anti-inflammatory and immunosuppressant glucocorticoid properties, which suppress the clinical manifestations of disease in a wide range of disorders.[1,2]



In the open literature, few methods were reported for the quantitative determination of deflazacort in biological samples using HPLC methods with different detection system.[3–8] The reported methods require solid-phase extraction or expensive equipments, which are not economically feasible for

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routine use in pharmacokinetic and pharmaceutical studies, where numerous samples should be analyzed. Because of their selectivity, sensitivity, and overall versatility, the development of reliable and validated HPLC methods has received considerable attention in the quality control of drugs and quantitative determination in pharmaceuticals and biological samples. Owing to the widespread use of HPLC in routine analysis, it is important that good HPLC methods are developed and that these are thoroughly validated.[9–14]

Dissolution tests have become important tools for real-time quality control in large-scale pharmaceutical production. Dissolution testing, a functionality test of the pharmaceutical formulation, a quite complex process in vitro tests, have been judged as suitable for process control. The dissolution process is sensitive to many parameters, such as temperature, stirring, solvent, shape of vessel, etc. For this reason, we used USP requirements in our equipment and solvent. The in-vitro dissolution of drug from dosage forms is employed either as a primary aid in the characterization of formulations, or as a quality control procedure for monitoring the uniformity and reproducibility of production batches, or both.[15] The main purpose of an oral solid dosage forms is to make available a certain and defined amount of active substance to the human body through the gastrointestinal system. Drug dissolution testing is an integral part of pharmaceutical development and routine quality control monitoring of drug release characteristics. The in vitro dissolution profiles obtained from dissolution rate studies have also been used in an attempt to characterize the in vivo behavior of drugs with success. Among all tests that can be performed on drug solids, dissolution testing is considered to be sensitive, reliable, and rational for predicting in vivo drug bioavailability behavior.

In the present study, simple, economical, accurate, reproducible, and fully validated analytical method with good detection ranges for estimation of DEF in pure form, in its solid dosage forms, and spiked human serum samples was developed. The proposed method was aimed at developing an easy and rapid assay method for DEF without any time-consuming sample preparation steps for routine analysis, to be adopted in quality control laboratories and, at the same time, ensure satisfactory recovery during drug estimation from pharmaceutical preparations. In the proposed HPLC method, there is no need to extract DEF from the excipients matrix of pharmaceutical dosage forms and endogenous substance in serum samples, thereby decreasing the error in quantitation. The tablet dosage form samples can be directly used after dissolving and filtration, and spiked human serum samples can be directly used after adequate dilution of supernatant liquid. The developed method was used to estimate the total drug content in commercially available tablet dosage forms of DEF and in spiked human serum samples. The results of the analysis were fully validated by statistics[16,17] and recovery studies were realized.

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EXPERIMENTAL

Apparatus

HPLC analyses were performed using a HP Chromatographic System (Hewlett Packard, Avondale, USA), consisting of a Model Agilent 1100 series with a Model Agilent series G-13158 DAD detector and a Model Agilent 1100 series G-1329 ALS auto sampler. Data analyses were done using an Agilent Technologies HP 1100 software. The separation was carried out at ambient temperature, on a reversed-phase Waters spherisorb column (250×4.6 mm; 5 μm particle size). The chromatographic separation was performed using an isocratic mode.

The dissolution rate studies forming the pharmaceutical formulation were performed on Caleva 7ST dissolution apparatus (GB. CALEVA Inc., UK).

The mobile phase was acetonitrile : methanol : 0.067 M KH_2PO_4 (27 : 20 : 53, v/v/v) adjusted to pH 6.5 with 3 M NaOH and delivered at a flow rate of 0.75 mL min^{-1}. The injection volume was 20 μL. The eluate was analyzed at a wavelength of 244 nm. Etodolac was used as an internal standard.

Chemicals and Reagents

Deflazacort and its tablet dosage forms were kindly provided Aventis-Pharma Ind. (Istanbul, Turkey) and internal standard Etodolac was kindly supplied by Mustafa Nevzat Pharm. Ind. (Istanbul, Turkey).

HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). All other chemicals were analytical reagent grade quality. Doubly distilled water was used for preparing mobile phase solutions.

Preparation of Standard Calibration Graph

A stock solution (1 mg mL^{-1}) of pure drug and internal standard was prepared by dissolving 10 mg DEF and 10 mg in a 10 mL volumetric flask with mobile phase, separately. The concentration of DEF was varied in the range of 10–30,000 ng mL^{-1} and the concentration of IS was maintained at a constant level of 2000 ng mL^{-1}. The prepared dilutions were injected serially. The calibration graph for this analysis was constructed by plotting the ratio of the peak area of the drug to that of internal standard against the drug concentration.

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Analysis of Marketed Formulation

Ten tablets were accurately weighed and crushed to a fine powder. Four accurately weighed quantities of this powder, equivalent to 10 mg of DEF, were taken in different 10 mL volumetric flasks and used for the assay. About 7 mL of mobile phase was added to them and the flasks were sonicated for 10 min. The solution was then diluted to 10 mL with mobile phase. This solution was centrifuged. Appropriate solutions were prepared by taking suitable aliquots of clear filtrate and adding the appropriate IS solution, diluting them with mobile phase in order to obtain a final solution. The amount of DEF per tablets was calculated from the related linear regression equation.

Recovery Studies

To keep an additional check on the accuracy of this developed assay method, recovery experiments were performed by adding known amounts of pure drug to pre-analyzed samples of pharmaceutical dosage forms. The percent analytical recovery was calculated by comparing the concentration obtained from spiked samples with actual added concentration. Thus, the effect of common formulation excipients on chromatograms (e.g., broading, tail, etc.) was investigated. Recovery experiments were also shown to be reliable and suitable with the proposed method. The known amounts of DEF and a constant level of IS were added to DEF tablet dosage form and the mixtures were analyzed by the proposed method. After five repeated experiments, the recovery results were calculated.

Recovery Studies in Spiked Human Serum Samples

Drug free serum samples, obtained from healthy individuals (after obtaining their written consent), were stored frozen until assay.

One milliliter of the thawed serum samples was spiked with 100 $\mu g\,mL^{-1}$ of DEF (dissolved in mobile phase), and 1 mL methanol (for precipitation of proteins). The tubes were tightly capped and vortex-mixed for 5 min and then centrifuged for 5 min at 5000*g*. The upper layer was transferred into a clean tube. The concentration of DEF was varied in the range of 25–30,000 $ng\,mL^{-1}$ in human serum samples, and the concentration of IS was maintained at a constant level of 2000 $ng\,mL^{-1}$.

Serum samples were injected into the column. The amount of DEF in spiked serum samples was calculated from the related linear regression equation.

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In-Vitro Drug Dissolution Rate Studies

Dissolution rate studies with one marketed product (Flantadin®) using USP 24 dissolution apparatus and the procedure for the single entity products, with use of a paddle-stirrer type of apparatus, in 900 mL of 0.1 M HCl (pH 1.2 gastric medium), at a stirring rate of 75 rpm was done. The temperature of the cell was maintained at $37 \pm 0.5^{\circ}C$ by use of a thermostatic bath. The samples (1.0 mL) were withdrawn at predetermined time intervals (0, 0.33, 0.66, 1, 1.33, 1.66, 2, 2.33, 2.66, 3, 3.33, 3.66, 4, 5, 10, 15, 30, 45 min), and at the same time an equivalent volume of buffer was replaced. Withdrawn samples were filtered (0.45 μm Millipore Syringe filter); 20 μL of each sample was injected for HPLC analysis. The concentrations of DEF in the dissolution medium were determined from the linear regression equation, which was proposed using a standard sample solution in mobile phase. The cumulative percentage of drug released in media was plotted against time, in order to determine the release profile of drugs from each formulation.

RESULTS AND DISCUSSION

To develop a rugged and suitable HPLC method for the quantitative determination of DEF, different mobile phase compositions and ratios were employed. Our preliminary trials using different compositions of mobile phases consisting of water, methanol, and acetonitrile and different ratios of this solution, did not give good peak shape. Introduction of KH_2PO_4 buffer (0.067 M) instead of water improved the peak shape of DEF. Finally, by fixing buffer at pH 6.5 and mobile phase composition consisting of a mixture of acetonitrile : methanol : 0.067 M KH_2PO_4 (27 : 20 : 53, v/v/v) DEF and IS were resolved to the baseline and obtained the best peak shape. This mobile phase composition was found to be optimal for good peak shape, as well as to achieve minimal background current. The proposed conditions were found suitable for the determination of DEF in bulk drugs, pharmaceutical dosage form, human serum samples, and drug dissolution studies.

For the HPLC method, precision and accuracy can often be enhanced by the use of an appropriate internal standard, which also serves to correct for fluctuations in the detector response. One of the main reasons for using an internal standard is for samples requiring significant pretreatment or preparation. Often, sample preparation steps that include reaction, filtration, precipitation, extraction, and so on, results in sample losses. When added prior to sample preparation, a properly chosen internal standard can be used to correct for these sample losses. The internal standard is a different compound from the analyte, but one that is well resolved in the separation. The chemical structure of etodolac is

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not similar to the DEF structure. However, it was chosen as the internal standard because it not only gave the best peak shape but also gave the better resolution and shorter retention time compared to other potential internal standards. Finally, using the conditions selected above, a satisfactory chromatographic peak resolution was obtained in a short analysis time, as can be seen in Fig. 1.

System suitability tests are an integral part of HPLC method development. It can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validation have been completed. The criteria selected will be based on the actual performance of the method, as determined during its validation. These parameters include relative standard deviations (RSD%) of retention times, tailing factor, resolution, capacity factor, selectivity factor, and RSD% of peak height or area for repetitive injections. Typically, at least two of these criteria are required to demonstrate that system suitability tests were carried out on freshly prepared standard stock solutions of DEF. Resolution and selectivity factors for this system were found as 6.38 and 2.44, respectively. The method has enabled good resolution of analytes, since values of resolution factors of adjacent peaks were greater than 1.0. Tailing and capacity factors were obtained as 1.09

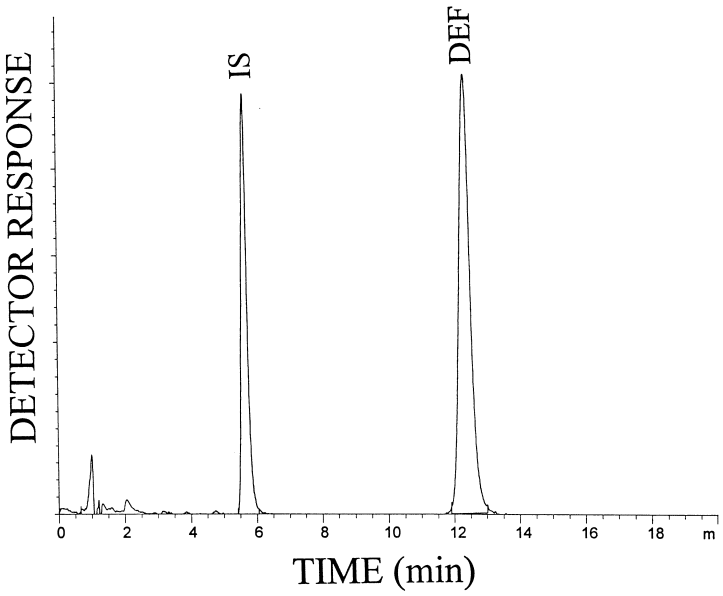

Figure 1. HPLC chromatogram of formulated DEF (5000 ng mL^{-1}) and IS (2000 ng mL^{-1}).

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and 11.26 for DEF and 1.17 and 4.61 for IS, respectively. The retention times in mobile phase were used as hold-up time for the capacity factor calculations. The presented chromatographic conditions ensure adequate retention of both compounds, since capacity factor values obtained satisfied the conditions (≥ 1.0). The retention times of DEF standard sample in mobile phase, in tablets, and in serum samples were 12.34, 12.36, and 12.32 min, respectively. The variation in retention time among five replicate injections of DEF reference solution was very little in raw material, tablets, and spiked serum samples, giving RSD% of 0.11, 0.15, and 0.16%, respectively. The results obtained from system suitability tests are in agreement with the USP requirements. The calibration curves for DEF in mobile phase and serum samples were drawn by plotting the peak area ratio of DEF to IS vs. concentration of DEF, and yielded the correlation coefficient (r) of 0.999 in both media over the concentration range 10–30,000 ng mL^{-1} and 25–30,000 ng mL^{-1}, respectively. Values obtained for the calibration curve and their related validation parameters are presented in Table 1. The low values of RSD% of slope and intercept, and greater than 0.999 correlation coefficient in both media, established the precision of the proposed method. Several approaches are given in the ICH guideline to determine the detection (LOD) and quantitation (LOQ) limits. In this study, LOD and LOQ were based on the standard deviation of the response and the slope of the corresponding calibration curve using the following equations: $LOD = 3\,s\,m^{-1}$; $LOQ = 10\,s\,m^{-1}$.

The stability of the reference substance and sample solutions were checked by analyzing a prepared standard solution of DEF in mobile phase aged at $+4^{\circ}C$ in the dark, against a sample freshly prepared. The results demonstrated that the working reference solutions were stable for up to 7 days. The DEF area ratio to IS for the assay reference solutions over 7 days did not considerably change.

The developed method was validated according to the standard procedures[16,17] and the results obtained are tabulated in Table 2. Accuracy,

Table 1. Characteristics of the linear regression analysis of DEF.

	Mobile phase	Human serum
Linearity range (ng mL^{-1})	10–30,000	25–30,000
Slope	5.86×10^{-4}	5.16×10^{-4}
Intercept	0.034	0.018
Correlation coefficient (r)	0.999	0.999
RSD% of slope	0.26	0.18
RSD% of intercept	0.89	0.56
Detection limit (ng mL^{-1})	2.05	4.06
Quantification limit (ng mL^{-1})	6.83	13.55

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Table 2. Within-day and between-day precision of DEF standards.

	In mobile phase				In serum samples			
	Within-day measured concentration ($ng\,mL^{-1}$)[a]		Between-day measured concentration ($ng\,mL^{-1}$)[b]		Within-day measured concentration ($ng\,mL^{-1}$)[a]		Between-day measured concentration ($ng\,mL^{-1}$)[b]	
Theoretical concentration ($ng\,mL^{-1}$)	Mean	RSD%	Mean	RSD%	Mean	RSD%	Mean	RSD%
75	74.92	0.45	75.16	0.59	74.62	0.99	74.24	1.05
250	249.51	0.63	249.14	0.92	248.86	1.21	246.27	1.48

[a]Mean values represent six different deflazacort standards for each concentration.
[b]Between-day reproducibility was determined from six different runs over a 2 weeks period.

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precision, and reproducibility of the proposed method were assessed by performing replicate analysis of the standard solutions in mobile phase and serum. Within calibration curves, two different concentrations were prepared in both media and assayed with related calibration curves to determine within-day and between-day variability. The within and between-day precision, accuracy, and reproducibility were determined as the RSD% and mean value and the results were shown in Table 2. According to the results of Table 2, the proposed method demonstrates good precision, accuracy, and reproducibility.

When working on standard solutions, and according to the obtained validation parameters, results encourage the use of the proposed method described for the assay of DEF in pharmaceutical dosage forms, spiked human serum samples, and drug dissolution studies.

The utility of the proposed method was verified by means of replicate estimations of marketed product and the results obtained were evaluated statistically (Fig. 1).

The results obtained from the analysis of tablet dosage form are summarized in Table 3. Recovery experiments were realized buy using the standard addition method. Recovery experiments using the developed assay procedure, further indicated the absence of interference from commonly encountered pharmaceutical excipients used in the selected formulation (Table 3).

In order to check the applicability of the proposed method to the human serum samples, the calibration equations were obtained in spiked serum samples. Recovery studies were also performed in human serum samples and the results were calculated using the related calibration equation. Calibration equation parameters were shown in Table 1. Related validation parameters and obtained recovery results of spiked serum samples were given in Table 2

Table 3. Results of the assay and the recovery analysis of deflazacort in tablets and spiked human serum samples.

	Tablets (mg)	Serum samples (ng mL^{-1})
Labeled claim (mg tablets^{-1})	30.00	—
Mean of amount found[a]	29.58	—
RSD% of amount found	0.276	—
Added	5.00	100.00
Recovered[b]	4.94	99.12
Recovery%	98.84	99.10
RSD% of recovery	0.483	0.583

[a]Each value is the mean of six experiments.
[b]Each value is the mean of five experiments.

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and Table 3, respectively. Analysis of drugs from serum samples by HPLC usually requires extensive time-consuming sample preparation, use of expensive organic solvents, and other chemicals.[18,20] In our proposed method, the serum proteins are precipitated by the addition of methanol, which is centrifuged at 5000*g*, and the supernatant is diluted, directly injected, and analyzed. Figure 2 shows the typical chromatogram obtained, the blank serum (a) and serum spiked with DEF and constant amount IS (b). As can be seen in Fig. 2, there are no extraneous peaks in chromatograms obtained for serum samples. The determined results and recoveries of known amounts of DEF added to serum samples are given in Table 3. The proposed method gives reproducible results, is easy to perform, and is sensitive enough to determine DEF in human serum samples.

The proposed method was also applied to the determination of DEF in dissolution rate studies of the samples obtained from the tablets. DEF tablet formulation, which includes 30 mg active compound, was investigated with the paddle dissolution method. It is essential to consider the in vitro dissolution tests as important criteria for the quality of the marketed dosage forms if

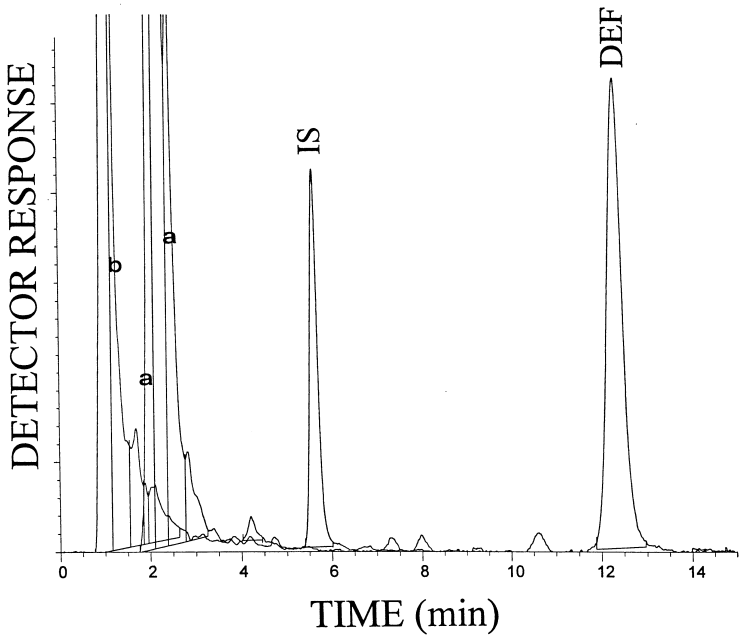

Figure 2. Chromatogram of (a) blank serum, (b) serum spiked with 5000 ng mL^{-1} of DEF and 2000 ng mL^{-1} of IS.

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obtained from various sources, and can judge the suitability of this formulation to deliver the required active substance properly to the patient. The cumulative percentage of drug released in dissolution media vs. time profile is shown in Fig. 3. As can be seen in Fig. 3, more than 90% drug dissolved in this media within 2.0 min. The release data were evaluated according to the different models, namely zero order, first order, Hixson-Crowell, Weibull distribution (RRSBW)[21,22] function, and Peppas equation.[23,24]. Table 4 shows all the kinetics, related rate constants, and parameters. According to the investigation of the kinetic assessment of the release data, the most proper release kinetic was found to be Weibull distribution according to the highest determination coefficient and lowest AKAIKEs information criteria. The release of DEF from tablets attained 63.2%, at the end of 1.00 min. According to the RRSBW kinetic, low shape factor value ($\beta < 1$) corresponds with a steeper initial slope followed by a flattened tail in the final part. Shape factor value was found to be lower than 1.0. The release of DEF from tablets tested was completed within 2.33 min in the proposed technique. In order to understand the magnitude of the diffusional exponent n of drug from tablets, dissolution data were fitted to the Peppas equation where n is a factor, which indicates the mechanism of the release; for instance, $n = 0.5$ for square root of time and $n = 1$ for zero order release. The values of n being greater than 0.5 indicate anomalous diffusion. Exponent value was found as 0.70 (Table 4). According to the Peppas equation, the results were to be not in agreement with $Q\sqrt{t}$ and zero order kinetics.

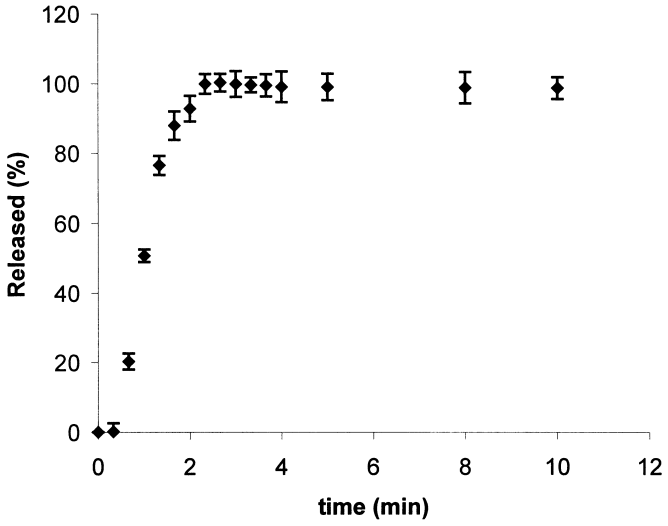

Figure 3. In-vitro drug release profile of marketed product ($n = 3$).

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Table 4. Kinetic parameters of release data of DEF tablets.

Zero order	k_{r_0}	14.84
	r^2	0.108
	SWSD	8.61
	AKAIKEs information criteria	40.05
First Order	k_r	6.27
	r^2	0.460
	SWSD	7.18
	AKAIKEs information criteria	29.70
Hixson-Crowell	k	7.15
	r^2	0.254
	SWSD	7.15
	AKAIKEs information criteria	30.07
Weibull distribution (RRSBW)	$T_{\min}$	1.00
	β	0.806
	r^2	0.500
	SWSD	8.48
	AKAIKEs information criteria	−17.89
Peppas equation	k_p	0.040
	n	0.703
	r^2	0.336

Note: k_r, Release rate constant of first order kinetic; k_{r_0}, Release rate constant of zero order kinetic; k, Release rate constant of Hixson-Crowell kinetic; k_p, Release constant of Peppas equation; r^2, Determination coefficient; SWSD, Sum of weighed squared deviations; β, Shape factor; $T_{\min}$, Value stands for the time for 63.2% release of the drug; n, Diffusional exponent.

CONCLUSIONS

The proposed method obviates the need of a conventional column using nearly neutral pH mobile phase. The proposed HPLC method is simple, selective, reproducible, easy, and fully validated for determination of DEF in raw material, marketed pharmaceutical dosage forms, and spiked serum samples, with good linearity, accuracy, and precision. The method was extensively validated. The proposed method used a simple serum deproteination step instead of extraction. No interferences from excipients for tablets and endogenous substances for biological sample were observed. The suggested technique can be used in quality control of formulations containing DEF.

The sample assay results and recoveries in pharmaceutical formulations were in good agreement with their respective label claims and added standard

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sample, and thus, suggested non-inference of formulation excipients in the estimation.

Moreover, the method can be used for the determination of DEF for monitoring its concentration in in-vitro dissolution studies. Hence, it can be easily adopted for the routine quality control analysis of DEF in pharmaceutical dosage forms and analysis of DEF in human serum, and it meets the requirement for applicability for clinical utility and for clinical and regulatory research.

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