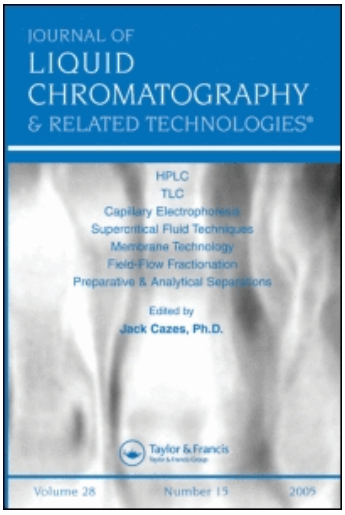

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CAPILLARY ELECTROPHORETIC BEHAVIOUR AND DETERMINATION OF ENOXACIN IN PHARMACEUTICAL PREPARATIONS AND HUMAN SERUM

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ABSTRACT

A validated capillary electrophoretic method with UV detection is described for the determination of enoxacin [ENX] in pharmaceutical preparation and human serum. The experiments were carried out in a fused-silica capillary (ID=75 μm, total 88 cm, effective 58 cm length) using a 20 mM borate buffer at pH 8.6, applying a potential of 30 kV, 1 s of injection. Acetylpipedimic acid was used as an internal standard (IS) and the detection was

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performed at 265 nm. The t_M±RSD% of ENX and IS were 4.8±0.9 and 5.5±1.2 minutes, respectively. A well-correlated calibration equation was obtained in the range of $3.1x10^{-6}$-$3.1x10^{-5}$ M ENX. Limit of detection (LOD) was $3.5x10^{-6}$ M (S/N = 3).

A modified reversed-phase HPLC was also conducted using a C-18 ODS column for the analysis of ENX to compare to its applicability with the CE method. An isocratic elution was performed using a mobile phase of 10 mM phosphate buffer (pH 4.0) and acetonitrile (85:15;v/v) detecting at 260 nm.

The determination of ENX in the pharmaceutical tablet formulation was carried out by both methods and the results of a single tablet (as mg with their RSD% values) was found to be 421.4±1.0 and 415.9±0.9 by CE and HPLC, respectively. ENX analysis was also performed by a standard addition method in serum, and the recoveries were found to be 89.7±0.6 (CE) and 78.8±4.9 (HPLC). It was concluded that capillary electrophoresis for the determination of ENX is a promising method for routine analysis and pharmacokinetic and bioavailability studies.

INTRODUCTION

Enoxacin, 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-1,8-naphthyridine-3-carboxylic acid, [ENX, Fig. 1] is a new broad spectrum fluorinated 4-quinolone antibacterial agent.(1) The 4-quinolone antibiotics have been used in the treatment of many soft tissue infections including bacterial prostatitis.(2)

ENX is excreted mainly in urine as an unchanged drug. It is metabolized by oxidation to oxo-enoxacin, conjugation with formic and acetic acid, ring opening, and deamination of the piperazinyl ring. Its major metabolite, oxo-enoxacin, accounts for 10-15% of the administered dose and each of other metabolites constitutes less than 1% of the dose.(3)

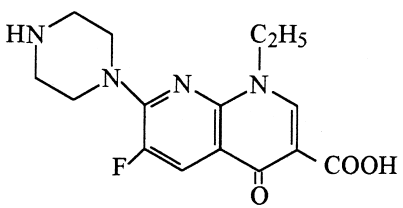

Figure 1. The chemical structure of ENX.

High performance liquid chromatography (HPLC) is the most commonly employed method for the determination of ENX and its metabolites in plasma, urine, and tissues with ultraviolet (UV) or fluorometric (FL) detection.(4-9) There are few reported methods based on UV/VIS spectrophotometry(10,11) and polarography.(12-14) A microbial method has also been developed.(15)

Capillary electrophoresis (CE) is becoming a reliable, preferable, and alternative method, especially for the analysis of drugs in biological matrices. CE offers some advantages such as rapidity, short analysis time, and low cost. This paper describes a rapid, precise, and accurate capillary electrophoretic method for the determination of ENX in the pharmaceutical formulation and human serum. The results obtained by the proposed method were statistically compared to those of a modified reversed-phase HPLC method.

EXPERIMENTAL

Apparatus

CE experiments were conducted using a Spectrophoresis 100 system equipped with Modular Injector, High Voltage Power Supply, and a model Spectra FOCUS scanning CE detector (Thermo Separation Products, CA, USA) cabled to a Model Etacomp 486 DX 4-100 computer processing the data using PC 1000 (Version 2.6) working under OS/2 Warp program (Version 3.0). The analysis was performed in a fused silica capillary with a total length of 88 cm and effective length of 58 cm having 75 μ ID (Phenomenex, CA, USA). The pH of the solutions were measured by using a Multiline P4 pH meter model with SenTix glass electrode (WTW, Weilheim, Germany). All the solutions were filtered using Phenex microfilter (25 mm, 0.45 μm) (Phenomenex, CA, USA) and degassed using a model B-220 ultrasonic bath (Branson, CT, USA).

HPLC experiments were carried out by using a Model 510 Liquid Chromatograph equipped with a Model 481 UV detector (Waters Associates, Milford, MA, USA). The chromatograms were processed by means of a chromatographic workstation (Baseline 810). Separation was performed on a reversed-phase Supelcosil LC-18 column (250x4.6 mm ID, 5 μm particle size) (Supelco, St. Louis, MO USA). Samples were injected in a 50-μL loop through a Rheodyne 7125 valve (Rheodyne, Cotati, CA, USA).

Chemicals

Enoxacin and its pharmaceutical preparation Enoksetin® Tablet (containing 400 mg active material) were generously provided by Eczacibasi Ilaç Sanayi ve

Ticaret A.S., Istanbul, Turkey. Acetonitrile, methanol (HPLC grade), ethanol, propanol, hydrochloric acid, sodium hydroxide, borax, acetylpipemidic acid (IS for CE), and 3,4-dihydroxybenzylamine HBr (IS for HPLC) were supplied from (Merck, Darmstadt, Germany). Blood samples were withdrawn from healthy volunteers after attaining their consent. The serum samples were separated by centrifuging for 10 min at 5000 x g. Double distilled water was prepared in our laboratory using all glassware. All the solutions were prepared in this water.

Procedure for CE Instrumentation

The fused silica capillary tubing was filled with the background electrolyte (pH 8.6; 20 mM borate). Both ends of the tube were dipped into the reservoir (8 mL) and the vial (1.1 mL) was filled with background buffer. The end part in which the sample (side of vial) was introduced was connected with a platinum electrode positive high voltage side of the power supply. The reservoir side at the detector end was connected with a platinum electrode to ground. Samples at a concentration $7.7x10^{-5}$ M for the optimization of CE parameters, were introduced by 1s of vacuum injection corresponding to almost 65 nL.

Before each run, the capillary was purged for 2 min with 0.1 M sodium hydroxide solution, followed by 2 min. double distilled water. It was then equilibrated by passing the background electrolyte for 5 min. prior to operation.

Procedures

A stock solution of ENX (10 mg/25 mL of methanol) was prepared. Dilutions were made in the range of $2.5x10^{-5}$ and $1.2x10^{-4}$ M, each containing 0.25 μmol IS for CE and $3.1x10^{-6}$ - $3.12x10^{-4}$ M ENX, each having 0.11 μmol IS for HPLC. All the dilutions for CE and HPLC were prepared in the background electrolyte and mobile phase, respectively.

Background electrolyte was 20 mM borate buffer at pH 8.6 for the CE experiments. They were analyzed by applying +30 kV potential, injecting the sample 1s, and detecting at 265 nm where ENX and acetylpipemidic acid (IS) absorb the monochromatic light equivalently.

During HPLC experiments, a 10 mM phosphate buffer (pH 4.0):acetonitrile (85:15, v/v) was used as a mobile phase. 3,4-Dihydroxybenzylamine HBr (IS) was found to be a suitable internal standard for the HPLC experiments. The flow rate was 1.5 mL min^{-1} and detection was carried out at 260 nm.

Analysis of ENX Tablets by CE and HPLC Methods

Ten ENX tablets were accurately weighed. The average weight of one tablet was calculated and then they were finely powdered in a mortar. A sufficient amount of tablet powder, equivalent to 10 mg of ENX, was accurately weighed, transferred to a 25 mL flask, and methanol was added to dissolve the active material. It was magnetically stirred for 10 min and made up to the final volume with related solvent. The solution was then centrifuged at 5000 x g for 10 min. The supernatant and fix amount of IS solutions were diluted with background electrolyte or mobile phase solutions to carry out either CE or HPLC assay.

Analysis of ENX in Serum

For CE analysis, 0.25 μmol ENX (in 1 mL) was added to a 1 mL serum and vigorously shaken. Then 3 mL ethanol were added and mixed well using a shaker. The precipitated proteins were separated by centrifuging for 10 min at 5000 x g. A definite amount of clear supernatant was transferred to a tube, IS solution was added, and the final solution was directly injected to CE in the same conditions

For HPLC the precipitation of proteins of 1-mL serum was achieved according to the methods described by Nangra et al.,(7) by adding a 50 μL $HClO_4$ (60% w/v), centrifuging for 10 min at 5000 x g, and the supernatant was directly injected into the column of the HPLC at conditions mentioned above.

RESULTS AND DISCUSSION

A background electrolyte consisting of borax was preferred to conduct the initial CE experiments because ENX has a carboxylic group on its structure. Several pH values were tested in the range from 8.45 – 9.95 using the concentration of 20 mM borax buffer. It was observed that ENX ($1.26x10^{-4}$ M) peak appeared in all the studied pH values, but the migration time of ENX, as expected, increased with increasing of pH.

Phosphate and citrate buffer of the same pH and concentration (8.6; 20 mM) were used to compare to the effect of the nature of buffer components. Migration time (t_M) of ENX was not affected by the buffer components, but the repeatability of peak areas decreased with the use of citrate and phosphate buffers. It is concluded, that some optimization studies were required if these buffer systems are to be used.

Influence of borax buffer concentration was investigated in the range from 10 – 100 mM. The sharpest peaks were obtained in the use of 10–30 mM concentrations and t_M of ENX was almost constant and an increase was observed in the use of concentration of borax above 30 mM, but also, peak deformation occurred due to the heat production by Joule effect.

In order to achieve optimization of the proposed analytical procedure, low buffer concentration was considered to decrease the electrophoretic mobility that corresponds with short analysis time. Based on the above results, the most convenient buffer system was 20 mM borate buffer at a pH 8.6 value.

Since the separation depends on the conditioning of the capillary inner surface in CE analysis, the t_M and peak integration values might be vary similar to HPLC techniques.

The electropherogram of ENX and acetylpipemidic acid (IS) in the background electrolyte is shown in Fig 2**.** The signal of electroosmosis and the migration time of the peaks of ENX and IS appeared at 3.8, 4.8, and 5.5 minutes, respectively. From the integration data, net mobility toward cathode (electroosmosis), ENX and IS toward anode (electrophoretic) were $7.56x10^{-4}$, $5.96x10^{-4}$, and $4.6x10^{-4}$ $cm^2.V^{-1}.s^{-1}$, respectively. The capacity factors were 3.82 (ENX) and 4.53(IS).

Certain evaluation methods were examined based on the quantification processes. These can be divided into three groups; employing only the values of peak normalization.

The effect of the use of IS and certain evaluation methods, such as correction of peak area (peak normalization (PN) was calculated by dividing the related peak area into (t_M) on the precision) was examined. These can be divided into three groups: (a) employing only the area values of peak normalization (PN no IS), (b) computing the ratio values (IS no PN), and (c) using the area values of peak normalized IS and ENX (IS and PN). The precision of peak areas were calculated as shown in Table 1.

The success of CE experiments from the analytical points of view depends on the conditioning of capillary surface. Therefore, cleaning and conditioning processes as explained in the Experimental, must be repeated after each injection to provide better resolution and reproducibility. The precision of peak area was also assessed by considering certain parameters, such as the effects of correction of peak area (normalization), which is found by the division of the related peak into the corresponding migration time; use of an internal standard was studied.

As seen from the table, the lowest RSD% values are obtained from those of IS and PN. Thus, such evaluation was considered throughout in the rest of the study.

A series of standard ENX solutions in the concentration range of $2.5x10^{-5}$ and $1.2x10^{-4}$ M and each containing 0.25 μmol at fixed concentration of IS were

Table 1. Precision of Peak Areas

Precision of Peak Areas (RSD%)	PN no IS	IS no PN	IS and PN
Repeatability (day=3; n=6)	2.80	0.99	0.99
Intermediate precision (day=3; n=6)	3.24	2.86	1.36

prepared; and they were injected (n=3). Linear regression lines were obtained by plotting the ratios of normalized peak areas to those of the internal standard versus the analyte concentration. The calibration equation was computed with the regression analysis program considering the ratio values versus the related concentrations. The results are presented in Table 2.

Method Validation

From the electropherogram in Fig. 2, no interference from the formulation excipients could observed at the migration times of ENX and IS. The limit of the detection (LOD) was 3.85×10^{-7} M, while the limit of quantification (LOQ) was 1.16×10^{-6} M. The results indicate a good precision.

Accuracy

Method accuracy was determined by analyzing a placebo (mixture of excipients) spiked with ENX at three concentration levels (n=6) covering the

Table 2. Linearity and Accuracy of the Method (Spiked Placebos)

	Regression Parameters		
	r^2	Intercept (Mean±SD)	Slope (Mean±SD)
Linearity	0.9998	$-0.02342 \pm 7.32 \times 10^{-3}$	9813.18±102.31
Accuracy	50 %	100 %	150 %
Mean recovery ± CI %	101.4 ± 2.12	101.1 ± 2.63	100.4 ± 2.25

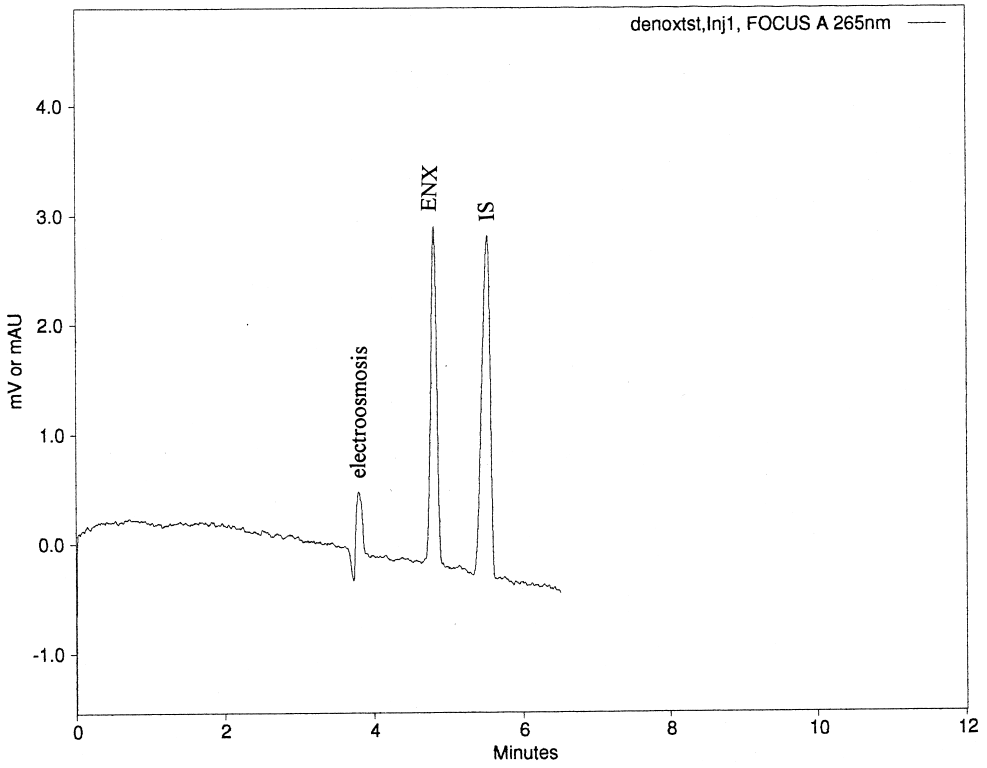

Figure 2. Typical electropherogram of standard ENX ($7.7x10^{-5}$ M) and IS (acetylpipemidic acid, $5.18x10^{-5}$ M). Conditions: 20 mM borate (pH 8.6); injection, hydrodynamically 1.0 s; applied voltage, +30 kV; capillary, uncoated fused silica, 75 µm I.D., 88-cm total and 58-cm effective length; detection wavelength, 265 nm.

Table 3. Precision of the Method (Spiked Placebos)

Concentration Levels (%)	50	100	150
Repeatability (day=3; n=6, RSD%)	2.0	2.5	2.1
Intermediate precision (day=3; n=6, RSD%)	3.3	3.0	3.1

same range as that used for linearity. Mean recoveries with 95% confidence intervals are given in Table 3.

HPLC of ENX

Under the described chromatographic conditions, ENX has a retention time of 10.03 min., whereas IS eluted at 2 min. Peak area ratios were linearly proportional to ENX concentrations in the range $3.12x10^{-6}$ - $3.12x10^{-4}$ M with a limit

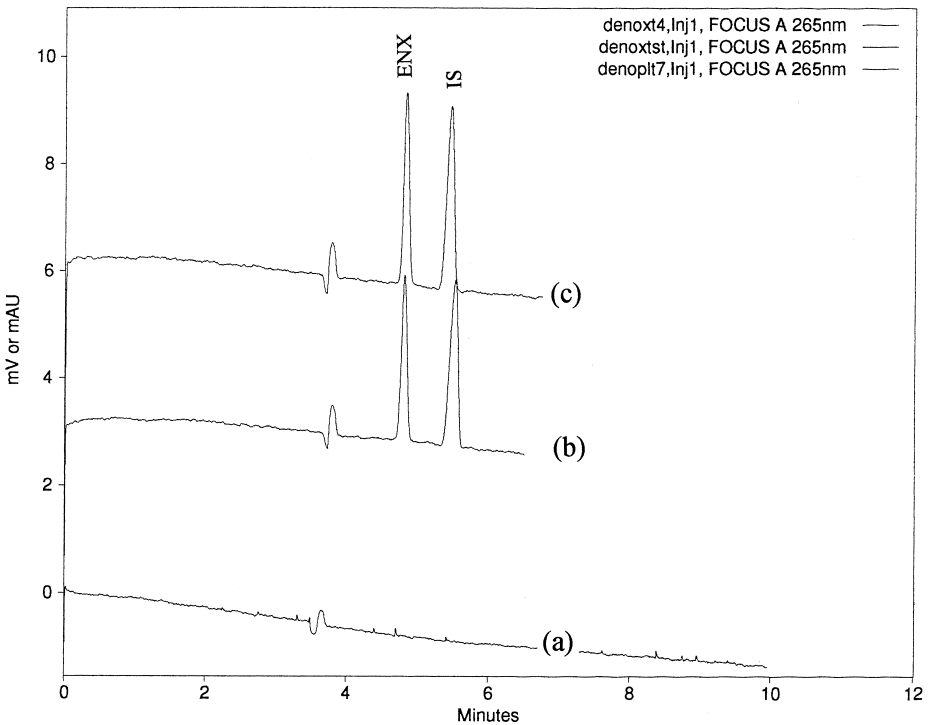

Figure 3. The electropherograms of: (a) Inactive ingredients of tablet solution of ENX; (b) The standard ENX ($7.7x10^{-5}$ M) and IS (acetylpipemidic acid, $5.18x10^{-5}$ M); and (c) Enoxetin ® Tablet solution containing IS. Conditions are the same as described in Figure 2.

detection of $1.56x10^{-6}$ M. The calibration equation was found to be [R= -0.51 + $2.1x10^{5}$ C(M) ; r=0.9992], where C(M) is the molar concentration of ENX.

Application of CE in the Analysis of ENX in Pharmaceutical Formulation

A typical electropherogram is shown in Figure 3, which indicates no interferences from the tablet excipients. In order to examine the applicability and the validity of the CE method, ENX pharmaceutical tablets were analyzed by CE and HPLC methods. Results of the comparative studies are demonstrated in Table 4.

The results indicate that both methods i.e., by CE and HPLC, show insignificant difference at the 95% probability level, and the ENX tablet formulations conform with the official requirements.(16)

CE Analysis of ENX in Serum

The determination of ENX in serum was performed by the standard addition method.

For the precipitation of proteins in serum, usually excess amounts of certain substances such as sulphosalycilic acid, trichloracetic acid, perchloric acid,

Table 4. Comparative Studies for the Determination of ENX in Enoksetin® Tablet[a]

No. of Experiment	Amount Found (mg) Using CE	Amount Found (mg) Using HPLC
1	419.7	410.1
2	428.5	417.4
3	422.3	414.9
4	417.1	419.9
5	419.4	417.4
Mean	421.4	415.9
RSD%	1.04	0.89
$t_{calculated}$	2.13	t_{table} 2.78 (p=0.05)

[a]Declared amount, 400 mg per tablet.

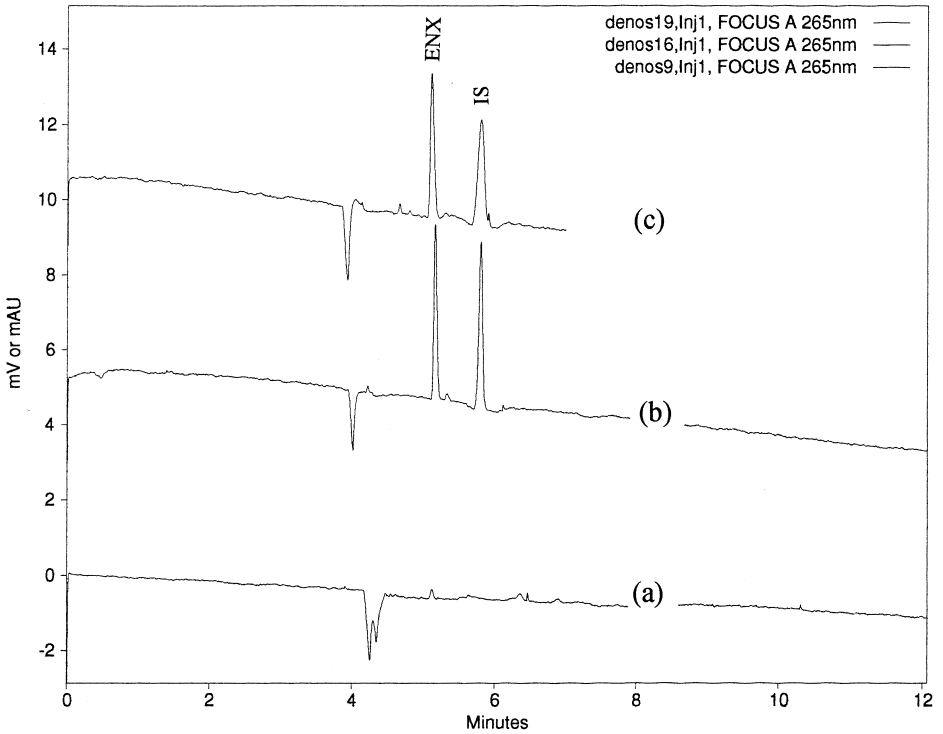

Figure 4. The electropherograms of: (a) Blank serum deproteinized with ethanol; (b) serum spiked with the standard ENX solution (0.25 μmol) and IS (0.15 μmol); and (c) water spiked with the standard ENX xolution (0.25 μmol) and IS (0.15 μmol). Conditions are the same as described in Figure 2.

and sodium hydroxide are used for this purpose. However, the excess part of the precipitating agents interfere with the peak or peaks of interest. The use of ethanol or ethanol-acetone mixture do not cause any interference in the electropherogram and, therefore, these reagents were successfully used for the analysis of certain compounds in the body fluids.(17) It was reported that some determinations have been carried out by directly injecting the supernatant of the homogenates and urine into the CE.(17) These kinds of applications shorten the total analysis time and is time-saving.

The analysis of ENX in serum was carried out as presented in the Experimental Section. Sharp and resolved peaks were obtained as shown in Figure 4.

Certain experiments were conducted to elucidate the recovery of ENX and to validate the CE studies. Three sets of experiments with definite amounts of ENX were added to serum and to the double distilled water and were analyzed. The same experiment was also performed without any ENX. The recovery was found to be 89.7±0.63 (RSD %).

The recovery experiments were also tested by HPLC and were found to be 78.8±4.94 (RSD%). The difference between the methods could be due to the different precipitation procedures applied.

These results show that the proposed CE method is simple, rapid, and low cost as compared to HPLC, specially for the quality control analysis of ENX. The method can also be applied for the bioavailability and pharmacokinetic studies.

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