High Performance Liquid Chromatographic Method for the Determination of Piroxicam, Naproxen, Diclofenac Sodium, and Mefenamic Acid in Bulk Drug and Pharmaceutical Preparations

Sarmad Bahjat Dikran **Ruaa Moayed Mahmood**

Chemistry Department, College of Education for Pure Science-Ibin Al-Haitham, University of Baghdad

Ruaa_Moayed@yahoo.com

Abstract

A simple accurate and rapid reversed phase high performance liquid chromatographic method has been developed and validated for the determination of four nonsteroidal anti-inflammatory drugs; Piroxicam(PX), Naproxen(NAP), Diclofenac sodium(DCL) and Mefenamic acid(MFNC) in their pure form and different commercial pharmaceutical formulation. The separation was performed on a NUCLEODUR® 100-5 C18ec (250 × 4.6 mm i.d.; particle size 5 µm) column as stationary phase with a mobile phase comprising of acetonitrile: deionized water acidified with 1% acetic acid in gradient mode. The flow rate was 1.5 $mL.min^{-1}$ at the temperature 35 ºC and detection was carried out at 264 nm. Separation has been completed within 8 min. The retention times of Piroxicam, Naproxen, Diclofenac sodium, and Mefenamic acid were 4.267 min, 4.785 min, 6.555 min, and 7.683 min respectively. The linearity for Piroxicam was in the range of 3–200 $\mu g.mL^{-1}$ and for Naproxen and Mefenamic acid was in the range 1-200 $\mu g.mL^{-1}$, while for Diclofenac sodium was in the range 1.5–200 $\mu g.mL^{-1}$. The proposed method was successfully applied for the determination of these drugs in their pure form and different pharmaceutical preparations (tablets, capsules, and ampoule) and there is no interference with additives.

Keywords*: High performance liquid chromatography, Piroxicam, Naproxen, Diclofenac sodium, Mefenamic acid.*

الخلاصة

تم تطوير طريقة كروماتوغرافيا السائل ذات الأداء العالي ذي الطور العكوس حساسة ودقيقة وسريعة، وتم اختبار صلاحيتها لتقدير أربعة عقاقير مضادة للالتهاب غير ستيرويدية هي: البيروكسيكام (PX) والنابروكسين (NAP) وديكلوفيناك صوديوم (DCL) وحامض الميفيناميك (MFNC) في شكلها النقي وبمستحضرات صيدلانية مختلفة. تم إجراء الفصل على عمود من نوع NUCLEODUR® 100-5 C18ec بأبعاد (4.6×250) وحجم جسيمات 5 µm كطور ساكن، أما الطور المتحرك فتألف من الأسيتونيتريل:الماء منزوع الأيونات محمض بحامض الخليك (1%) وبأسلوب الاسترداد التدريجي. كان معدل الجريان 1.5 $mL.min^{-1}$ عند درجة حرارة 35 ^{0}م وأجري الكشف عن الحزم المسردة عند 264 nm وكان الزمن اللازم لانتهاء الفصل هو ٨ دقائق. امكن بهذه الطريقة استرداد العقاقير بأزمان احتجاز هي 4.259 min، 4.774 min، 6.544 min، 7.672 min لكل من البيروكسيكام والنابروكسين وديكلوفيناك الصوديوم وحامض الميفيناميك على التوالي

وتراوحت المديات الخطية للبيروكسيكام بين 3-200 $\mu g.mL^{-1}$ وللنابروكسين ولحامض الميفيناميك بين 1-200 $\mu g.mL^{-1}$، في حين كان مدى الخطية لديكلوفيناك الصوديوم هو 1.5-200 $\mu g.mL^{-1}$. تم تطبيق الطريقة المقترحة بنجاح لتقدير هذه العقاقير في شكلها النقي وفي المستحضرات الصيدلانية مختلفة (أقراص، كبسولات، وأمبولة) وبدون أن يظهر أي تداخل من المواد المضافة في المستحضرات الصيدلانية.

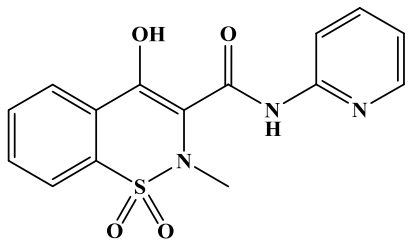
الكلمات المفتاحية: كروماتوغرافيا سائلة عالية الأداء ، بيروكسيكام ، نابروكسين ، ديكلوفيناك صوديوم ، حمض ميفيناميك.

Introduction

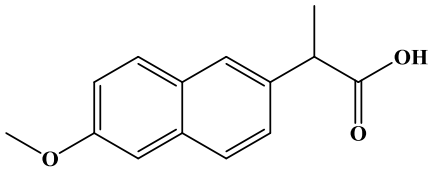
Piroxicam, Naproxen, Diclofenac sodium, and Mefenamic acid (Figure 1), are nonsteroidal anti-inflammatory drugs (NSAID) which display a potent analgesic activity and are effective in the treatment of rheumatoid arthritis, osteoarthritis, and other joint diseases. These drugs are related to inhibition of cyclo-oxygenase (Cox), a key enzyme of prostaglandin biosynthesis at the site of inflammation [(1)].

Different analytical methods are reported for estimation of these drugs in pharmaceuticals individually or in mixture with other drugs [(2-4)]. Various chromatographic techniques are also available in the literature. These include gas-liquid chromatography [(5)], liquid chromatographic [(6)], and HPLC [(7-13)]; however, there is no RP-HPLC method reported for simultaneous determination of the four cited drugs.

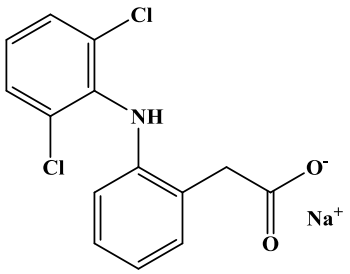
The aim of the work is to develop a HPLC method for determination of piroxicam, naproxen, diclofenac sodium, and mefenamic acid in mixture of the four drugs in their pure forms and in pharmaceutical preparation.



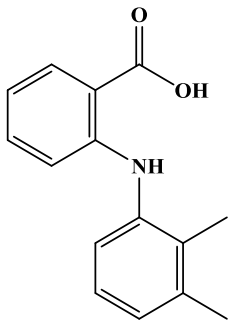
Piroxicam



Naproxen



Diclofenac sodium



Mefenamic acid

Figure 1. Chemical structures of the studied drugs.

Experimental

Materials and reagents

The solvents which are used (acetonitrile, methanol) were of HPLC grade and analytical grade acetic acid supplied from Sigma-Aldrich company (Germany) and water was of HPLC grade supplied from Scharlau company (Spain). The investigated samples of piroxicam, naproxen, diclofenac sodium, and mefenamic acid were obtained from S.D.I-Iraq. Tablets, capsules, and ampule were purchased from local pharmacy.

Chromatographic system

The HPLC system was composed of two LC-20AD pumps, an SPD-20A UV-VIS detector, an SIL-20AC auto injector, a DGU-20A5 degasser, an CBM-20A system controller, and CTO-20A column oven (all from Shimadzu, Kyoto, Japan). An NUCLEODUR® 100-5 C18 ec (250 × 4.6 mm i.d.; particle size 5 µm) column (MACHEREY-NAGEL Germany) was used for separation. The chromatographic and the integrated data were recorded using Shimadzu's LCsolution software operated on a Pentium-4 computer system.

Chromatographic conditions

Chromatographic separation was achieved using a RP- NUCLEODUR® 100-5 C18 ec column. The mobile phase consisted of acetonitrile:deionized water acidified with 1 % acetic acid in a ratio (50:50, v/v). It was filtered through a 0.45µm filter, degassed by ultra-sonication for 10 min before use. A steady flow rate of 1.5 $mL.min^{-1}$, temperature 35 ºC and injection volume 30 µL were used to carry out the separation in gradient mode and the detection was made at 264 nm. Complete resolution of the peaks with clear baseline was obtained Figure 5. Peak areas and peak height were measured for the quantitation of the analytes.

Preparation of drug standard, formulation and matrix solutions

Standard stock solution of each drugs was made in methanol containing 1000$\mu g.mL^{-1}$. A mixture of four drugs containing 10$\mu g.mL^{-1}$ was prepared and was stored at 4 ºC and protected from light. The solutions of formulation were prepared in a similar way. Ten weighed tablets of each formulation were finely powdered (or the content of ten capsules) and homogenized in a mortar. An amount equivalent to content of the active ingredient per tablet (or capsule) was taken into a 10 mL volumetric flask, dissolved in methanol, ultra-sonicated for approximately 10 min, and made up to the mark with methanol. The supernatant liquid was collected and filtered through a 0.45-micron filter for use and three concentrations 200, 100 and 10 $\mu g.mL^{-1}$ were prepared for each preparation by serial dilution. On the other hand, 1000 $\mu g.mL^{-1}$ solution of DCL was prepared by diluting the content of an ampoule (75mg/3mL) with methanol, and other working solutions were prepared by serial dilution with methanol. All solutions were stored at 4 ºC and protected from light.

Method validation

The proposed method was validated with respect to suitability of the method, linearity, accuracy, and precision, the limit of detection (LOD) and, limit of quantification (LOQ).

Suitability of the method

The column efficiency, resolution, and selectivity were calculated for the standard solutions. The values obtained demonstrated the suitability of the system for analysis of these drugs.

Linearity

The linearity of the response, expressed as peak area, to different concentrations of PX, NAP, DCL, and MFNC was studied and the data were subjected to statistical analysis using a linear-regression model.

Accuracy

Accuracy of the method was performed by estimating the relative error percent of the method experiments. The relative error percent of PX, NAP, DCL, and MFNC were determined for three replicates by spiking each at three different levels 10, 100, and 200 $\mu g.mL^{-1}$. Similarly, recovery studies were carried out for the four drugs by calculating the recovery (n=3) at three different levels of drug concentration.

Precision

Drug solutions were prepared at different concentrations, and precision of the method was studied using repeatability. Repeatability was the intra-day variation in assay obtained at different concentration levels of PX, NAP, DCL, and MFNC and expressed in terms of relative standard deviation (RSD) calculated for each day.

LOD and LOQ

LOD and LOQ represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ, respectively. LOD and LOQ were determined by measuring the magnitude of analytical background, i.e. by injecting a series of blank solutions (n=5) and calculating the signal-to-noise ratio for each compound.

Method development

A mixture of PX, NAP, DCL, and MFNC was subjected to separation by RP-HPLC on C18 columns. Initially, deionized water acidified with acetic acid were tried using methanol and acetonitrile as organic modifiers. However, the compounds exhibited incomplete separation and a characteristic tailing on all columns. This could be due to their ability to form strong hydrogen bonding with the residual silanols of the C18 materials. To overcome these problems several mobile phase compositions, concentration of acetic acid, flow rates, isocratic and gradient modes were tried. Moreover, to minimize the peak tailing, the column temperature was increased (25 ºC – 50 ºC).

Results and discussion

The present study aimed to develop a chromatographic method for separation and determination of PX, NAP, DCL, and MFNC Figure 1. The HPLC conditions were optimized by studying the effects of concentration of organic modifier, concentration of acetic acid, mobile phase flow rate and column temperature.

Effect of organic modifier

Isocratic mode elution using deionized water acidified with 1% acetic acid and methanol or acetonitrile as organic modifiers on an RP- NUCLEODUR® 100-5 C18 ec (250 mm × 4.6 mm) column maintained at 25 ºC. Using acetonitrile as a modifier gave a reasonably accepted separation. This could be attributed little ability of acetonitrile to accept weak hydrogen bond than methanol and a high dipole moment which enables him to participate in selective dipole-dipole interaction with certain solutes [14]. Moreover, the mobile phase becomes less viscosus than the corresponding methanol/water mixtures; It thus enables faster flow rates develops faster separation methods [15]. Therefore, acetonitrile was selected as an organic modifier. Further studies were carried out to determine the effect of acetonitrile concentration on separation. Several proportions were tried, and the degree of the separation was followed by checking the values of chromatographic parameters in addition to the shape of chromatographic bands. It was found that increasing the percent of acetonitrile, above 50%, leads to decrease in the analysis time as well as an increase in the peak heights, but no-good resolution of peaks was observed. The study shows a significant difference in the separation of the investigated drugs when a mobile phase composition was varied from 50:50 water acidified with 1% acetic acid: methanol to 50:50 water acidified with 1% acetic acid: acetonitrile Table 1.

Table 1. The effect of organic modifier on efficiency and retention factors

Organic modifier (%)	k'				N			
	PX	NAP	DCL	MFNC	PX	NAP	DCL	MFNC
48	2.71	3.38	7.89	13.51	3085.2	3897.7	6441.5	8997.2
50	2.41	3.03	6.79	11.40	3340.3	4520.5	6742.6	9639.4
55	1.92	2.17	4.50	7.40	3506.5	4075.4	6600.7	9189.1
60	1.54	1.54	2.98	4.78	2774.5	2774.5	6419.6	8966.9
65	1.23	1.23	2.18	3.45	1997.5	1997.5	6362.9	8729.2
70	0.95	1.06	1.60	2.49	1611.7	2942.7	6091.6	8643.0
75	0.71	0.90	1.22	1.86	1597.4	1819.5	5650.4	7825.1
85	0.52	0.69	0.77	1.12	1146.9	4473.5	4936.8	6960.4
90	0.47	0.64	0.64	0.92	1808.0	2033.1	2033.1	6033.7
95	0.44	0.55	0.55	0.78	2391.8	2180.3	2180.3	3543.5

Concentration of Acetic acid

The effect of the concentration of CH_3COOH on the separation was studied by maintaining all other factors constant. Figure 2 shows that there is no significant effect of the concentration of acetic acid on separation of peaks as well as run analysis when the concentration of the acid is varied from 0.25 % to 1.0%. . Hence, 1% acetic acid was used in all the subsequent experimental work.

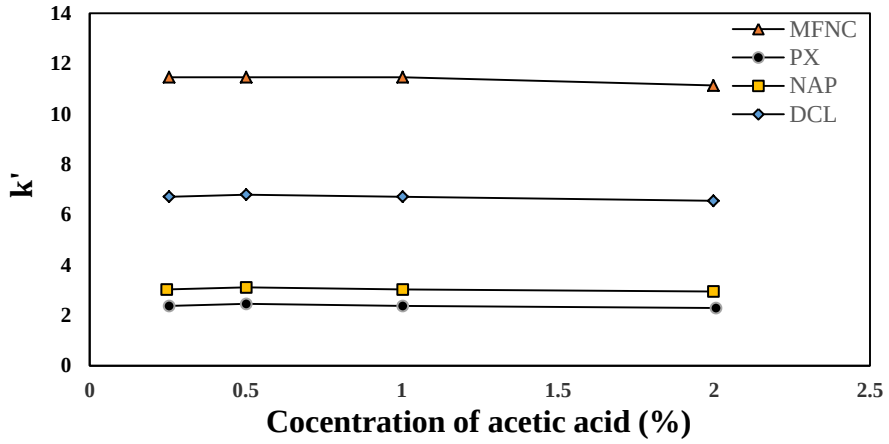


Figure 2. The effect of acetic acid percentage on retention factors (*k'*).

Effect of Flow rate of the mobile phase

Different flow rates of mobile phase ranged between 0.8 to 1.7 mL.min^{-1} were tried to choose the optimum flow rate that achieve complete analysis within a short time and reasonable sensitivity and high column efficiency. Results in Figure 3 indicated that the suitable flow rate of the mobile phase for separation of the studied drugs is 1.5 mL.min^{-1}.

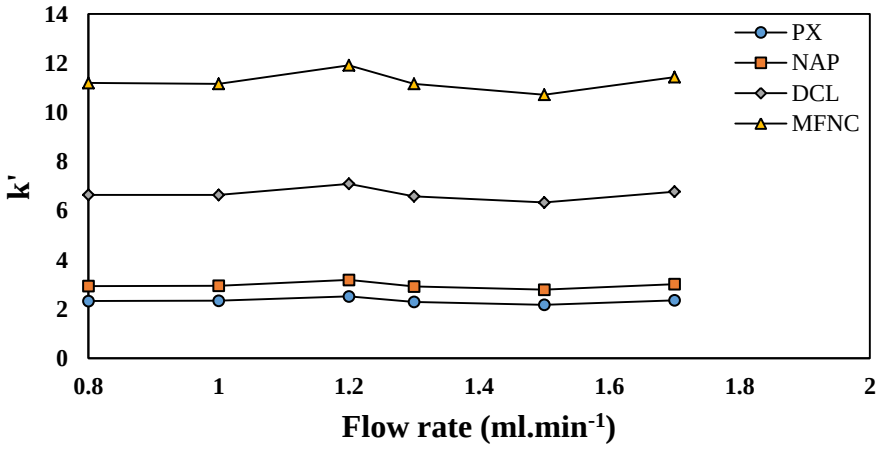


Figure 3. The effect of flow rate on retention factors (*k'*).

Effect of temperature

The column was maintained at different temperatures ranging from 25 ºC – 50 ºC in a thermostated oven. Generally increasing column temperature in RP- chromatography decreases the retention times of the separated bands and increases column efficiency by decreasing mobile phase viscosity, which in turn lowering the column head pressure. Table 2 show the values of retention times, retention factors, selectivity, efficiency N and resolution Rs for each drug at different temperature. Finally, separation was carried out by maintaining the column at 35 ºC. At this temperature a good shape and resolution of the separated bands of the drugs were obtained Figure 4.

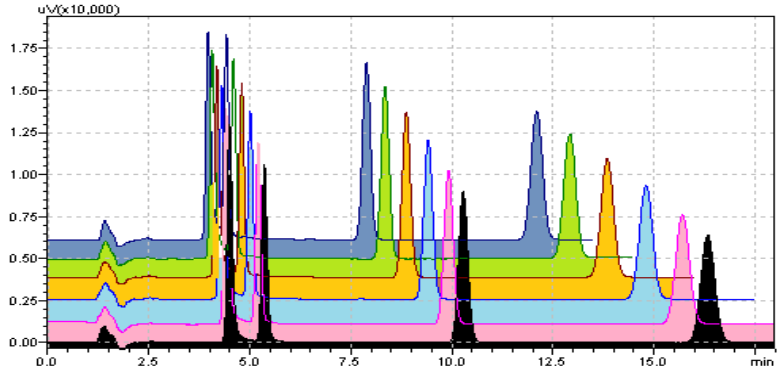


Figure 4. Overlaid chromatogram of four drugs at different temperature.

Table 2. The Effect of Temperature on retention times, retention factors, selectivity, efficie and resolution in Optimized Conditions.

Temperature (^{0}C)	Drug	t_R	k`	α	N	Rs
25	**PX**	4.495	2.22	1.28	5012.12	3.13
	NAP	5.356	2.84	2.24	7111.67	10.70
	DCL	10.275	6.37	1.68	8883.69	8.98
	MFNC	16.332	10.72		9890.04	
30	**PX**	4.411	2.13	1.26	4855.40	2.97
	NAP	5.205	2.69	2.24	6923.21	10.62
	DCL	9.915	6.03	1.68	8547.26	8.93
	MFNC	15.707	10.13		9452.04	
35	**PX**	4.304	2.03	1.28	4656.96	3.05
	NAP	5.010	2.59	2.22	6679.63	10.46
	DCL	9.412	5.75	1.67	8326.32	8.82
	MFNC	14.804	9.62		9108.47	
40	**PX**	4.184	1.97	1.22	4382.69	2.48
	NAP	4.796	2.41	2.20	6579.25	10.27
	DCL	8.869	5.30	1.67	7981.10	8.70
	MFNC	13.844	8.83		8649.80	
45	**PX**	4.067	1.86	1.20	4173.08	2.23
	NAP	4.593	2.23	2.18	6181.74	10.05
	DCL	8.344	4.86	1.66	7714.23	8.57
	MFNC	12.917	8.08		8265.55	
50	**PX**	3.964	1.83	1.18	4023.42	1.99
	NAP	4.415	2.15	2.15	5826.56	9.84
	DCL	7.883	4.62	1.65	7292.59	8.44
	MFNC	12.100	7.62		7753.21	

Development of the gradient HPLC method

Although, a reasonable separation of the cited drugs was achieved via isocratic elution, gradient elution was tried to decrease the analysis time and improve the resolution since mode of elution increase quasi-efficiency of the column [16].

Figure 5 shows that quality separations in terms of, resolution, reasonable run time, and peak symmetry were obtained using the gradient steps shown in Table 3.

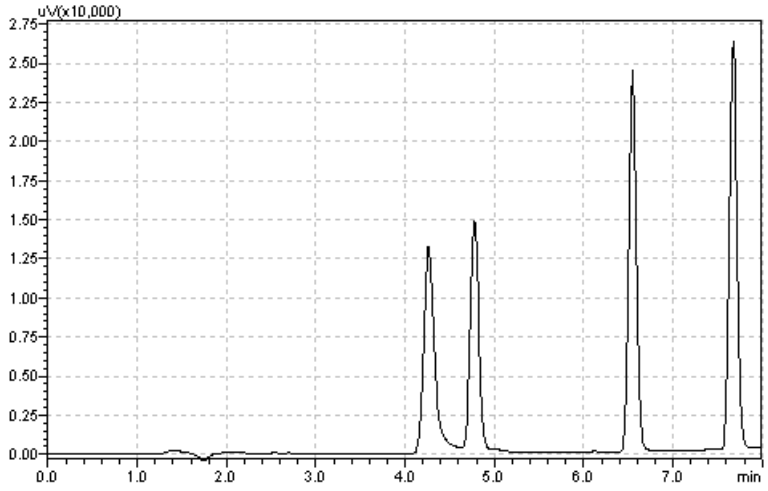


Figure 5. Chromatogram of mixture of four drugs (10 µg.mL^{-1}) in gradient elution system.

Table 3. Gradient elution program for separation of four drugs using RP-HPLC.

Time (min)	Module	Action	Value
0.0	Pumps	Acetonitrile Conc	0
8.0	Pumps	Acetonitrile Conc.	100
8.1	Controller	stop	

Validation

System suitability

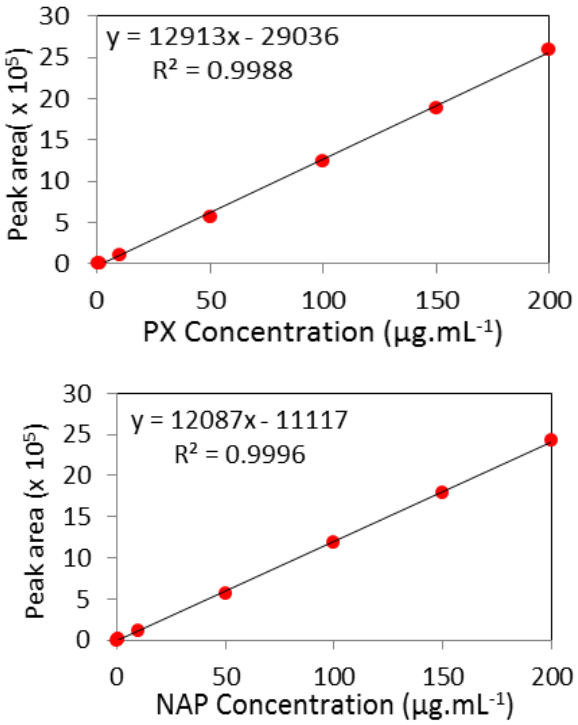
The column efficiency, resolution, and selectivity were calculated for the standard solutions and the results are expressed in Table 4. The values obtained demonstrated the suitability of the system for analysis of these drugs.

Table 4. System suitability Data.

System suitability parameter	PX	NAP	DCL	MFNC
Retention time (t_R), min	4.259	4.774	6.544	7.672
Retention factors (k`)	2.01	2.38	3.63	4.43
Theoretical plate number (N)	5504.06	10817.13	27406.05	37556.34
Resolution factor (R_s)	2.44	9.35	6.62	
Selectivity (α)	1.18	1.53	1.22	

Linearity, LOD, and LOQ

The peak areas of PX, NAP, DCL, and MFNC were linear with respect to the concentrations over the range of 3-200 µg.ml^{-1} for PX, 1.5-200 µg.ml^{-1} for DCL and 1-200 µg.ml^{-1} for NAP and MFNC Figure 6. Statistical analysis of data were carried out using a linear-regression model. The linear regression equations and determination coefficients (r^2) indicate good linearity. The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the linearity curve and are shown in Table 5.



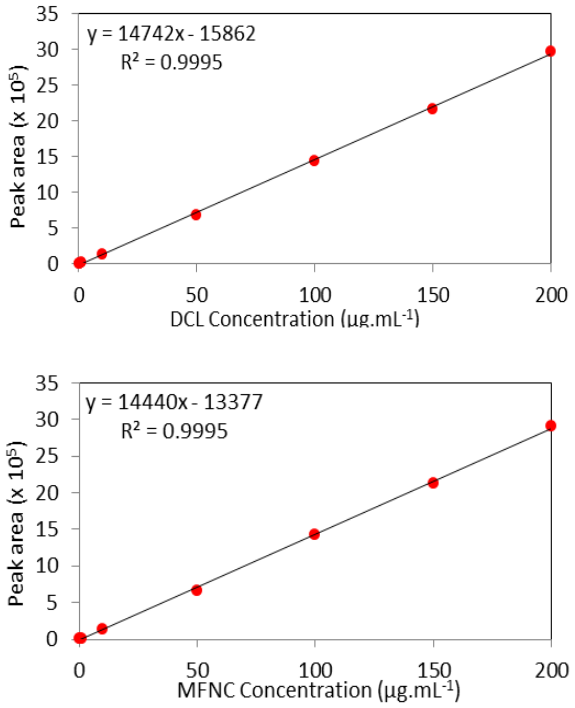


Figure 6. Calibration graphs, concentration (µg.ml^{-1}) vs. peak area for PX, NAP, DCL, and MFNC.

Table 5. Calibration Data.

Drug	Linearity range (µg.mL^{-1})	Regression equation	r^2	Slop	Intercept	LOD (µg.mL^{-1})	LOQ (µg.mL^{-1})
PX	3-200	***y=12913 x-29036***	0.9988	12913	29036	0.281	0.926
NAP	1-200	***y=12087 x-11117***	0.9996	12087	11117	0.300	0.989
DCL	1.5-200	***y=14742 x-15862***	0.9995	14742	15862	0.246	0.811
MFNC	1-200	***y=14440 x-13377***	0.9995	14440	13377	0.251	0.828

Analysis of bulk drugs and formulations

The contents of PX, NAP, DCL, and MFNC obtained from the measurement of their concentrations in different commercial tablet, capsule, and ampul dosage are shown in Figure 7 and Table 6. The results show good correlation between the declared and determined values of cited drugs for all analyzed samples, which indicates a reasonable efficacy and selectivity of the method used.

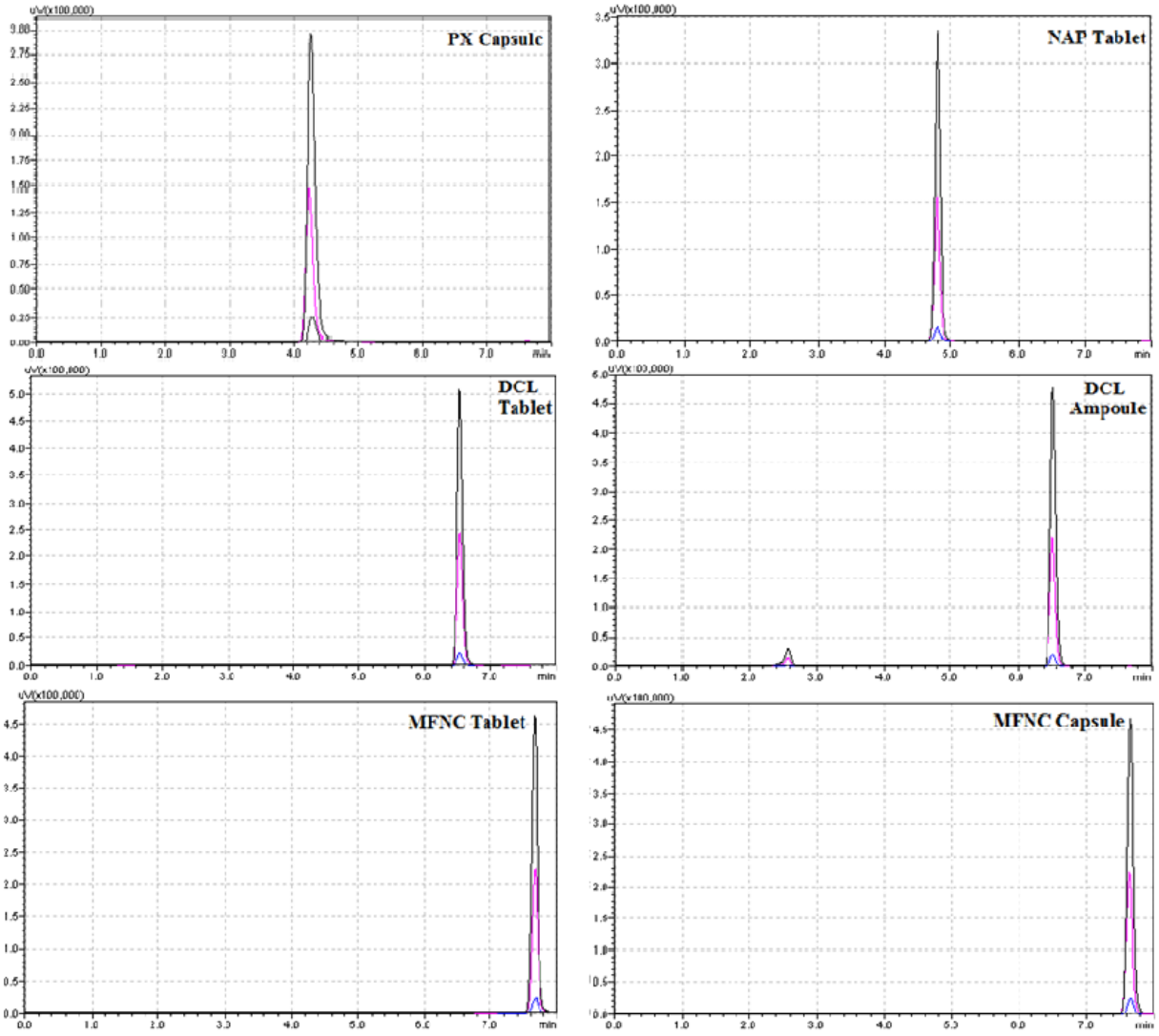


Figure 7. Typical overlaid chromatogram of four drugs in different pharmaceutical preparations at 200,100 and 10 µg.mL^{-1}.

Table 6. Application of the method to the drugs concentration measurements in different pharmaceutical preparations.

Sample	Conc. taken (µg.mL^{-1})	Weight* found (mg/formulation)	Recovery %	RSD %
PX capsule 20mg	10	20.89	104.3	0.1224
	100	20.17	100.7	0.1529
	200	20.82	104.2	0.0681

NAP tablet 500mg	10	485.19	97.0	0.0442
	100	483.52	96.7	0.1075
	200	481.52	96.3	0.3964
DCL tablet 50mg	10	53.98	107.9	0.2256
	100	50.91	101.8	0.1907
	200	52.71	105.4	0.2811
DCL ampoule 75mg/3mL	10	73.02	97.4	0.1258
	100	68.69	91.6	0.3327
	200	73.41	97.9	0.2884
MFNC tablet 500mg	10	489.28	97.9	1.2276
	100	475.92	95.2	0.6002
	200	489.42	97.9	0.3018
MFNC capsule 250mg	10	245.51	98.2	0.3401
	100	249.21	99.7	0.1214
	200	246.86	98.7	0.3880

**Average of three determinations.*

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