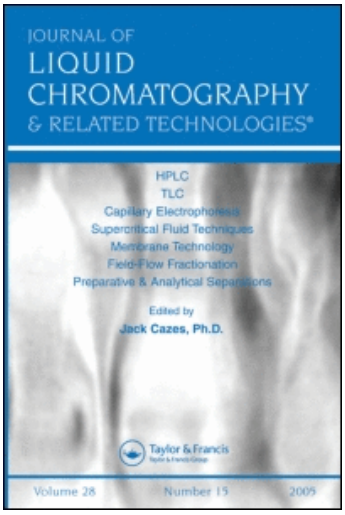

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TLC Separation of Fluoroquinolones: Searching for Better Selectivity

Irena Maria Choma[a]
[a] Department of Chemical Physics, University of M. Curie-Skłodowska, Lublin, Poland

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TLC Separation of Fluoroquinolones: Searching for Better Selectivity

Irena Maria Choma*

Department of Chemical Physics, University of M. Curie-Skłodowska, Lublin, Poland

ABSTRACT

Fluoroquinolones are a relatively new group of chemotherapeutics used worldwide. They are approved for use as therapeutic agents and as feed additives in food producing animals in many countries. The misuse of these antibiotics in human and veterinary medicine has led to the loss of their efficacy and to the emergence of drug-resistant bacteria. Therefore, there is a need for developing analytical methods to monitor the levels of fluoroquinolone residues in biological fluids and edible animal tissues. In this paper, the optimal conditions for thin-layer chromatography (TLC) analysis of six veterinary fluoroquinolones, i.e., difloxacin, ciprofloxacin, norfloxacin, sarafloxacin, enrofloxacin, and flumequine are established. Good separation was achieved using 2-dimensional TLC on silica gel. Retention parameters for various chromatographic systems are compared. Chromatographic properties of four sorbents, i.e., plane silica

*Correspondence: Irena Maria Choma, Department of Chemical Physics, University of M. Curie-Skłodowska, M. Skłodowska Sq. 3, 20–031 Lublin, Poland; E-mail: ichoma@hermes.umcs.lublin.pl.

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gel and silica gel with bonded diol-, amino-, and cyanopropyl chains are discussed.

Key Words: TLC; Fluoroquinolones; Silica; Diol-silica; Amino-silica; Cyano-silica.

INTRODUCTION

The fluoroquinolones are a class of synthetic antimicrobial agents, 6-fluorinated derivatives of 4-oxo-1,4-dihydroquinoline. The progenitor of the so-called 4-quinolones, nalidixic acid, was introduced in 1962. This narrow spectrum, nonfluorinated chemotherapeutic was used mainly in treating urinary tract infections. The antibacterial activity was enhanced significantly by addition of 6-fluoro and 7-piperazinyl groups to the quinoline skeleton. Since introduction of flumequine in 1973, norfloxacin in 1978, ciprofloxacin in 1983, and others, fluoroquinolones have became the most popular antimicrobials used not only in treating urinary tract infections, but also in a variety of other infections such as pneumonia, bronchitis, otitis, sinusitis, prostatitis, enteric infections, or sexually-transmitted diseases. Their efficacy, good pharmacokinetic properties, and tolerability have made them useful worldwide.[1,2]

Besides clinical applications, fluoroquinolones are also widely used in veterinary medicine in cattle, swine, poultry, and fish.[3] Some drugs such as enrofloxacin and sarafloxacin were specially developed for that purpose; some others such as flumequine, norfloxacin, or ofloxacin are used both as human and animal drugs. The extensive use of fluoroquinolones in treating animals and as growth promoters, as well as their misuse in human medicine, has caused development of drug-resistant bacteria.[4] The biggest risk is connected with the drugs used both in human and veterinary medicine and with those that produce similar metabolites. The major metabolite of enrofloxacin is its human counterpart, ciprofloxacin, one of the most popular human antibiotics in the world. There is a need for developing analytical methods to monitor the levels of fluoroquinolone residues in biological fluids and edible animal tissues.

Chromatography, mainly high-performance liquid chromatography (HPLC), is the most important method of quinolone antibiotic analysis.[5–7] Thin-layer chromatography (TLC) is less sensitive than HPLC but offers high sample throughput and limited sample pretreatment. Despite this, examples of separation of quinolones by TLC are scarce.[8–20]

Fluoroquinolones are polar, mostly amphoteric compounds that interact strongly with silanol groups and with metal impurities of siliceous sorbents.

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In spite of this, most TLC methods use silica gel 60 TLC plates, plane or impregnated with Na_2EDTA or K_2HPO_4. Multicomponent organic mobile phases are used, usually with the addition of aqueous solutions of ammonia or acids. There is also an example of applying ion-association systems.[15]

In this paper, the optimal conditions for separation of six veterinary fluoroquinolones, i.e., difloxacin (D), ciprofloxacin (C), norfloxacin (N), sarafloxacin (S), enrofloxacin (E), and flumequine (F) are established. The structures of the drugs are presented in Fig. 1.

EXPERIMENTAL

Equipment and Reagents

DS sandwich chambers[21] were purchased from Chromdes, Lublin, Poland. Precoated silica gel TLC plates $Si60F_{254}$ 10 cm × 20 cm, HPTLC $Si60F_{254}$, $DiolF_{254}$, CNF_{254}, and NH_2F_{254} 10 cm × 10 cm were purchased from E. Merck, Darmstadt, Germany. Ammonia (25%), acetone, chloroform, dichloromethane, 2-propanol, and tetrahydrofuran, analytical grade, were purchased from P.O.Ch. Gliwice, Poland; HPLC grade methanol from Merck, Darmstadt, Germany. Fluoroquinolones were supplied by Sigma (St. Louis, MO, U.S.A.).

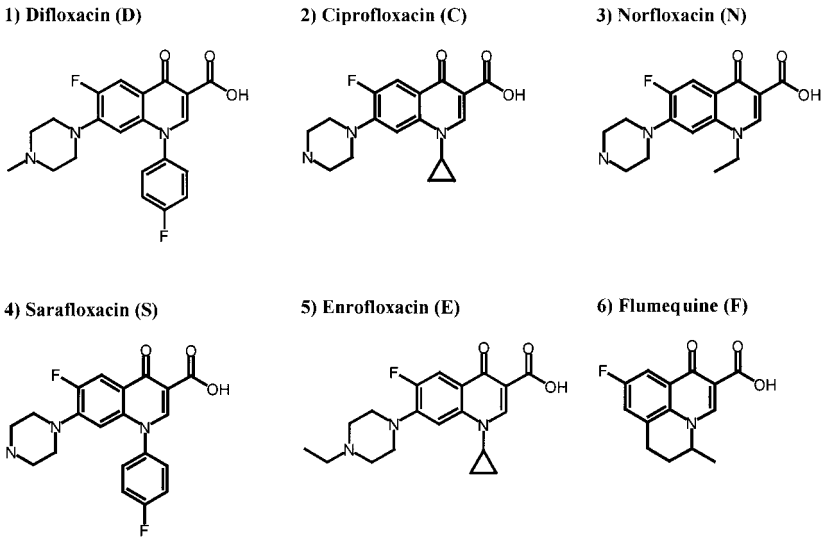

Figure 1. The structures of the fluoroquinolones analyzed.

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Methods

Preparation of Antibiotics Solutions

Portions of 0.01 g of each of the fluoroquinolones were weighed accurately in 10-mL volumetric flasks, dissolved in 9 mL of 1% Na_2CO_3 and then diluted to the volume with water. The working solutions were the mixtures, prepared by the dilution of 100 µL of each stock-solutions in 9.9 mL of methanol to produce 0.01 mg/mL standards. The standards of fluoroquinolones were applied to the TLC plates using a Hamilton microsyringe (Bonaduz, Switzerland). After air drying, fluoroquinolones spots were detected at 366 or 254 nm and flumequine only at 254 nm by UV lamp with dual-wavelength (HA-05 Haland, Warsaw, Poland).

RESULTS AND DISCUSSION

Difloxacin, ciprofloxacin, norfloxacin, sarafloxacin, and enrofloxacin belong to the so-called 7-piperazinylflouroquinolones. They possess both the acidic carboxylic group, as well as the basic, amine group of piperazinyl substitution. It means that below pK_{a1} they are cationic, above pK_{a2} they are anionic, and between these values—zwitterionic. The values of pK_{a1} and pK_{a2} for 7-piperazinylquinolones vary from 5.5 to 6.6 and from 7.2 to 8.9, respectively.[7] Flumequine is neutral below pH 6.3 (the value of pK_a) and anionic above that value.[22] Organic mobile phases containing ammonia possess pH values above 10, so all piperazinylfluoroquinolones and flumequine are in the anionic form.

Basic pH controlled by addition of ammonia, prevents appearance of the double spots on the plate resulting from different ionic forms of the drugs. Additionally, ammonia hinders adsorption of fluoroquinolones on silanol groups and metal impurities of silica gel. It is important not only when Si60 plates are used, but also when plates with bonded stationary phases are used.

Ten mobile phases were tested for the separation of the six fluoroquinolones analyzed. They are denoted by Roman numbers and listed below.

I. Dichloromethane/methanol/2-propanol/25% aqueous ammonia 4 : 4 : 5 : 2.
II. Dichloromethane/methanol/2-propanol/25% aqueous ammonia 3 : 3 : 5 : 2.
III. Dichloromethane/methanol/2-propanol/25% aqueous ammonia 2.5 : 2.5 : 5 : 2.

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IV. Dichloromethane/methanol/2-propanol/25% aqueous ammonia 2 : 2 : 5 : 2.
V. Dichloromethane/2-propanol/25% aqueous ammonia 4 : 5 : 2.[13]
VI. Methanol/2-propanol/25% aqueous ammonia 5 : 5 : 2.[14]
VII. Chloroform/methanol/2-propanol/25% aqueous ammonia 4 : 2 : 5 : 1.
VIII. Acetone/methanol/25% aqueous ammonia 5 : 3 : 2.[14]
IX. Dichloromethane/2-propanol/tetrahydrofuran/25% aqueous ammonia 4 : 6 : 3 : 3.[14]
X. Methanol/acetone/1 M citric acid/triethyloamine 2.8 : 2 : 0.2 : 0.5.[14]

Some of the solvents were used by other authors for separation of quinolones (but different than described in this paper). They are indicated by literature references.

Table 1 contains R_F and R_M that is $\log(1 - R_F/R_F)$ values of the drugs for those solvents on TLC silica gel plates. For all 10 phases the elution order is the same: the highest R_F values are for difloxacin, the next to elute are sarafloxacin, enrofloxacin, and flumequine and the lowest R_F are for ciprofloxacin and norfloxacin. The most difficult for separation are sarafloxacin, enrofloxacin, and flumequine, especially the latter two. This is reflected by separation factors α presented in Table 2. Generally, it is difficult to separate pairs of drugs that differ only slightly in their structure, like difloxacin and sarafloxacin (only in CH_3 group), ciprofloxacin and enrofloxacin (only in C_2H_5 group), and ciprofloxacin and norfloxacin (cyclopropyl group is replaced by ethyl group). Surprisingly, this behavior seems not to concern the pair of enrofloxacin and its metabolite ciprofloxacin, as it is separated very well in most of the systems discussed. It is odd that it is difficult to separate the compounds that differ significantly in their structure such as sarafloxacin, enrofloxacin, and flumequine (see α values for the pairs S/E and E/F), but not norfloxacin and flumequine. The phase III seems to be very effective in separation of C/E and N/F pairs but separation of N/C is unsatisfactory. Of the 10 solvents, the best for separation of the six fluoroquinolones appears to be solvents IV and II. Separation factors are higher for the solvent II but spots are more compact for solvent IV. The solvents IX and X are useless for separation of the six fluoroquinolones, because of strong demixing, especially for the solvent IX.

Selectivity is usually better for high-performance TLC plates. Apart from that, it depends not only on the kind of solvent but also on the character of the sorbent. Tables 3 and 4 demonstrate separation factors for HPTLC Si60 plates, HPTLC DIOL (silica with bonded $(CH_2)_3$—O—CHOH—CHOH groups), HPTLC CN (cyanopropyl silica), and HPTLC NH_2 (aminopropyl silica) plates and solvents IV and VIII, respectively. Selectivity can be assessed

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Table 1. R_F and R_M values of the analyzed fluoroquinolones obtained on TLC silica gel plates with various mobile phases (I–X).

	D	C	N	S	E	F
I						
R_F	0.5	0.28	0.23	0.48	0.43	0.38
R_M	0.00	0.42	0.53	0.03	0.12	0.22
II						
R_F	0.54	0.26	0.21	0.45	0.41	0.40
R_M	−0.06	0.45	0.58	0.08	0.15	0.17
III						
R_F	0.78	0.33	0.30	0.70	0.60	0.58
R_M	−0.54	0.30	0.36	−0.37	−0.18	−0.13
IV						
R_F	0.57	0.32	0.27	0.51	0.44	0.41
R_M	−0.13	0.33	0.44	−0.02	0.11	0.15
V						
R_F	0.54	0.37	0.32	0.53	0.49	0.47
R_M	−0.07	0.24	0.32	−0.05	0.01	0.05
VI						
R_F	0.27	0.08	0.05	0.19	0.16	0.20
R_M	0.44	1.09	1.26	0.64	0.73	0.61
VII						
R_F	0.37	0.25	0.21	0.35	0.34	0.32
R_M	0.24	0.48	0.59	0.26	0.28	0.33
VIII						
R_F	0.55	0.36	0.31	0.50	0.41	0.41
R_M	−0.09	0.25	0.35	0.01	0.16	0.16
IX						
R_F	0.61	0.56	0.53	0.60	0.59	0.59
R_M	−0.20	−0.10	−0.05	−0.18	−0.17	−0.17
X						
R_F	0.66	0.31	0.25	0.54	0.62	0.63
R_M	−0.29	0.35	0.47	−0.07	−0.21	−0.22

by correlation plots of retention parameters, e.g., R_F or R_M values for two different chromatographic systems. Figures 2 and 3 present correlations between hR_F values obtained on HPTLC silica gel plates and hR_F values obtained on various bonded phases, i.e., Diol, CN, and amino for the solvents VIII and IV, respectively.

The most similar to silica gel seems to be amino-silica gel. For the solvent VIII points hR_F SiO_2 vs. hR_F amino are only a little above and parallel to the $x = y$ line. Hence, separation factors for amino plates are lower

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Table 2. Separation factors for pairs of the analyzed fluoroquinolones obtained on TLC silica gel plates with various mobile phases (I–X).

	$\alpha_{S/D}$	$\alpha_{C/E}$	$\alpha_{N/C}$	$\alpha_{N/F}$	$\alpha_{E/S}$	$\alpha_{F/E}$
I	1.08	1.99	1.28	2.03	1.23	1.25
II	1.38	2.00	1.36	2.59	1.18	1.05
III	1.49	3.07	1.13	3.11	1.54	1.12
IV	1.29	1.65	1.29	1.94	1.33	1.09
V	1.06	1.67	1.22	1.87	1.15	1.09
VI	1.57	2.31	1.46	4.37	1.23	1.30
VII	1.06	1.57	1.29	1.80	1.04	1.12
VIII	1.25	1.24	1.26	1.57	1.41	1.00
IX	1.04	1.17	1.12	1.31	1.04	1.00
X	1.67	3.61	1.32	4.97	1.38	1.04

(see Tables 3 and 4). Good separation factors are obtained on diol-silica, especially for the pairs C/E and N/F. Yet, difloxacin, sarafloxacin, enrofloxacin, and flumequine migrate almost simultaneously. This results from forming fronts of solvent demixing on diol plates. The fronts can be observed when the plate is illuminated with UV light. R_F values for CN phase are very high (close to 1 for the phase VIII). Similar relationships are observed for the solvent IV. Now, fluoroquinolones have almost the same retention both on the amino and silica gel phase. It seems that amino-silica possesses adsorption properties similar to those of silica gel. Higher hR_F values are observed on diol-silica, which is in agreement with earlier observation that diol is similar to deactivated silica gel.[23] The least polar of the sorbents is cyano-silica,

Table 3. Separation factors for pairs of the analyzed fluoroquinolones obtained on various HPTLC plates with the phase dichloromethane/methanol/2-propanol-2/25% aqueous ammonia 2 : 2 : 5 : 2 (the phase IV).

	HPTLC			
	Si60	DIOL	NH_2	CN
$\alpha_{S/D}$	1.30	1.20	1.26	1.66
$\alpha_{C/E}$	1.69	1.98	1.40	1.46
$\alpha_{N/C}$	1.38	1.28	1.12	1.25
$\alpha_{N/F}$	2.37	2.52	1.47	1.31
$\alpha_{E/S}$	1.26	1.09	1.03	0.94
$\alpha_{F/E}$	1.02	1.00	1.07	1.39

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Table 4. Separation factors for pairs of the analyzed fluoroquinolones obtained on various HPTLC plates with the phase acetone/methanol/25% aqueous ammonia 5 : 3 : 2 (the phase VIII).

	HPTLC			
	Si60	DIOL	NH_2	CN
$\alpha_{S/D}$	1.39	1.20	1.34	1.00
$\alpha_{C/E}$	1.30	2.62	1.12	1.00
$\alpha_{N/C}$	1.35	1.28	1.22	1.00
$\alpha_{N/F}$	2.15	4.00	1.53	1.00
$\alpha_{E/S}$	1.44	1.04	1.24	1.00
$\alpha_{F/E}$	1.23	1.20	1.12	1.00

which retains the drugs very weakly, but what is rather odd is that the spots developed on the CN phase with solvent IV are tailing.

From the above results, it can be concluded that the best separations of veterinary fluoroquinolones are using HPTLC silica gel plates and solvents I–IV. The only disadvantage of this system is that they slightly differentiate enrofloxacin from flumequine. The chromatograms of six standards of those drugs, as well as of the mixture of them, are presented in Figs. 4–6. For all chromatograms flumequine migrates together with enrofloxacin.

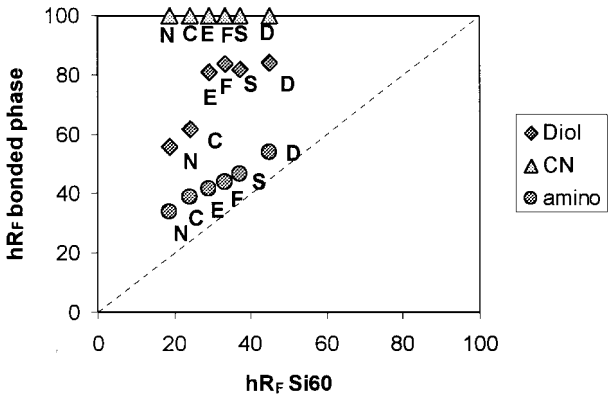

Figure 2. Correlation between hR_F values obtained on HPTLC silica gel plates and hR_F values obtained on amino-, diol-, and cyano-silica for the solvent VIII, i.e., acetone/methanol/25% aqueous ammonia 5 : 3 : 2.

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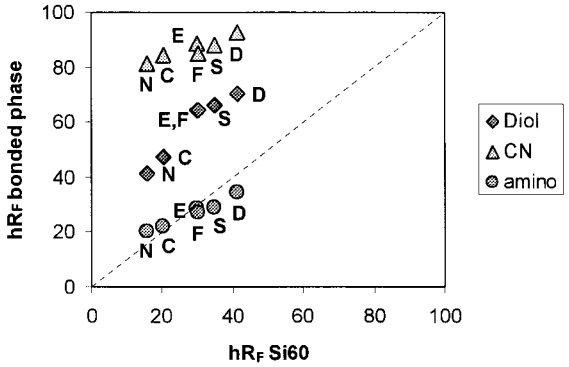

Figure 3. Correlation between hR_F values obtained on HPTLC silica gel plates and hR_F values obtained on amino-, diol-, and cyano-silica for the solvent IV, i.e., dichloromethane/methanol/2-propanol/25% aqueous ammonia 2 : 2 : 5 : 2.

Searching for the mobile phases for TLC of fluoroquinolones, I had to exclude the toluene/ethyl acetate/80% formic acid (60 : 30 : 10) phase, applied by Vega et al. for oxolonic and nalidixic acid, as well as for flumequine determination in fish and their feed.[8] Of the six fluoroquinolones, flumequine is the only one to be eluted by this phase. What previously seemed to be a

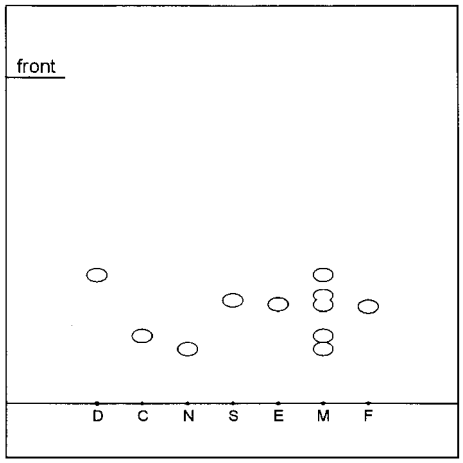

Figure 4. The chromatogram of six fluoroquinolone standards and the mixture of them (M) obtained on HPTLC SI60 plate with dichloromethane/methanol/2-propanol/25% aqueous ammonia 2 : 2 : 5 : 2.

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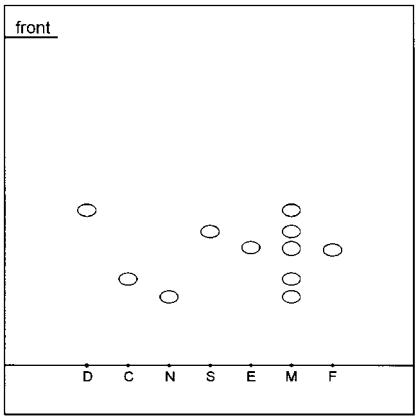

Figure 5. The chromatogram of six fluoroquinolone standards and the mixture of them (M) obtained on HPTLC Si60 plate with dichloromethane/methanol/2-propanol/25% aqueous ammonia 3 : 3 : 5 : 2.

great disadvantage now appears to be a benefit. It was possible to develop chromatograms 4–6 in the second direction with the toluene/ethyl acetate/80% formic acid (60 : 30 : 10) phase to obtain good separation of all the analyzed fluoroquinolones. Figure 7 shows a 2-dimensional chromatogram of the mixture of the antibiotics. In the first direction, it was developed with the

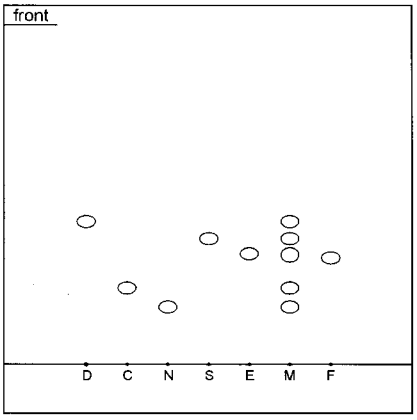

Figure 6. The chromatogram of six fluoroquinolone standards and the mixture of them (M) obtained on HPTLC Si60 plate with dichloromethane/methanol/2-propanol/25% aqueous ammonia 4 : 4 : 5 : 2.

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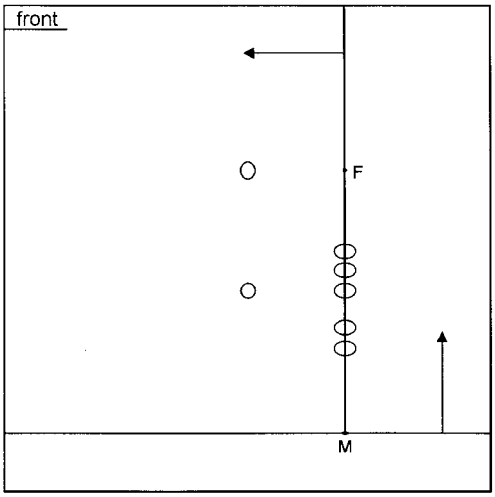

Figure 7. The 2-dimensional chromatogram of the mixture of six fluoroquinolones on HPTLC Si60. The first development with dichloromethane/methanol/2-propanol/25% aqueous ammonia 3 : 3 : 5 : 2. The second, perpendicular to the first, development with toluene/ethyl acetate/80% formic acid (60 : 30 : 10). Additionally, the standard of flumequine was spotted.

phase II, i.e., dichloromethane/methanol/2-propanol/25% aqueous ammonia 3 : 3 : 5 : 2, and after 1 h of air-drying, in the second direction with toluene/ethyl acetate/80% formic acid (60 : 30 : 10). Additionally, the standard of flumequine was spotted for the second development. This method can be applied for all systems in which flumequine elutes together with another fluoroquinolone.

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MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016