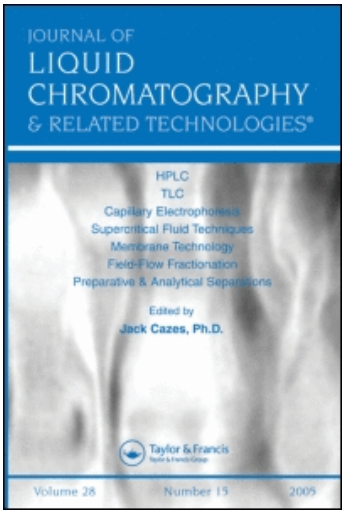

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation and Determination of Carbadox, Nitrofurazone, Nitrofurantoin, Furazolidone, and Furaltadone in their Mixtures by Thin Layer and High Performance Liquid Chromatography

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To cite this Article Kaniou, I. , Zachariadis, G. , Kalligas, G. , Tsoukali, H. and Stratis, J.(1994) 'Separation and Determination of Carbadox, Nitrofurazone, Nitrofurantoin, Furazolidone, and Furaltadone in their Mixtures by Thin Layer and High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 17: 6, 1385 — 1398

To link to this Article: DOI: 10.1080/10826079408013771

URL: http://dx.doi.org/10.1080/10826079408013771

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(6), 1385-1398 (1994)

SEPARATION AND DETERMINATION OF CARBADOX, NITROFURAZONE, NITROFURANTOIN, FURAZOLIDONE, AND FURALTADONE IN THEIR MIXTURES BY THIN LAYER AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The separation and quantitative determination of five drugs, namely carbadox, nitrofurazone, nitrofurantoin, furazolidone and furaltadone, in their various mixtures of 3, 4 or 5 components was investigated. Two types of mobile phases were examined for the TLC separation of the drugs, chloroform/acetonitrile/formic acid and chloroform/acetone. Two other mobile phases were also examined for the high performance liquid chromatographic determination of them, acetonitrile/sodium acetate and acetonitrile/sodium dihydrogen phosphate. The resolution of the chromatograms was studied in both cases and

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also the regression lines of the quantitative determination were described. The absolute detection limits of the determination were in the range of 0.2-1.6 ng for the five compounds.

INTRODUCTION

Among the drugs that are used extensively in veterinary medicine there are some groups of artificial substances with antimicrobial action either on gram positive or gram negative microorganisms. Nitrofurazone (2-[(5-nitro-2-furanyl) methylene]-hydrazinecarboxamide), nitrofurantoin (1-(5-nitro-2-furfurylidene-1-amino) hydantoin), furazolidone (3-(5-nitrofurfurylideneamino)oxazolidinone) and furaltadone (5-morpholinomethyl-3-(5-nitrofurfurylideneamino)-2-oxazolidinone) belong to the group of nitrofuranes and they are chemically characterized by the presence of 5- nitro groups in their molecules as a requisite for antimicrobial activity [1]. In figure 1 the chemical formulas of these substances are given, emphasizing the similarity of their chemical structure with the exception of carbadox. In the following text the substances will be referred by numbers as in fig. 1.

Carbadox ((2-quinoxalinylmethylene) hydrazine- carboxylic acid methyl ester N,N' -dioxide) is used to increase the rate of weight gain, improve feed efficiency and prevent swine dysentery and bacterial enteritis (treponema hyodysenteria) [2], but many times it is used during unnecessary prolonged periods. carbadox and its metabolites (desoxycarbadox) are suspected carcinogens and their use should be restricted to a bare minimum [3]. Within EEC the upper level of carbadox concentration permitted by the Feed Additives Committee is 0.0050 % (50 $g.ton^{-1}$) [4].

Furazolidone and nitrofurazone are fed to poultry at low levels (5-50 $g.ton^{-1}$) as growth promoters. At intermediate levels (50-200 $g.ton^{-1}$) they are used in both poultry and swine husbandry for the prevention of diseases such as fowl cholera, coccidiosis blackhead and swine enteritis. Nitrofurazone, furazolidone and furaltadone are also used in the control and treatment of mastitis in dairy cattle [5-8].

Several methods have been reported in the literature for the separation and determination of carbadox or nitrofurans in animal feed or biological tissue

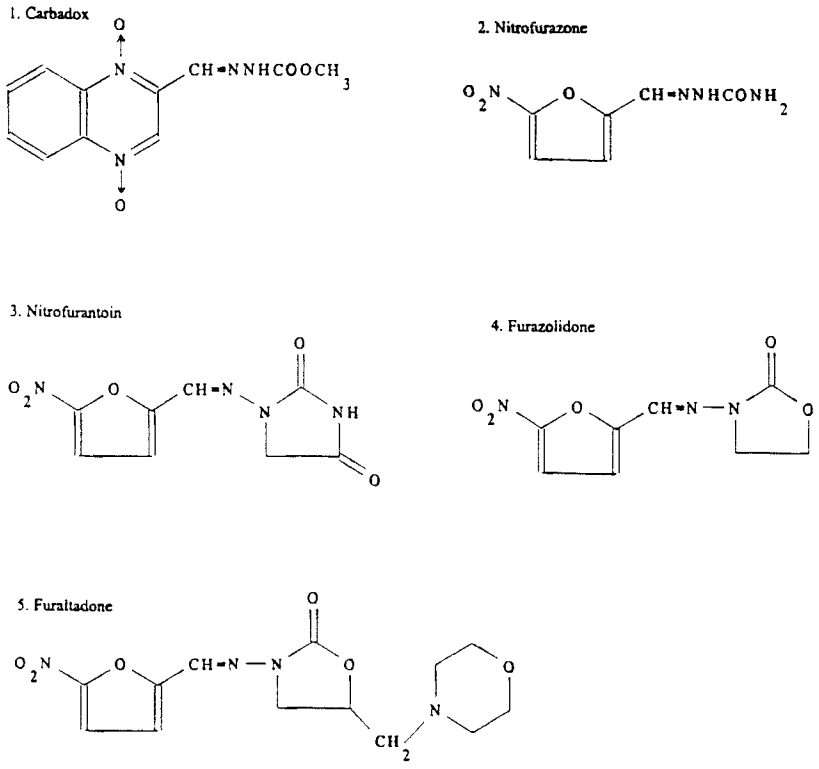

Fig. 1. Formulas of the compounds examined with HPTLC and HPLC.

matrices. They can be classified to spectroscopic methods (as the colorimeric methods for carbadox and furazolidone-nitrofurazone in feeds determination approved by AOAC) [9] and liquid chromatographic methods. The former have some drawbacks like time consuming procedures and poor specificity. In addition the later (LC methods) have better reproducibility and lead to higher recoveries.

Another approach to the analysis of nitrofurans is the application of gas chromatography [10]. Practically, a method of analysis must be capable of reliably determining some hundredths of ppm to be suitable for routine analysis of such compounds [2,11-13].

In this work a comparative study of two chromatographic techniques is described for the determination of the above set of drugs. Thin layer chromatography with two different mobile phases has been applied for the separation of the five compounds in their mixtures and high pressure liquid chromatography with two other mobile phases for the determination of these five compounds in their mixtures.

MATERIALS AND METHODS

Reagents and solutions

The stock solutions of 1, 2, 3, and 5 were prepared by dissolving these compounds (Sigma) in ethanol and the stock solution of 4 was prepared in chloroform (HPLC grade). The concentration of the compounds in all the above solutions was 500 $mg.l^{-1}$ and they were stored in the dark at -18°C. It must be noted that the solutions must be protected from UV and fluorescent light during storage and handling.

The standard working solutions were prepared in a concentration range between 0.1 to 5.0 $mg.l^{-1}$ by dilution of the stock solutions with chloroform. Various mixtures of the compounds were prepared in the same way, with three (1,2,4), (3,4,5), (1,4,5), four (1,2,4,5) or five compounds (1,2,3,4,5) for the TLC separation experiments.

Instrumental Conditions for TLC

The mobile phases examined were the folowing:

i) chloroform / acetonitrile / formic acid, 87/10/3 (v/v) and

ii) chloroform / acetone, 70/30 (v/v).

The plates were Merck No 5631 type, 10X10cm covered with silica gel.

The volume of the solutions was 1 µl or 2 µl and was injected at 2 cm from the lower edge of the plate.

The chromatograms were developed by spraying the spots with pyridine vapours and the spots were detected at 366 nm by means of a Gamag TLC Scanner II cabinet.

Instrumental Conditions for HPLC

The mobile phases examined were the folowing:

i) acetonitrile / sodium acetate 0.01 M, 20/80 (v/v), pH = 5 and

ii) acetonitrile / sodium dihydrogen phosphate 0.05 M, 20/80 (v/v), pH = 4.

A Jasco 880-PU High Pressure Liquid Chromatograph was used for all the determinations. The flow rate was 1.6 $ml.min^{-1}$ for mobile phase (i) and 0.75 $ml.min^{-1}$ for mobile phase (ii).

The chromatographic column was a Lichrospher RP-18, 5 μm particle size, stainless steel, with dimensions 250X4 mm, kept at 30°C. The detector was a Jasco 870-UV spectrophotometer operated at 365 nm.

RESULTS AND DISCUSSION

Thin Layer Chromatographic Separation

Two different experiments were carried out to check the TLC behavior of the five drugs. In the first experiment mixtures of standard solutions prepared freshly were examined (concentration of each compound 1 or 5 $mg.l^{-1}$) and in the second experiment simple standard solutions prepared one day before were examined to detect the appearance of dissociation products (concentration of each compound 5 $mg.l^{-1}$).

In the first experiment the chloroform / acetone (mobile phase ii) was proven more efficient than the chloroform / acetonitrile / formic acid (mobile phase i) for the separation of the drugs in their various mixtures. In figs. 2 and 3 the resulting plates are shown schematically. Ternal and quaternaly mixtures are well separated with both mobile phases but the quintiple mixture of the drugs was sufficiently separated only with mobile phase (ii). With the mobile phase (i) the spots of carbadox and nitrofurantoin were partially overlapped. It must be noted here that carbadox is a self fluorescent compound that can be seen directly by eye on the plate when present in a significant concentration (more than 1 $mg.l^{-1}$). No second spots were developed when the solutions were freshly prepared and rapidly injected on the plates. In the second experiment two spots from each substance were developed, the second being probably dissociation products. With the first mobile phase (fig. 4) the two spots of furaltadone were not sufficiently distinguished while with the second

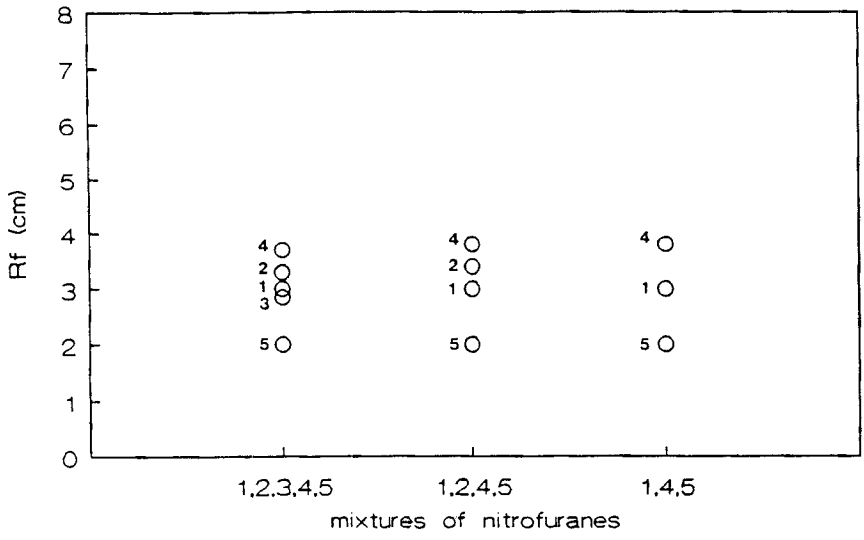

Fig. 2. TLC plates from various mixtures of the drugs. The mobile phase (i) consisted from chloroform, acetonitrile and formic acid. The numbers of drugs as in fig. 1.

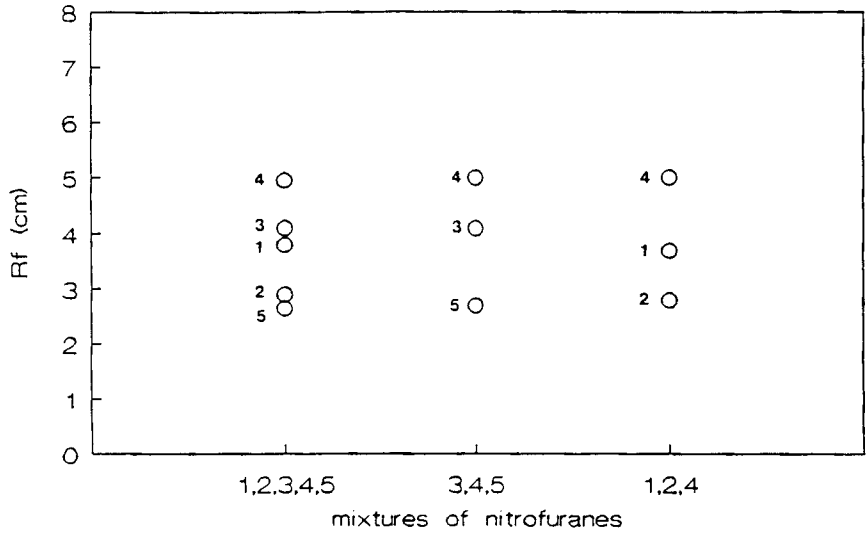

Fig. 3. TLC plates from various mixtures of the drugs. The mobile phase (ii) consisted from chloroform and acetone. The numbers of drugs as in fig. 1.

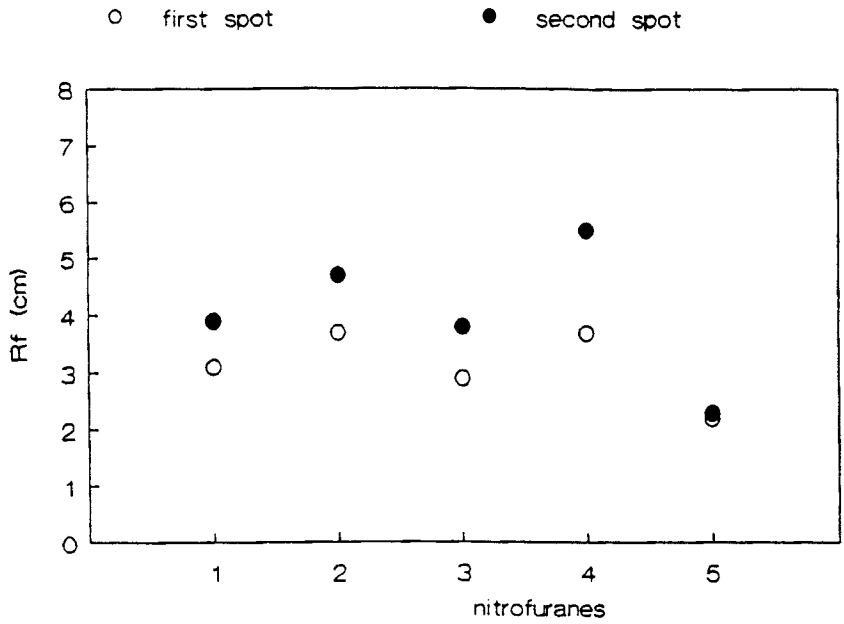

Fig. 4. TLC plates from standard solutions of the drugs. The mobile phase (i) consisted from chloroform, acetonitrile and formic acid. The numbers of drugs as in fig. 1.

mobile phase (fig. 5) carbadox showed no second spot and all the others were clearly separated.

Characteristics of the HPLC Chromatograms

In this mode two different mobile phases were examined also. In fig 6 two different chromatograms obtained from a mixture of the five substances (1 mg. l^{-1} each) and a mixture of four substances (drugs 1 and 2, 0.5 mg.l^{-1} each, furazolidone and furaltadone 1 mg.l^{-1} each, no nitrofurantoin) are given. The mobile phase used was acetonitrile / sodium dihydrogen phosphate (mobile phase ii) and it was proved that the peaks of nitrofurantoin and furazolidone were not resolved at all although when present one of them the resolution was good. On the other hand no secondary peaks from dissociation products were appeared during the time needed for the elution of the drugs.

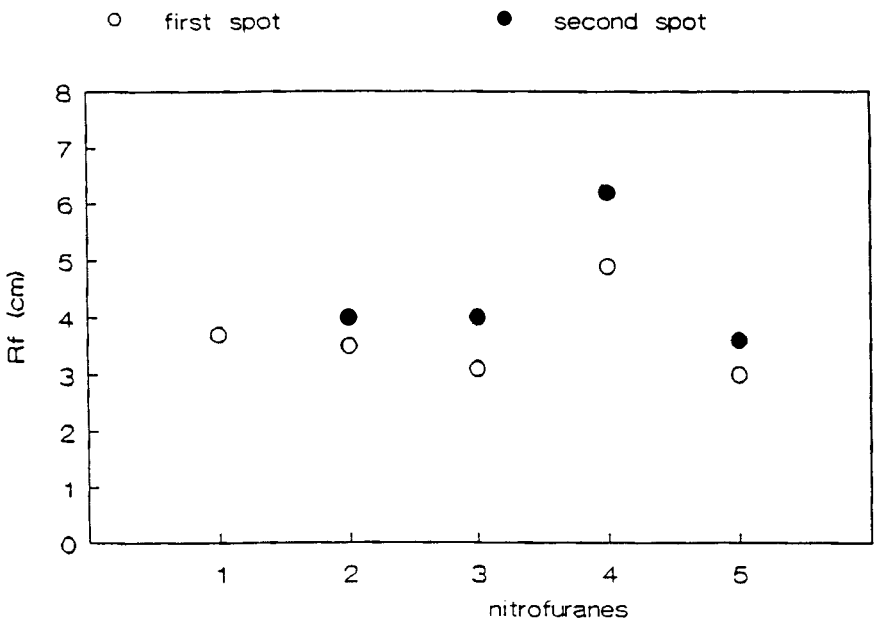

Fig. 5. TLC plates from standard solutions of the drugs. The mobile phase (ii) consisted from chloroform and acetone. The numbers of drugs as in fig. 1.

In fig. 7 two typical chromatograms of a mixture of the five substances (1 mg. l^{-1} each) developed with acetonitrile / sodium acetate (mobile phase i) are given. The two runs were taken from the same mixture with an hour delay between each other in order to examine the effect of light on the degradation of the drugs. From the second run it was obvious that the four last peaks (i.e. nitrofurazone, nitrofurantoin, furazolidone and furaltadone) had lost about 20 % of their heights whereas the carbadox peak seemed to be stable. In addition, two secondary peaks appeared just before the second and third peak, indicating probably the presence of at least two different products. This was explained because nitrofurans undergo photochemical reactions which result to the production of other chemical compounds. These products from nitrofurazone and nitrofurantoin in many cases develope a second peak during the elution while furazolidone remains with a single peak. However even furazolidone gives a dissociation product but its retention time is the same as the parent drug

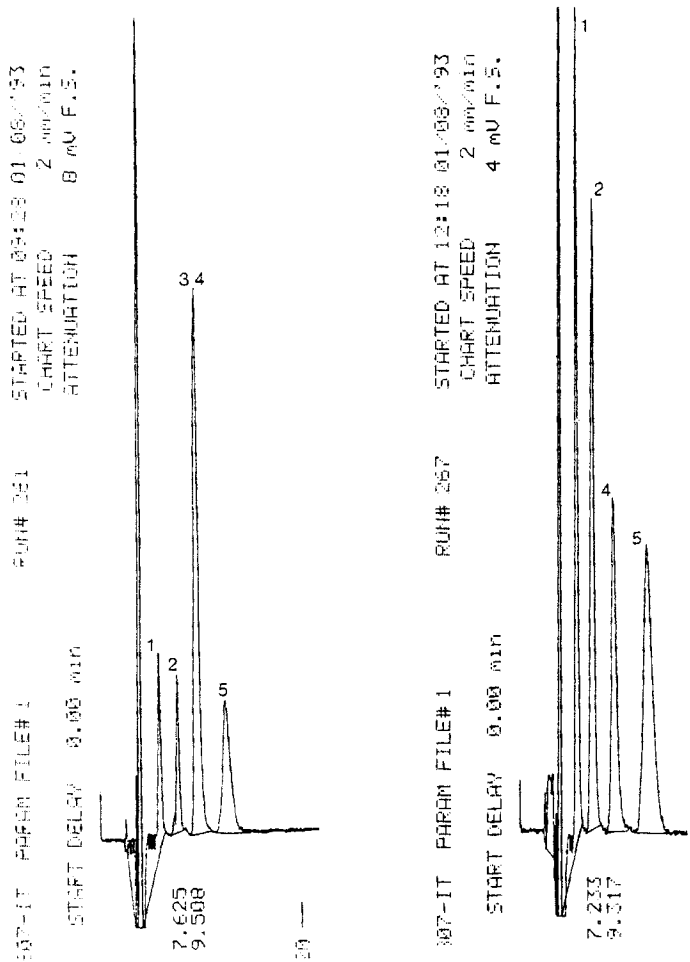

Fig. 6. HPLC chromatograms from quintiple mixture of the drugs. The mobile phase (i) consisted from acetonitrile and CH_3COONa. The numbers of drugs as in fig. 1. The second elution was done 1 hour later than the first.

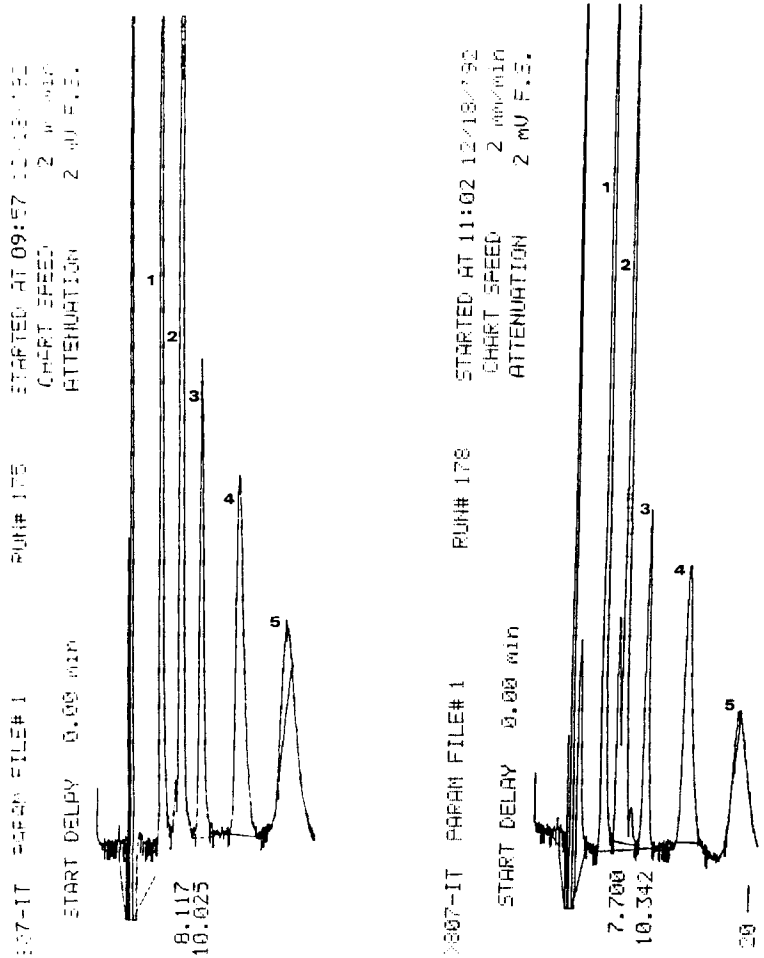

Fig. 7. HPLC chromatograms from quintiple and quaternaly mixtures of the drugs. The mobile phase (ii) consisted from acetonitrile and NaH_2PO_4. The numbers of drugs as in fig. 1. The first elution shows the ovelapped peaks of nitrofurantoin and furazolidone. The second elution was the quaternaly mixture without nitrofurantoin.

TABLE 1

Retention times and Resolution factors of the HPL Chromatographic separation of mixtures of five and four drugs with equal concentrations (1 mg. l^{-1}) for the two mobile phases respectively.

Substance	Retention time (min)		Resolution factor	
Mobile phase	(i)	(ii)	(i)	(ii)
1. carbadox	5.9	5.8	-	-
2. nitrofurazone	7.5	7.5	1.6	1.5
3. nitrofurantoin	9.8	9.4	2.0	1.4
4. furazolidone	13.0	9.4	2.1	0
5. furaltadone	17.2	13.3	1.6	1.4

with the mobile phase used. This behavior has been proposed [14] even as a distinguishing technique between nitrofurazone and furazolidone.

The resolution factors calculated for the two mobile phases (for 4.4 % of the peak height) are given in table 1. For mobile phase (i), it was proved that the resolution between the five substances of the mixture was very good and practically for all of them baseline separations were achieved. For mobile phase (ii) it was proved that no resolution can be achieved between nitrofurantoin and furazolidone while for the rest of the substances baseline resolution is achieved.

Regression Analysis of HPLC Determination of the Drugs

Various standard solutions of the drugs in the concentration range 0.1-5.0 mg. l^{-1} were injected in the chromatographic column to prepare the calibration curves of the separate determinations while passing the mobile phase (i). The regression data calculated are given in details in table 2, and the respective regression lines are plotted in the diagram of fig. 8.

The sensitivity of the determinations decreases in the order carbadox, nitrofurazone, nitrofurantoine, furazolidone and furaltadone, which is similar to the order of their elution. Also the linearity is better for carbadox and

TABLE 2

Regression data of the HPL chromatographic determination of the five drugs for mobile phase (i).

Substance	Regression equation	Correlation coefficient	Confid. limits of slope (95%)
1. carbadox	Y = 10.5 + 221X	0.998	210-233
2. nitrofurazone	Y = 14.4 + 138X	0.989	123-154
3. nitrofurantoin	Y = 14.6 + 66X	0.996	55-76
4. furazolidone	Y = 15.1 + 34X	0.977	27-40
5. furaltadone	Y = 10.4 + 21X	0.963	18-25

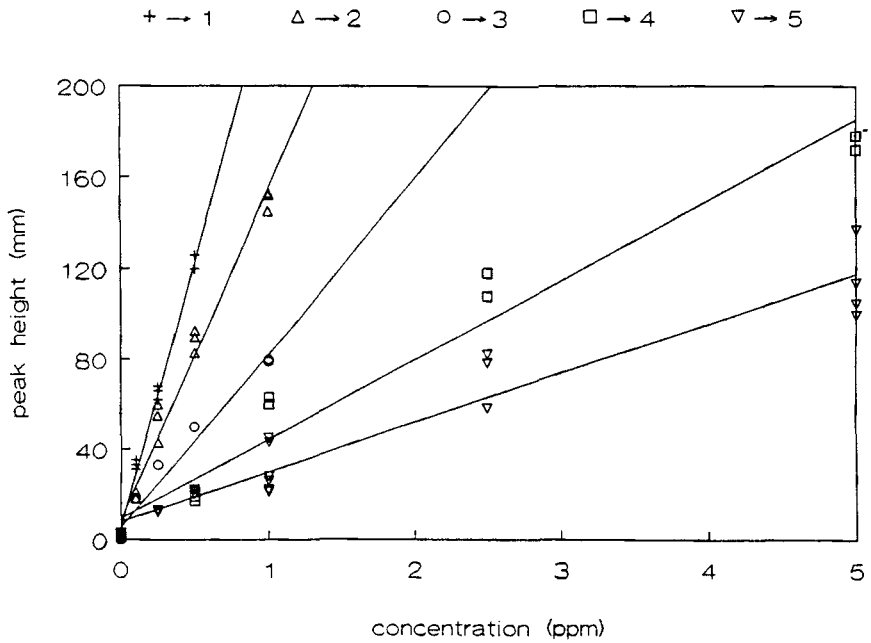

Fig. 8. Regression lines and experimental points of the HPLC determination.

nitrofurantoin than for the other drugs. The detection limits expressed by means of concentration in the injected solution were calculated as follows: carbadox 0.011 mg. l^{-1}, nitrofurazone 0.015 mg. l^{-1}, nitrofurantoine 0.022 mg. l^{-1}, furazolidone 0.060 mg. l^{-1} furaltadone 0.084 mg. l^{-1}. The corresponding absolute detectable quantities for 20 μl injection volume were as follows: carbadox 0.2 ng, nitrofurazone 0.3 mg, nitrofurantoine 0.4 ng, furazolidone 1.2 ng, furaltadone 1.6 ng.

ACKNOWLEDGMENTS

We gratefully acknowledge Assoc. Prof. J.N. Papadoyannis for helpfull discussion on the text of this paper.

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Received: October 10, 1993
Accepted: October 19, 1993