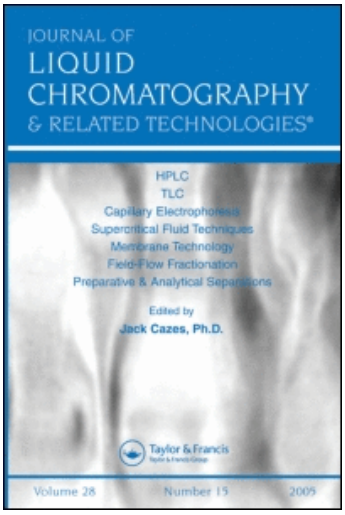

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Large Volume Injection of Biological Samples Dissolved in a Non-Eluting Solvent: A Way to Increase Sensitivity and a Means of Automating Drug Determination Using Hplc

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LARGE VOLUME INJECTION OF BIOLOGICAL SAMPLES DISSOLVED IN A NON-ELUTING SOLVENT : A WAY TO INCREASE SENSITIVITY AND A MEANS OF AUTOMATING DRUG DETERMINATION USING HPLC

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ABSTRACT

The injected volume of a sample dissolved in the mobile phase of an HPLC system must be maintained as small as possible so as to minimize the loss in efficiency. Generally this requirement limits the sensitivity of HPLC methods devoted to trace quantity determinations of drugs in biological fluids. In order to avoid this limitation and to increase the effective sensitivity of HPLC methods for determination of drugs such as antrafenine, nifuroxazide and cipropride, the samples were dissolved in a non-eluting solvent and a large volume (> 100 µl) was injected on to the chromatographic column.

The above-mentioned compounds and their internal standards were dissolved in a series of eluting and non-eluting solvents and increasing volumes (5 to 1000 µl) were injected. Peaks corresponding to injections made in an eluting solvent showed retention times independent of the injection volume but their variances increased with the volume injected. In contrast, peaks corresponding to injections made in a non-eluting solvent, similar to the mobile phase, had a variance independent of the injection volume but their retention times increased linearly with the injection volume. The repeated injection of such non-eluting solvents had no influence on chromatographic behaviour. Peaks corresponding to compounds injected in a non-eluting solvent made with components different from those of the mobile phase had a variance independent of the injection volume but their retention times varied both with the injection volume and with the interval between injection.

The application of non-eluting solvents has been defined theoretically and it has been demonstrated that solutions composed

of 25 % of the mobile phase diluted with the least eluting of its components act as non-eluting solvents and can be injected in large volume without loss in efficiency. This feature could be used to inject all the samples volume or only part of it, manually or automatically, since any automatic injector can be used with large volumes.

Thus, using the relatively simple procedure of making injections with a non-eluting solvent it is possible to increase both sensitivity and the rate of sample analysis.

INTRODUCTION

The interest of high pressure liquid chromatography (HPLC) as a useful tool for drug determination in biological samples is demonstrated by the very large number of papers published monthly. However, despite this apparent success, HPLC is sometimes limited by lack of sensitivity and the rate of routine analysis.

In most of the HPLC methods described, part of the lack of sensitivity is due to the method used to introduce the sample on the column. At the end of the purification and concentration step, generally an extraction by an organic solvent, the sample is evaporated to dryness. Then, in order to inject the extract on the column, it is dissolved in an organic solvent or in the mobile phase (which are both eluents for the column). Although some authors use less, at least 50 to 100 µl of liquid is needed to easily obtain a complete and reproducible dissolution of the extracted sample. Apart from a small number of drugs which normally have a high concentration in biological samples with high U.V. absorbance or high fluorescence, the size of the injection volume is a problem. The injection of all the diluted sample will involve a loss of efficiency, this is because if it is injected in an eluent, the sample will begin to elute before the end of the injection. This extra column effect creates a loss in efficiency (then in selectivity) and a relative decrease in sensitivity (1 to 5). The injection of an aliquot small enough to maintain the efficiency (and selectivity) will involve a loss of sensitivity. In front of this dilema, the chromatographer generally uses a compromise in order to obtain the least loss of efficiency.

In order to solve these problems of sensitivity and rate of analysis, an easy alternative was investigated during the development of automated sensitive analytical HPLC methods for a series of drugs in biological fluids (6, 7). The main idea was to dissolve the extracted sample in a large volume of a "non-eluting" solvent and to inject all this volume manually or part of it by means of an automatic injector.

In this study the concept of "non-eluting" solvent is further defined and its experimental consequences are considered. For this purpose, the investigation was divided into three parts :

A) Influence of the injected volume of eluting or "non-eluting" solvent on retention volume and peak broadening.

B) Influence of repetitive injection of non-eluting solvents upon the chromatographic behaviour.

C) Definition of a non-eluting solvent as close as possible to the mobile phase.

EQUIPMENT, SOLVENTS AND STANDARDS

The analyses were carried out either on a Micromeritics 7000 B liquid chromatograph with manual injection valve (volume of loop 1000 µl), U.V.visible spectrophotometer and Perkin Elmer 56 recorder or on a laboratory-built automated liquid chromatograph comprising the following system : Micromeritics 725 Automatic Injector (with a 500 µl loop), LDC Constametrics II G pump, Micromeritics 785 or LDC Monitor III U.V. spectrophometer and Perkin Elmer Sigma 10 Chromatography Data Station.

The columns, used in this study, were all identical stainless steel tubing (L = 15 cm, int. $\emptyset$ = 4.6 mm) and ZDV reducers. They were all packed in the laboratory with Spherisorb ODS 5 µ. In order to measure the variations only due to the injection process, the columns were thermostatically controlled by a water jacket (Touzard et Matignon, Vitry, France) and a circulating water bath FE2 (Haake, Karlsruhe, West Germany).

Analytical grade orthophosphoric acid (H_3PO_4), sodium acetate (AcONa) and "Lichrosolv" acetonitrile used for the mobile phases were all purchased from Merck (Darmstadt, West Germany).

Apart from methylmercadone which was kindly supplied by Fumouze S.A. (France), all the pure compounds used in this study were synthesized in the laboratories of Synthélabo.

CHROMATOGRAPHIC CONDITIONS

The chromatographic conditions developed to analyse the drugs in biological extracts were the following :

. Antrafenine :

Mobile phase : MeCN/AcONa 0.1 M (72.5:27.5 v/v)

Flow rate : 2.00 to 1.25 $ml.min^{-1}$ depending on "the age of the column" (6)

U.V. detection : 353 nm Internal standard : EFQB.

. Nifuroxazide :

Mobile phase : MeCN/H_3PO_4 pH 2.5 (30:70 v/v)

Flow rate : 1 $ml.min^{-1}$

U.V. detection : 362 nm Internal standard : methylmercadone.

. Cipropride :

Mobile phase : MeCN/AcONa 0.1 M (62:38 v/v)

Flow rate : 1 $ml.min^{-1}$

U.V. detection : 230 nm Internal standard : flubepride.

The chemical structure of these drugs and their internal standards are shown in Figure 1 and a typical chromatograph of each pair of compound is displayed in Figure 2.

A- INJECTION OF SAMPLES DISSOLVED IN INCREASING VOLUMES OF ELUTING AND NON-ELUTING SOLVENTS

Standard solutions of nifuroxazide and methylmercadone were prepared in the following solvents :

a) pure acetonitrile

b) mobile phase

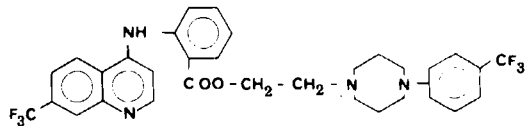
c) non acidified mobile phase : MeCN/H_2O (30:70 v/v)

d) MeCN/H_3PO_4 pH 2.5 (10:90 v/v)

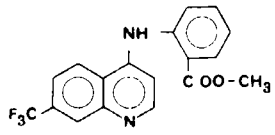
e) MeCN/H_2O (10:90 v/v)

which corresponded to the different ways to dissolving the dry extract :

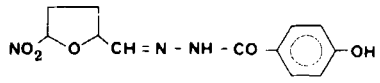
(i) - small volume of a very good solvent (which is often a very good eluent, in reverse phase HPLC) : pure acetonitrile.



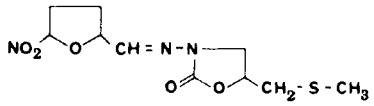
ANTRAFENINE



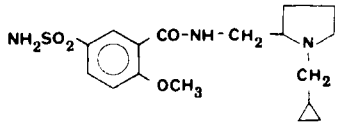
EFQB



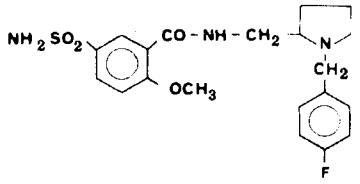
NIFUROXAZIDE



METHYLMERCADONE



CIPROPRIDE



FLUBEPRIDE

Figure 1: Chemical structures of Compounds used in the study.

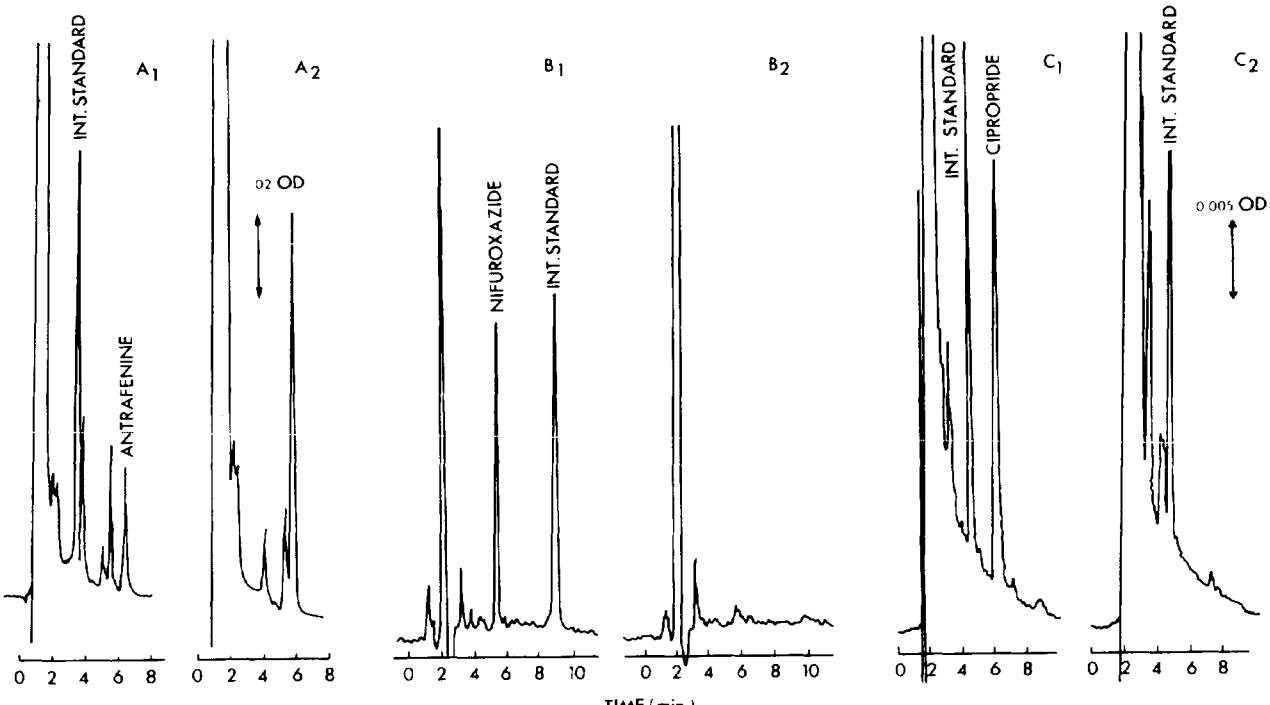


Figure 2: Chromatograms of plasma extracts for determination of drugs in biological samples:

a_1) spiked plasma — a_2) blank plasma for antrafenine

b_1) " " — b_2) " " for nifuroxazide

c_1) " " — c_2) " " for cipropide

Conditions: see the text.

(ii) - larger volume (50 to 200 µl) of the mobile phase or of a similar solvent (which is obviously an eluent) : MeCN 30 % (b and c).

(iii) - larger volume (100 to 1000 µl) of a solvent considerably less eluting than the mobile phase : MeCN 10 % (d and e).

Increasing volumes (10 to 1000 µl) of each solution were injected on the column.

Results and Discussion

The values of retention times of nifuroxazide and methylmercadone are reported in Table 1. The variation of $b_{0.5}$ (width at 1/2 height of peak) and reduced H.E.T.P. are displayed in Figures 3 and 4, respectively.

For standards injected in MeCN, shoulders on the peaks profiles appeared for a 50 µl injection. The retention times (t_R) did not vary between 5 to 10 µl but decreased rapidly when 25 µl of MeCN standard solutions was injected. The value of $b_{0.5}$ increased with the injected volume from 5 to 25µl. These reversed variations of t_R and $b_{0.5}$ explain the very rapid increase of reduced H.E.T.P. with the volume of MeCN injected. Similar results were noticed for antrafenine and EFQB injected in MeCN. The efficiencies were maintained on the column only when the injected volume was equal to or lower than 5 µl. With such a small volume, it is not reasonable to expect a total and reproducible dissolution of any dry extract and the use of an automatic injector is impossible. In addition, care must be taken in the choice of such solvents : Wu and Wittick (8) noticed a large tailing in the peaks of vitamins B_{12} and D_2 when injected in 5 µl of methanol, ethanol or acetonitrile. As a consequence, the use of a small volume of eluting liquid as a solvent for the dry extract cannot be a worthwile way of improving sensitivity and increasing the rate of analysis.

For standards injected in mobile phase or in MeCN/H_2O 30:70, the retention times seemed to be independent of the injected volume from 1 to 500 µl. An increase was noticed for injection of 1,000 µl of MeCN/H_2O 30:70. The values of $b_{0.5}$ were almost constant from 1 to 100 µl of sample injected but increased rapidly after

TABLE 1

Variation of Retention Times with the Injected Volume : Influence of the Solvents

INJECTED VOLUME (µl)	NIFUROXAZIDE					METHYLMERCADONE				
	INJECTION LIQUID					INJECTION LIQUID				
	a	b	c	d	e	a	b	c	d	e
1	-	-	4.8	-	4.8	-	-	8.4	-	8.4
5	4.6	4.8	4.7	4.6	4.7	8.2	8.4	8.3	8.0	8.3
10	4.7	4.7	4.7	4.6	4.7	8.3	8.4	8.3	8.0	8.3
25	3.7	4.7	4.7	4.6	4.7	6.4	8.2	8.3	8.0	8.3
50	-	4.7	4.7	4.6	4.8	-	8.3	8.3	8.0	8.3
100	-	4.7	4.8	4.6	4.7	-	8.2	8.4	8.0	8.3
200	-	4.8	4.8	4.7	4.8	-	8.3	8.3	8.0	8.3
300	-	4.8	4.8	4.8	5.0	-	8.3	8.3	8.2	8.5
400	-	4.8	4.8	5.2	5.0	-	8.3	8.3	8.8	8.5
500	-	5.0	4.8	5.2	5.3	-	8.4	8.3	8.8	8.8
1000	-	-	5.3	5.7	5.7	-	-	8.6	9.2	9.2

100 µl and explained the variations of reduced H.E.T.P. The samples injected in MeCN/H_2O 30:70 differed from those injected in the mobile phase only by the pH. However for equal injected volume, the $b_{0.5}$'s related to MeCN/H_2O were a littler smaller than those related to the mobile phase, whereas the retention times remained similar. This difference could be explained by a decrease in the eluting character due to the variation of pH. Nevertheless it is not sufficient to maintain the efficiency of the column at its best. As a consequence, even if the dissolution of the dry extract were total and reproducible in 100 and 200 µl of such solutions, even if some automatic injectors could inject the largest part of these volumes, the best sensitivity and the best efficiencies are

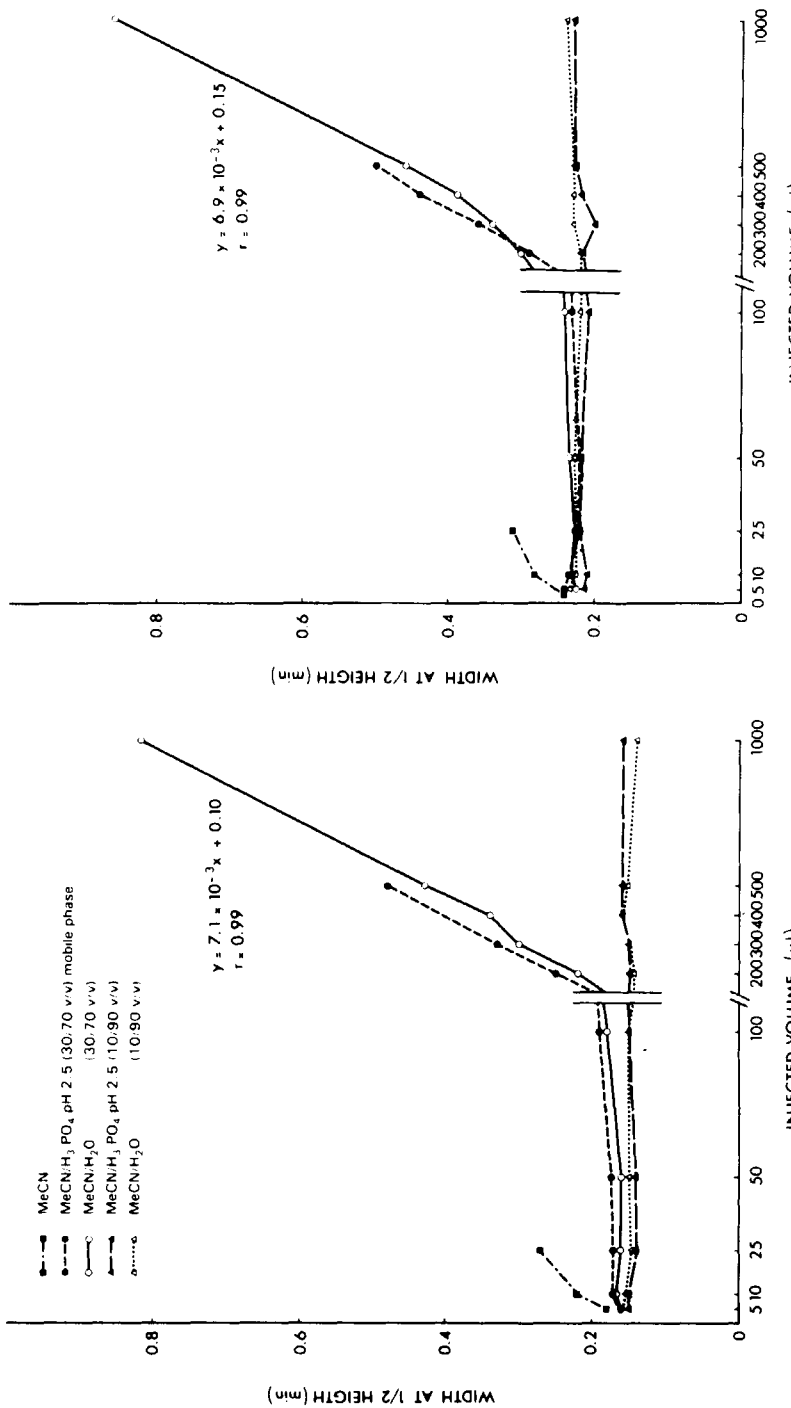


Figure 3: Variation of $b_{0.5}$ of nifuroxazide (a) and methylmercadone (b) with the injected volume: influence of the composition of the injected liquid.

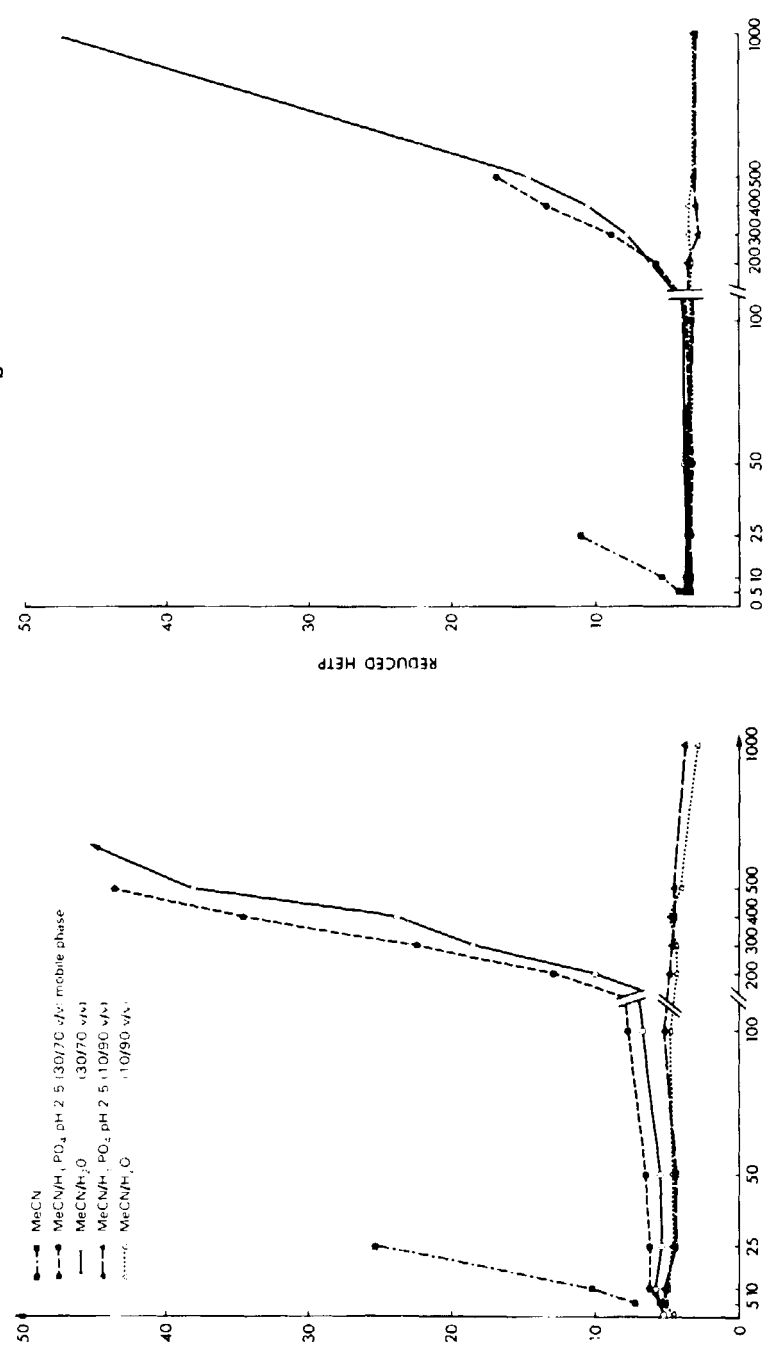


Figure 4: Variation of the reduced H.E.T.P. of nifuroxazide (a) and methylmercadone (b) with the injected volume: influence of the composition of the injected liquid.

not reached when the dry extract is dissolved in the mobile phase or in a similar solvent.

The values of $b_{0.5}$ for standards injected in solvent containing only 10 % of MeCN were quite independent of the injected volumes and of the pH. The retention times were also independent of the pH but increased linearly with the injected volume (Figure 5). These variations of t_R explained the small improvment of reduced H.E.T.P. observed with samples injected in 10 % MeCN (Figure 4). Similar results were noticed for Antrafenine and EFQB injected in MeCN/AcONa 25:75. As $b_{0.5}$ was independent from the injected volume, V_{inj}, the dry extract could be dissolved in a large volume and totally injected on the column, leading to the best sensitivity which can be expected. As V_{inj} can be large, most of the automatic samplers commercially available become usable to introduce the sample on the column. The rate of routine analysis increases as the analyses can be performed without human intervention.

Aside from their advantages in increasing the possibilities of HPLC for drug determination in biological samples, some results obtained during this study must be considered at a more general level. During these last five years, several theoretical or pratical papers have been published, dealing with extra column effects in high performance liquid chromatography (1 to 4). It is usually assumed that the variance of the chromatographic peak, σ^2_{tot} is equal to the sum of the variance of the chromatographic process itself and of the variances of all the parameters contribution to broadening, in particular that of the injection process σ^2_{inj}. Therefore, the total variance can be written as :

$$\sigma^2_{tot} = \sigma^2_{inj} + \Sigma\sigma^2 \qquad (1)$$

where $\Sigma\sigma^2$ reprensents the variance of all the other parameters.

The variance of a plug sample injection, expressed in volume, is given by :

$$\sigma^2_{inj} = \frac{V^2_{inj}}{K^2} \qquad (2)$$

where V_{inj} is the injected volume and K a constant depending on the solute and on the injected technique. Then,

$$\sigma^2_{tot} = \frac{V^2_{inj}}{K^2} + \Sigma\sigma^2 \quad (3)$$

or in term of $b_{0.5}$ expressed in volume :

$$b^2_{0.5} = 8 \text{ Log2 } (\frac{V^2_{inj}}{K^2} + \Sigma\sigma^2) \quad (4)$$

In a study similar to that described above, Westerlund et al. (9) reported a linear relationship between $b_{0.5}$ and V_{inj}. In a first approximation, the values of $b_{0.5}$ measured in the present investigation confirmed the observation of Westerlund ; linear correlations were found between $b_{0.5}$ and V_{inj} when nifuroxazide and methylmercadone were injected in the mobile phase and in a similar solvent (Figure 3). The correlation coefficients were equal to 0.99 in both cases. However, no reason could be found to explain the observed correlations between 100 and 1,000 µl but not below 100 µl.

Assuming that all the contributions to band broadening, except that due to V_{inj}, were constant, and assuming that the contributions due to small V_{inj} (< 25 µl) were negligible compared with the sum of all the others, $\{b^2_{0.5} (V_{inj} < 25 \text{ µl}) \simeq 8 \text{ Log2 } \Sigma\sigma^2 = b^2_{0.5}(0)\}$, $b^2_{0.5} - b^2_{0.5}(0)$ was computed versus V^2_{inj} according to equation (4).

The experimental correlations were found to be as follows :

- for injection made in mobile phase :

  $b^2_{0.5} - b^2_{0.5}(0) = 0.207\ V^2_{inj} + 0.068$ ($r = 0.995$) for methylmercadone

  $b^2_{0.5} - b^2_{0.5}(0) = 0.206\ V^2_{inj} + 0.037$ ($r = 0.998$) for nifuroxazide

- and for injection made in $MeCN/H_2O$ (30:70) :

  $b^2_{0.5} - b^2_{0.5}(0) = 0.172\ V^2_{inj} + 0.019$ ($r = 0.999$) for methylmercadone

  $b^2_{0.5} - b^2_{0.5}(0) = 0.162\ V^2_{inj} - 0.061$ ($r = 0.999$) for nifuroxazide

whatever the values of V_{inj} between 25 and 1,000 µl.

All these considerations concerned injections of sample diluted in mobile phase or a similar solvent.

The experimental data concerning injections made in (MeCN 10 %) solutions did not satisfy equation (2). $b_{0.5}$ was independent of V_{inj}. Therefore, it is realistic to assume that σ_{inj} is a constant, or

$$\sigma^2_{inj} = C^{ste} \quad (5)$$

The parameters which were related to V_{inj} were the retention volumes. Considering the linear relationship between t_R and V_{inj} (Figure 5), the same variation of 1 minute was measured in the retention time of both standards injected in both solvents when injection volumes varied from 1 to 1,000 µl. As the injections were made through a loop at a flow rate of 1 ml.min^{-1} (see chromatographic conditions), the retention times increased with the duration of the injection, t_{inj}, (assuming a square wave plug injection). This can be translated by the equation :

$$t_R = t_{Ro} + t_{inj} \quad (6)$$

or in terms of retention volumes :

$$V_R = V_{Ro} + V_{inj} \quad (7)$$

where t_R and V_R are the retention time and the retention volume corresponding to an injection of volume V_{inj}, and t_{Ro} and V_{Ro} the retention time and the retention volume corresponding to a zero injection volume.

Equations (5) and (7) define the theoretical aspect of the injection of samples dissolved in a non-eluting solvent and at the same time the concept of non-eluting solvent.

Whatever the injected volume is, its contribution to band broadening is effectively zero. All the sample is concentrated at the top of the column during the time of the injection. With the general trend towards short times of analysis on short columns packed with microparticules (5 µ or less) this result is particularly important as it avoids band broadening due to the injection volume.

B - INFLUENCE OF REPETITIVE INJECTIONS OF LARGE VOLUMES OF NON-ELUTING SOLVENT ON THE CHROMATOGRAPHIC BEHAVIOUR

The repeated injection of a large volume of solvent different from the mobile phase could disturb the equilibria existing inside

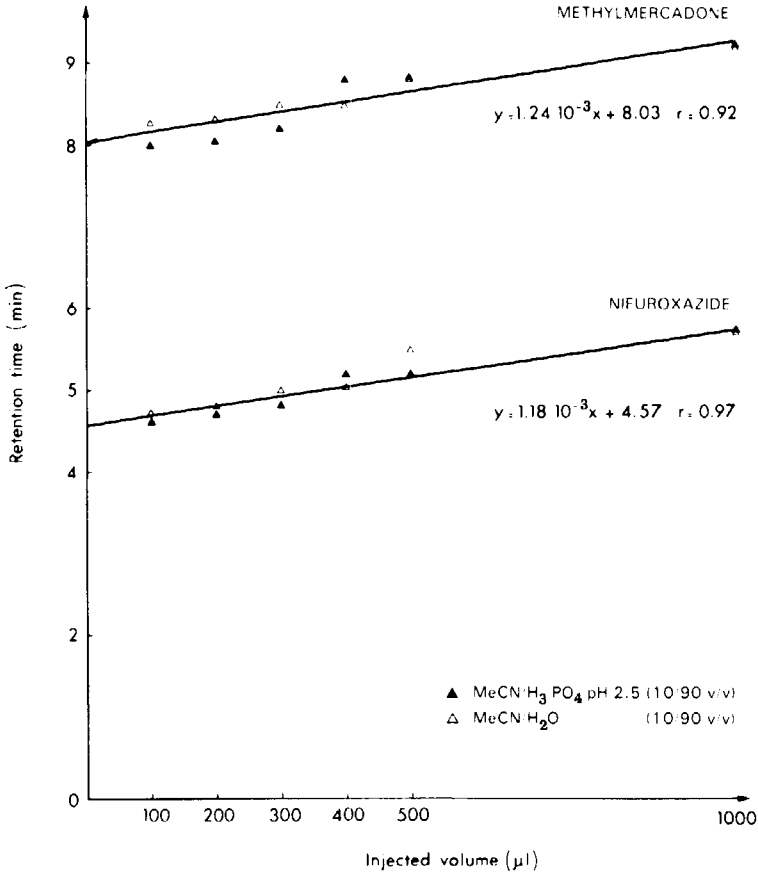


Figure 5: Injection of solutes dissolved in a non-eluting solvent: variation of retention times with the injected volume.

the column between the stationary and mobile phases and the solute, leading to erratic retention time. The stability of retention times had to be checked before developing a repetitive analytical method involving such injections either with a manual valve or with an automatic injector.

Experimental

Repeated injections of 500 µl of standard dissolved in non-eluting solvent were made following the general schedule described below :

- n_1 injections were made each time interval 1 ΔT ; n_2 injections, each 2 ΔT ; n_3 injections, each 3 ΔT ; n_4 injections, each 4 ΔT; n'_1 injections, each 1 ΔT. ΔT was dependent on the standards and on the chromatographic conditions used. In each series, the values of ΔT was set on the automatic injector as the time between two consecutive injections to make each injection ΔT. In order to make the injections at intervals of 2, 3 and 4 ΔT, 1, 2 and 3 vials filled with mobile phase were placed on the rack between two vials filled with standard solutions.

The standard solutions were prepared as described in Table 2. The value of ΔT and n_i for each series of standards are given in Table 3. Two standards solutions of nifuroxazide and methylmercadone were prepared in MeCN/H_3PO_4 pH 2.5 and in MeCN/H_2O in order to explore the effect of pH.

Standards of cipropride and flubepride were prepared either in MeCN/AcONa 0.1 M and in HCl 0.01 M in order to investigate the solvent totally different from the mobile phase.

TABLE 2

Influence of Repeated Injections on Chromatographic Behaviour : Composition of Standard Solutions

MAIN COMPOUND	CONCENTRATION ($\mu g.ml^{-1}$)		NON-ELUTING SOLVENT
ANTRAFENINE	ANTRAFENINE	0.92	MeCN/AcONa 0.1 M 50/50
	EFQB	0.57	
NIFUROXAZIDE	NIFUROXAZIDE	0.06	MeCN/H_3PO_4 pH 2.5 10/90
	METHYLMERCADONE	0.17	MeCN/H_2O 10/90
CIPROPRIDE	CIPROPRIDE	0.29	MeCN/AcONa 0.1 M 10/90
	FLUBEPRIDE	0.29	HCl 0.01 M

TABLE 3

Influence of Repeated Injections on Chromatographic Behaviour : Experimental Parameters and Mean Retention Times

ΔT (min)	θ (o_c)	n ΔT	n_i	MEAN RETENTION TIME ± S.D. (C.V.) (min)	
Antrafenine				Antrafenine	E F Q B
8.2		1	12	5.977 ± 0.008 (0.13 %)	3.263 ± 0.005 (14 %)
		2	5	5.976 ± 0.014 (0.25 %)	3.256 ± 0.009 (0.3 %)
		3	5	5.968 ± 0.222 (0.4 %)	3.258 ± 0.004 (0.14%)
		4	5	5.962 ± 0.018 (0.3 %)	3.244 ± 0.005 (0.17%)
		1	7	5.941 ± 0.013 (0.23 %)	3.247 ± 0.013 (0.4 %)
Nifuroxazide in H_3PO_4				Nifuroxazide	Methylmercadone
10.2	2.9	1	13	4.872 ± 0.004 (0.08 %)	8.067 ± 0.006 (0.08%)
		2	5	4.874 ± 0.005 (0.1 %)	8.076 ± 0.005 (0.07%)
		3	4	4.878 ± 0.005 (0.1 %)	8.088 ± 0.005 (0.06%)
		4	5	4.882 ± 0.004 (0.09 %)	8.096 ± 0.009 (0.1 %)
		1	13	4.892 ± 0.004 (0.09 %)	8.107 ± 0.009 (0.1 %)
Nifuroxazide in H_2O				Nifuroxazide	Methylmercadone
9.6	28	1	10	4.816 ± 0.005 (0.1 %)	7.946 ± 0.008 (0.1 %)
		2	5	4.826 ± 0.005 (0.1 %)	7.956 ± 0.005 (0.06%)
		3	5	4.824 ± 0.005 (0.1 %)	7.954 ± 0.005 (0.07%)
		4	5	4.826 ± 0.005 (0.1 %)	7.960 ± 0.000 -
		1	13	4.828 ± 0.008 (0.2 %)	7.963 ± 0.003 (0.09%)
Cipropride in MeCN				Flubepride	Cipropride
8.8	28.5	1	7	3.633 ± 0.012 (0.3 %)	6.274 ± 0.022 (0.3 %)
		2	5	3.606 ± 0.005 (0.15 %)	6.228 ± 0.008 (0.13%)
		3	5	3.602 ± 0.016 (0.5 %)	6.202 ± 0.023 (0.4 %)
		4	6	3.610 ± 0.013 (0.35 %)	6.220 ± 0.015 (0.25%)
		1	12	3.608 ± 0.009 (0.26 %)	6.230 ± (2 measures)
Cipropride in dilute HCl				Flubepride	Cipropride
9.2	28	1	10	4.563 ± 0.005 (0.1 %)	6.219 ± 0.009 (0.14%)
		2	5	4.32 ± 0	6.413 ± 0.010 (0.15%)
		3	5	4.230 ± 0.012 (0.3 %)	6.434 ± 0.015 (0.20%)
		4	7	4.183 ± 0.008 (0.18 %)	6.439 ± 0.007 (0.11%)
		1	12	4.588 ± 0.008 (0.18 %)	6.301 ± 0.006 (0.09 %)

Results and discussion

The mean values of retention times of each compound are shown in Table 3 apart from cipropride and flubepride injected in HCl 0.01 M. The reported values correspond in this case to mean values of retention times after equilibration. For all the other experiments, the repeated injections of a large volume of a non-eluting solvent similar to the mobile phase did not disturb the chromatographic behaviour : the retention times remained rigorously constant whatever the interval between injections. In the case of nifuroxazide and methylmercadone, the difference in pH between the two standard solutions did not appear to have an influence on the retention times or on chromatographic behaviour. The very small difference noticed between the retention times (< 0.06 min.) could also be due to the difference in temperature (1°C) between the two experiments.

The variation in retention times of cipropride and flubepride injected in HCl 0.01 M versus time are displayed in Figure 6.

These results indicated that the chromatographic behaviour was disturbed by the injection of large volume of solvent totally different from the mobile phase. If the injections are repeated at a constant interval, the chromatographic equilibria are modified until a different stabilization occurring after some injections : at this stage, the retention times remain constant as long as the interval between two injections remains constant. Modification of chromatographic equilibria can have an opposite influence on compounds as close together as cipropride and flubepride : the retention time of flubepride increased while that of cipropride decreased. In a preliminary study, carried out on a column packed with a different batch of Spherisorb ODS 5 µ, an inversion of retention time between cipropride and flubepride occurred during the "saturation period".

As a consequence of these results, the repeated injection of large volume of eluting solvent can be used to inject diluted samples either manually or automatically for routine analysis. If the injected solvent is similar to the mobile phase, no precaution

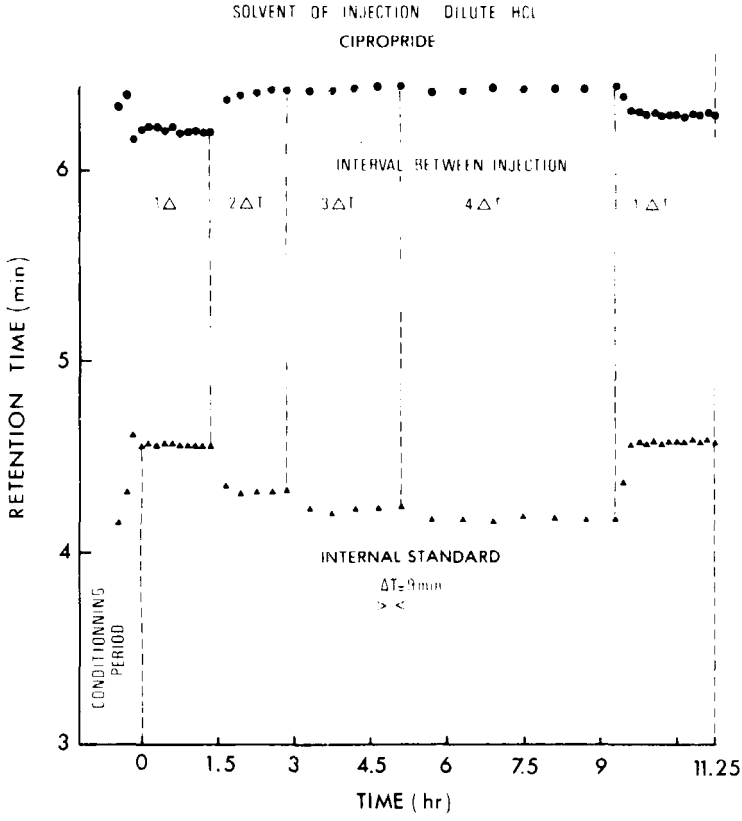


Figure 6: Injection of solutes dissolved in a non-eluting solvent different from the mobile phase: effect of the repeated injection on retention times.

is needed ; if the solvent is different, the first sample must be injected after a "saturation period" made with some injections of the solvent used for the following analysis. The interval between two consecutive injections must remain constant.

C - EXPERIMENTAL DETERMINATION OF A NON-ELUTING SOLVENT SIMILAR TO THE MOBILE PHASE

The non-eluting character of the injection solvent is obviously dependent from the composition of the mobile phase. It could be

TABLE 4

Injection of Samples Dissolved in a Large Volume of Non-eluting Solvent : Influence of the Composition of the Solvent on Retention Time and $b_{0.5}$

X %	ANTRAFENINE		E F Q B		NIFUROXAZIDE		METHYLMERCADONE		FLUBEPRIDE		CIPROPRIDE	
	t_R	$b_{0.5}$	t_R	$b_{0.5}$	t_R	$b_{0.5}$	t_R	$b_{0.5}$	t_R	$b_{0.5}$	t_R	$b_{0.5}$
0					5.43	0.15	9.17	0.23	3.65	0.12	6.31	0.26
2.1	6.06	0.19	3.37	0.10								
10.0									3.65	0.12	6.27	0.26
16.6					5.43	0.14	9.14	0.25	3.65	0.12	6.29	0.26
21.0	6.06	0.19	3.39	0.10								
33.3					5.36	0.15	9.07	0.26	3.61	0.12	6.24	0.28
34	6.09	0.20	3.37	0.10								
48	6.04	0.20	3.35	0.10								
50									3.55	0.12	6.21	0.30
60	6.00	0.20	3.29	0.10								
66.6					5.33	0.19	9.06	0.29	3.51	0.16	6.22	0.34
69	5.95	0.20	3.27	0.12								
76	5.92	0.21	3.23	0.15								
83.3	5.89	0.23	3.20	0.18	5.24	0.29	8.97	0.41	3.43	0.26	6.22	0.46
90	5.86	0.36	3.15	0.23								
97	5.76	6.34	3.09	0.32					3.34	0.40	6.21	0.56
100					5.03	0.50	8.35	0.53				

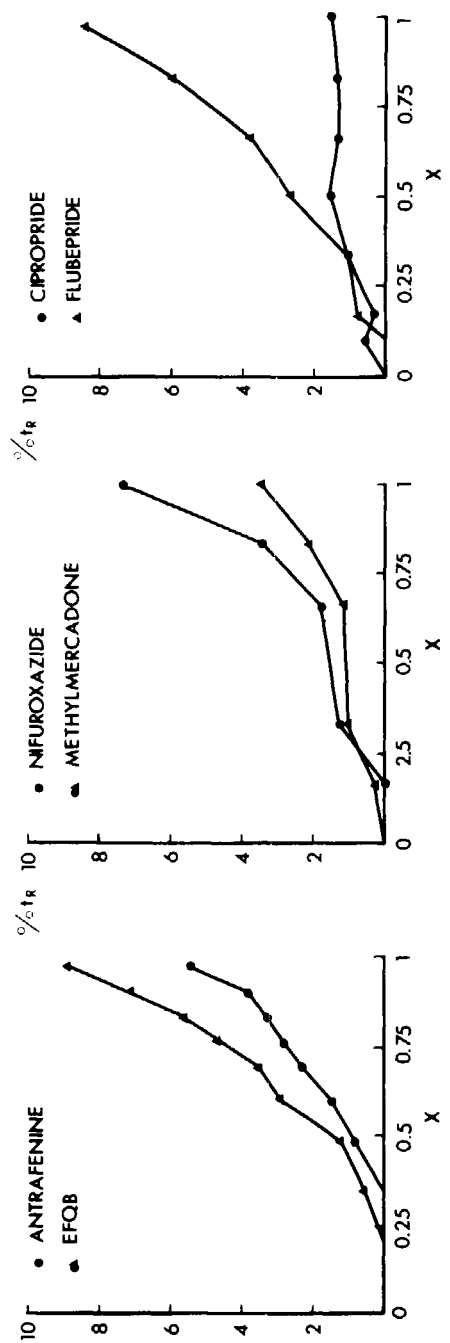


Figure 7: Injection of solutes dissolved in a non-eluting solvent: influence of the composition of the solvent on retention time.

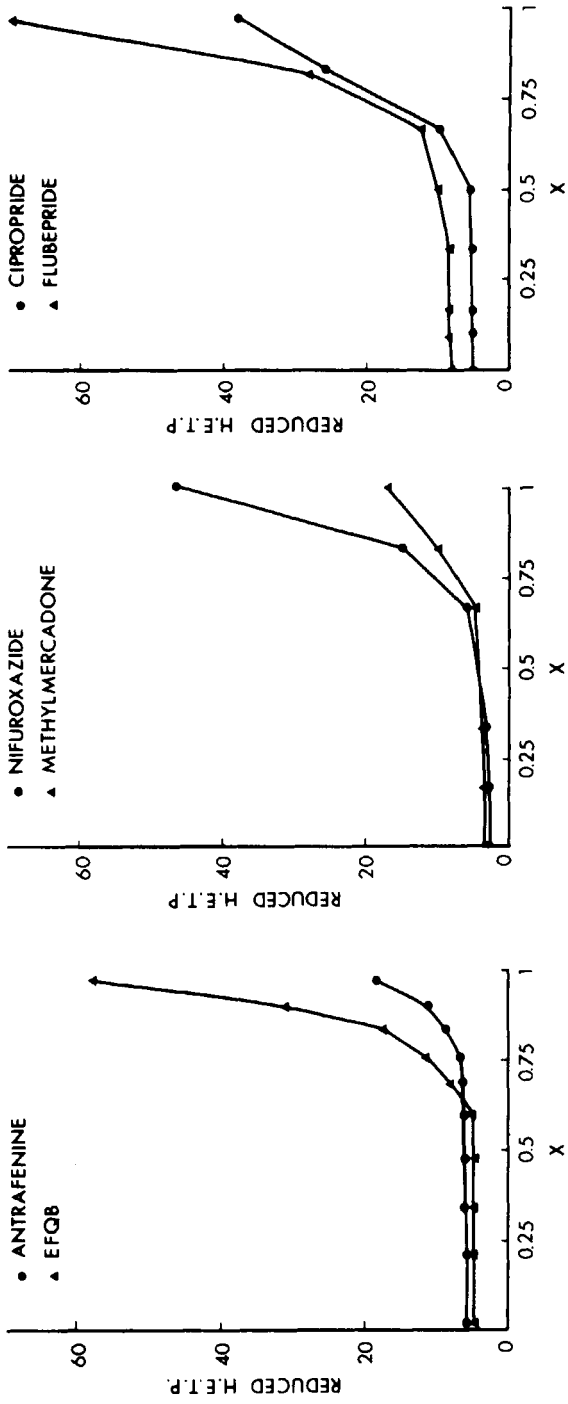


Figure 8: Injection of solutes dissolved in a non-eluting solvent: influence of the composition of the solvent on reduced H.E.T.P.

determined independently by considering the fraction of mobile phase X diluted in its weakest component to make the injection solvent. A non-eluting solvent can be determined experimently by measuring the range of X for which equations 5 and 7 remained true.

Experimental

For each pair of compounds, standard solutions were prepared in a series of solvents similar to the mobile phase and containing increasing amounts of MeCN. 500 µl of these solutions were injected on the column and eluted using the chromatographic conditions related to each compound.

Results and discussion

The retention times and the corresponding $b_{0.5}$ are reported in Table 4. The variations of retention time, expressed in per cent of t_R (corresponding to the lowest value of X) displayed in Figure 7, showed that in all the cases, the variations of t_R remained lower than 1 % for X lower than 0.25. Apart from cipropride, t_R of which remained constant for X varying between 0.5 and 1,% t_R increased with X : the largest variations were observed for the lowest retention times. Apart from methylmercadone $b_{0.5}$ appeared to be independent of X for X values lower than 0.16 to 0.20. For higher values of X, $b_{0.5}$ increased with X, the largest variations being due to the least retained compound. The variation of the reduced H.E.T.P. with X, displayed in Figure 8, remained negligible for values of X between 0 and 0.5 but increased rapidly with larger values of X ; the largest variations were noticed for the lowest retention time. Finally, for values of X lower than 0.25, equations (5) and (7) were satisfied in all cases, allowing the determination of the non-eluting solvent composition limit which may give the maximum efficiency and selectivity.

CONCLUSION

The injection of samples dissolved in a non-eluting solvent has been demonstrated as an easy and powerful way to minimize the losses of efficiency related to the injected volume. As a consequence, the sensitivity and rate of analysis of HPLC methods for trace amounts determination are considerably improved. This

injection method has already been successfully used in several automatic HPLC methods for drug determination in biological samples where concentrations as small as 5 $ng.ml^{-1}$ were routinely assayed.

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