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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC BEHAVIOUR OF SOME PHARMACEUTICALLY IMPORTANT THIAZIDE, LOOP AND POTASSIUM-SPARING DIURETICS

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SUMMARY

This paper deals with the specific identification of several thiazide, potassium-sparing and loop diuretics. The liquid chromatographic behaviour of these compounds is studied. Different organic modifiers (methanol, acetonitrile and tetrahydrofuran) are compared in terms of selectivity for the thiazide diuretics. An acetonitrile–water (40:60) eluent can be used to identify the thiazide diuretics. The loop and potassium-sparing diuretics are well chromatographed at an acidic pH in the presence of propylamine hydrochloride. This study enables us to select the right mobile phase composition for any given selectivity or resolution.

The determination of the dead volume in different chromatographic systems is also discussed. A mixture of organic solvent and deuterium oxide in the same volume ratio as the eluent is used as dead volume marker. The signal is monitored with a UV detector at low wavelength.

INTRODUCTION

Thiazide, potassium-sparing and loop diuretics, alone or in combination, are often used as antihypertensive drugs. The specific identification and quantitation of these compounds is pharmaceutically very important. This paper fits into the study of patient compliance for diuretics.

Several chromatographic techniques have been used to tackle this problem, including paper and thin-layer chromatography[1,2] and high-performance liquid chromatography[3–6]. Most of these publications consider only some diuretics. Honigberg *et al.*[5] obtained chromatographic data for some diuretics and antihypertensive drugs by using two columns with different selectivities and various mobile phases. Moskalyk *et al.*[6] reported retention data for thiazide and other antihypertensive diuretics but no chromatograms. Triamterene, as mentioned in their paper, elutes as a very broad peak. Therefore, we investigated the liquid chromatographic behaviour of the thiazide, potassium-sparing and loop diuretics. We used a reversed-phase LiChrosorb C_{18} column. Different parameters were tested in order to obtain good resolution of

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all thiazide diuretics: the kind of organic modifier (methanol, acetonitrile or tetrahydrofuran), the volume fraction of water in the eluent, and the column temperature.

The group of the potassium-sparing and the loop diuretics contains basic as well as acidic compounds. An excellent separation of all these compounds was obtained after testing other parameters of the mobile phase: the pH of the eluent, the propylamine hydrochloride concentration in the eluent, and the molarity of the buffer.

This study enables us to select the right eluent composition for chromatographing any of these diuretic compounds. Special attention was paid to the determination of the column dead volume. In order to evaluate the k' values in the different eluent systems tested, we needed a good and reproducible dead volume marker. Many different methods have been described in the literature[7–13,15–19]. A mixture of organic solvent and deuterium oxide in the same volume ratio as the eluent was chosen, as suggested by Engelhardt[7]. The deuterium oxide signal was measured with a UV detector at low wavelength.

EXPERIMENTAL

Apparatus

A SP 8770 isocratic pump (Spectra-Physics, Darmstadt, F.R.G.) was equipped with a HP 1040A UV spectrophotometric detector (Hewlett-Packard, Palo Alto, CA, U.S.A.), a HP 85 computer, a HP 82901M flexible disc drive and a HP 3390A integrator. The HP 1040A UV detector contains a photodiode array. This detector can

TABLE I

THE DIURETICS STUDIED

*No.**	*Abbreviation*	*Diuretic*	*Origin*
1	HCT	Hydrochlorothiazide	Merck, Sharp & Dohme
2	HFM	Hydroflumethiazide	Squibb & Sohns
3	TCM	Trichloromethiazide	Essex
4	CTA	Chlorthalidone	Ciba Geigy
5	MCT	Methylclothiazide	Abbott
6	EP	Epithiazide	R.I.T.
7	ALT	Althiazide	Searle
8	BUT	Butizide	Boehringer farma
9	PT	Polythiazide	Pfizer
10	BHF	Bendroflumethiazide	Squibb & Sohns
11	CYT	Cyclothiazide	Eli Lilly
12	CPT	Cyclopenthiazide	Ciba Geigy
13	MEBU	Mebutizide	CCP Thyssen
14	FUR	Furosemide	Hoechst
15	ETA	Ethacrynic acid	Merck, Sharp & Dohme
16	KCAN	Potassium canrenoate	Searle
17	TRI	Triamterene	R.I.T.
18	AMI	Amiloride hydrochloride	Merck, Sharp & Dohme
19	SPIR	Spironolactone	Searle
20	CAN	Canrenone	Searle

* The numbers of the diuretics are indicated in the text in parentheses.

follow up to eight wavelengths at the same time. The wavelength selection was changed automatically by the computer program: from 275 nm to 238 nm for the detection of SPIR and CAN (Table I). The refractive index detector (Varian, Walnut Creek, CA, U.S.A.) was coupled to the outlet of the HP 1040A UV detector and equipped with a recorder (BD 80 Kipp en Zonen, Delft, The Netherlands).

The eluent was filtered through a 5-μm filter and degassed with helium. A Valco six-port injection valve with a 10-μl sample loop was used. The column was thermostatted with a water-bath.

Chromatographic procedure

A LiChrosorb RP C_{18} column (Chrompack, Middelburg, The Netherlands) was used. The chromatographic conditions listed in Table II were used unless otherwise specified.

Stock solutions of the diuretics in methanol are diluted with water to obtain the same ratio of organic solvent–water. The concentration of the injected solutions varied between 0.05 mg/ml and 0.25 mg/ml.

Reagents and chemicals

All reagents were of analytical grade. Acetonitrile was of HPLC grade. Propylamine hydrochloride (Janssen, Beerse, Belgium) had a purity of at least 99%. Table I lists the diuretics studied, and their structures are shown in Table III.

Eluents

The organic solvent–water ratios of the eluents are given as volume ratios. Mobile phases containing various amounts of tetrahydrofuran were prepared by mixing the stated volume percentages. In these eluents the methanol–water ratio was kept constant, while the percentage of tetrahydrofuran was increased. The acetonitrile–buffer eluents were prepared by mixing the stated volumes. The molarity of the buffer and the pH refer to the water phase. The phosphate buffers were prepared by mixing sodium dihydrogen phosphate with sodium hydroxide (pH 7–6–5) or by mixing phosphoric acid with sodium hydroxide (pH 4–3) until the desired pH was obtained.

The acetonitrile–buffer eluents containing propylamine hydrochloride were prepared as follows: the amine and phosphoric acid were dissolved in water; the pH of the solution was adjusted; then the stated volumes of acetonitrile and the water

TABLE II

CHROMATOGRAPHIC CONDITIONS

Column, LiChrosorb RP-18, 5 μm, 150 × 4.6 mm I.D.
Eluent, see text or figures
Flow-rate, 1 ml/min
Temperature, 25°C
UV detector wavelength, 275 nm for the diuretics, 238 nm for SPIR and CAN, 210 nm for dead volume measurements
Recorder chart speed, 0.5 or 1 cm/min
Sample loop, 10 μl

phase were mixed. The indicated molarity of the amine refers to the molarity in the total eluent.

RESULTS AND DISCUSSION

Determination of the dead volume

The determination of the dead volume (V_0) in liquid chromatography is of prime importance for the calculation of the capacity factors (k'). The k' values of the diuretics are evaluated in different mobile phases. Therefore we need a dead volume marker applicable in various eluent systems with different compositions and selectivities.

The dead volume is determined by measuring the retention volume of an unretained compound. Snyder and Kirkland[8] mention the centre of the first band or baseline disturbance. As we did not know how this dead volume measurement would evaluate in mobile phases with different compositions and selectivities we preferred not to use this approach.

UV-absorbing non-retained ions seemed to be a good approach. Indeed, inorganic ions (sodium nitrate, sodium nitrite and lithium nitrate) are recommended by different authors[9–11]. The problem with these ions is the Donnan exclusion effect[12]: the exclusion of the anion out of the porous structure of the RP materials. Wells and Clark[9] tested different concentrations of sodium nitrate in buffered and non-buffered

TABLE III

CHEMICAL STRUCTURES OF THE INVESTIGATED DIURETICS

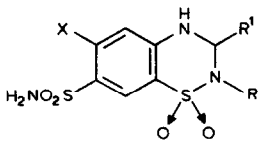
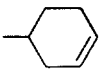
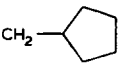
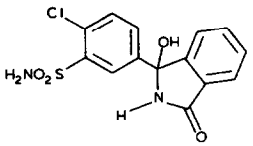
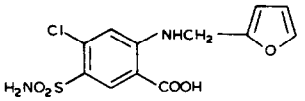
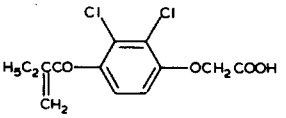
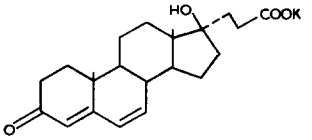
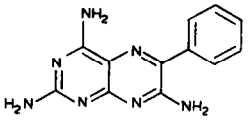
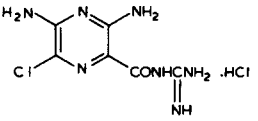
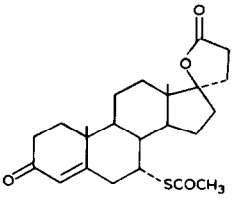
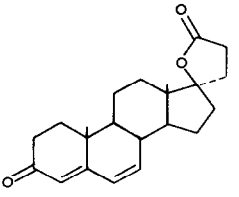
Abbreviation	*No.*	*X*	*R*	*R*[1]
Hydrothiazides				
HCT	1	Cl	H	H
HFM	2	CF_3	H	H
TCM	3	Cl	H	$CHCl_2$
MCT	5	Cl	CH_3	CH_2Cl
EP	6	Cl	H	$CH_2SCH_2CF_3$
ALT	7	Cl	H	$CH_2SCH_2CH=CH_2$
BUT	8	Cl	H	$CH_2CH(CH_3)_2$
PT	9	Cl	CH_3	$CH_2SCH_2CF_3$
BHF	10	CH_3	H	$CH_2C_6H_5$
CYT	11	Cl	H	
CPT	12	Cl	H	
MEBU	13	Cl	H	$CH(CH_3)CH(CH_3)CH_2CH_3$

TABLE III (*continued*)

Abbreviation	*No.*	*Structure*
Other diuretics		
CTA	4	
FUR	14	
ETA	15	
KCAN	16	
TRI	17	
AMI	18	
SPIR	19	
CAN	20	

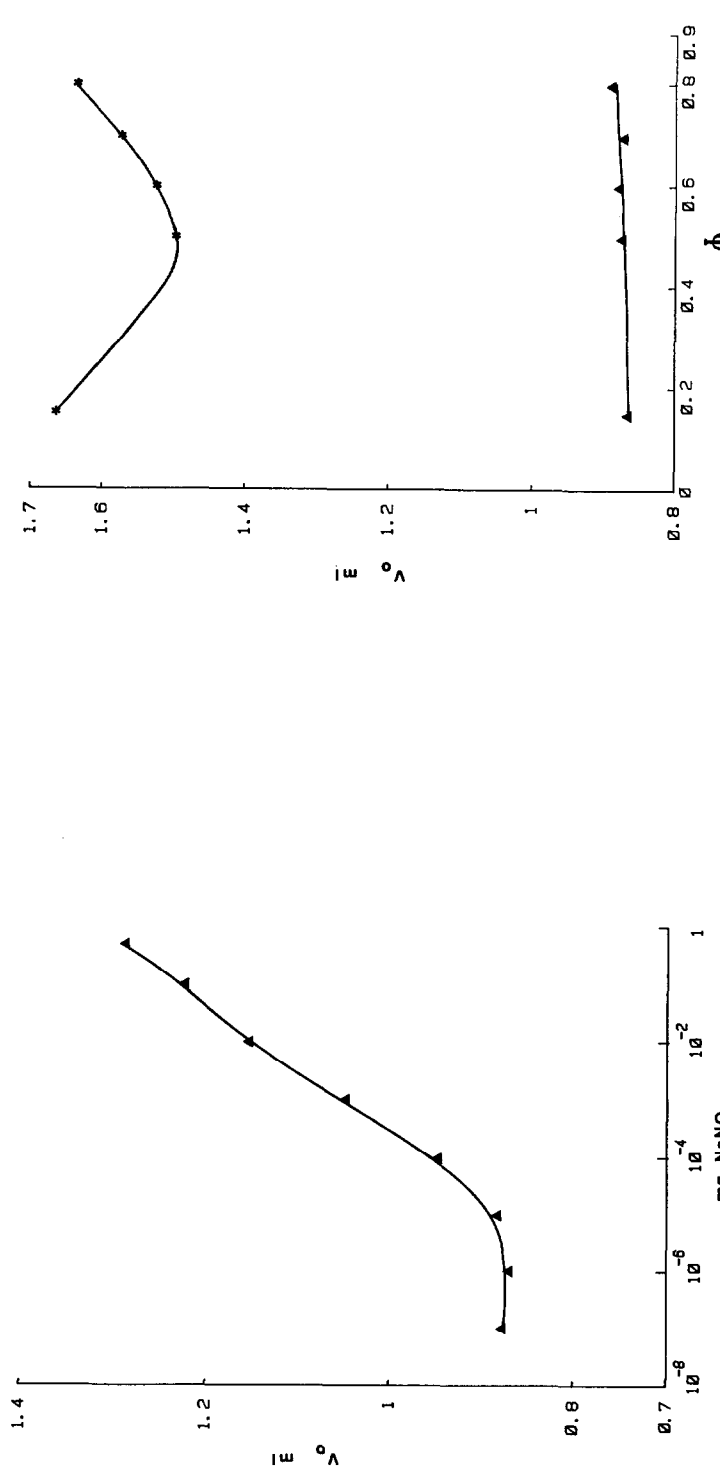


Fig. 1. Plot of the elution volume of sodium nitrate *versus* the amount injected. Mobile phase, acetonitrile–water (50:50); chromatographic conditions as in Table II.

Fig. 2. Plots of the elution volume of the deuterium oxide signal (*) and the sodium nitrate signal (▲) *versus* the volume fraction of water (Φ) in the acetonitrile–water eluent system. The amount of sodium nitrate injected is $3 \cdot 10^{-6}$ mg. Chromatographic conditions as in Table II.

eluents. When larger amounts of sodium nitrate are injected, its retention volume increases and levels out at *ca.* $3 \cdot 10^{-6}$ moles (0.25 mg) injected. We also injected different amounts of sodium nitrate, and obtained a similar curve (Fig. 1). Large amounts of sodium nitrate (0.1–0.5 mg) cause peak fronting. Highly concentrated salt solutions (potassium iodide) are also recommended by Berendsen *et al.*[13]. However, Papp *et al.*[14] noted that when they used this dead volume marker, the retention times of aromatic amines became irreproducible for a fairly long period of time. For all these reasons we preferred not to use a highly concentrated sodium nitrate solution as dead volume marker. Low amounts of sodium nitrate (less than 10^{-5} mg) are suitable for determining the exclusion volume.

Some articles evaluate different methods of determining V_0[9,13,15–19]. Berendsen *et al.*[13] divided the methods into three general categories: unretained compounds, static methods and linearization time for homologous series. The static method gives an upper limit for the total column porosity. The most accurate hold-up time is derived from the linearization of the logarithmic net retention times of a homologous series. This procedure is quite time-consuming.

McCormick and Karger[16] studied the distribution phenomena of mobile phase components in eluents containing different organic modifiers. The elution behaviour is explained partially by the gas chromatographic distribution isotherm. They state that on injecting "deuterium oxide enriched mobile phase" the original injection band is detected, which is not the case on injecting water-enriched mobile phase. Engel-

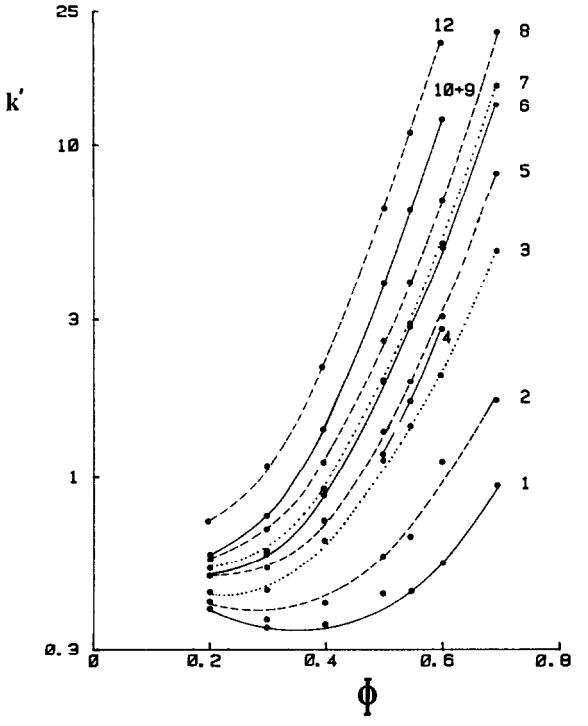


Fig. 3. Plots of the log k' value of the thiazide diuretics *versus* the amount of water in a methanol–water eluent system. Chromatographic conditions as in Table II. Key as in Table I.

hardt[7] suggests that, for methanol–water eluents, a methanol–deuterium oxide mixture of the same composition as the eluent is used, with deuterium oxide serving as the unretained compound. The deuterium oxide signal thus corresponds to the original injection band. This method was chosen for determining the dead volume. The only disadvantage, the need for a refractive index detector, was overcome by measuring the signal with a UV detector at low wavelength (210 nm). Jinno *et al.*[18] ascribed the signal on the UV detector to a possible change of the refractive index rather than to UV absorption of deuterium oxide in their small detector cell (less than 100 nl)[20]. The volume of the detector cell we used was 4.5 μl.

We used an organic modifier–deuterium oxide (10–50%) in water mixture in the same volume ratio as the eluent to determine V_0. Fig. 2 shows the plot of the dead volume *versus* the water content in an acetonitrile–water eluent system. As the concentration of the organic modifier is increased from 0 to 50%, the amount of modifier extracted into the stationary phase increases, resulting in a decrease in the column dead volume. This curve does correspond to the curve found by McCormick and Karger[16].

Chromatographic behaviour of the diuretics

Retention characteristics of the thiazide diuretics in different liquid chromatographic systems. We studied the influence of three organic modifiers: methanol, acetonitrile and tetrahydrofuran.

The behaviour of the thiazide diuretics in methanol–water eluents is shown in Fig. 3. All the diuretics follow a similar elution profile.

The dead volume in these experiments was determined with very small amounts of sodium nitrate. These experiments were performed before we selected deuterium oxide as dead volume marker. Therefore, the k' values of the slightly retained com-

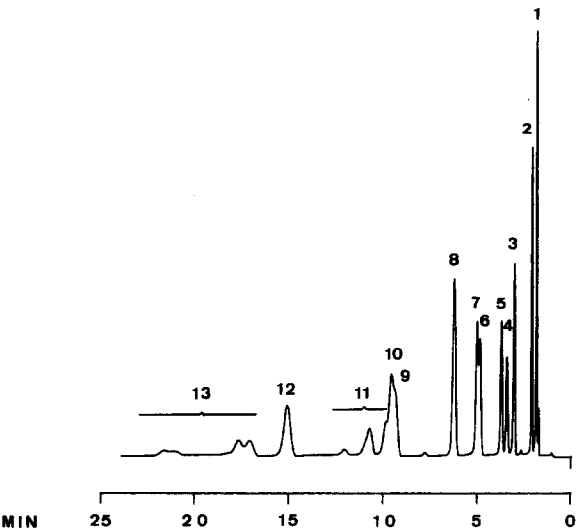


Fig. 4. Chromatogram of the separation of some thiazide diuretics with an eluent consisting of methanol–water (45:55). Chromatographic conditions as in Table II. Key as in Table I. Detector sensitivity, 0.4 a.u.f.s.

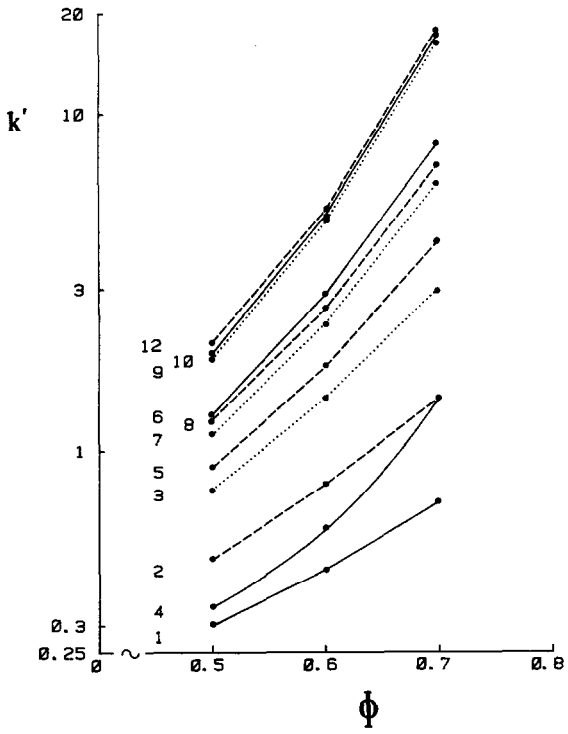


Fig. 5. Plots of the log k' value of the thiazide diuretics *versus* the amount of water in an acetonitrile–water eluent system. Chromatographic conditions as in Table II. Key as in Table I.

pounds are overestimates. Fig. 4 shows a chromatogram of the separation of thirteen thiazide diuretics with a methanol–water (45:55) eluent. The separation between EP (6) and ALT (7) is not satisfactory. However, for identification purposes the difference (0.15 min) in retention time between the two compounds is large enough. Only PT (9), BHF (10) and CYT (11) cannot be distinguished accurately. We therefore tried other mobile phase compositions.

MEBU (13) and CYT (11) give several peaks on elution with methanol–water (45:55). This is probably due to the presence of stereoisomers. Tisdall *et al.*[4] have previously noted this phenomenon for CYT. Pure samples of each compound could not be obtained for this study. We investigated the UV spectrum of the different compounds in CYT and MEBU. The HP 1040A detector is a high speed spectrophotometric detector: it takes UV spectra in less than 1 sec. This enables us to take UV spectra of the compounds as they elute from the column. The spectra can be stored on a disk, and evaluated after the chromatographic run. The stored spectra can also be plotted over one other to see if they coincide. This is especially useful when looking for stereoisomers, the spectra of which should coincide.

The three compounds observed for CYT (11) (Fig. 4) showed the same UV maxima (224, 272 and 312 nm) and minimum (243 nm). When the spectrum of the first compound (k' 6.8) was plotted over the second compound (k' 7.5) only a minor deviation in the absorbance band at 272 nm was observed. MEBU (13) is separated

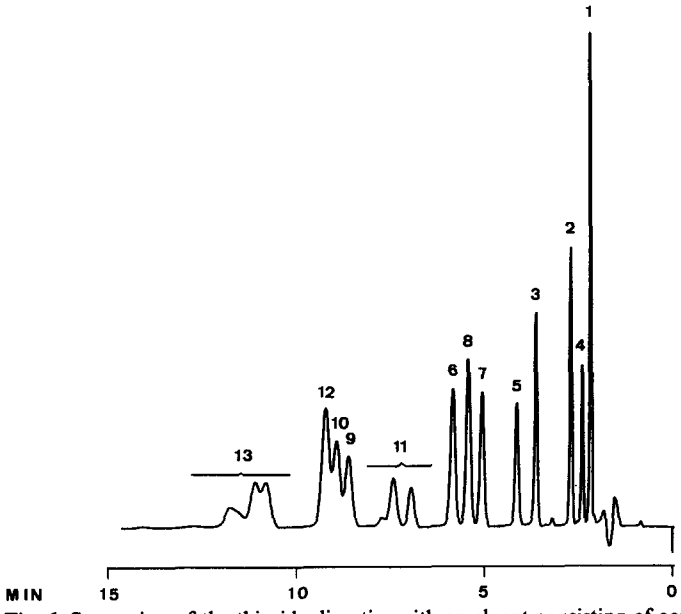


Fig. 6. Separation of the thiazide diuretics with an eluent consisting of acetonitrile–water (40:60). Chromatographic conditions as in Table II. Key as in Table I. Detector sensitivity, 0.1 a.u.f.s.

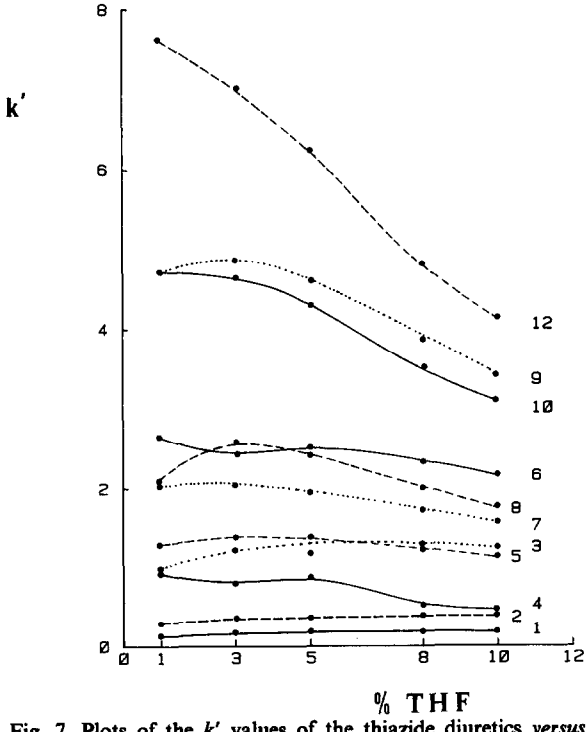


Fig. 7. Plots of the k' values of the thiazide diuretics *versus* the percentage of tetrahydrofuran in a methanol–water (45:55) eluent. Chromatographic conditions as in Table II. Key as in Table I.

into two different groups each containing two compounds (Fig. 4). Again the same UV maxima (226, 272 and 312 nm) and minimum (243 nm) were observed. Again, the spectra of the different compounds were found to coïncide, indicating that the compounds are stereoisomers.

The elution behaviour in acetonitrile–water eluents is shown in Fig. 5. All thiazide diuretics show the same elution characteristics except CTA (4). This might be due to the different chemical structure of CTA, which does not have the typical hydrothiazide structure. Fig. 6 shows a chromatogram of a separation of thirteen diuretics in an acetonitrile–water (40:60) eluent. Compared with the methanol–water eluent a better overall resolution is obtained in a shorter time. A change in elution order is observed for CTA (4), EP (6) and CYT (11). This eluent can be used to identify all thirteen diuretics.

The influence of a third mobile phase component, tetrahydrofuran, is shown in Fig. 7. When the tetrahydrofuran content is increased with the methanol water ratio kept constant, there is a decrease in retention for nearly all the diuretics. This is due to an increase in the eluotropic strength of the eluent. There is an increase in the retention of TCM (3), but the retention of HCT (1) and HFM (2) remains nearly constant. McCormick and Karger[21] showed that tetrahydrofuran concentrates in the stationary phase at the expense of methanol. Tetrahydrofuran retards acidic compounds because it is more basic than methanol. This results in a different selectivity.

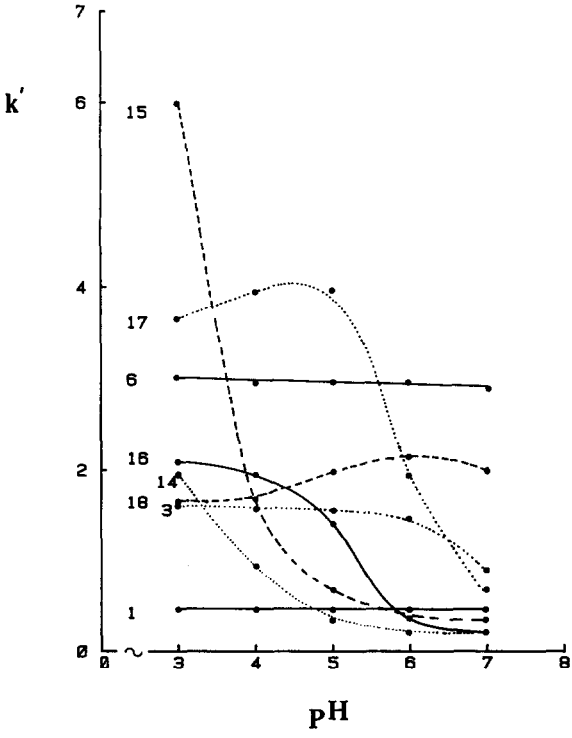


Fig. 8. Influence of the pH of the mobile phase on the retention of some thiazide, loop and potassium-sparing diuretics. Mobile phase, acetonitrile–0.05 *M* phosphate buffer (40:60). Chromatographic conditions as in Table II. Key as in Table I.

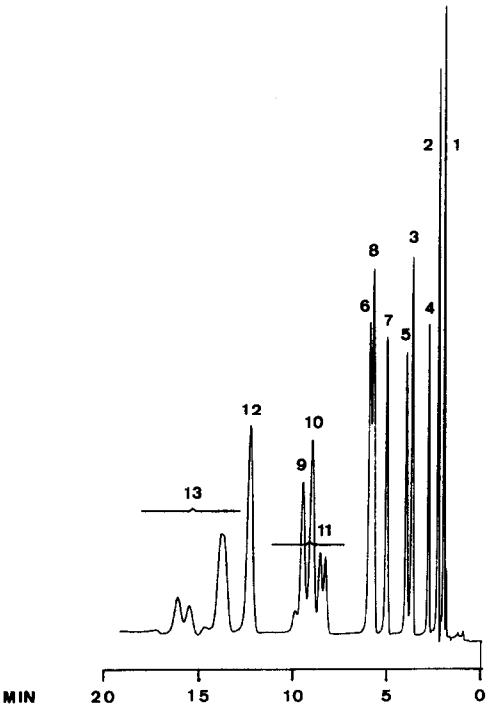


Fig. 9. Chromatogram of the influence of tetrahydrofuran on the separation of the thiazide diuretics. Mobile phase, methanol–water (45:55) containing 5% tetrahydrofuran. Chromatographic conditions as in Table II. Key as in Table I. Detector sensitivity, 0.04 a.u.f.s.

TCM is more acidic (pK_a 8.6) than most other diuretics (pK_a 8–10). This probably explains the different behaviour of TCM. Indeed, as can be seen from Fig. 8, the retention of TCM is influenced by the change in pH of the eluent, whereas the retention of EP (6) and HCT (1) is not. The retention of the other thiazide diuretics didn't vary either. As the pH is decreased, TCM is retarded (increased solvophobic retention).

There is a remarkable difference in selectivity obtained for BHF (10) and PT (9) (Fig. 7). These compounds can be separated with just 3% tetrahydrofuran in the eluent, although the separation is not possible with methanol–water. Fig. 9 shows the separation of the thirteen diuretics in a methanol–water (45:55) eluent containing 5% tetrahydrofuran.

The influence of the temperature was also investigated in an eluent consisting of acetonitrile–water (40:60) (Fig. 10). On increasing the temperature an almost linear decrease in the k' values was observed. The linearity of the plots of ln k' *vs.* $1/T$ was calculated according to the Van't Hoff equation: correlation coefficients higher than 0.9996 were obtained for most diuretics. Only HCT (1) (r = 0.9930) and CTA (4) (r = 0.9963) had somewhat smaller values. This linear relationship again indicates that only solvophobic forces are responsible for the retention in this water-rich eluent. The increase in temperature enhances the solubility in the mobile phase, and diminishes the solvophobic effect.

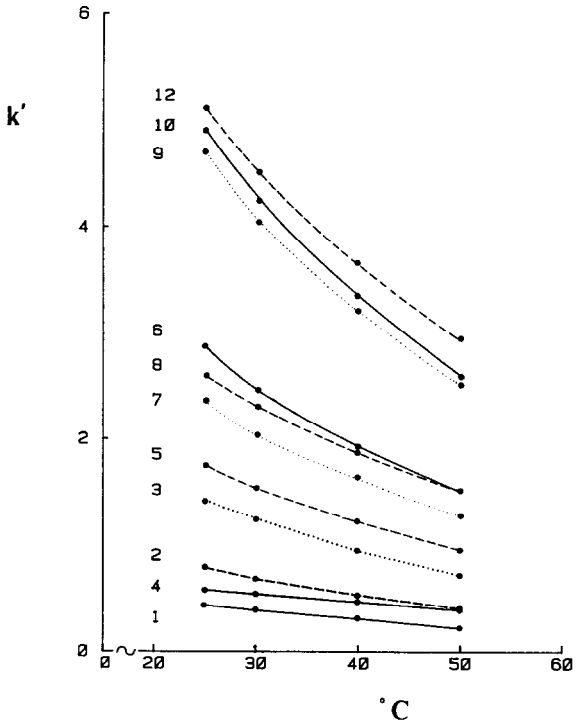


Fig. 10. Influence of the column temperature on the retention of the thiazide diuretics. Mobile phase, acetonitrile–water (40:60). Chromatographic conditions as in Table II. Key as in Table I.

Chromatography of some potassium-sparing and loop diuretics. Some of the thiazide diuretics are administered in combination with potassium-sparing diuretics: HCT is used in combination with AMI and TRI, EP is used in combination with TRI, ALT is used in combination with SPIR. Some other currently used diuretics, FUR, ETA (loop diuretics) and KCAN (a potassium-sparing diuretic) are also investigated.

We started by using the best mobile phase for the thiazide diuretics: acetonitrile–water (40:60). Obviously because of the chemically different structures of the compounds the chromatogram obtained was not satisfactory: no retention of the acidic compounds and a too-strong retention of the basic compounds ($k' > 20$), while the neutral compounds SPIR and CAN eluted with a k' of *ca.* 10. We therefore investigated the influence of the pH of the mobile phase (Fig. 8).

The retention of the acidic compounds ETA (15), FUR (14) and KCAN (16) increases as the pH decreases. Indeed, at low pH the acids are in the uncharged form; they are subject to solvophobic expulsion out of the eluent. The plots of the k' factor *versus* the pH show the expected sigmoidal behaviour[22].

The retention of the basic compounds is governed by electrostatic and silanophilic interactions[23]. Silanophilic interactions are said to be the strongest in water-lean eluents[24]. Nevertheless, we still observed a strong dependence of the retention of the basic diuretics on this type of interaction in a water-rich eluent system. The difference in the plot of the k' value *versus* the pH of AMI (18) and TRI (17) (Fig.

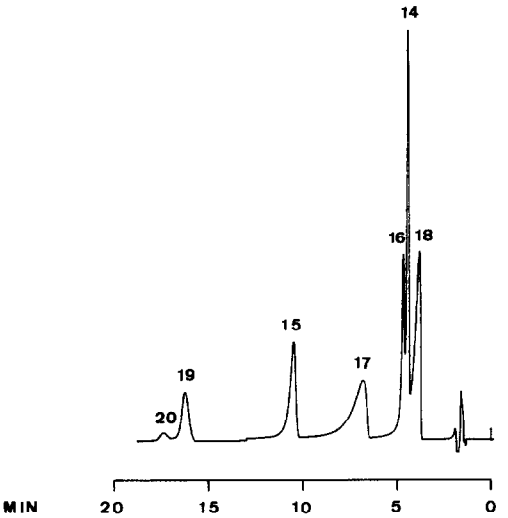


Fig. 11. Chromatogram of the separation of some potassium-sparing and loop diuretics. Mobile phase, acetonitrile–0.05 *M* phosphate buffer (pH 3) (40:60). Chromatographic conditions as in Table II. Key as in Table I. Detector sensitivity, 0.2 a.u.f.s. The wavelength changed automatically after 13 min from 275 nm to 238 nm.

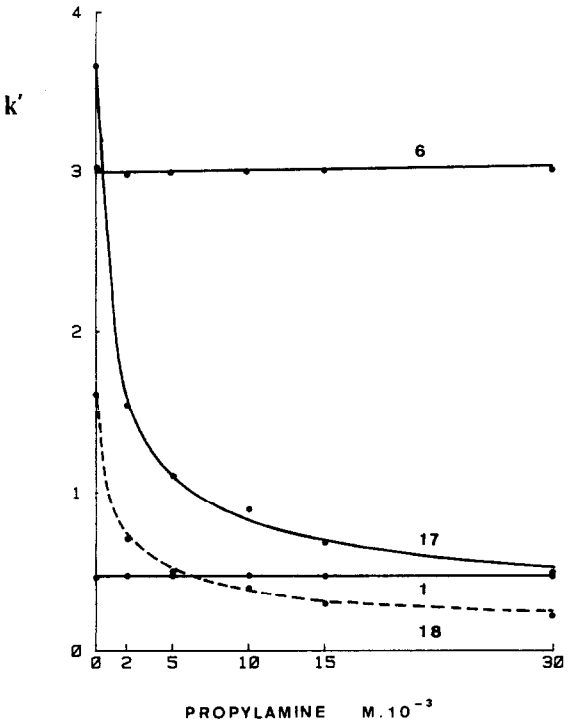


Fig. 12. Influence of the concentration of propylamine hydrochloride in an eluent consisting of acetonitrile–0.05 *M* phosphate buffer (pH 3) (40:60) on the retention of the basic diuretics and some thiazide diuretics. Chromatographic conditions as in Table II. Key as in Table I.

8) is due to the difference in basicity. AMI with a pK_a of 8.7[25] is positively charged until pH 6. As the pH increases the retention increases owing to a stronger silanophilic interaction. On the other hand, TRI is a weaker base with a pK_a of 6.2[25]. TRI follows the behaviour of the weak bases in a water-lean eluent as described by Papp *et al.*[14], despite the use of a water-rich eluent system here. When the pH is increased, the positively charged TRI is more retained by an increased ionic interaction with the silanol groups. When the pH is further increased TRI becomes less positively charged, which results in a decrease in silanophilic interaction and faster elution.

None of the thiazide diuretics except TCM (3) (as mentioned earlier) is influenced by the pH change of the eluent, because they are very weak acids (pK_a 8–10) and are neutral compounds in the pH range investigated.

By selecting the appropriate pH it is possible to chromatograph thiazide, potassium-sparing and loop diuretics in a suitable way. Fig. 11 shows a chromatogram of the potassium-sparing and loop diuretics at pH 3. AMI (18) and TRI (17) elute as broad asymmetric peaks, probably owing to silanophilic interaction even at this low pH. Indeed, LiChrosorb RP 18 is a non-end-capped column material.

In order to enhance the peak symmetry, an organic competing base was added to the acidic mobile phase. We added increasing amounts of propylamine hydrochloride (Fig. 12). This had no effect on the retention of the non-ionized compounds: the thiazide diuretics, the acidic potassium-sparing and loop diuretics and the neutral diuretics. A remarkable decrease in retention was observed for the basic compounds.

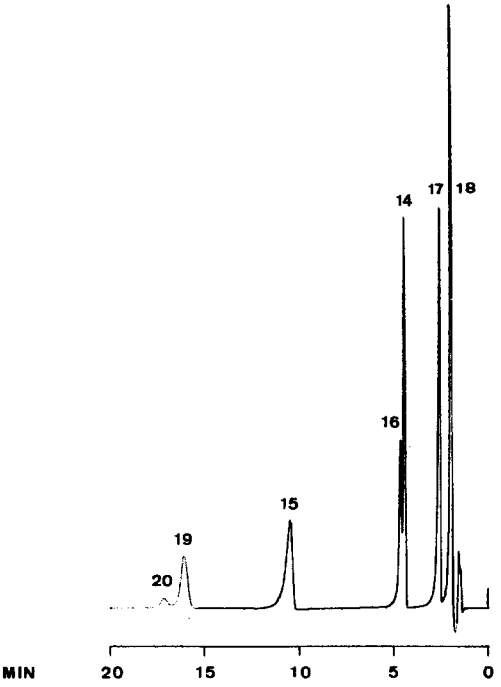


Fig. 13. Separation of some loop and potassium-sparing diuretics. Mobile phase, acetonitrile–0.05 *M* phosphate buffer (pH 3) (40:60) containing $15 \cdot 10^{-3}$ *M* propylamine hydrochloride. Key and chromatographic conditions as in Fig. 11. Detector sensitivity, 0.2 a.u.f.s.

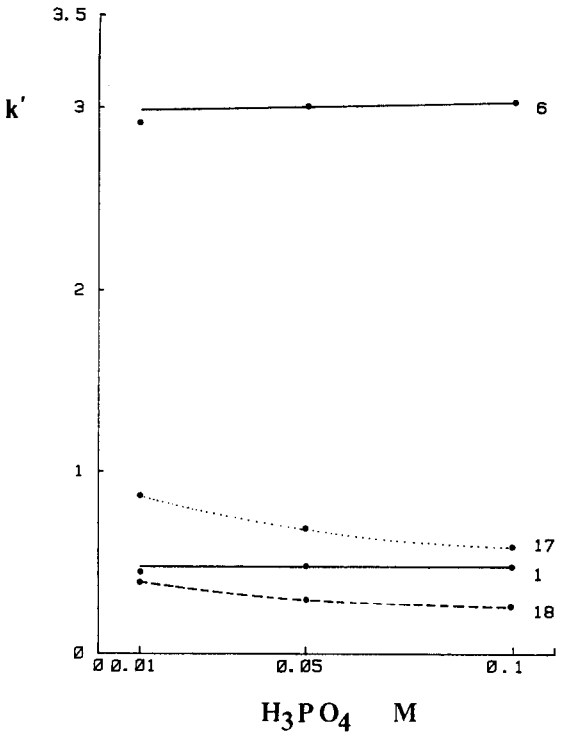


Fig. 14. Plots of the k' values of some diuretics *versus* the molarity of the phosphate buffer in the eluent. Mobile phase, acetonitrile–phosphate buffer (pH 3) (40:60) containing $15 \cdot 10^{-3}$ *M* propylamine hydrochloride. Chromatographic conditions as in Table II. Key as in Table I.

This is due to the competition of propylamine hydrochloride with the positively charged basic compounds for the residual silanol groups, and also to the electrostatic repulsion of the solute ions out of the positively charged primary ion layer of the stationary phase[26]. A better peak symmetry is obtained for AMI and TRI, not only owing to the addition of propylamine hydrochloride to the eluent but also owing to the faster elution of AMI and TRI (Fig. 13).

The molarity of the buffer was also investigated (Fig. 14). A small decrease in the k' factor was obtained for the basic compounds when the molarity was increased. The phosphate buffer also diminishes the interaction of the samples with the "residual" silanol groups[27].

CONCLUSION

These experiments show that any given selectivity or resolution can be obtained in the chromatography of thiazide, potassium-sparing and loop diuretics by selecting the appropriate mobile phase composition. Indeed, acetonitrile–water mixtures are suitable for chromatography of the thiazide diuretics. The use of tetrahydrofuran as a third mobile phase component can be useful when a different selectivity is needed. For chromatography of the loop and potassium-sparing diuretics, additional control

of the pH of the eluent is necessary. A good peak symmetry of the basic diuretics is obtained only after propylamine hydrochloride has been added to the acidic eluent. On adjusting the pH of the eluent and/or the concentration of propylamine, a remarkable change in the retention of the acidic compounds can be obtained while the retention of the thiazide diuretics remains nearly constant. This is a suitable way for chromatographing thiazide and potassium-sparing diuretics, which are frequently administered together, with the necessary selectivity and resolution. With a mobile phase of acetonitrile–0.05 *M* phosphate buffer (pH 3) containing 15 m*M* propylamine hydrochloride (40:60), an excellent separation of the loop and potassium-sparing-diuretics was obtained.

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