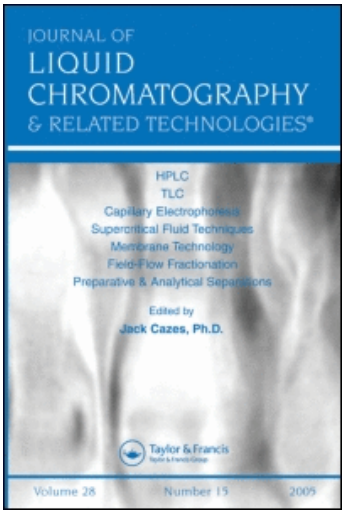

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EVALUATION OF THE USE OF LITHIUM NITRATE AS A TEST SUBSTANCE FOR THE DETERMINATION OF THE HOLD-UP TIME ON A REVERSED-PHASE PACKING

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ABSTRACT

The use of lithium nitrate as a test substance for the determination of the hold-up time on μBondapak C18 columns has been evaluated by comparison with the hold-up time calculated with the homologous series of n-alcohols. The influence of charge exclusion effects on the retention time of lithium nitrate is investigated. The addition of phosphoric acid to the eluent appears to be an effective method to reduce charge exclusion effects. The existence of deviations from linearity are demonstrated for the homologous series of n-alcohols in eluents with a low methanol content.

INTRODUCTION

In the investigations of the relation between structure and retention or between eluent composition and retention, retention is usually expressed as the capacity factor or the logarithm of the capacity factor.

For the calculation of the capacity factor the hold-up time is used. Large errors in the value of the capacity factor can result from imprecise and/or inaccurate measurements of the hold-up time, particularly in the case of compounds which show little retention.

Lithium nitrate has been used in the laboratory of the authors for the measurement of the hold-up time in reversed-phase high performance liquid chromatography (HPLC) for a number of

years. It had been chosen because of its good solubility in water-methanol mixtures and its detectability with UV detectors. However, some doubts arose about its use due to the fact that differences in retention times were observed between some very polar compounds, the constituents of the eluent and lithium nitrate. It was then decided to compare the retention times of those substances with the theoretical hold-up time calculated from the homologous series of n-alcohols, a method which has been in use for a long time in gas chromatography (1-7), and which has more recently been described for HPLC (8-10).

In a recent publication Berendsen et al. (8) objected to using salt solutions for the determination of the hold-up time. They describe a strong dependence of the retention time of bromide on the quantity injected. They explain this phenomenon by the charge exclusion effects from the pores of the column packing and the suppression of this effect at higher salt concentrations.

For this reason the dependence of the retention time of lithium nitrate on the quantity injected was also investigated. As charge exclusion effects will depend on the charge of the packing, i.e. the dissociation of the free silanol groups, the effect of the injection of acidic and basic samples (i.e. solutions of phosphoric acid and ammonium carbonate in the eluent) as well as the addition of phosphoric acid or lithium chloride to the eluent on the retention time of lithium nitrate were included in this study. The results of these investigations are given together with the previous ones.

In this study some attention is also paid to the occurrence of deviations from the well-known linear relationship between the logarithm of the capacity factor and the number of carbon atoms for the series of n-alcohols in eluents with low methanol content.

EQUIPMENT AND CHEMICALS

Chromatographic experiments were performed using a 6000A solvent delivery system, a U6K injection system and an R401 differential refractometer (all from Waters Assoc., Milford, MA, U.S.A.).

The 2 ml sample loop of the U6K injector was replaced by a loop of approximately 100 µl in order to reduce errors in the retention time measurements caused by decompression and subsequent compression of the contents of the sample loop upon injection of the sample. A µBondapak C18 column, 30 cm x 3.9 mm I.D., particle size 10 µm, (Waters Assoc.) was used. The column was thermostatted by means of a metal block which was shielded from the surroundings by isolating material and which was connected in series with the housing of the refractive index detector to a circulating waterbath. Due to the limited column life time not all experiments were carried out using the same column. When data were obtained on different columns this is indicated in the tables. The temperature of the column and the refractive index detector was kept at 25° C.

The retention times were measured and recorded by the combination of an SP 4000 central processor, an SP 4020 data interface and an SP 4050 printer plotter (all from Spectra Physics, Santa Clara, CA, U.S.A.).

The pH shifts in the eluent were studied with a Radiometer (Copenhagen, Denmark) G 299 A capillary glass pH electrode which was connected to the outlet of the refractive index detector. The outlet of the capillary glass electrode was connected to a small glass vessel (provided with an outlet for the excess of eluted solvent) in which a Type 373-90 reference electrode (Ingold, Zürich, Switzerland) was placed. The capillary glass electrode and the reference electrode were connected to a Radiometer PHM 64 pH meter. The jacket around the capillary glass electrode was filled with potassium nitrate solution which was electrically connected to the reference electrode to avoid instability of the signal of the pH electrode (11, 12).

Water was purified by deionization and subsequent double destillation from glass. Methanol (pro analysis) was obtained from Merck (Darmstadt, G.F.R.). The other chemicals were purchased from various suppliers.

INSTRUMENTAL AND ANALYTICAL PROCEDURES

Retention times were corrected for the extra column volume by replacing the column by a low dead volume union and measuring the retention time of an equal volume of a test substance. All retention times were corrected by subtraction of the "extra column hold-up time". Retention times were measured using the SP 4000 integrator. The peak width parameter and the slope sensitivity for the integration process were adapted in such a way that precision in the retention time measurements of 0.1 to 0.2% could be obtained.

To avoid cavitation in the pump heads methanol and water were filtered directly before the preparation of the eluents by vacuum filtration through filters with a pore size of 0.2 µm. After mixing the eluents were degassed further ultrasonically. In order to avoid long term flow fluctuations the pump was continuously operated at the same flow (1.0 ml/min).

The hold-up time was studied with the eluents methanol, methanol-water (8:2, w/w), methanol-water (6:4, w/w), methanol-water (4:6, w/w), methanol-water (2:8, w/w) and water. A limited number of experiments has also been done with eluents containing 50% w/w methanol.

The theoretical value of the hold-up time was calculated from the retention times of the alcohols methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol and 1-decanol.

The following test substances for the determination of the hold-up time were compared: water, methanol, formamide, glycerol, 1,3,5-trihydroxybenzene (THB) and lithium nitrate.

In the eluents containing water-methanol (5:5, w/w), water-methanol (8:2, w/w), and water, lithium nitrate, urea and thiourea were compared. All compounds were dissolved in or diluted with the eluent. Solutions of lithium nitrate were prepared in a concentration of 10^{-1} gmol·kg^{-1}.

Sufficiently symmetrical peaks and constant retention times were obtained by injection of approximately 20 µl. The other compounds were injected in such quantities that they could be well detected

at 8 to 16x attenuation on the refractive index detector. The amounts injected were generally 100 to 200 µg.

MATHEMATICAL PROCEDURES

The calculation of the hold-up time is based upon the assumption that in reversed-phase liquid chromatography the relationship between the retention of the members of the homologous series of n-alcohols and their number of carbon atoms is given by the following well-known equation

$$t_R - t_d = t_d \times 10^{(A+BxN)} \qquad (I)$$

(in which t_d, t_R, N, A and B are the hold-up time, the retention time of the alcohol, its number of carbon atoms and two constants respectively).

After measurement of the retention times of the n-alcohols the values of the hold-up time and both constants that fit best in the equation are computed. For the calculation of the hold-up time a calculation method based upon minimizing of the function

$$\sum_m \left(\frac{t_{R_m} - t_d - t_d \times 10^{A+BxN_m}}{t_{R_m}} \right)^2 \qquad (II)$$

according to Levenberg and Marquard (13,14) was used. The calculation was done by means of a program based upon the FORTRAN subroutine ZXSSQ (15). This method should be preferred to other possible techniques for in this calculation method the separate retention times contribute to the calculated hold-up time according to the reproducibility in their measurements.

The accuracy in the hold-up time calculated from the retention time of a number of members of a homologous series depends on the following factors:

1. The number of homologues involved in the calculation
2. The number of carbon atoms of the homologue involved in the calculation

3. The precision in the retention times measured (which is a function of the number of measurements)
4. The values of the constants A and B in Equation I
5. Deviations from the assumed valid relationship of Equation I.

The computer program used for the calculation of the hold-up time also calculates the standard deviation in the hold-up time. This standard deviation results from the observed differences between the measured retention times and the calculated relationship of Equation I.

It therefore results both from the reproducibility of the retention times measured and possible systematic deviations from Equation I. When the assumption of the validity of Equation I is correct, the following results should be obtained on the calculation of the hold-up time:

1. The measured retention times should exhibit a random distribution round the values obtained with the calculated relationship of Equation I
2. This distribution should have a standard deviation which corresponds with the precision of the measurement of the retention times of the homologues
3. No significant differences should occur between the hold-up times calculated from different combinations of the members of the homologous series.

When one or more of these results are not obtained, deviations of Equation I are likely to occur.

RESULTS AND DISCUSSION

Calculation of the hold-up time from the homologous series of n-alcohols

In the eluents with lower methanol contents only the lower homologues of the series of alcohols have been chromatographed, because the higher members of the series gave too long retention times and were only detectable if rather large quantities were injected.

TABLE 1

Retention Times of Alcohols

	Eluent Composition % w/w Methanol					
	100%	80%	60%	40%	20%	0%
Methanol		158.1	154.9	156.4	163.1	200.9
Ethanol	166.8	168.6	171.9	183.7	205.6	301.2
Propanol	167.0	174.3	188.5	225.9	305.4	667
Butanol	169.6	181.2	214.9	320.9	603	2042
Pentanol	172.1	192.8	262.5	538	1525	
Hexanol	175.1	208.8	336.3	1038		
Heptanol	178.9	231.3	462.6			
Octanol	182.4	261.1				
Decanol	193.0	358.8				

The reproducibility in the measurements of the retention times was found to be dependent on the solvent composition. The following relative standard deviations were found for the eluents investigated: For the eluents containing 100% and 80% methanol 0.2%, for 60% methanol 0.4%, for 40% methanol 0.5-0.7%, for 20% methanol 1-1.5% and for 0% methanol 1-2%. These higher relative standard deviations with the eluents with a lower methanol content are mainly caused by long term fluctuations in the retention times. Short term reproducibility was found to be considerably better. The long term fluctuations may possibly be caused by the increased solubility of air in the eluents at lower methanol contents, resulting in an increased tendency to cavitation in the pump head. The retention times found for the n-alcohols are given in Table 1.

In the literature deviations from Equation I for the lower members of a homologous series have been described (16). Although it is possible that such observations result from the use of a wrong value for the hold-up time, this is not necessarily the cause of this deviation. It is therefore necessary to evaluate the validity of Equation I.

TABLE 2

Hold-up Times Calculated from the Retention Times of Alcohols

Eluent Composition % w/w Methanol	I	II
100%	———	155.2 (1.5)
80%	148.8 (2.2)	152.8 (0.9)
60%	138.9 (3.7)	146.8 (1.2)
40%*	142.6 (3.6)	151.0 (0.3)
20%*	147.2 (4.6)	157.9 (1.3)
0%*	164.8 (1.5)	169

I Hold-up time calculated from all alcohols
II Hold-up time calculated from all alcohols except methanol
* These values have been obtained on a second column

The effect of the inclusion of the retention time of methanol in the calculation is shown in Table 2.

The standard deviations in Table 2 are obtained with the computer program. The strong decrease in the standard deviation when the retention time of methanol is excluded from the calculation reflects the contribution of methanol to the deviation from Equation I.

It was therefore concluded that the retention time of methanol should not be included in the calculation of the hold-up time.

This phenomenon of systematic deviations due to methanol is not unexpected as methanol is in these experiments both solute and modifier.

The methanol in the eluent is known to be incorporated in the stationary phase according to a non-linear distribution isotherm (17,18). The retention time of the concentration pulse caused by the injected methanol will be influenced by the curvature of this distribution isotherm.

This will result in a chromatographic behaviour which deviates from the relationship of Equation I. When methanol was excluded from the calculation no indication for deviations from Equation I were

TABLE 3

Average Hold-up Times Calculated from 12 Series of Retention Times of n-Alcohols with the Eluent containing 40% MeOH

Hold-up Time calculated from	Averaged Hold-up Time	Standard Deviation in the Averaged Hold-up Time
Methanol-Hexanol	142.6	0.3
Ethanol-Hexanol	151.0	0.3
Propanol-Hexanol	152.0	0.3
Butanol-Hexanol	153.7	0.7
Ethanol-Pentanol	150.5	0.2
Propanol-Pentanol	150.7	0.3
Ethanol-Butanol	150.3	0.2

obtained with eluents with a methanol content higher than 40%. With eluents containing 40% methanol, however, indications for a slight non-linearity were obtained. For further examination of this phenomenon 12 series of retention times of n-alcohols from methanol to hexanol were determined (the injected quantity was 100-200 µg). For each of the series the hold-up time was calculated from different combinations of alcohols. The corresponding hold-up times were averaged and the standard deviations in the averaged hold-up time were calculated. The results obtained are given in Table 3. The data clearly indicate the existence of a deviation from Equation I, even when methanol is excluded from the calculations. The data also suggest that the non-linearity is more likely to be caused by deviation of hexanol than deviations of ethanol.

(The standard deviations in Table 3, in contrast to those in Table 2, result only from the reproducibility of the retention times measured and not from systematic deviations from Equation I).

Analogous results were obtained with 14 series of retention times of series of alcohols from ethanol to pentanol with an eluent containing 20% methanol (Results not shown). With eluents containing

0% methanol no such investigations could be done because only the retention times of ethanol, propanol and butanol were available for calculation.

Examination of the peak shape of the n-alcohols revealed that the peaks showed an increased tendency to tail when the methanol content of the eluents was lowered from 40% to 20% and 0%. This tendency to tail was stronger with the alcohols with longer chains.

The change in peak shape on changing the quantities injected for the eluents containing 20% and 0% methanol is illustrated by Fig. 1. The chromatograms were recorded in such a way that the changes in the quantities injected were compensated by a change in the

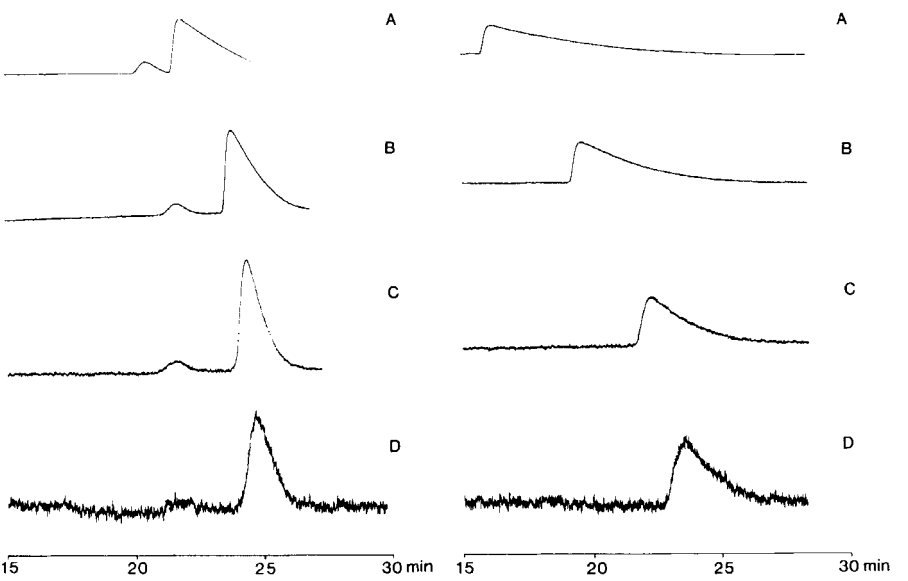

FIGURE 1

Influence of amounts injected on the peak shape and the retention time of pentanol (left, eluent 20% methanol w/w) and butanol (right, eluent water). The amounts injected and the detector attenuation are a: 1280 µg, 64; b: 320 µg, 16; c: 80 µg, 4 and d: 20 µg, 1.

attenuation of the refractive index detector. In this way a better comparison of the peak shape is possible.

The changes in the retention times and the peak shapes on varying the quantities injected clearly indicate that some overload ing occurs. (For the peak chape improves on decreasing the quantity injected and the retention times measured seem to approach a constant value).

Although reduction of the quantities improves the linearity, no sufficient linearity (i.e. no constant retention times) could be obtained with the eluents containing 0% and 20% methanol, due to the limited sensitivity of the refractive index detector, which made it impossible to reduce the quantities injected beyond the range studied. In the eluents containing 40% methanol the linearity may be regarded as acceptable when quantities injected are kept sufficiently low and when the retention of hexanol is excluded from the calculation.

The possibility that the phenomena observed arose from imperfections in an individual column or from instrumental imperfections could be excluded by demonstrating that peaks with identical shape were obtained on other commercial μBondapak C18 columns, user packed μBondapak C18 columns and user packed (10 μm) Lichrosorb RP 18 columns and that no imperfections in peak shape were observed when the column was replaced by a low dead volume union. The suggestion of Berendsen et al. (8) that n-alcohols can be used for the calculation of the hold-up time even with eluents containign 0% methanol seems therefore not to be justified at least not for μBondapak C18 columns. A careful check whether deviations from linearity of Equation I occur is therefore necessary whenever the hold-up time is calculated from a series of n-alcohols. This makes this method for the determination of the hold-up time a very time-consuming and tedious one. In our opininion it should therefore only be regarded as an independent method to evaluate whether a test substance has a retention time which corresponds to the actual hold-up time.

Determination of the hold-up time with test substances

The use of test substances is particularly important when frequent determinations of the hold-up time are necessary to eliminate the effect of long term fluctuations in the flow, when very precise measurements of capacity factors are needed.
To determine the hold-up time by means of a test substance this test substance should not be retained by, nor be excluded from the column packing.

Very polar uncharged test substances can be expected to interact with residual silanol groups. Such interactions should be expected to be more important when the eluent is less polar i.e. contains more organic modifier. When an uncharged test substance is less polar, hydrophobic retention should be expected to occur (at least with eluents containing little or no modifier). It is debatable, whether an uncharged test substance for the hold-up time can be at the same time both sufficiently polar (not to be retained by hydrophobic effects) and at the same time be sufficiently apolar (not to be retained by adsorption at residual silanol groups). It seems to be even more debatable that such an uncharged test substance can fulfil these requirements at all possible concentrations of the modifier. With charged test substances no retention should be expected to occur unless the ions involved have a sufficiently hydrophobic character to be adsorbed with the formation of an electrical double layer or as ion pairs.

However, when the packing itself is charged, charge exclusion effects for ions of the same charge, and ion exchange effects for ions of opposite charge should be expected to occur. Reversed-phase packings have been demonstrated to possess considerable ion exchange properties (due to the dissociation of residual silanol groups) over a large range of pH values (12). This dissociation results in a negative charge at those pH values.

Charged test substances can be used only for the determination of the hold-up time when the charge of the dissociated silanol groups can be masked by large concentrations of electrolyte.

The following factors should therefore be regarded as important for the behaviour of test substances:

1. The amount of residual silanol groups
2. The degree of dissociation of the residual silanol groups
3. The affinity of the test substance for the residual silanol groups
4. The affinity of the components of the eluent for the residual silanol groups and their concentration in the eluent
5. The hydrophobicity of the test substance
6. The nature and the concentration of the modifier
7. The presence of components in the eluent which modify the character of the hydrophobic layer.

From these factors it appears that test substances have to be evaluated (with an independent method) for each particular set of experimental conditions. Comparison with the hold-up time calculated from a homologous series should be regarded as a standard method of evaluation (provided that no deviations from Equation I are observed).

The retention times of some test substances are given in Table 4 together with the calculated hold-up times. From this

TABLE 4

Retention Times of Test Substances and the Values Calculated for the Hold-up Time from the Series of n-Alcohols

	Eluent Composition % w/w Methanol					
	100%	80%	60%	40%*	20%*	0%*
Water	166.8	160.3	157.5	157.5	162.6	-
Methanol	-	158.1	154.9	156.4	163.1	200.9
Formamide	162.5	160.7	160.8	156.8	159.9	178.4
Glycerol	158.9	158.7	155.5	153.6	156.8	175.1
THB	153.6	155.8	152.8	168.8	222.9	779.5
Lithium Nitrate	153.4	152.7	144.0	149.8	150.9	155.4
Calculated	155.1	152.8	146.8	150.5	157.9**	169**

*These values have been obtained on a different column
**These values have been calculated from tailing peaks

table it appears that the retention time of lithium nitrace corresponds well with the calculated hold-up times at methanol concentrations above 20%.
According to the concentration pulse concept no differences in the retention times of methanol and water should occur.
The differences found might possibly be explained by the dependence of the retention time of the vacancy peak on the concentration of the modifier in the injected sample as described by McCormack and Karger (17).

The values of the retention times of the concentration pulses of the components of the eluents, water and methanol, are found to be significantly higher than the calculated hold-up time, which is in accordance with the well-established fact that the modifier is incorporated in the stationary phase (17-21) and which corresponds with the results obtained by Berendsen et al. (8).
The differences between the calculated hold-up time and the retention times of formamide and glycerol could possibly also be explained by hydrophobic effects or by adsorption at residual silanol groups.
Water was found to be retained considerably with the eluent containing 100% methanol. Although this effect may be of less importance with eluents with lower methanol contents deuterium oxide was therefore not considered to be a safe marker for the hold-up time. It was therefore not included in this study.

The possibility of incorporation of components of the eluent into the stationary phase makes total porosity measurements (based upon the differences in weight after filling the column with solvents of different densities) useless as a method for the determination of the hold-up time. No such measurements are therefore presented in this study.
Differences in the incorporation of the components of the eluents at different eluent compositions may explain the variation of the calculated hold-up time on the variation of the eluent composition. The fact that the retention time of lithium nitrate follows the same pattern of variation as the calculated hold-up time strongly sug-

gests that its retention time corresponds indeed with the actual hold-up time.

At high methanol contents the retention time of THB corresponds with the retention time of lithium nitrate and the calculated hold-up time. At low methanol contents THB is obviously retained by a hydrophobic retention mechanism.

The influence of charge exclusion effects on the retention time of lithium nitrate

As charge exclusion effects have extensively been described by Berendsen et al. (8), it was investigated whether such effects occurred with the quantities injected in this study and whether the retention times measured were influenced by them.
As charge exclusion effects will depend on the charge of the column packing (i.e. the degree of dissociation of the redisual silanol groups) such effects should be expected to be dependent on the pH of the eluent when buffered eluents are used. When non-buffered eluents are used the charge of the column packing will be influenced by previously used eluents and/or the nature of previously injected samples.

When charge exclusion effects are studied the condition of the column packing should therefore be well defined in this respect. In this study this has been done by injection of measured quantities of solutions of phosphoric acid or ammonium carbonate in the eluent onto the column before the series of injections of lithium nitrate. The retention times measured are given in Table 5. The data in this table indicate that only with the injection of small amounts of lithium nitrate charge exclusion effects are observed. These effects are more pronounced when basic samples have been injected previously.
On the injection of 140 µg lithium nitrate (∿ 20 µl 0.1 M) no significant charge exclusion effects are observed.
Upon addition of a small amount of phosphoric acid to the eluent no charge exclusion effects could be observed over the entire range of quantities studied.

TABLE 5

Retention Time of Lithium Nitrate

µl injected (10^{-1} M. $LiNO_3$ in the Eluent)	Eluent Composition I a)	I b)	II a)	II b)	III
100	154.9	153.2	153.7	153.1	153.9
50	154.1	150.7	153.7	151.1	153.7
20	152.4	143.7	151.4	144.4	152.1
10	151.8	135.4	149.8	139.2	153.0
5	141.1	120.5	146.0	131.2	153.3
2	125	111.6	139.3	125.5	152.9
1	117	105.2	138.3	121.7	153.3

I 50% methanol w/w
II 50% methanol w/w containing 10^{-3} mol·kg^{-1} lithium chloride
III 50% methanol w/w containing 10^{-3} mol·kg^{-1} phosphoric acid
a) series measured after two injections of 100 µl 0.1 M. phosphoric acid in the eluent
b) series measured after two injections of 100 µl 0.1 M. ammonium carbonate in the eluent

These effects are more pronounced when basic samples have been injected previously.

On the injection of 150 µg lithium nitrate no significant charge exclusion effects are observed.

Upon addition of a small amount of phosphoric acid to the eluent no charge exclusion effects could be observed in the range of quantities studied.

When the elution of injected lithium nitrate was monitored with a flow-through pH electrode a decrease of the pH was observed during the elution of the peak of lithium nitrate. Obviously some lithium ions are exchanged against protons. The liberated protons will influence the charge of more distant parts of the column thereby reducing charge exclusion effects. This mechanism will be one of the causes for the variation of the retention times of salts with the injected quantity. Whether this mechanism is more important than the suppression of the charge exclusion effect by

ionic strength of the injected sample cannot be concluded from the data in this study. However, the data in Table 5 indicate that addition of phosphoric acid to the eluent is much more effective than the addition of an equivalent amount of lithium chloride.

It is therefore probable that the effect of buffers to suppress charge exclusion effects will in some cases be largely due to the reduction of dissociated silanol groups instead of the masking of their charge as has been suggested by Parker et al. (23) and by Wells and Clark (24).

Buffers of low pH should therefore be expected to be more effective than those of high pH. This was confirmed by an experiment in which the retention time of lithium nitrate was measured in eluents composed of equal weights of methanol and 0.01 M. aqueous phosphate buffers of different pH.

When buffers of pH 4.5 , 5.0 and 5.5 were used the retention times of lithium nitrate were found to be constant. With buffers of a higher pH lower retention times were found and the precipitation of lithium phosphate occurred.

The non-linearity of the chromatographic distribution process in eluents containing 20% and 0% methanol makes it impossible to use the calculation of the hold-up time as an independent method to determine whether the retention time of lithium nitrate does correspond with the actual hold-up time or not. The fact that no increase in the retention time with increasing amounts of injected lithium nitrate could be found for these eluents suggests, however, that these retention times indeed correspond with the actual hold-up times.

Comparison of lithium nitrate with urea and thiourea as test substances for the hold-up time

In a recent publication the use of thiourea has been proposed as a test substance for the determination of the hold-up time (10). We have therefore made a comparison between the retention times of thiourea, urea, lithium nitrate and the hold-up time calculated from a series of n-alcohols in eluents containing 50%, 20% and 0% methanol. The values obtained are given in Table 6.

TABLE 6

Comparison of the Retention Times of Lithium Nitrate, Urea and Thio Urea and the Hold-up Time Calculated from a Series of n-Alcohols

Eluent Composition % w/w Methanol	Hold-up Time Calculated	Lithium Nitrate	Urea	Thiourea
50%	158.3	160.7	164.7	168.1
20%	-	167.7	168.6	177.0
0%	-	166.8	179.2	200.0

From the values in Table 6 it appears that the retention time of thiourea is significantly higher than those of urea with all three eluents. It is therefore highly improbable that the retention time of thiourea corresponds with the hold-up time. The fact that the difference between the retention times of thiourea and urea increases with decreasing methanol content strongly suggests that the retention of thiourea is hydrophobic in character.
The fact that urea gives significantly higher retention times than lithium nitrate in eluents containing 0% and 50% methanol makes its use as a test substance rather dubious. For the latter eluent this is confirmed by comparison with the hold-up time calculated from the n-alcohols.

CONCLUSION

Lithium nitrate was found to be the only test substance for the hold-up time that gave correct values over the whole range of eluents investigated, provided a sufficient quantity was injected to overcome charge exclusion effects. The occurrence of charge exclusion effects was found to be dependent on the history of the column, i.e. pretreatment with acidic or basic samples. The occurrence of charge exclusion effects could be reduced considerably by the addition of small amounts of phosphoric acid in the eluent.

The retention time of lithium nitrate being the correct value for the hold-up time could be confirmed by comparison with the

hold-up time calculated from the homologous series of n-alcohols for eluents with a methanol content of 40% or more.
In eluents with a low methanol content of 20% and 0% significant deviations from Equation I were found, probably due to a non-linear chromatographic distribution process.

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