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Journal of Chromatography A, 734 (1996) 271–276

JOURNAL OF CHROMATOGRAPHY A

Effect of the eluent pH on the thermospray molecular ion intensity of nucleosides

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Received 16 October 1995; revised 29 November 1995; accepted 30 November 1995

Abstract

The protonated molecular ion intensities of 15 nucleosides obtained by thermospray ionisation have been measured using 0.1 *M* ammonium acetate mobile phase at neutral and acidic pH. To explain the dependence of the molecular ion intensity on the mobile phase pH, the hydrophobicity, the pK_a values and reversed-phase high-performance liquid chromatographic retention data ($\log k'$) at neutral and acidic pH values were studied. Significant correlations (above 95% probability level) were found between the change in the protonated molecular ion intensity and the hydrophobicity as well as the pK_a values of the compounds. The reversed-phase chromatographic retention parameter ($\log k'$) obtained at pH 3.5, showed significant correlation together with the pK_a values to the molecular ion intensity change caused by decreasing the mobile phase pH.

None of the investigated nucleosides showed an increased molecular ion intensity change at low pH when more than 5% methanol was present in the mobile phase.

Keywords: pH effects; Thermospray ionization; Molecular ion intensity; Mobile phase composition; Nucleosides

1. Introduction

Thermospray ionisation was first introduced as a method of interfacing HPLC to mass spectrometry by Blakley et al., in 1980 [1]. Intense protonated molecular ions were observed with a few structurally significant fragment ions. It was recognised that volatile compounds present in the eluent, such as formic acid or ammonium acetate provided conditions under which the molecular ion formation was preferred [2]. Thermospray high-performance liquid chromatography–mass spectrometry (HPLC–TSP-MS) has been extensively used for trace analysis of pharmacologically active compounds from complex mixtures [3]. The ionisation mechanism in thermospray buffer ionisation has been widely studied and reviewed [4]. Much debate has been focused on whether thermospray is a liquid or gas phase ionisation method. The work of Iribarne and co-workers [5,6] showed that preformed ions were evaporated from droplets when buffer and/or salt levels were low. The presence of a volatile buffer appears to change the ionisation to a gas phase process [7]. As most thermospray ionisation is performed in the presence of buffer, it is generally held that gas phase ionisation predominates [8–10].

The effect of the mobile phase pH on the protonated molecular ion intensity has previously been studied [7,10] and no effect was noted.

In an earlier study [11], a significant increase in

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0021-9673/96/$15.00
SSDI 0021-9673(95)01306-7

the protonated molecular ion intensity was observed on decreasing the mobile phase pH for a nucleoside analogue compound (3′-chloro-2′-deoxythymidine). In this study the protonated molecular ion intensities of 15 nucleoside analogues have been investigated as a function of the mobile phase pH with ammonium acetate buffer, with or without the presence of organic modifiers (methanol or acetonitrile). The reversed-phase HPLC retention data were also measured by varying the mobile phase pH to characterise the hydrophobicity of the compounds at various pH. The octanol/water partition coefficients (log P) were calculated and the pK values were used for characterising their proton affinity in aqueous solutions. Multiple regression analysis was carried out on the collected data in order to reveal the molecular properties influencing the ion formation process at various pH values.

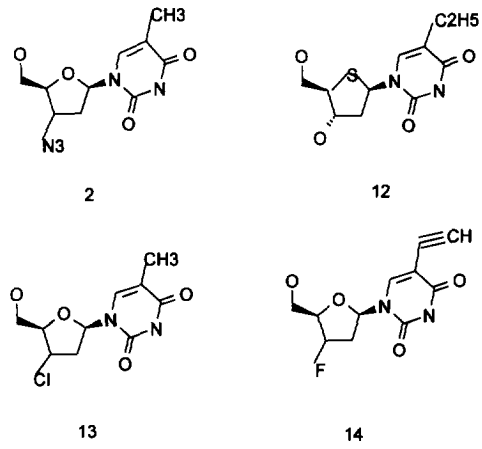

Fig. 1. The chemical structures of the commercially not available nucleoside derivatives.

2. Experimental

The investigated compounds are listed in Table 1. The nucleosides were obtained from Aldrich Chemical (Dorset, UK) with the highest available purity. The remaining nucleoside analogues (structures shown in Fig. 1) were synthesised at Wellcome Research Laboratories (Beckenham, UK) and their purity was chromatographically tested.

The octanol/water partition coefficients (log P) and the acid and base pK values (pK_a and pK_b) were

Table 1
The observed changes in molecular ion intensity [MII (%)] by lowering the mobile phase pH

Compound	MII (%)					
	Methanol (%)					ACN[a] (5%)
	0	5	15	50	85	
1. 2′-Deoxycytidine	−70	−36	−88	−45	−74	0
2. AZT[b]	36	−59	−56	−86	−96	−29
3. Cytidine	−50	−87	−86	−33	−74	−47
4. Uridine	−10	−44	−50	−79	−84	−63
5. Guanosine	−15	−84	−40	7	−65	−67
6. 2′-Deoxyuridine	+10	−78	−87	−71	−82	−33
7. 2′-Deoxyguanosine	−90	+4	−67	24	−59	−68
8. Thymidine	+80	+107	−67	−68	−87	−6
9. Adenosine	0	−9	−80	−59	−91	+21
10. 2′-Deoxyadenosine	0	−5	−55	−51	−91	−2
11. 5-Ethyl-2′-deoxythiouridine[b]	+100	+147	−53	−81	−85	+176
12. Cl-thymidine[b]	+100	+18	−48	−88	−92	+93
13. F-Vynyldeoxyuridine[b]	+80	−51	−66	−68	−76	+30
14. Ethyluracil	+50	+73	−73	−91	−90	+245
15. Methyluridine	−50	−77	−94	−81	−80	+21

[a] ACN = acetonitrile.
[b] The chemical structures of the compounds are shown in Fig. 1.

calculated by PrologP and pKalc programs obtained from CompuDrug Chemistry (Budapest, Hungary).

2.1. HPLC–TSP-MS experiments

Six series of mobile phases were used: 100% water; acetonitrile–water (5:95); methanol–water (5:95); methanol–water (15:85); methanol–water (50:50) and methanol–water (85:15). Ammonium acetate (HPLC grade, Fisons, Loughborough, UK) was added at a concentration of 0.1 *M* to all mobile phases. These mobile phases represented neutral pH as the measured pH was 6.9–7.0. For the lower pH mobile phases, 0.1 *M* ammonium acetate was again added and a portion of the water was replaced with glacial acetic acid (analytical grade, BDH, Poole, UK). The resultant pH values were: [water–glacial acetic acid (93:7)]+0.1 *M* ammonium acetate, pH 3.5; [acetonitrile–water–glacial acetic acid (5:88:7)]+0.1 *M* ammonium acetate, pH 3.5; [methanol–water–glacial acetic acid (5:88:7)]+0.1 *M* ammonium acetate, pH 3.5; [methanol–water–glacial acetic acid (15:75:10)]+0.1 *M* ammonium acetate, pH 3.4; [methanol–water–glacial acetic acid (50:25:25)]+0.1 *M* ammonium acetate, pH 3.4; [methanol–glacial acetic acid (85:15)]+0.1 *M* ammonium acetate, pH 4.4.

A Hewlett-Packard Model 1050 (Hewlett-Packard, Waldbronn, Germany) pump unit, ultraviolet detector and autosampler were used. No column was used and the HPLC effluent passed through a thermospray interface with 1.0 ml/min flow to a Fisons VG TRIO-1000 mass spectrometer (Fisons, Manchester, UK). Positive ion thermospray ionisation was used for all nucleosides and the molecular ion intensity was measured as the $(M+H)^+$ ion. The nucleosides were dissolved in the appropriate water–methanol solvent (no ammonium acetate or acetic acid) to 0.1 mg/ml concentrations. Three injections of 10 μl were made into the HPLC–MS system for each nucleoside at both pH values for each solvent system. The average counts of the three injections were taken. The change of the molecular ion intensity was expressed as a percentage of the obtained value at pH 3.5 in comparison to that of obtained at pH 7 and signed as MII (%). The mass spectrometer conditions were: source temperature 230°C; repeller voltage 140 V and the mass spectrometer scanned from m/z 100 to 400. All other source parameters were tuned on the background ions. The nozzle temperatures used were: 100% water, 248°C; methanol–water (5:95), 245°C; acetonitrile–water (5:95), 245°C; methanol–water (15:85), 240°C; methanol–water (50:50), 205°C; methanol–water (85:15), 190°C.

2.2. HPLC measurements

Waters (Milford, MA, USA) 2×510 pumps with automated gradient controller were used together with a Waters 712 Wisp autosampler and Waters 490e programmable multiwavelength detector. A Zorbax C_{18} 150×4.6 mm I.D. column was purchased from DuPont Instruments (Wilmington, DE, USA) and was maintained at 30°C by an oven unit obtained from Jones Chromatography (Hengoed, Mid-Glamorgan, UK). The mobile phase was 0.1 *M* ammonium acetate for compounds 1, 3, 4, 6 and 10. The mobile phase was modified by adding 2% acetonitrile to the buffer for compounds 5, 7, 8, 9, 11, 15 and 16, and 8% acetonitrile for compounds 2, 12, 13 and 14. The mobile phase pH was decreased by adding glacial acetic acid to the buffer down to pH 3.5. Detection was carried out by UV at 270 nm. Aliquots of 15 μl from the 0.1 mg/ml solutions of the compounds were injected onto the column which contained 0.1% sodium nitrate to detect the dead time value. The log k' values of the compounds were calculated at each pH value.

3. Results and discussion

The observed changes of the molecular ion intensity by decreasing the pH [MII (%)] of the compounds are listed in Table 1. When methanol or acetonitrile was present in greater than 5% concentration lowering the pH resulted in a lower molecular ion intensity in almost all cases. It can be seen that when no organic solvents were present, the positive molecular ion intensity increased on lowering the pH for several analogues. An increase in molecular ion intensity has been observed for nucleosides [7] in the absence of volatile buffer. In the presence of buffer this increase is not expected to occur, as gas phase ionisation is predominating. The

Table 2
The calculated log P, pK_a and measured reversed-phase retention parameters of the investigated nucleosides

Compound	log P	pK_a	pK_b	log $k'_{pH\ 7}$	log $k'_{pH\ 3.5}$
1. 2′-Deoxycytidine	−2.19	13.0[b]	4.2[b]	0.263	−0.835
2. AZT[a]	−3.37	9.5	0	0.910	0.658
3. Cytidine	−2.66	12.3[b]	4.2[b]	0.009	−0.920
4. Uridine	−2.25	9.2b	0	0.230	−0.965
5. Guanosine	−2.56	12.4[b]	1.6[b]	0.523	−0.737
6. 2′-Deoxyuridine	−1.78	9.3	0	0.475	−0.789
7. 2′-Deoxyguanosine	−2.09	12.8[b]	2.5[b]	0.702	−0.536
8. Thymidine	−1.47	9.8	0	0.822	−0.425
9. Adenosine	−1.70	12.5[b]	3.5[b]	0.885	0.168
10. 2′-Deoxyadenosine	−1.24	13.8[b]	3.8[b]	0.976	0.259
11. 5-Ethyl-2′-deoxythiouridine[a]	−0.31	9.6	0	0.729	0.418
12. Cl-Thymidine[a]	−0.15	9.5	0	0.969	0.718
13. F-Vynyldeoxyuridine[a]	−0.82	8.5	0	0.721	0.404
14. Ethyluracil	−0.20	9.6	5.5	0.814	0.005
15. Methyluridine	−1.94	9.5	0	0.584	−0.613

[a] The chemical structures of the compounds are shown in Fig. 1.
[b] Measured values from Ref. [13].

lower pH, and hence a higher proton concentration in the liquid phase, can enhance the positive molecular ion intensity when the liquid phase ionisation dominates the ion formation.

Thymidine analogues which have an alkyl or alkynyl substituent at position 5 and do not have a hydroxyl group at the 2′ position of the sugar showed higher molecular ion intensity at lower pH than at neutral pH (compounds: 2, 8, 11, 12, 13).

The hydrophobicity of the compounds was characterised by their calculated octanol/water partition coefficients (log P). The proton affinity of the compounds was characterised by their literature [12] or calculated pK_a values. These parameters are listed in Table 2. The partition coefficient of the compounds were modelled also by their reversed-phase chromatographic retention data (log k') measured at pH 7 and pH 3.5. The log k' values were extrapolated to the 0% acetonitrile for all compounds to have more comparable relative retention parameters. The retention parameters of the compounds are listed in Table 2.

Step-wise linear regression analysis was carried out on the data using the molecular intensity change [MII (%)] values as dependent variables and the calculated log P, pK_a and the measured log k' values as independent variables.

The correlation coefficients between the variables are summarised in a correlation matrix in Table 3. Significant correlations (above the 95% probability level) were found with the log P, log $k'_{pH\ 3.5}$ and pK_a values of acidic groups. From these simple correlations it can be seen that the more hydrophobic are the compounds the higher the increase in the molecular ion intensity at low pH can be expected. A longer retention time (higher log $k'_{pH\ 3.5}$) means a higher hydrophobicity at low pH. The presence of weak acidic group also enhance the chance for the highly

Table 3
The correlation coefficients between the investigated parameters based on the data of 15 compounds listed in Tables 1 and 2

	MII (%)	log P	pK_a	pK_b	log $k'_{pH\ 7}$	log $k'_{pH\ 3.5}$
MII (%)	1.00	0.65	−0.67	0.43	0.55	0.70
log P		1.00	−0.32	0.01	0.46	0.49
pK_a			1.00	0.67	−0.10	−0.25
pK_b				1.00	−0.15	−0.17
log $k'_{pH\ 7}$					1.00	0.82
log $k'_{pH\ 3.5}$						1.00

positive [MII (%)] value. Applying multiple regression calculations and including both the hydrophobicity and pK_a values, the best correlations obtained are described by Eq. 1 and Eq. 2.

$$\text{MII (\%)} = 31.5(\pm 11.4)\log P - 16.7(\pm 5.8)\,pK_a + 244.6 \quad (1)$$

$n = 15,\ r = 0.812,\ s = 38.8,\ F = 11.6$

$$\text{MII (\%)} = 58.0(\pm 15.2)\log k'_{\text{pH 3.5}} - 17.1(\pm 4.9)\,pK_a + 208.0 \quad (2)$$

$n = 15,\ r = 0.865,\ s = 33.4,\ F = 17.8$

Fig. 2 shows the fit of the measured and recalculated MII (%) values by Eq. 2. Both equations suggest, that the more hydrophobic are the compounds, the higher will be the molecular ion intensity increase upon lowering the mobile phase pH.

It is of interest to note that the compounds, which showed the domination of liquid phase ionisation have two structural features: they are substituted at the 5 position of the uracil base ring and have a 2′-deoxy sugar attached. It has been reported [13] that uridine type nucleosides have intramolecular hydrogen bonding between the 2′-hydroxyl group and the 2-carbonyl group on the base. This hinders intermolecular hydrogen bond formation by the carbonyl group. In the absence of the 2′-hydroxyl group, intermolecular hydrogen bonding becomes possible and may result in strongly hydrogen-bonded solvent clusters, particularly with water.

In order to reveal that these structural features are essential to observe positive MII (%) values, another 3 compounds (structures are shown in Fig. 3) were investigated. On the basis of their calculated pK_a and $\log P$ values, a positive effect was predicted for compounds 16 and 18 (95% and 101%), although they do not show the above-mentioned structural criteria for the positive MII (%) value. A negative MII (%) was predicted for compound 17 (−29%). The actual measured values (104, 12 and 46% for compound 16, 17 and 18, respectively) were within the 95% confidence interval based on the standard error of the estimate of Eq. 1. This implies that the correlation equation provides acceptable prediction.

Considering the MII (%) values obtained by applying 5% acetonitrile in the mobile phase (see Table 1) Eq. 3 was obtained:

$$\text{MII (\%) (5\% ACN)} = 60.2(\pm 13.7)\log P + 23.4(\pm 8.2)\,pK_b - 25.1(\pm 9.3)\,pK_a + 349.9 \quad (3)$$

$n = 15,\ r = 0.901,\ s = 44.1,\ F = 15.8$

The equation suggests that the $\log P$ values and both the pK_a and pK_b values have a significant impact on the pH effect of the molecular ion

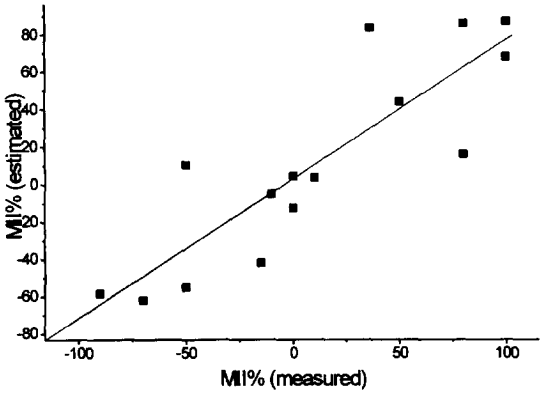

Fig. 2. The plot of the measured and estimated MII (%) values on the basis of Eq. 2.

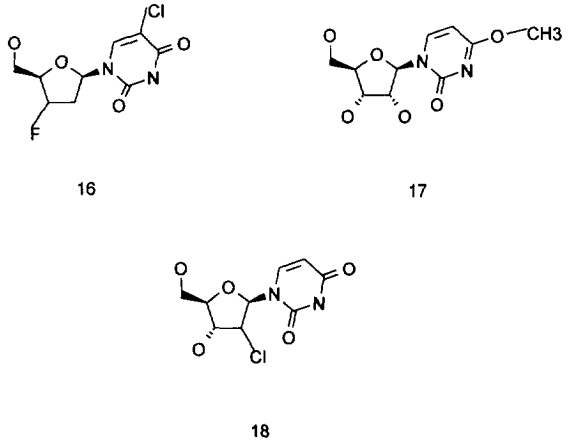

Fig. 3. The chemical structures of the trial compounds.

intensity in the presence of 5% acetonitrile. When MII (%) values obtained with 5% methanol were investigated, only the log P values showed significant impact ($r=0.672$, $F=10.7$).

At higher methanol concentrations all of the compounds showed similar negative MII (%) values, which means a decrease of the molecular ion intensity by decreasing the mobile phase pH. No correlations with the physico-chemical parameters of the compounds were observed in these instances.

From the results it can be concluded that the hydrophobic and acidic properties of the compounds have a high impact on the positive molecular ion formation in thermospray ionisation mode in the presence of ammonium acetate buffer in the mobile phase with lower than 5% organic phase concentration. In the case of hydrophobic nucleosides with lower pK_a values the positive molecular ion intensity can be increased by decreasing the mobile phase pH. This facilitates the sensitivity of the HPLC–TSP-MS detection of such compounds and suggests that liquid phase ionisation dominates the ion formation process.

The presence of methanol or acetonitrile significantly influenced the ionisation process. Some of the nucleoside derivatives showed negative MII (%) values at any methanol concentration (i.e. cytidine), some of them showed an increased positive MII (%) value by increasing the methanol concentration to 5% (i.e. thymidine) and some showed a significant decrease of MII (%) value by adding methanol in the mobile phase (i.e. F-vynyldeoxyuridine).

From the above results it can be concluded that the positive MII (%) values reflect the dominance of the liquid phase ionisation process. The ionisation process is strongly influenced by the presence of the organic modifier which changes the liquid phase solvation which can be related to the hydrophobicity (log P) values of the compounds.

In conclusion, in the case of some nucleoside derivatives the mobile phase pH and organic modifier content influenced the positive molecular ion formation in thermospray ionisation with the presence of ammonium acetate buffer. The hydrophobic character and the pK values of the compounds showed correlation with the mobile phase effect indicating the importance of the solvation and the proton affinity of the nucleosides.

Acknowledgments

The authors would like to thank Professor J.L. Holmes, University of Ottawa, for his helpful comments and suggestions with this study.

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