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Nitrobenzenes in reversed-phase liquid chromatography. New candidates for internal standards

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Abstract

The utility of nitrobenzenes as candidates for internal standards in reversed-phase HPLC was investigated. Among many kinds of commercial reagents, 21 nitrobenzenes were selected as candidates, based on the chromatographic results obtained under various conditions. These have higher UV absorption within a wider range of wavelengths than the phenols previously reported. The wavelength range extends up to at least 310 nm and the molar absorptivity is more than $500 \ 1 \ mol^{-1} \ cm^{-1}$. The twenty-one nitrobenzenes showed a constant order of elution regardless of the separation conditions. The mobile phases consisted of 20–70% aqueous–organic solutions (organic solvent = acetonitrile, methanol, tetrahydrofuran and mixtures thereof). The packing materials used were hydrocarbon-bonded silica gels (C_1, phenyl, C_8 and C_{18} columns). Moreover, the capacity factors of the 21 nitrobenzenes were independent of the acidity and basicity of mobile phases within the pH range 2.5–12.0 with an octadecyl-bonded polymer gel.

1. Introduction

Our previously reported 31 phenols are very useful in systematically searching for a suitable internal standard in RP-HPLC using a hydrocarbon-bonded silica gel column [1,2] because they show a constant elution order over a wide range of HPLC conditions. We have been using them as first-choice candidates for internal standards, and the most suitable internal standard can usually be selected from among them. However, some phenols do not show very high UV absorption over a wide range of wavelengths owing to their spectrophotometric characteristics. Therefore, the detectable wavelength range is restricted to within a narrow range when using

several phenols, e.g., halogenated phenols and alkyl parabens.

Detection at long wavelengths is useful for eliminating interferences from other components of a complex mixture or the background of the mobile phase.

On the other hand, the capacity factor of each phenol is independent of the pH of mobile phase (within the pH range 2.0–7.0). In general, it is recommended that the mobile phase should be controlled in the acidic to neutral range in RP-HPLC using the chemically bonded silica gel columns. In recent years, chemically bonded silica gels that are stable even with alkaline (up to pH 10) mobile phases have been developed and are now commercially available [3]. Another

type of packing material, the support of which is not silica gel, can be obtained and is stable in alkaline (pH 13) mobile phases [4]. Using these packing materials, alkaline mobile phases can be utilized in RP-HPLC. However, at pH >7.0, the capacity factor of each phenol changes in accordance with its pK_a (7.5–10.2). Alkyl 4-hydroxybenzoates are not stable in alkaline solutions.

As new candidates for internal standards in RP-HPLC, we have searched for other benzene derivatives that should satisfy the following conditions: (1) have high UV absorption within a wide wavelength range, at least up to 320 nm; (2) have no dissociating group; and (3) be commercially available and usable without further purification.

Nitrobenzene derivatives satisfy these criteria. Nitrobenzene has a high UV absorption within a wide wavelength range, owing to the resonance between the phenyl ring and nitro group, which is a chromophore. It contains no dissociating group. There are several reports relevant to aromatic nitro compounds [5–7] and 1-nitroalkanes as retention index standards [8] in RP-HPLC. In this paper, we report the results for a series of 21 nitrobenzenes that are potential internal standards in RP-HPLC and their comparison with phenols.

2. Experimental

2.1. Chemicals

Nitrobenzenes were purchased from Wako (Osaka, Japan), Tokyo Kasei (Tokyo, Japan) and Lancaster (Morecambe, UK) and were used as received.

2.2. Apparatus

The HPLC system consisted of a Model 510 pump, a Model 440 detector fixed at 254 and 280 nm and a WISP 710B automatic sample injector (Waters, Milford, MA, USA). Chromatograms were recorded on a LabChart 180 (System Instrument, Tokyo Japan). For measurement of the UV spectra of nitrobenzenes, a U-3200 spectrophotometer (Hitachi, Tokyo Japan) was used.

Table 1
HPLC conditions employed for evaluating the chromatographic behaviour of nitrobenzenes and influence of packing material and mobile phase

Stationary phase				Mobile phase[b]
Material	Bonded group	Carbon content (%)[a]	End-capping	
Nucleosil 5C$_{18}$	C$_{18}$	14	Treated	ACN–water (20:80, 30:70, 40:60, 50:50, 60:40)
				McOH–water (30:70, 40:60, 50:50, 60:40, 70:30)
				ACN–MeOH–water (20:20:60)
				ACN–THF–water (30:10:60)
				ACN–20 mM Na$_2$SO$_4$ (40:60)
				ACN–5 mM sodium octanesulfonate (pH 2.5) (40:60)
μBondapak C$_{18}$	C$_{18}$	10	Treated	ACN–water (40:60)
Zorbax ODS	C$_{18}$	10	Untreated	ACN–water (40:60)
Nucleosil 5C$_8$	C$_8$	9	Untreated	ACN–water (40:60)
Cosmosil 5Ph	C$_6$H$_5$	9	Treated	ACN–water (40:60)
Shodex TMS	C$_1$	5	Treated	ACN–water (40:60)
Asahikak ODP-50	C$_{18}$	17	–	ACN–water (40:60)
				ACN–10 mM KH$_2$PO$_4$ (pH 2.5, 7, 12) (40:60)

[a] Values taken from manufacturers' catalogues.
[b] ACN = acetonitrile; MeOH = methanol; THF = tetrahydrofuran.
[c] pH adjusted with 85% phosphoric acid.

2.3. Analytical columns and mobile phases

In the chromatography of nitrobenzenes, the analytical columns and mobile phases used in this study were equivalent to those reported previously [1]. The analytical conditions are summarized in Table 1. In the chromatography using alkaline mobile phase, the following conditions were used: column, Asahipak ODP-50 (15 cm × 4.6 mm I.D.) (Asahikasei, Tokyo, Japan) [4]; mobile phase, acetonitrile–10 mM KH_2PO_4 (pH 2.5, 7.0 and 12.0, adjusted with 85% phosphoric acid or 1 M sodium hydroxide) (40:60); and flow-rate, 0.5 ml min^{-1}.

2.4. Sample preparation and chromatographic procedure

A 20-mg amount of each nitrobenzene was weighed accurately and dissolved in methanol to make exactly 100 ml [0.02% (w/v) solution]. A 5-μl volume of sample solution was injected for HPLC determination. The flow-rate was adjusted to between 0.5 and 1.0 ml min^{-1} to keep the column pressure below 2000 p.s.i. at 25°C. The retention time of uracil was taken as the column void volume and used for the calculation of the capacity factor of each nitrobenzene.

2.5. UV spectrophotometric procedure

Sample solutions for HPLC were diluted exactly fivefold with methanol and were scanned from 210 to 500 nm with reference to methanol.

3. Results and discussion

UV spectra of phenol and nitrobenzene in methanol are shown in Fig. 1. Apparently, nitrobenzene has advantageous spectrophotometric properties with respect to sensitivity and detectable wavelength range.

The capacity factors of all the nitrobenzenes decreased with increase in the content of organic solvent (acetonitrile or methanol) and increased in order of the hydrophobicity of the bonded alkyl groups ($C_{18} > C_8 \approx$ phenyl > methyl).
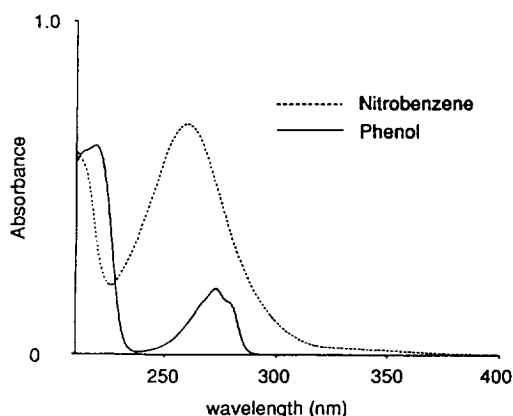

Fig. 1. UV spectra of (dotted line) nitrobenzene and (solid line) phenol in methanol solution. Concentration of each solution is 0.001 mg/ml.

These results agreed with the general retention tendencies in RP-HPLC.

Many nitrobenzenes were rejected as candidates for internal standards on the basis of the

Table 2
Series of nitrobenzene derivatives selected as potential internal standards in RP-HPLC

No.	Derivative	Substituent	λ (nm)[a]
1	1,3-Diamino-4-nitrobenzene	2,4-$(NH_2)_2$	450
2	4-Nitroaniline	4-NH_2	440
3	3-Nitroaniline	3-NH_2	430
4	4-Nitroacetanilide	4-$NHCOMe$	370
5	2-Amino-4-nitrobenzene	3-NH_2-4-Me	430
6	4-Nitroacetophenone	4-COMe	330
7	2,4-Dinitroaniline	3-NO_2-4-NH_2	430
8	Nitrobenzene	H	310
9	2-Amino-3-nitrotoluene	2-NH_2-3-Me	480
10	2-Nitrotoluene	2-Me	320
11	4-Nitrotoluene	4-Me	320
12	3-Nitrotoluene	3-Me	320
13	4-Bromonitrobenzene	4-Br	320
14	3-Bromonitrobenzene	3-Br	320
15	2,4-Dimethylnitrobenzene	2,4-Me_2	350
16	4-Chloro-2-nitrotoluene	2-Me-5-Cl	320
17	2-Chloro-4-nitrotoluene	4-Me-3-Cl	320
18	3,5-Dichloronitrobenzene	3,5-Cl_2	320
19	2-Nitromesitylene	2,4,5-Me_3	350
20	2,4,5-Trichloronitrobenzene	2,4,5-Cl_3	320
21	4-tert-Butylnitrobenzene	4-tert-Bu	320

Maximum available detectable wavelength at which the molar absorptivity is more than 500 l mol^{-1} cm^{-1}.

same criteria as reported previously [1] and also toxicological data [9–11].

We selected 21 nitrobenzenes as the candidates for potential internal standards in RP-HPLC, and they are listed in Table 2 in order of elution with the practically available maximum detection wavelengths at which the molar absorptivities are more than 500 l mol^{-1} cm^{-1}. The capacity factors (k') of the 21 nitrobenzenes are given in Table 3 and 4. The nitrobenzenes were eluted every 2–5 min within a 20-min operating period under the conditions given in Table 1.

1,3-Diamino-4-nitrobenzene, 4-nitroanaline, 3-nitroaniline, 2-amino-4-nitrotoluene, 2,4-dinitroaniline and 2-amino-3-nitrotoluene each have one or two amino groups (dissociating group), being aniline derivatives and basic compounds. Their peak shapes were sharp and symmetrical, and showed no tailing under all the HPLC conditions summarized in Table 1, even though

uncapped columns where residual silanol groups were not treated, Zorbax ODS and Nucleosil 5C$_8$, were used. As expected, their capacity factors were independent of the pH (2.5, 7.0 and 12.0) of the mobile phase in which the concentration of acetonitrile was fixed at 40% on the Asahipak ODP-50 column.

These phenomena can be explained as follows. Anilines with a nitro group bonded at the position of the benzene ring have low pK_a values, owing to the electron-inductive effect of the nitro group and a direct resonance effect between the amino and nitro groups. For example, the pK_a values of 4- and 3-nitroaniline are 1.02 and 2.50, respectively. Hence the basicity of the amino group in nitrobenzenes is very weak, and the pK_a may not be more than 3.0. Accordingly, the interaction between amino and residual silanol groups seems to be very slight or negligible.

The retention times and peak shapes of the 21

Table 3
Capacity factors (k') of 21 nitrobenzenes on Nucleosil 5C$_{18}$ column

| Nitrobenzene[a] | Mobile phase | | | | | | | | | |
| | Acetonitrile–water[b] | | | | | Methanol–water[b] | | | | |
	20	30	40	50	60	30	40	50	60	70
2,4-(NH$_2$)$_2$	2.335	1.327	0.853	0.576	0.331	1.675	0.897	0.463	0.260	0.141
4-NH$_2$	5.665	3.047	1.767	1.101	0.574	3.565	1.974	1.037	0.578	0.304
3-NH$_2$	7.015	3.895	2.221	1.342	0.695	4.170	2.454	1.368	0.765	0.413
4-NHCOMe	7.951	3.439	1.767	1.044	0.546	8.595	4.062	2.047	1.064	0.554
3-NH$_2$-4-Me	13.98	6.561	3.423	1.918	0.924	9.814	5.088	2.500	1.274	0.658
4-COMe	13.50	6.444	3.337	1.886	0.939	10.47	5.366	2.716	1.402	0.728
3-NO$_2$-4-NH$_2$	14.32	6.357	3.135	1.677	0.796	10.73	5.206	2.884	1.534	0.799
H	15.33	8.058	4.307	2.449	1.185	11.59	6.686	3.547	1.912	1.011
2-NH$_2$-3-Me	21.76	9.526	4.589	2.456	1.170	20.37	9.062	4.405	2.137	1.065
2-Me	34.92	15.12	7.018	3.551	1.578	27.58	13.95	6.426	3.039	1.467
4-Me	37.36	16.58	7.577	3.779	1.673	30.35	14.35	6.958	3.304	1.555
3-Me	41.17	17.45	7.878	3.968	1.740	32.57	16.36	7.600	3.627	1.707
4-Br	50.96	21.89	9.601	4.589	1.950	35.46	18.15	8.600	3.966	1.848
3-Br	56.19	23.40	10.13	4.823	2.058	45.54	20.54	10.62	4.980	2.326
2,4-Me$_2$	–	31.50	12.29	5.551	2.276	–	32.75	13.59	5.623	2.440
2Me-5-Cl	–	36.25	14.17	6.291	2.501	–	39.10	16.33	6.931	3.000
4-Me-3-Cl	–	39.72	15.48	6.791	2.743	–	39.47	18.85	8.078	3.494
3,5-Cl$_2$	–	42.67	16.79	7.405	2.934	–	42.89	19.73	8.706	3.897
2,4,5-Me$_3$	–	63.69	21.44	8.519	3.159	–	67.14	25.93	9.456	3.744
2,4,5-Cl$_3$	–	69.26	23.83	9.532	3.455	–	72.94	28.44	11.29	4.663
4-tert-Bu	–	110.6	33.60	12.20	4.171	–	109.7	41.17	14.17	5.244

[a] Each nitrobenzene is expressed using abbreviations of substituents (see Table 2).
[b] The contents (%) of organic solvents in the mobile phases are given.

Table 4
Retention data for nitrobenzenes in acetonitrile water (40:60) with various columns

Nitrobenzene[a]	Column						
	Nucleosil 5C$_{18}$	μ Bondapak C$_{18}$	Zorbax ODS	Nucleosil 5C$_8$	Cosmosil 5Ph	Shodex TMS	Asahipak ODP-50
2,4-(NH$_2$)$_2$	0.853	0.799	0.660	0.781	0.868	0.764	2.096
4-NH$_2$	1.767	1.557	1.359	1.415	1.500	1.254	3.664
3-NH$_2$	2.221	1.872	1.861	1.620	1.743	1.501	4.477
4-NHCOMe	1.767	1.480	1.357	1.446	1.297	1.210	1.795
3-NH$_2$-4-Me	3.423	2.710	2.838	2.220	2.330	1.941	5.900
4-COMe	3.337	2.652	2.848	2.124	2.282	1.974	3.867
3-NO$_2$-4-NH$_2$	3.135	2.587	2.409	2.030	2.214	1.891	6.144
H	4.307	3.323	3.915	2.573	2.643	2.152	5.624
2NH$_2$-3-Me	4.589	3.456	3.865	2.689	2.743	2.217	7.459
2-Me	7.018	5.077	6.450	3.686	3.784	3.076	8.211
4-Me	7.577	5.443	6.949	3.814	3.820	3.186	8.755
3-Me	7.878	5.747	7.330	3.995	3.962	3.228	9.296
4-Br	9.601	6.787	9.053	4.405	4.617	3.699	15.123
3-Br	10.135	7.049	9.620	4.615	4.562	3.620	14.847
2,4-Me$_2$	12.288	8.508	11.578	5.472	5.363	4.517	13.095
2-Me-5-Cl	14.165	9.617	13.554	5.990	5.878	4.743	17.370
4-Me-3-Cl	15.478	10.304	15.195	6.144	5.840	4.809	18.760
3,5-Cl$_2$	16.791	11.179	17.588	6.299	6.172	5.006	23.039
2,4,5-Me$_3$	21.435	14.051	20.376	8.200	7.987	6.597	19.950
2,4,5-Cl$_3$	23.828	15.781	23.617	8.537	8.918	6.738	34.033
4-tert-Bu	33.601	21.881	32.435	11.181	10.980	9.247	31.373

[a] Each nitrobenzenes is expressed using abbreviations of substituents (see Table 2).

nitrobenzenes were independent of the counter ion and ionic strength of the mobile phase. They maintained a constant elution order on all the columns studied with the exception of the Asahipak ODP column. The retention behaviour of the 21 nitrobenzenes on the latter column was different from that on the hydrocarbon bonded silica gel columns. The capacity factors of the 21 nitrobenzenes are plotted logarithmically in Fig. 2. ODP gel can be obtained by introduction of octadecyl groups in place in hydroxyl groups in vinyl alcohol copolymers [4]. The difference in selectivity between the two types of packing materials may be mainly due to the difference in the supports (silica gel vs. vinyl alcohol copolymer). No satisfactory explanation could be found for this behaviour.

The difference in retention behaviours of several nitrobenzenes between a ODS column and a polystyrene gel column, using Smejkal et
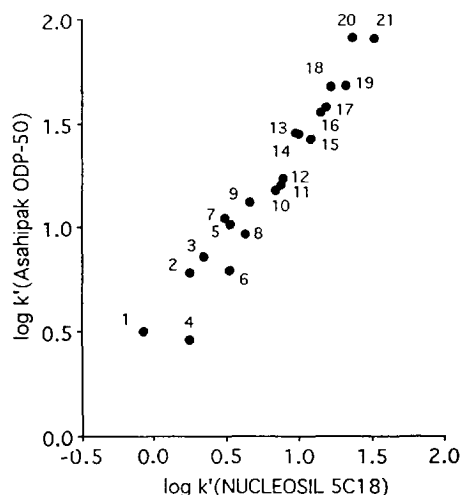

Fig. 2. Log k'(Nucleosil 5C$_{18}$) vs. log k'(Asahipak ODP) of nitrobenzenes in acetonitrile–water(40:60). Nitrobenzenes are expressed using the numbers listed in Table 2.

al's retention data [7], could be discussed, but this is omitted in this paper.

4. Conclusion

A large number of commercial nitrobenzenes were chromatographed under different conditions including alkaline mobile phases, and 21 nitrobenzenes were selected as candidates for internal standards in RP-HPLC. These maintained a constant elution order over a wide range of RP-HPLC conditions, including alkaline mobile phases (up to pH 12).

Compared with the previously reported phenols, the nitrobenzenes have the following advantages: (1) nitrobenzenes maintain a constant elution order even at mobile phase pH values in the range 2.5–12.0; (2) the practically available detection wavelength range is at least 200–310 nm. We expect that it will become easier and quicker to choose a suitable internal standard from these 21 nitrobenzenes in combination with the previously reported phenols.

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