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Phenols as internal standards in reversed-phase high-performance liquid chromatography in pharmaceutical analysis

SHIRO YAMAUCHI* and HIDEO MORI
Analytical Development, Formulation Research Institute, Otsuka Pharmaceutical Co., Ltd., 463–10 Kagasuno, Kawauchi-cho, Tokushima 771-01 (Japan)

ABSTRACT

To find a series of compounds for use as internal standards in reversed-phase high-performance liquid chromatography, about 70 commerically available phenols were chromatographed under various conditions. The stationary phases used were hydrocarbon chemically bonded silica gels (phenyl, C_8 and C_{18} columns). The mobile phases consisted of 10–60% aqueous–organic solutions (organic solvents, methanol, acetonitrile, tetrahydrofuran and mixtures thereof; aqueous phases, water, buffer solutions of pH 2 and 7, 20 m*M* sodium sulphate or water containing 5 m*M* of counter ion). A series of 30 phenol derivatives showed a constant order of elution regardless of the separation conditions and are useful as potential internal standards.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) using hydrocarbon chemically bonded silica gels has widely been used for the determination of a large number of drugs and chemicals. An internal standard technique has often been employed for the determination of drug substances and finished drug products as errors in the analytical measurements are usually reduced.

Finding a suitable internal standard, however, is empirical and often involves extensive trial-and-error experiments[1]. Moreover, an internal standard chosen for a particular set of conditions is sometimes useless under other conditions and a new one must be found again. The reason is that the retention times of target compounds vary and the profiles for their impurities or degradation products are often inconsistent. If a series of commercially available compounds that elute every few minutes with a consistent order under different separating conditions could be found, then choosing an internal standard would be facilitated because a new one could be found promptly as required among the established series.

With similar aims, systematic approaches to finding internal standards were reported by Verzele *et al.*[2] using anilides and by Kikta and Stange[3] using alkyl aryl

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ketones. However, most of the anilides selected were not commercially available and the alkyl aryl ketones were not applicable to the analysis of compounds that elute with mobile phases containing less than 50% of methanol.

There have been many studies aimed at finding standard compounds for retention indices applicable to the identification of drugs, such as Smith's work on alkyl aryl ketones[4,5], Baker and Ma's work on 2-ketoalkanes[6] and Bogusz and Aderjan's work on 1-nitroalkanes[7]. These studies were successful at finding scales of retention indices, and these reference standards for retention index scaling also seem to be useful as internal standards. Wells and Clark[8–10] also reported the retention behaviours of alkylbenzamides, but most of them are not commercially available.

As retention behaviour is very dependent on chemical structure[11,12], we considered that compounds with a fixed functional group that serves as a retention time determinant should be explored. Alkyl 4-hydroxybenzoates, which are a group of phenol derivatives, are often used as internal standards in official analytical methods for pharmaceutical products[13]. Retention behaviours of phenol derivatives have been reported by Miyake *et al.*[11], Hanai and Hubert[14] and Callmer *et al.*[15], and it is well known that phenol derivatives are eluted with a wide range of mobile phases. We therefore selected them as model compounds in a search for widely applicable internal standards.

In addition, many phenol derivatives are soluble in aqueous organic solvents and have dissociation constants (pK_a) higher than 7.5. This property is of great importance as the pH of mobile phases in RP-HPLC using chemically bonded silica gel as stationary phases is usually kept within the range 2–7 (ref. 16). Phenols respond well to UV spectrophotometric detectors, which are widely used in HPLC. Other advantages of phenols are that they are commercially available in high purity and are generally inexpensive. This is an important point as the analytical methods developed by us are often used in different locations.

With the aim of finding a series of compounds as practical internal standards useful for quality control analyses, we studied the chromatographic behaviour of commercial phenols with estimated pK_a values of more than 7 (refs. 17 and 18). We report here studies that resulted in finding a series of 30 phenol derivatives that are potential internal standards in RP-HPLC.

EXPERIMENTAL

Chromatographic apparatus

Two HPLC systems were used. One consisted of a Model 510 solvent delivery system, a WISP 710A automatic sample injection system and a Model 440 fixed-wavelength UV detector (Waters Assoc., Milford, MA, U.S.A.). The detector was set at 254 and 280 nm. The other consisted of a Model M600 solvent delivery system, a WISP 710B automatic sample injection system and a Model 481 variable-wavelength UV detector operated at 254 nm (Waters Assoc.). Chromatograms were recorded on either a Chromatopak C-R3A integrator (Shimadzu, Kyoto, Japan) or a SIC 7000A integrator (System Instruments, Tokyo, Japan).

Chemicals

All phenol derivatives were obtained from Tokyo Kasei (Tokyo, Japan) or

Wako (Osaka, Japan) and used without further purification. PIC B-8 reagent for ion-pair chromatography was obtained from Waters Assoc.

Analytical columns

The commercially available prepacked columns shown in Table I were used.

Mobile phase

LC-grade methanol (MeOH; Me = CH_3), acetonitrile (ACN) and tetrahydrofuran (THF) were used. Water was deionized and purified with a Milli-R/Q water purifier (Millipore, Bedford, MA, U.S.A.).

The mobile phases were 10–60% aqueous organic solutions consisting of the above organic solvents or mixtures thereof and aqueous phases which were water, 10 m*M* KH_2PO_4 solution (pH 2), 10 m*M* Na_2HPO_4 solution (pH 7), 20 m*M* sodium sulphate solution (ionic strength 0.06) or water containing a counter ion (5 m*M* octylsulphonate). The 10 m*M* KH_2PO_4 and 10 m*M* Na_2HPO_4 solutions were adjusted to pH 2–7 with 85% phosphoric acid.

Sample preparation and chromatographic procedure

A 50-mg amount of each phenol derivative was weighed accurately and dissolved in methanol to make exactly 100 ml (0.05% solution). A 10-μl volume of each sample solution was injected for HPLC determination. The flow-rate was adjusted to between 0.5 and 1.0 ml/min to keep the column pressure below 2000 p.s.i. at about 25°C.

The retention behaviours were evaluated by correlations of the logarithmic plots of capacity factors under different conditions. The retention time of uracil was taken as t_0 and was used to determine the capacity factors of the phenol derivatives.

RESULTS AND DISCUSSION

The phenol derivatives listed in Table II were chromatographed under various HPLC conditions as summarized in Table III. These conditions were set up to examine the effects of the separation conditions on the chromatographic behaviours of the phenols, *e.g.*, the structure of the bonded group, the degree of unreacted silanol groups

TABLE I

ANALYTICAL COLUMNS USED

Stationary phase	*Dimensions*	*Manufacturer*
Nucleosil $5C_{18}$ (5 μm)	15 cm × 4.6 mm I.D.	Macherey, Nagel & Co. (Düren, F.R.G.)
YMC Pack ODS-A, A-302 (5 μm)	15 cm × 4.6 mm I.D.	Yamamura Chemical (Kyoto, Japan)
μBondapak C_{18} (10 μm)	30 cm × 3.9 mm I.D.	Waters Assoc. (Milford, MA, U.S.A.)
Chemcosorb 5-ODS-H (5 μm)	15 cm × 4.6 mm I.D.	Chemco Scientific (Tokyo, Japan)
Zorbax ODS (5 μm)	25 cm × 4.6 mm I.D.	DuPont (Wilmington, DE, U.S.A.)
Cosmosil 5Ph (5 μm)	15 cm × 4.6 mm I.D.	Nakarai Tesque (Kyoto, Japan)
Chemcosorb $10C_8$ (10 μm)	15 cm × 4.6 mm I.D.	Chemco Scientific

TABLE II

PHENOL DERIVATIVES USED

The structure and name of each phenol are expressed using abbreviations of substituents: Me = CH_3–; Et = C_2H_5–; *iso*-Pr = $(CH_3)_2CH$–; *n*-Pr = $CH_3(CH_2)_2$–; *sec.*-Bu = $CH_3CH_2CH(CH_3)$–; *n*-Bu = $CH_3(CH_2)_3$–; *tert.*-Bu = $(CH_3)_3C$–; *iso*-Am = $(CH_3)_2CH(CH_2)_2$–; *n*-Hex = $CH_3(CH_2)_5$–; *n*-Hep = $CH_3(CH_2)_6$–; Oc = $CH_3(CH_2)_3CH(C_2H_5)CH_2$–; *n*-No = $CH_3(CH_2)_8$–; Ph = C_6H_5–.

2-OMe	3-OMe	4-OMe	2-OEt
4-O*n*-Bu	2-OCH_2Ph	4-OCH_2Ph	4-*iso*-Pro
4-*tert.*-Bu	2,3-Me_2	2,4-Me_2	2,6-Me_2
2-CH_2Ph	4-Ph	2,3,5-Me_3	2-*tert.*-Bu-4-OMe
3-Me-6-*tert.*-Bu	2-Me-4-OH	2-CH_2OH	H
4-CH_2OH	2-NHCOMe	3-NHCOMe	4-NHCOMe
3-OH	3-OH-5-Me	2-OMe-4-CHO	4-Cl
4-Br	2,3-Cl_2	2,4-Cl_2	2,5-Cl_2
2,4-Br_2	2,4,6-Cl_3	4-Cl-2-NO_2	2-NO_2
3-NO_2	4-NO_2	2-CN	4-CN
2-COMe	3-COMe	4-COMe	4-COEt
4-CO*n*-Bu	2-CHO	3-CHO	4-CHO
2-COOMe	2-COOEt	2-COO*iso*-Pr	2-COO*sec.*-Bu
2-COO*n*-Bu	2-COOPh	2-$COOCH_2Ph$	4-COOMe
4-COOEt	4-COO*iso*-Pr	4-COO*n*-Pr	4-COO*iso*-Bu
4-COO*sec.*-Bu	4-COO*n*-Bu	4-COOPh	4-$COOCH_2Ph$
4-COO*iso*-Am	4-COO*n*-Am	4-COO*n*-Hex	4-COO*n*-Hep
4-COOOc	4-COO*n*-No		

TABLE III

HPLC CONDITIONS EMPLOYED FOR EVALUATING THE CHROMATOGRAPHIC BEHAVIOUR OF PHENOLS: INFLUENCE OF ANALYTICAL COLUMNS AND MOBILE PHASES

Stationary phase				*Mobile phase*
Material	*Bonded group*	*Carbon content (%)*[a]	*End-capping*	
Nucleosil $5C_{18}$	C_{18}	14	Treated	ACN–water (10:90, 20:80, 30:70, 40:60, 50::50, 60:40) MeOH–water (40:60, 50:50, 60:40) ACN–MeOH–water (20:20:60) ACN–THF–water (25:5:70) ACN–MeOH–THF–water (10:10:10:70) ACN–20 m*M* Na_2SO_4 (30:70) ACN–10 m*M* phosphate buffer (pH 2.5) (30:70) ACN–10 m*M* phosphate buffer (pH 7.0) (30:70) ACN–5 m*M* octylsulphonic acid (30:70)
YMC Pack ODS-A, A-302	C_{18}	17	Treated	ACN–water (20:80, 30:70, 40:60, 50:50)
μBondapak C_{18}	C_{18}	10	Treated	ACN–water (30:70)
Chemcosorb 5-ODS-H	C_{18}	20	Treated	ACN–water (30:70)
Zorbax ODS	C_{18}	10	Untreated	ACN–water (30:70)
Cosmosil 5Ph	C_6H_5	9	Treated	ACN–water (30:70), MeOH–water (30:70)
Chemcosorb $10C_8$	C_8	9.5	Treated	ACN–water (30:70)

[a] Values taken from manufacturers' catalogues.

of the stationary phases, type and content of organic solvents in mobile phases, pH and ionic strength of the mobile phases[19] and counter ion added to the mobile phases.

The capacity factors of all the phenols decreased with increase in concentration of organic solvent in the mobile phase, and increased in order of the hydrophobicity of the bonded alkyl group ($C_{18} > C_8 >$ phenyl). These results seem to agree with general retention tendencies in RP-HPLC.

Many phenols were rejected as candidates for internal standards, as they showed undesirable peak shapes such as leading or tailing or impurity peaks of more than 1%. The phenols of which the capacity factors varied with pH, ionic strength or counter ion of the mobile phase were also rejected. In addition, phenols with deviations from the correlation line for logarithmic plots were rejected. However, three bicyclic compounds (4-hydroxybiphenyl, 4-hydroxybenzophenone and benzyl 4-hydroxybenzoate) were not rejected as the deviations from the correlation line for logarithmic plots, due to an increase in π–π interactions between the compounds and phenyl stationary phase, were only small.

Finally a series of 30 phenol derivatives were selected and are listed in Table IV. Typical elution patterns of these phenols are shown in Fig. 1 [operating conditions: column, Nucleosil $5C_{18}$ (15 cm × 4.6 mm I.D.); mobile phase, ACN–water (20:80, 30:70, 40:60, 50:50, 60:40); flow-rate, 1.0 ml/min]. These phenols elute every 2–5 min within a 20-min operating period.

We applied this series of 30 selected phenols as potential internal standards to the determination of a new antibacterial agent, OPC-7251. The operating conditions indicated in Fig. 2 were set to separate all impurities and degradation products. OPC-7251 showed a retention time of about 8 min under these conditions. It is therefore desirable that an internal standard should have a retention time of about 10–20 min. As the acetonitrile concentration in the mobile phase was 35%, we referred to the second and third columns of Fig. 1 and selected ethyl 4-hydroxybenzoate,

TABLE IV

SERIES OF 30 PHENOL DERIVATIVES SELECTED AS POTENTIAL INTERNAL STANDARDS IN RP-HPLC

Derivative	*Substituent*	*Derivative*	*Substituent*
4-Hydroxyacetanilide	4-NHCOMe	2,3-Dichlorophenol	2,3-Cl_2
3-Hydroxyacetanilide	3-NHCOMe	2,5-Dichlorophenol	2,5-Cl_2
2-Hydroxyacetanilide	2-NHCOMe	4-Isopropylphenol	4-*iso*-Pr
4-Hydroxyacetophenone	4-COMe	*sec.*-Butyl 4-hydroxybenzoate	4-COO*sec.*-Bu
3-Hydroxyacetophenone	3-COMe	4-Hydroxybiphenyl	4-Ph
Methyl 4-hydroxybenzoate	4-COOMe	*n*-Butyl 4-hydroxybenzoate	4-COO*n*-Bu
4-Hydroxypropionphenone	4-COEt	4-*tert.*-Butylphenol	4-*tert.*-Bu
3-Nitrophenol	3-NO_2	Benzyl 4-hydroxybenzoate	4-$COOCH_2Ph$
Ethyl 4-hydroxybenzoate	4-COOEt	Thymol	3-Me-6-*iso*-Pr
4-Chlorophenol	4-Cl	Isoamyl 4-hydroxybenzoate	4-COO*iso*-Am
4-Bromophenol	4-Br	*n*-Amyl 4-hydroxybenzoate	4-COO*n*-Am
4-Hydroxybenzophenone	4-COPh	*n*-Hexyl 4-hydroxybenzoate	4-COO*n*-Hex
Isopropyl 4-hydroxybenzoate	4-COO*iso*-Pr	*n*-Heptyl 4-hydroxybenzoate	4-COO*n*-Hep
n-Propyl 4-hydroxybenzoate	4-COO*n*-Pr	2-Ethylhexyl 4-hydroxybenzoate	4-COOOc
4-Hydroxyvalerophenone	4-CO*n*-Bu	*n*-Nonyl 4-hydroxybenzoate	4-COO*n*-No

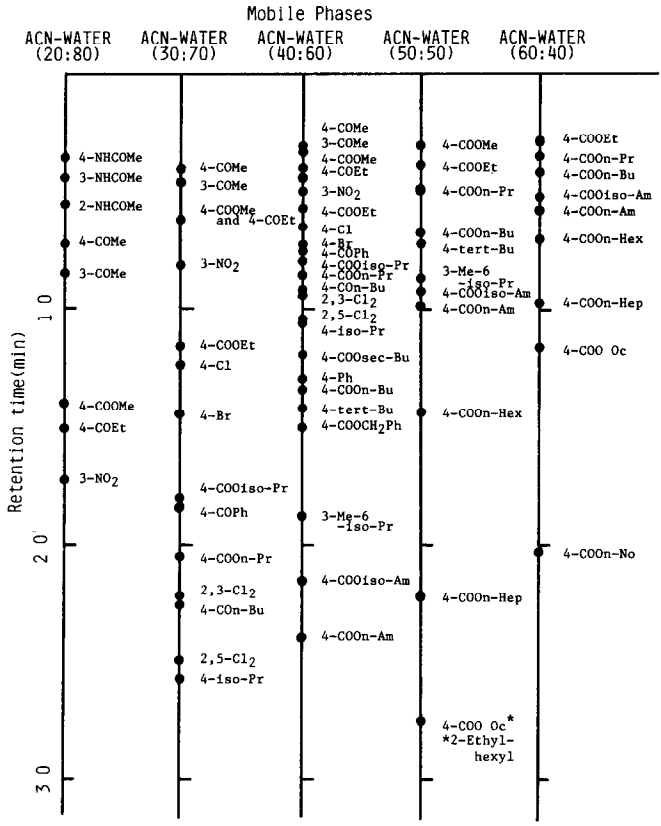

Fig. 1. Typical elution patterns of selected phenols. Column, Nucleosil $5C_{18}$ (5 μm) (15 cm × 4.6 mm I.D.); mobile phase, ACN–water (20:80 to 60:40); flow-rate, 1.0 ml/min; t_0 was found to be 1.9 min. All phenols are expressed using abbreviations of substituents (see Tables II and IV).

4-bromophenol, 4-hydroxybenzophenone, isopropyl 4-hydroxybenzoate, *n*-propyl 4-hydroxybenzoate and 4-hydroxyvalerophenone as candidates for the internal standard. These compounds were chromatographed under the conditions shown in Fig. 2. 4-Bromophenol, 4-hydroxybenzophenone, *n*-propyl 4-hydroxybenzoate and 4-hydroxyvalerophenone were well separated from OPC-7251 and did not overlap with any related substances. 4-Bromophenol showed the shortest retention time of these four compounds and was therefore selected as the internal standard. Sample solutions were prepared so that each injection contained 0.2–1.2 μg of OPC-7251 and 4.5–5.5 μg of the internal standard in 10 μl. A calibration graph was plotted of relative peak area *versus* the amount of OPC-7251 (μg). Over this range, the calibration graph showed excellent linearity (correlation coefficient 0.99999) and the precision was 0.4% (relative standard deviation). Sample solutions were found to be stable for at least 6 days at room temperature without precautions. The relative retention of OPC-7251 with respect to the internal standard was consistent even when using three C_{18} columns supplied by different manufacturers.

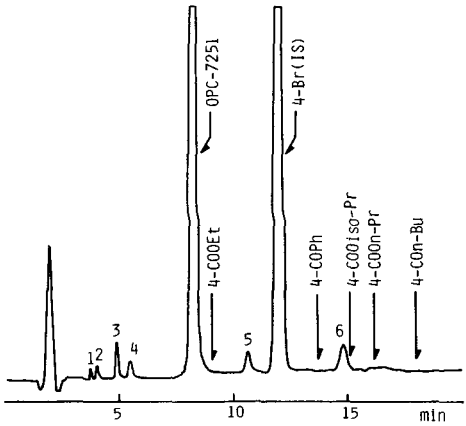

Fig. 2. Typical chromatogram showing the separation of OPC-7251 and 4-bromophenol as an internal standard spiked with 0.5% of substances related to OPC-7251 (1–6). Candidate phenols for the internal standard are expressed using abbreviations of substituents (see Tables II and IV). Column, TSKgel ODS 80 (5 μm) (15 cm × 4.6 mm I.D.); mobile phase, ACN–water–acetic acid (70:130:1); flow-rate, 1.0 ml/min; detection, 280 nm.

CONCLUSION

About 70 commercial phenol derivatives were chromatographed under various RP-HPLC conditions in order to find a series of compounds as candidates for internal standards. Of these, 30 were selected from logarithmic plots of capacity factors and other chromatographic parameters. These 30 phenols maintain an almost constant elution order under different operating conditions which are typical for RP-HPLC methods commonly used in analytical and quality control laboratories.

REFERENCES

1 C. Guillemin, J. Gressin and M. Caude, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 3 (1982) 128.
2 M. Verzele, L. Use and M. Van Kerrebroeck, *J. Chromatogr.*, 289 (1984) 333.
3 E. J. Kikta, Jr. and A. E. Stange, *J. Chromatogr.*, 138 (1977) 41.
4 R. M. Smith, *J. Chromatogr.*, 236 (1982) 313.
5 R. M. Smith, *Trends Anal. Chem.*, 7 (1984) 186.
6 J. K. Baker and C.-Y. Ma, *J. Chromatogr.*, 169 (1979) 107.
7 M. Bogusz and R. Aderjan, *J. Chromatogr.*, 435 (1988) 43.
8 M. J. M. Wells and C. R. Clark, *J. Chromatogr.*, 235 (1982) 31.
9 M. J. M. Wells and C. R. Clark, *J. Chromatogr.*, 243 (1982) 263.
10 M. J. M. Wells and C. R. Clark, *J. Chromatogr.*, 244 (1982) 231.
11 K. Miyake, N. Mizuno and H. Terada, *Chem. Pharm. Bull.*, 34 (1986) 4787.
12 N. Tanaka, H. Goodell and B. L. Karger, *J. Chromatogr.*, 158 (1978) 233.
13 N. Kyokai (Editor), *Pharmacopoeia of Japan*, Hirokawa Press, Tokyo, 11th ed., 1986.
14 T. Hanai and J. Hubert, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 6 (1983) 20.
15 K. Callmer, L.-E. Edholm and B. E. F. Smith, *J. Chromatogr.*, 136 (1977) 45.
16 S. Hara, S. Mori and T. Hanai, *Kuromatogurafi-Bunri Sisutemu* (*Chromatographic Separation System*; in Japanese), Maruzen, Tokyo, 1981, p. 177.
17 G.-Z. Ceng, *Acta Chim. Sin.*, 32 (1966) 107.
18 P. D. Bolton, K. A. Fleming and F. M. Hall, *J. Am. Chem. Soc.*, 94 (1972) 1033.
19 J. L. M. van de Venne and J. L. H. M. Hendrikx, *J. Chromatogr.*, 167 (1978) 1.