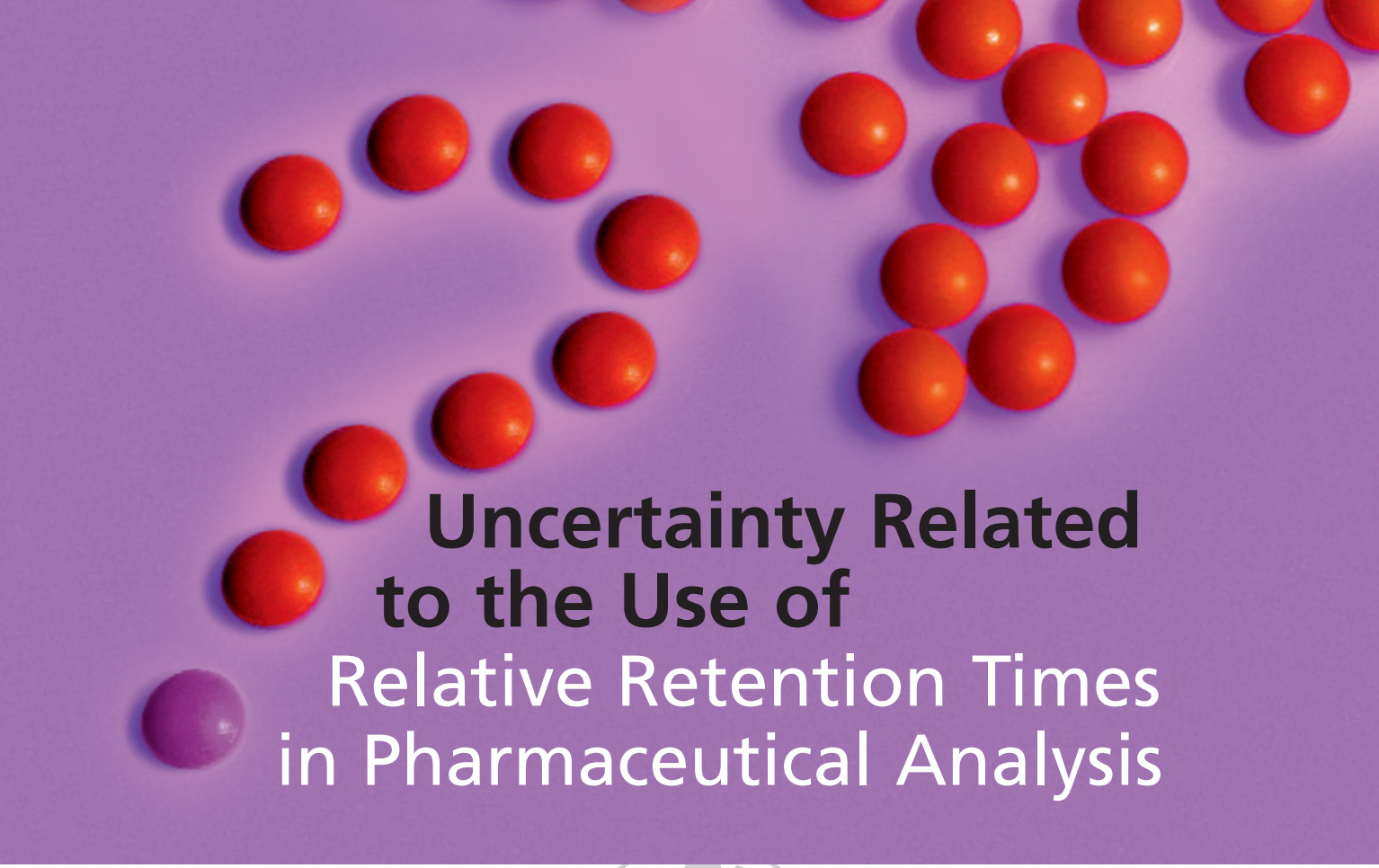


# Uncertainty Related to the Use of Relative Retention Times in Pharmaceutical Analysis

**Pieter Dehouck,**[a] **Dora Visky,**[a,b] **Zsuzsanna Kovács,**[a,b] **Béla Noszál,**[b] **Erwin Adams**[a] **and Jos Hoogmartens**[a]
[a]Laboratory for Pharmaceutical Chemistry and Drug Analysis, Leuven, Belgium,
[b]Department of Pharmaceutical Chemistry, Semmelweis University, Budapest, Hungary.

**One way to identify peaks in a chromatogram is by their relative retention time (RRT). However, because of a lack of harmonization in the calculation of RRT, interpretation of results may be difficult. In both the *European Pharmacopoeia* (*Ph. Eur.*) and the *United States Pharmacopeia*, much confusion remains because no method for the determination of hold-up times is prescribed. Also, the influence of the stationary phase must be taken into account. In this study, five separations prescribed by the *Ph. Eur.* were performed on 59 different reversed-phase liquid chromatography C18 columns and the variability of RRTs between them was studied. It is shown that the RRT alone is not sufficient for correct peak identification.**



## Introduction

Peaks corresponding to specified impurities must be identified in a chromatogram. One way to achieve this is through the relative retention time (RRT). The RRT can be defined as the retention relative to a standard, or

$$r = (t_R - t_M) / (t_{R(st)} - t_M) \quad [1]$$

where $t_R$ is the retention time of the component of interest, $t_{R(st)}$ the retention time of the standard and $t_M$ the hold-up time.[1] The main peak in the chromatogram is usually considered to be the standard (= reference peak). The hold-up time $t_M$ can be defined as the time it takes for a small, unretained compound that completely permeates the stationary-phase pores, to be eluted from the chromatographic column. In fact, the ratio used for the calculation of $r$ is similar to the ratio of mass distribution ratios. The *United States Pharmacopeia* (*USP*) has used Equation 1 for some time.[2]

However, $r$ is not always calculated this way. Until 2002 the *European Pharmacopoeia* (*Ph. Eur.*) defined $r$ as the ratio of the two retention times of the considered peaks, or

$$r_{a/b} = t_{r,b} / t_{r,a} \quad [2]$$

with $t_{r,b}$ the retention time of the peak of interest and $t_{r,a}$ the retention time of the reference peak.[3] From 2002, $t_M$ has been taken into account and a formula equal to Equation 1 is described.[4] Unfortunately, RRT values in monographs were not adapted accordingly and it is not always certain that the new Equation 1 has been applied in monographs published since 2002.

This lack of harmonization around the calculation of RRT has led to much confusion among analysts. Many texts still describe $r$ as a ratio of two retention times without considering the subtraction of $t_M$. However, even when Equation 1 is applied, confusion remains over how to measure $t_M$. Different

compounds, such as thiourea, uracil, nicotinic acid and nitrate, have been cited in the literature as markers of $t_M$. Nevertheless, no harmonized procedure for its measurement can be found and even prominent compendia, such as *Ph. Eur.* and *USP* fail to prescribe the appropriate method.[5–8] The determination of $t_M$ is subject to much controversy.[8]

In addition to the uncertainty surrounding the calculation of $t_M$, the influence of the stationary phase must also be taken into account. Many liquid chromatographic (LC) methods are described in the *Ph. Eur.* and *USP*. These methods often use reversed-phase (RP)-LC C18 columns. However, the description of an LC method in the *Ph. Eur.* or *USP* never mentions the brand of stationary phase that can or must be used. Some information on columns that provide adequate separations is available through other channels, such as *Pharmeuropa*. However, because hundreds of different RP-LC C18 column brands are available, different columns will be selected for the same analysis. Does RRT remain constant when the same analysis is performed on different column brands? One might think that, even if retention times vary on different columns, the RRT does not. However, this is not true. Identification based on the RRT may lead to difficult or incorrect peak assignments, resulting in uncertainty of results. Imagine the consequences if impurities are misidentified in a chromatographic method that applies different impurity limits. The same problem occurs in methods in which peak areas must be corrected, depending on differences in specific absorbances. In these instances, incorrect peak identification can lead to inaccurate content determinations and/or incorrect decisions regarding compliance of the substance examined.

This study focuses on uncertainty related to use of the RRT when separations are performed on different stationary phases. Five separations, prescribed by the *Ph. Eur.*, were performed on 59 different RP-LC C18 columns. For each separation, the RRT values for all components were calculated and the intercolumn variability studied.

## Experimental

**Samples:** Buflomedil hydrochloride, 4-(pyrrolidin-1-yl)-1-(2-hydroxy-4,6-dimethoxyphenyl)butan-1-one (impurity A) and 4-(pyrrolidin-1-yl)-1-(4-hydroxy-4,6-dimethoxyphenyl)butan-1-one (impurity B) were obtained from the *Ph. Eur.* laboratory (Strasbourg, France). Chloramphenicol and chloramphenicol disodium dissuccinate were purchased from the *Ph. Eur.* and chloramphenicol sodium succinate from Pharm-Inter (Brussels, Belgium). Nimesulide, N-(2,4-dinitro-6-phenoxyphenyl)methanesulphonamide (impurity A), N-(2-phenoxyphenyl)methanesulphonamide (impurity B), 2-phenoxyaniline (impurity C), 4-nitro-2-phenoxyaniline (impurity D) and N,N-bis(methylsulphonyl)-2-phenoxyaniline (impurity E) were obtained from the *Ph. Eur.* laboratory. Acetylsalicylic acid (ASA), salicylsalicylic acid (SSA), 4-hydroxybenzoic acid (HBA), 4-hydroxyisophthalic acid (HIPA) and acetylsalicylsalicylic acid (ASSA) were obtained from Acros Organics (Beerse, Belgium), and salicylic acid (SA) was purchased from Merck (Darmstadt, Germany). Acetylsalicylic anhydride (ASAN) was prepared in the laboratory according to a described method.[9] Lincomycin, lincomycin B, 7-epilincomyin and 7-epiclindamycin were obtained from Pharmacia Upjohn (Kalamazoo, Michigan, USA), and clindamycin from Alpha Pharma (Zwevegem, Belgium).

**Imagine the consequences if impurities are misidentified in a chromatographic method that applies different impurity limits.**

**Chromatography:** Analyses were performed using a Varian (Walnut Creek, California, USA) 9010 LC pump, 9100 autosampler and 9050 UV-VIS detector, with ChromPerfect 4.0 software (Justice Laboratory Software, Fife, UK) for data acquisition. Column temperature was maintained by immersion in a water bath. The injection volume was 20 µL in all analyses.

The analyses of buflomedil,[4] chloramphenicol,[4] acetylsalicylic acid,[4] nimesulide[4] and clindamycin[10] were performed in accordance with the corresponding *Ph. Eur.* monograph. All solvents and reagents were of *Ph. Eur.* quality. The 59 stationary phases used are listed in Table 1. For each separation, columns that did not comply with the prescription of the *Ph. Eur.* monograph or according to the results of the system suitability test (SST) were eliminated.

The mobile-phase composition was kept constant for all columns, as was the flow-rate of 1.0 mL/min. No attempts were made to improve the separations by adapting the composition. During preliminary experiments uracil was used as a marker for the hold-up time.[11] A 0.01 mg/mL solution of uracil in mobile phase was injected three times before each sample analysis. Chromatographic conditions for the different separations are summarized below:

**Separation of buflomedil:** The mobile phase consisted of acetonitrile–9.25 g/L potassium dihydrogen phosphate adjusted to pH 2.5 with phosphoric acid (35/65 v/v). The column was maintained at 40 °C and the detection wavelength was 210 nm. The sample contained buflomedil (92% m/m), impurity A (3%) and impurity B (5%), and 30 mg of this sample was dissolved in 10.0 mL of mobile phase.

**Separation of chloramphenicol:** The mobile phase consisted of methanol–phosphoric acid–water (40/5/55 v/v/v). The column was maintained at 25 °C and detection was performed at 275 nm. The sample contained chloramphenicol sodium succinate (93% m/m), chloramphenicol (3%) and chloramphenicol disodium disuccinate (4%), and 2.5 mg of this sample was dissolved in 10.0 mL of mobile phase. Chloramphenicol sodium succinate is a mixture of chloramphenicol-1-sodium succinate and chloramphenicol-3-sodium succinate.

**Separation of nimesulide:** The mobile phase consisted of acetonitrile–1.15 g/L ammonium dihydrogen phosphate adjusted to pH 7.0 with ammonia (35/65 v/v). The column was maintained at 25 °C and detection was at 230 nm. The sample contained nimesulide (29% m/m), impurity A (3%), impurity B (11%), impurity C (21%), impurity D (23%) and impurity E (13%), and 10 mg of this sample was dissolved in 10.0 mL of acetonitrile–water (40/60 v/v).

**Separation of acetylsalicylic acid:** The mobile phase consisted of phosphoric acid–acetonitrile–water (2/400/600 v/v/v). The column was maintained at 30 °C and the detection wavelength was 237 nm. The sample solution contained 0.3 mg of HBA, 0.1 mg of HIPA, 0.2 mg of SA, 2.5 mg of ASA, 0.5 mg of ASSA, 0.7 mg of SSA and 0.3 mg of ASAN in 50.0 mL of acetonitrile. The sample was prepared daily because some of the compounds are unstable in solution.

**Table 1:** Stationary phases involved in this project.

| Column number | Name of the column | Length (mm)* | Particle size (µm) | Manufacturer/Supplier | Type of silica | End-capped | Base deactiv. | Polar embed. | Pore size |
|---|---|---|---|---|---|---|---|---|---|
| 1 | ACE 3 C18 | 150 | 3 | Advanced Chrom. Tech./Achrom | new | ✓ | ✓ | ✘ | 100 |
| 2 | ACE 5 C18 | 250 | 5 | Advanced Chrom. Tech./Achrom | new | ✓ | ✓ | ✘ | 100 |
| 3 | Alltima C18 3 | 150 | 3 | Alltech | new | ✓ | ✓ | ✘ | 120 |
| 4 | Alltima C18 5 | 250 | 5 | Alltech | new | ✓ | ✓ | ✘ | 120 |
| 5 | Apex Basic | 250 | 5 | Jones Chromatography/Sopachem | old | ✓ | ✓ | ✘ | 100 |
| 6 | Apex ODS II | 250 | 5 | Jones Chromatography/Sopachem | old | ✓ | ✘ | ✘ | 100 |
| 7 | Aqua | 150 | 5 | Phenomenex/Bester | new | ✓ | ✘ | ✘ | 125 |
| 8 | µBondapak | 250 | 10 | Waters | old | ✓ | ✘ | ✘ | 125 |
| 9 | Brava BDS 3 | 150 | 3 | Alltech | new | ✓ | ✓ | ✘ | 145 |
| 10 | Brava BDS 5 | 250 | 5 | Alltech | new | ✓ | ✓ | ✘ | 145 |
| 11 | Chromolith | 100 | – | Merck | new | ✓ | ✘ | ✘ | - |
| 12 | Discovery C18 | 250 | 5 | Supelco | new | ✓ | ✘ | ✘ | 180 |
| 13 | Genesis C18 3 | 100 | 3 | Jones Chromatography/Sopachem | new | ✓ | ✓ | ✘ | 100 |
| 14 | Hypersil BDS 5 | 250 | 5 | ThermoQuest | old | ✓ | ✓ | ✘ | 130 |
| 15 | Hypersil ODS 5 | 250 | 5 | ThermoQuest | old | ✓ | ✘ | ✘ | 120 |
| 16 | HyPURITY Elite 3 | 150 | 3 | ThermoQuest, SerCoLab | new | ✓ | ✓ | | 200 |
| 17 | HyPURITY Elite 5 | 150 | 5 | ThermoQuest, SerCoLab | new | ✓ | ✓ | | 200 |
| 18 | Kromasil (MN) | 250 | 5 | Macherey-Nagel/Filter Service | new | ✓ | ✘ | ✘ | 100 |
| 19 | Kromasil (EKA) | 250 | 5 | Akzo Nobel/SerCoLab | new | ✓ | ✘ | ✘ | 100 |
| 20 | LiChrospher | 250 | 5 | Merck | old | ✘ | ✓ | ✘ | 100 |
| 21 | Luna | 150 | 5 | Phenomenex/Bester | new | ✓ | ✘ | ✘ | 100 |
| 22 | Nucleosil 5 | 250 | 5 | Macherey-Nagel/Filter Service | old | ✓ | ✘ | ✘ | 100 |
| 23 | Nucleosil HD | 250 | 5 | Macherey-Nagel/Filter Service | new | ✓ | ✘ | ✘ | 100 |
| 24 | Nucleosil Nautilus | 250 | 5 | Macherey-Nagel/Filter Service | new | ✘ | ✘ | ✓ | 100 |
| 25 | OmniSpher | 250 | 5 | Varian | new | ✘ | ✘ | ✘ | 110 |
| 26 | Pecospher C18 | 83 | 3 | PerkinElmer | new | ✓ | ✘ | ✘ | 80 |
| 27 | Platinum C18 3 | 150 | 3 | Alltech | new | ✓ | ✓ | ✘ | 100 |
| 28 | Platinum C18 5 | 250 | 5 | Alltech | new | ✓ | ✓ | ✘ | 100 |
| 29 | Platinum EPS C18 3 | 150 | 3 | Alltech | new | ✘ | ✓ | ✘ | 100 |
| 30 | Platinum EPS C18 5 | 250 | 5 | Alltech | new | ✘ | ✓ | ✘ | 100 |
| 31 | Prodigy | 100 | 3 | Phenomenex/Bester | new | ✓ | ✘ | ✘ | 100 |
| 32 | Purospher | 250 | 5 | Merck | new | ✓ | ✘ | ✘ | 80 |
| 33 | Purospher endcapped | 250 | 5 | Merck | new | ✓ | ✓ | ✘ | 80 |
| 34 | Purospher STAR e | 250 | 5 | Merck | new | ✓ | ✓ | ✘ | 80 |
| 35 | SPHERI-5 | 250 | 5 | PerkinElmer | new | ✘ | ✘ | ✘ | 80 |
| 36 | Spherisorb ODS2 5 | 250 | 5 | Waters | old | ✓ | ✘ | ✘ | 80 |
| 37 | Supelcosil LC-18 | 250 | 5 | Supelco | old | ✘ | ✘ | ✘ | 120 |
| 38 | Supelcosil LC-18 DB 3 | 150 | 3 | Supelco | new | ✘ | ✓ | ✘ | 120 |
| 39 | Supelcosil LC-18 DB 5 | 250 | 5 | Supelco | new | ✘ | ✓ | ✘ | 120 |
| 40 | Superspher | 250 | 4 | Merck | new | ✓ | ✘ | ✘ | 100 |
| 41 | Symmetry 5 | 250 | 5 | Waters | new | ✓ | ✘ | ✘ | 100 |
| 42 | TracerExcel ODS A-3 | 150 | 3 | Teknokroma/SerCoLab | NA | NA | NA | NA | 120 |
| 43 | TracerExcel ODS A-5 | 250 | 5 | Teknokroma/SerCoLab | NA | NA | NA | NA | 120 |
| 44 | TSKgel ODS-80TS | 150 | 5 | TosoHaas/SerCoLab | new | ✓ | ✘ | ✘ | 80 |
| 45 | TSKgel Super ODS | 100 | 2 | TosoHaas/SerCoLab | new | ✓ | ✘ | ✘ | 110 |
| 46 | Uptisphere 3 HDOC18 | 100 | 3 | Interchrom/Achrom | new | ✓ | ✘ | ✘ | 120 |

**Table 1: (continued).**

| Column number | Name of the column | Length (mm)* | Particle size (µm) | Manufacturer/Supplier | Type of silica | End-capped | Base deactiv. | Polar embed. | Pore size |
|---|---|---|---|---|---|---|---|---|---|
| 47 | Uptisphere 5 HDOC18 | 250 | 5 | Interchrom/Achrom | new | ✓ | ✘ | ✘ | 120 |
| 48 | Uptisphere 3 ODB | 100 | 3 | Interchrom/Achrom | new | ✓ | ✘ | ✘ | 120 |
| 49 | Uptisphere 5 ODB | 250 | 5 | Interchrom/Achrom | new | ✓ | ✘ | ✘ | 120 |
| 50 | Validated C18 | 250 | 5 | PerkinElmer | new | ✓ | ✘ | ✘ | 100 |
| 51 | Wakosil C18 HG 5 – 10 | 100 | 5 | SGE/Achrom | new | ✓ | ✘ | ✘ | 120 |
| 52 | Wakosil C18 HG 5 – 25 | 250 | 5 | SGE/Achrom | new | ✓ | ✘ | ✘ | 120 |
| 53 | Wakosil C18 RS 3 – 10 | 100 | 3 | SGE/Achrom | new | ✓ | ✘ | ✘ | 125 |
| 54 | YMC-Hydrosphere C18 | 150 | 5 | YMC Sep. Tech./ThermoQuest | new | ✓ | ✘ | ✘ | 120 |
| 55 | YMC-Pack Pro C18-3 | 150 | 3 | YMC Sep. Tech./ThermoQuest | new | ✓ | ✓ | ✘ | 120 |
| 56 | YMC-Pack Pro C18-5 | 150 | 5 | YMC Sep. Tech./ThermoQuest | new | ✓ | ✓ | ✘ | 120 |
| 57 | Zorbax Eclipse XDB-C18 | 250 | 5 | Agilent Technologies | new | ✓ | ✘ | ✘ | 80 |
| 58 | Zorbax Extend C18 | 250 | 5 | Agilent Technologies | new | ✓ | ✘ | ✘ | 80 |
| 59 | Zorbax SB-C18 | 250 | 5 | Agilent Technologies | new | ✓ | ✘ | ✘ | 80 |

* The internal diameter is always 4.6 mm.

**Separation of clindamycin:** The mobile phase consisted of acetonitrile–6.8 g/L potassium dihydrogen phosphate adjusted to pH 7.5 with a 250 g/L solution of potassium hydroxide (40/60 v/v). The column was maintained at 30 °C and the detection wavelength was 210 nm. The clindamycin hydrochloride sample contained clindamycin (88.6% m/m), lincomycin (5.0%), lincomycin B (0.9%), 7-epilincomyin (3.0%) and 7-epiclindamycin (2.5%), and 15 mg of this sample was dissolved in 10.0 mL of mobile phase.

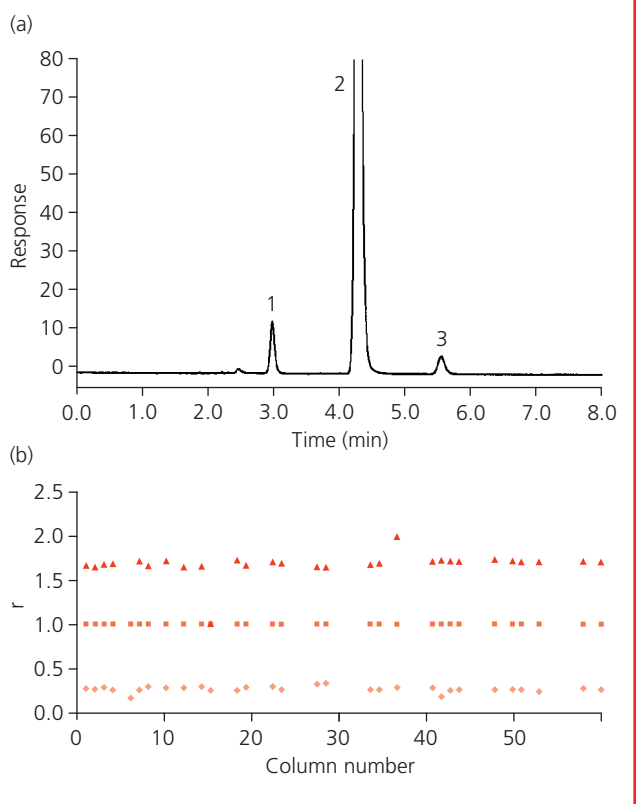
Each chromatogram was recorded three times. Mean RRT values were calculated using the formula $r = (t_R - t_M) / (t_{R(st)} - t_M)$, where $t_R$ is the retention time of the component of interest, $t_{R(st)}$ the retention time of the standard and $t_M$ the hold-up time. The standard or reference peak corresponded to the substance to be examined.

## Results

The first separation shown is that of buflomedil. The order of elution is: impurity B, buflomedil and impurity A (Figure 1(a)). The monograph prescribes the use of a 250 × 4.6 mm column packed with endcapped 5 µm octadecylsilyl silica gel. However, the *Ph. Eur.* allows adaptation of the column length by 70%, the internal diameter by 25% and the particle size (reduction only) by 50%. Columns without endcapping (see Table 1), column 11 (a monolithic column) and column 45 (2 µm particle size) did not meet these *Ph. Eur.* requirements and were removed. Columns that did not comply with the SST, which asks for a resolution of at least 5.0 between buflomedil and impurity B, were removed as well. The RRT values on 30 suitable columns are shown in Figure 1(b). The peak corresponding to buflomedil is taken as the reference peak ($r = 1$). Although the peak identification in this separation is simple, it can be noticed that $r$ varies from 0.171 to 0.331 for impurity B and from 1.000 to 1.981 for impurity A. Column 15 shows a coelution of buflomedil and impurity A.

A typical chromatogram of the separation of chloramphenicol sodium succinate is shown in Figure 2(a). The elution order of this separation is: chloramphenicol, chloramphenicol-1-sodium succinate, chloramphenicol-3-sodium succinate and chloramphenicol-1,3-disodium disuccinate. Results for the analysis are shown in Figure 2(b). The *Ph. Eur.* monograph prescribes the use of a 250 × 4.6 mm

**Figure 1:** (a) Typical chromatogram of buflomedil on column 47; (b) RRT values for the separation of buflomedil and its impurities for the columns that comply with *Ph. Eur.* requirements.

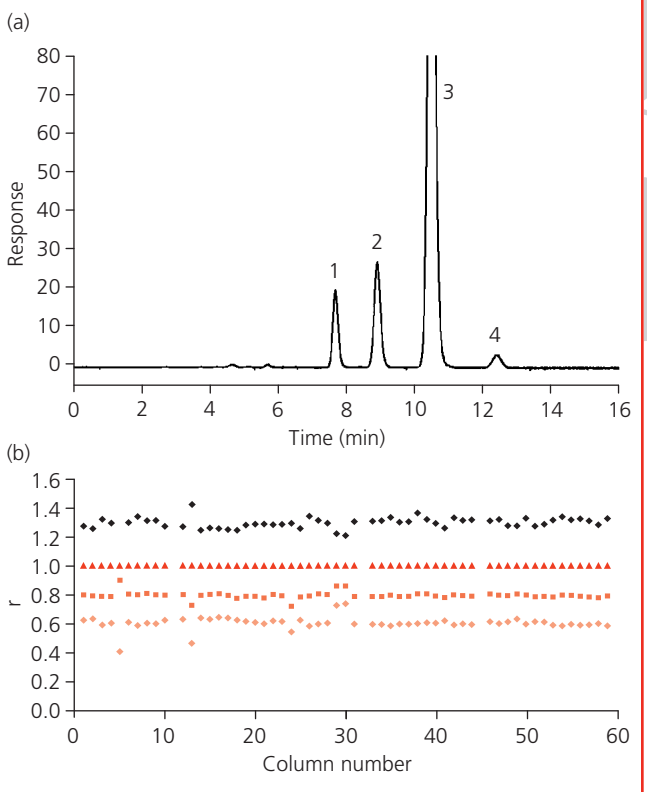



**Peaks:** 1(◆) = impurity B, 2(■) = buflomedil, 3(▲) = impurity A.

column packed with 5 µm octadecylsilyl silica gel. To comply with the SST, the peaks corresponding to chloramphenicol and chloramphenicol disodium disuccinate must be clearly separated from the two peaks corresponding to chloramphenicol-1-sodium succinate and chloramphenicol-3-sodium succinate. All columns presented in Figure 2(b) comply with these requirements and only three columns were removed. The peak corresponding to chloramphenicol-3-sodium succinate was taken as reference peak ($r = 1$). Compared with the previous separation, a somewhat larger variation in $r$ can be observed. For the chloramphenicol peak, $r$ varies from 0.407 to 0.738 and for the peak corresponding to chloramphenicol-1-sodium succinate from 0.723 to 0.901. These ranges overlap, indicating a serious problem for peak identification when based on the RRT alone. Indeed, peaks with an RRT of around 0.73 may correspond to chloramphenicol or chloramphenicol-1-sodium succinate, depending on the stationary phase used. As the *Ph. Eur.* limits the chloramphenicol content to 2.0%, but does not limit the content of chloramphenicol-1-sodium succinate, a clear identification is important. Therefore, the *Ph. Eur.* monograph prescribes the use of a chloramphenicol chemical reference substance (CRS) solution to identify this peak. The RRT of the peak corresponding to chloramphenicol-1,3-disodium disuccinate varies from 1.209 to 1.422.

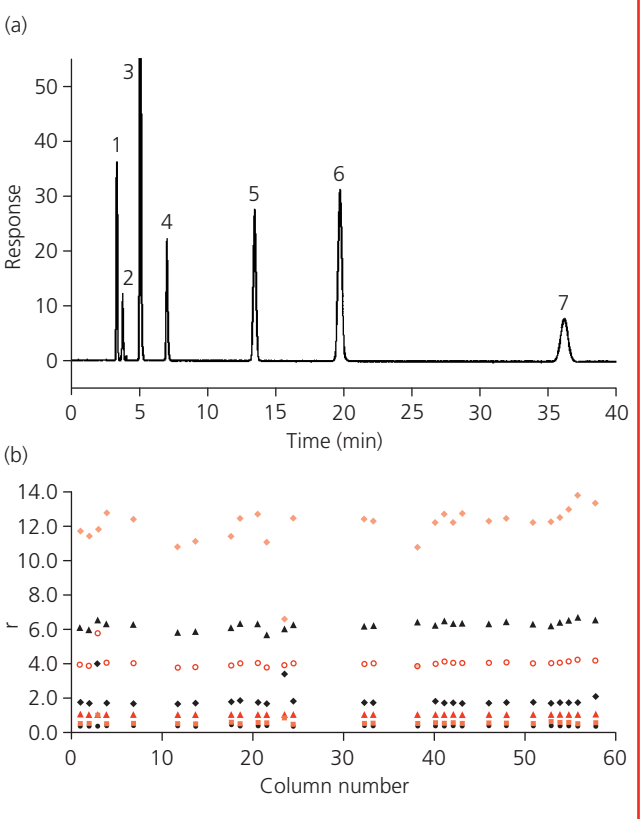
A third analysis is that of ASA. On most RP-LC C18 columns the order of elution is: HBA, HIPA, ASA, SA, ASSA, SSA and ASAN. A typical chromatogram is shown in Figure 3(a). The monograph prescribes the use of a 250 × 4.6 mm column packed with 5 µm octadecylsilyl silica gel. To comply with the SST, the resolution between the ASA and SA peaks must be at least 6. Figure 3(b) shows the RRT values for all columns (28 out of a total of 59) that comply with these requirements. The peak corresponding to ASA was taken as the reference peak ($r = 1$). For HBA, $r$ varies from 0.353 to 0.815 and for HIPA from 0.452 to 1.000. On column 24, HBA coelutes with HIPA. Column 3 shows a coelution of HIPA and ASA. If columns 3 and 24 are eliminated, the ranges become $r_{HBA}$: 0.353–0.412 and $r_{HIPA}$: 0.452–0.565. The RRT for SA varies between 1.649 and 3.968, for ASSA between 3.753 and 5.750, for SSA between 5.620 and 6.656, and for ASAN between 6.493 and 13.755. An overlap of the adjacent ranges above is observed for all these impurities. Although this is the result of some columns behaving very differently, it does show how careful one should be with the identification of peaks based on the RRT alone. When $r = 6.0$ would be prescribed as an indicative RRT for SSA, an analyst using column 3 could identify ASSA as SSA, while an analyst using column 24 could identify ASAN as SSA. It should be noticed that the stationary

**Figure 2:** (a) Typical chromatogram of chloramphenicol sodium succinate on column 14; (b) RRT values for the separation of chloramphenicol-3-sodium succinate and its impurities for the columns that comply with *Ph. Eur.* requirements.




**Peaks:** 1(◆) = chloramphenicol, 2(■) = chloramphenicol-1-sodium succinate, 3(▲) = chloramphenicol-3-sodium succinate, 4(◆) = chloramphenicol-1,3-disodium disuccinate.

**Figure 3:** (a) Typical chromatogram of ASA on column 43; (b) RRT values for the separation of ASA and its impurities for the columns that comply with *Ph. Eur.* requirements.



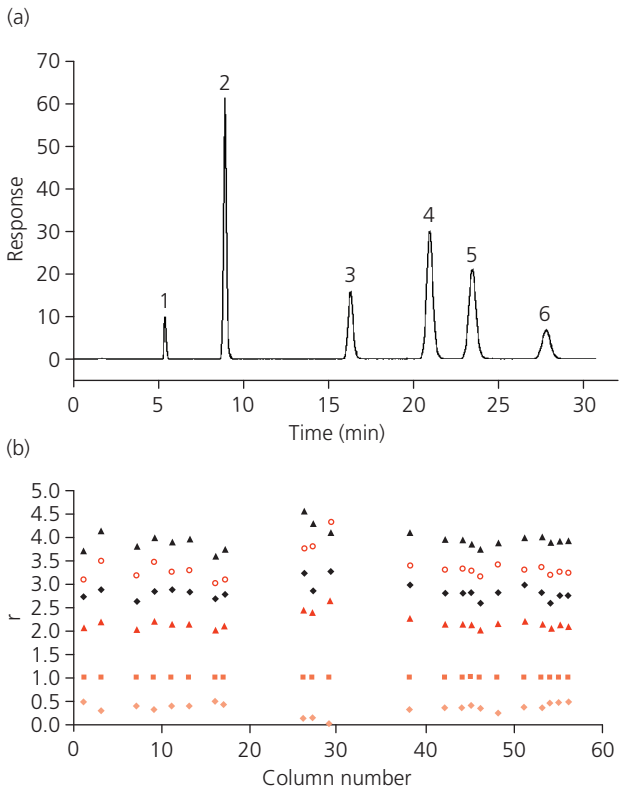

**Peaks:** 1(●) = impurity HBA, 2(■) = impurity HIPA, 3(▲) = impurity ASA, 4(◆) = impurity SA, 5(○) = impurity ASSA, 6(▲) = impurity SSA, 7(◆) = impurity ASAN.

phases that give somewhat different RRT values for this separation are not the same as those that give different RRT values for the separation of chloramphenicol.

A typical chromatogram of the separation of nimesulide is shown in Figure 4(a). On most LC-RP C18 columns, nimesulide and its impurities are eluted as follows: impurity A, nimesulide, impurity B, impurity C, impurity D and impurity E. Results for the separation of nimesulide are presented in Figure 4(b). For this separation, the *Ph. Eur.* prescribes the use of a 125 × 4.0 mm column packed with octadecyl silica gel. The SST asks for a minimum resolution of 2.0 between the two peaks corresponding to impurity C and D. The 22 columns presented in Figure 4(b) comply with these requirements. For impurity A, *r* varies from 0.012 to 0.515, for impurity B from 2.017 to 2.638, for impurity C from 2.595 to 3.275, for impurity D from 3.005 to 4.243 and for impurity E from 3.601 to 4.572. An overlap of ranges is observed for impurities B and C, impurities C and D, and impurities D and E. This leads to difficult peak identification based on the RRT alone. An RRT of 3.0 may correspond to impurity C or D, while an RRT of 3.6 may correspond to impurity D or E, depending on the stationary phase used. Moreover, the elution order on column 29 has changed. Although this column shows sufficient resolution between impurity C and D and thus passes the resolution test, the elution order of impurities D and E is reversed. Therefore, an identification of the peaks, based on the RRT alone, will result in incorrect peak identification. Again, this shows the importance of the use of chemical reference substances. The *Ph. Eur.* monograph prescribes the use of nimesulide impurity C CRS and nimesulide impurity D CRS. Indeed, these are critical peaks regarding peak identification.

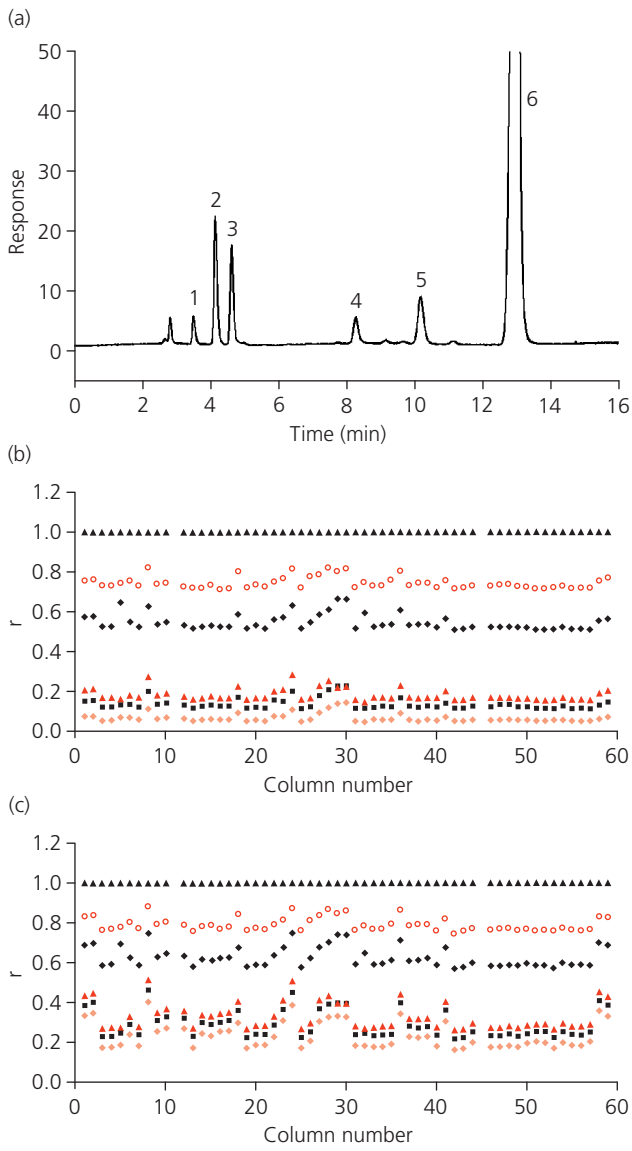
The separation method used for clindamycin was based on the *Ph. Eur.* monograph,[10] which is a monograph that uses RRT values in the SST. The *USP* uses the same chromatographic method. A typical chromatogram is shown in Figure 5(a). The monograph prescribes the use of a 250 × 4.6 mm column packed with 5 µm octadecylsilyl silica gel. Columns 11

**Figure 4:** (a) Typical chromatogram of nimesulide on column 16; (b) RRT values for the separation of nimesulide and its impurities for the columns that comply with *Ph. Eur.* requirements.




**Peaks:** 1(◆) = impurity A, 2(■) = nimesulide, 3(▲) = impurity B, 4(◆) = impurity C, 5(○) = impurity D, 6(▲) = impurity E.

**Figure 5:** (a) Typical chromatogram of clindamycin on column 12; (b) RRT values for the separation of clindamycin and its impurities; (c) $r_{a/b}$ values for clindamycin and its impurities for the columns that comply with *Ph. Eur.* requirements.




**Peaks:** 1(◆) = lincomycin B, 2(■) = lincomycin, 3(▲) = 7-epi-lincomycin, 4(◆) = clindamycin B, 5(○) = 7-epi-clindamycin.

and 45 were removed as they did not comply with these requirements. Both *Ph. Eur.* and *USP* report relative retentions with reference to clindamycin, for impurity A (lincomycin) about 0.4, for impurity B (clindamycin B) about 0.65 and for impurity C (7-epi-clindamycin) about 0.8. One would expect RRT values for different columns to be distributed randomly around these values. However, when RRT values were calculated, not one column showed an RRT around 0.4 and very few around 0.65 and 0.8 (Figure 5(b)). Moreover, when the results in Figure 5(b) are examined, it can be observed that the RRT values for lincomycin B, lincomycin and 7-epi-lincomycin show large variations when analysed on different stationary phases. The RRT values for lincomycin B vary from 0.051 to 0.146, for lincomycin from 0.111 to 0.225 and for 7-epi-lincomycin from 0.145 to 0.285. In this separation a peak with $r = 0.145$ may correspond to three different impurities, depending on the stationary phase used. The RRT values for clindamycin B vary from 0.512 to 0.666 and for 7-epi-clindamycin from 0.714 to 0.821.

The difference between the prescribed and the experimentally observed RRT values found is most likely the result of the formula used. Indeed, it is supposed that the RRT values prescribed in the *Ph. Eur.* and *USP* texts were obtained using Equation 2, which does not take into account the hold-up time. Therefore RRT values were also calculated using this equation. Results are shown in Figure 5(c). It can be observed that $r_{a/b}$ values are much better distributed around the values indicated in the monograph. When the results presented in Figure 5(c) are examined, the $r_{a/b}$ values for lincomycin B vary from 0.164 to 0.402, for lincomycin from 0.214 to 0.459, for 7-epi-lincomycin from 0.255 to 0.510, for clindamycin B from 0.568 to 0.748, and for 7-epi-clindamycin from 0.748 to 0.878. This corresponds better to the prescribed RRT. Still, it remains clear that it is not possible to identify the peaks using the RRT only.

Such discrepancies between the formula prescribed in the general part of the *Ph. Eur.* and the values in the monographs can be assumed to exist in many monographs. This is because the RRT formula was adapted when the 4th edition of the *Ph. Eur.* was published, while the RRT values in the monographs were not. It is still possible that many chromatographers use Equation 2 to calculate the RRT, and report results that may then be used to draft official monographs. This is probably why the problem is also encountered in the *USP*, although it has been using Equation 1 for some time.

## Conclusion

These experiments show that reproducibility of RRT values is uncertain on different stationary phases. Therefore, peak identification based on RRT alone may lead to misjudgements. It was also shown that inadequate use of formulas can lead to very different results. To make the official formulas fully applicable, it is necessary that instructions are given for the determination of hold-up times. This study was performed on 59 different column brands, but in a market offering more than 600 column brands, the problems are obviously even greater. Given the fact that different limits may be prescribed for the different impurities in a substance examined, and that impurity surface areas often have to be corrected, the unambiguous identification of peaks is of major importance. The use of appropriate chemical reference substances or the use of a reference sample, spiked with impurities, together with a typical chromatogram is in many instances the only way for peak identification with sufficient certainty.

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**Pieter Dehouck** is a PhD student at K.U. Leuven, Laboratory of Pharmaceutical Chemistry and Drug Analysis, Leuven, Belgium, working in the field of uncertainty determination in pharmaceutical analysis and column classification.
**Dora Visky** is a post-doc researcher from the Semmelweis University, Department of Pharmaceutical Chemistry, Budapest, Hungary, working in the field of column classification, LC–MS and CE–MS.
**Zsuzsanna Kovács** is a PhD student at Semmelweis University, Department of Pharmaceutical Chemistry, Budapest, Hungary, using separation techniques (HPCE, HPLC), electroanalytical and spectroscopic (NMR, UV) methods to characterize and analyse drugs and biomolecules.
**Béla Noszál** is Professor of and Head of Pharmaceutical Chemistry at Semmelweis University, Budapest, working in the fields of pharmaceutical and biomolecular analysis and microspeciation.
**Erwin Adams** is a post-doctoral fellow of the Fund for Scientific Research – Flanders (Belgium), actually working at K.U. Leuven, Laboratory of Pharmaceutical Chemistry and Drug Analysis, Leuven, Belgium. He is mainly working in the field of LC–MS and column classification.
**Jos Hoogmartens** is Professor of and Head of the Laboratory of Pharmaceutical Chemistry and Drug Analysis, K.U. Leuven,