Inert Column Hardware for the Separation of "Difficult" Samples in RP-HPLC

Wirth H.J., Gooley A. - SGE Analytical Science, 7 Argent Street, Ringwood, Vic. 3134, Australia

Abstract

We investigate the role of metal surfaces on the broadening and tailing of some peaks in reversed phase chromatography. Analytes containing high amounts of oxygen such as ortho-polyphenols or ß-hydroxy ketones have the potential to form chelates with iron resulting in poor peak shape when analyzed with HPLC. The largest source of these non specific interactions is the stationary phase due to its large surface area but other surfaces such as the frit, the column wall and the connection capillaries contribute as well.

We developed a range of column hardware with coated surfaces (either glass- or PEEK™-lined stainless steel) and metal-free frits to minimise non-specific binding of analytes. These columns were then packed with highly inert C18 silica to optimize column performance.

We show that eliminating all sources of metal in the flow path significantly improves the peak shape of various pharmaceutically active compounds, such as the antifungal drug ciclopirox, flavanoids and polyphenols or tetracycline antibiotics.

Why Metal-Free Chromatography?

The ability to interact with transition metals such as iron is common in a large number of pharmaceutically active substances. These metal interactions form undesired artefacts such as excessive peak tailing when analyzed by liquid chromatography due to the presence of metal in the flow path or as trace impurities in the silica matrix. Our aim was to combine the best possible stationary phase in a hardware format that excludes all contact of the sample with metal.

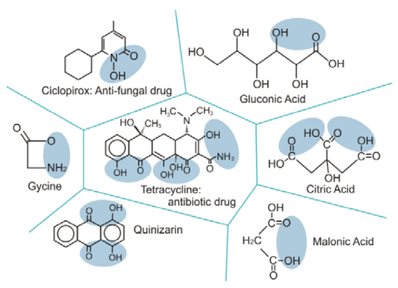

Figure 1: Examples of potentially metal chelating compounds

Column Design

Columns in the SGE ProteCol™ range feature a unique tamper-free design. The inner wall of the column is either coated with PEEK™ or glass and a porous PEEK™ frit is used. The design ensures that the sample never gets in contact with a metal surface (figure 2).

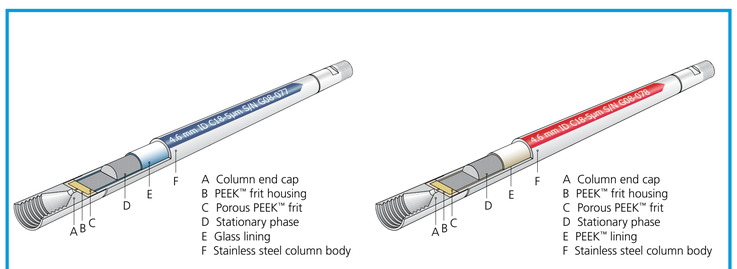

Figure 2: Schematic view of the PEEK™- and glass-lined columns

The same principle is used for capillary format. ProteCol™ capillary columns are made of PEEKsil™ (PEEK™ coated fused silica) with integrated connection tubing (figure 3).

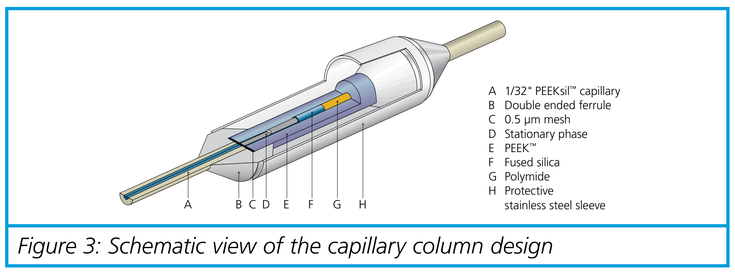

Figure 3: Schematic view of the capillary column design

The Stationary Phase

The columns are packed with a highly inert 5 µm C18 stationary phase with 100 Å pore size (C18 HQ105). Particles have 440 m²/g surface area and 1.1 ml/g pore volume. The surface is end-capped to restrict non-specific interaction with active silanol groups. The carbon content is ~17 %. The columns were tested with the National Institute of Standards and Technology (NIST) Standard Reference Method (SRM) 870.

NIST SRM870

The NIST SRM870 test mix contains five compounds (figure 4) and is designed to provide maximum information about the properties of the stationary phase with one simple test.

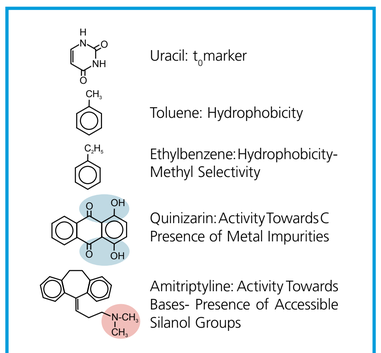

Figure 4: Test components in the NIST SRM870 test mix

NIST Results and Comparison

The column was tested with the following conditions:

Sample:	Uracil (28 µg/g), Toluene (1400 µg/g), Ethylbenzene (1700 µg/g), Quinizarin (94 µg/g), Amitriptyline (2800 µg/g) in methanol	**Mobile Phase:**	4 mM phosphate pH7.0 in 80 % methanol
		Flow rate:	1.0 ml/min
		Injection volume:	1 µl
		Detection:	254 nm
		Temperature:	23 °C
Column:	ProteCol™-P C18 HQ105 250 mm x 4.6 mm ID	**LC system:**	Shimadzu Prominence 20AC

Figure 5 shows the chromatogram achieved under the described conditions in comparison with a competitors column and a column packed with type I silica.

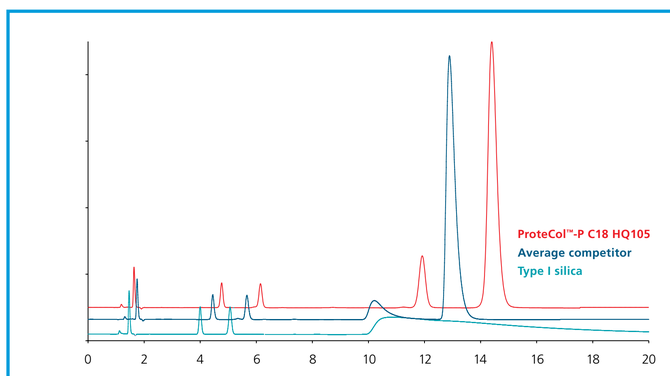

Figure 5: Chromatograms of the NIST SRM870 test mix on a variety of columns

In the test mix, uracil is a non-retained compound and marks t_o, a value needed for further calculations. The capacity factors (k') for toluene and/or ethylbenzene allow the estimation of the hydrophobicity of the column (strength of the column) while the relative retention between ethylbenzene and toluene gives an estimate of the selectivity of the column. The symmetry of the quinizarin and amitriptyline peaks highlight the amount of non-specific binding to metal- and silanol groups respectively.

The chromatogram on the ProteCol™-P C18 HQ105 column shows symmetrical elution profiles for both quinizarin and amitriptyline indicating that there are no non-specific interactions with either silanol groups or metals.

Plotting the asymmetry of the amitriptyline peak vs. the asymmetry of quinizarin results in a map for column inertness and allows for an easy comparison between columns. A column on the (1|1) coordinate of the map would be ideal. The US Pharmacopeia (USP) has published the NIST SRM870 results of more than 100 commercially available columns shown in figure 6. (http://www.usp.org/uspnf/columns.html)

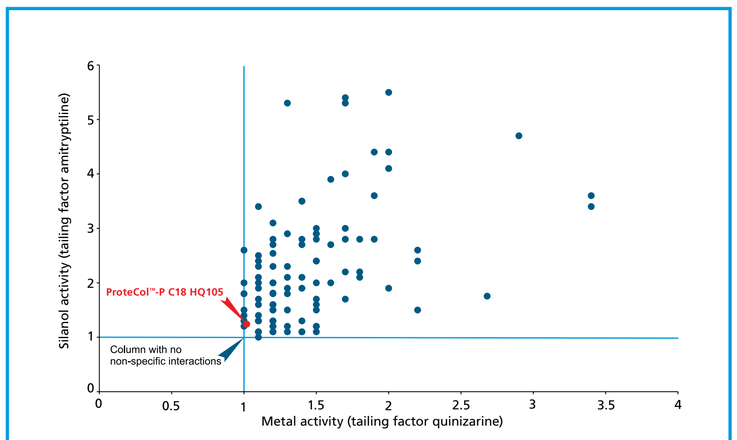

Figure 6: Non-specific interactions of the NIST SRM870 probe molecules on commercially available C18 columns

Application: N-Hydroxypyridine-2-on

2-Hydroxypyridine-N-on is the metal chelating part of the ciclopirox molecule, an anti-fungal drug. 2-Hydroxypyridine-N-on exhibits some very strong metal affinity and is therefore a useful probe molecule to investigate non-specific metal binding.

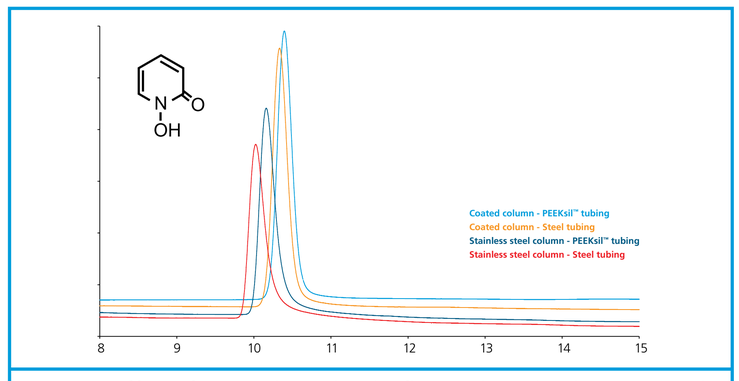

Figure 7: Effect of exposed metal in the flow path on the chromatography of chelating compounds using C18 HQ105 stationary phase

The results shown in figure 7 highlight the importance of excluding any exposed metal of getting in contact with the sample - connection capillaries, frits, column body and low activity stationary phase.

Application: Tetracycline Antibiotics

Tetracycline is a member of a group of antibiotic drugs commonly used in human and veterinary medicine. The molecule has three potential chelating sites for iron aligned at one site. The drugs are known to bind metal ions as dietary calcium and iron can render them ineffective.

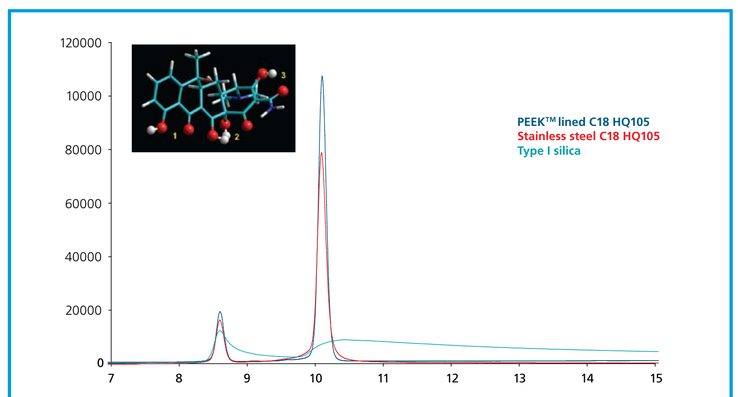

Figure 8: Chromatogram of tetracycline and its major degradation product

The results shown in figure 8 demonstrate that changing from stainless steel column to a PEEK™ coated column increased the sensitivity (peak height) of the tetracycline peak by 35 %. There is also less peak broadening on the base of the peak resolved on the PEEK™ lined column.

Application: Anti-Oxidants in Cranberry Juice

Cranberries are a rich natural source of polyphenolic anti-oxidants and flavonoids, some of which have the ability to form metal chelates. Cranberry juice was analysed with detection at 350 nm to selectively show the flavonoids.

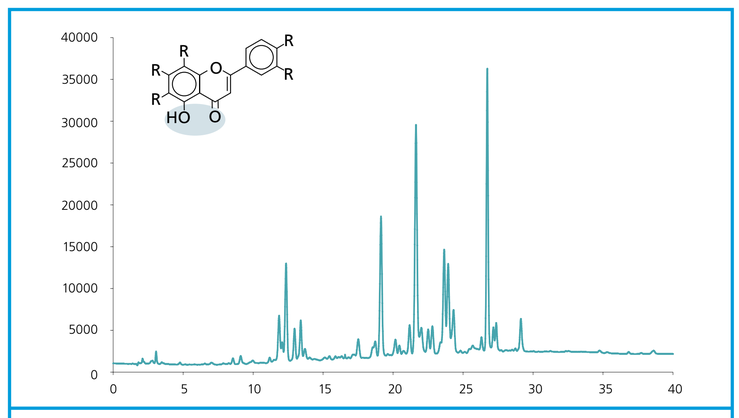

Figure 9: ProteCol-P C18 HQ105 chromatogram of flavonoids in Cranberry juice

Conclusions

Coating the internal metal surfaces of the LC column using either glass or PEEK™ reduces the amount of exposed metal in the flow path of an analyte. The resulting peak shape of some analytes can be improved significantly leading to an increase in sensitivity (sharper peaks are higher peaks) and improved resolution (sharper peaks are less likely to overlap).

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