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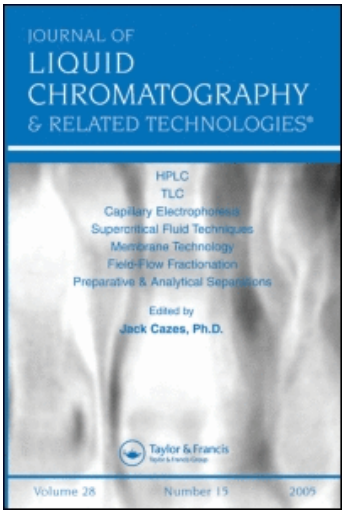

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UNUSUAL ANALYTE ADSORPTION EFFECTS ON INERT LC COMPONENTS

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Abstract

The use of injector loops made of the inert polymer PEEK™ was studied for the analysis of non-steroidal anti-inflammatory drugs (NSAID) using microbore LC with peak compression. Surprisingly it was found that the use of PEEK not only failed to prevent adsorption of analyte in the loop but, in this instance, actually exacerbated the problem. The effect of loop overfill volume, competing NSAID in the sample solution and percentage organic component in the mobile phase on this phenomenon was investigated. Comparison was also made between PEEK™ and other materials used for injector loops.

INTRODUCTION

There is increasing awareness that loss of analyte through adsorption in the LC injection system is a possible problem when the solvent used to dissolve the sample is weaker in elution strength than the LC mobile phase. (1-4) In particular

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this is an issue in the use of low dispersion forms of LC, where often it is necessary to use a "weak" solvent to bring about peak compression (5).

Injection of samples in solutions of low eluting strength in comparison with the mobile phase may be carried out in order to induce on-column focusing or "peak compression" of the sample band on injection of the sample onto the analytical column. By use of this technique the sample band is concentrated on the top of the analytical column before subsequent elution by the mobile phase. In this way peak dispersion is reduced thus allowing the injection of larger sample volumes than would be the case with the injection of samples in mobile phase solution (6-8). Peak compression is of particular importance when using narrow-bore columns, since in its absence, very small injection volumes are necessary to avoid the problem of volume overload of the column.

In view of the adsorption problems experienced when using peak compression using sample injectors fitted with conventional Vespel™ valve rotor seals and stainless steel sample loops (5), it follows that when working in this area, efforts should be made to use inert, non-absorbing materials. In this respect the use of a TEFZEL™ (ETFE) valve rotor seal had already been adopted (5). It was also decided that the use of injector loops made from PEEK™ should be investigated for microbore LC of NSAID

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MATERIALS

Apparatus

The chromatographic system consisted of a Shimadzu LC-10AD pump, SPD-6AV UV/visible detector fitted with an 8 µl flow cell and C-R5A integrator (all Dyson Instruments, Houghton-le-Spring, U.K.). Stainless steel connecting tubing was 0.007" x 9 cm pre-column and 0.007" x 9 cm post-column. A Rheodyne 7125 injection valve was used and fitted with a Tefzel™ (ETFE) rotor seal (Anachem, Luton, U.K.). External sample loops of 20 µl volume were used. The stainless steel and fused silica loops were cut to required lengths in - house. Fused silica tubing (uncoated) 0.25 mm i.d. x 0.4 mm o.d. was supplied by Chrompack (U.K.) Ltd., London, UK and the 20 µl PEEK™ loop was supplied by Whatman (Millipore (UK) Ltd.) Watford, U.K. Spherisorb ODS2 (5 µm), 12 cm x 2.1 mm

i.d. and Spherisorb ODS2 (5 μm), 12 cm x 4.6 mm i.d. stainless steel columns were supplied by Capital HPLC, Bathgate, U.K. and were used with a Spherisorb (10 μm), 10 cm x 4.6 mm i.d. silica pre-column in line before the injector. The column temperature was maintained at 25 °C with a water jacket and Tecam TE-7 Tempette pump/heater supplied by B.D.H., Poole, U.K.

Chemicals

Water was glass distilled and de-ionised (MilliQ purification system, Millipore (UK) Ltd. Watford, U.K.). Methanol was supplied by Rhone-Poulenc Ltd., Manchester, U.K. Nitric acid GPR, 69-72% v/v, Di-sodium hydrogen orthophosphate, AR grade and orthophosphoric acid (85%), GPR, were supplied by B.D.H., Poole, U.K. Indomethacin, flufenamic acid and naproxen were supplied by Sigma, Poole, U.K.

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METHODS

Mobile phase and sample solution preparation

The mobile phase, methanol - 0.02 M phosphate buffer (pH 7.0) (58:42, v/v), was filtered and degassed in an ultra-sonic bath before use. The flow rate used throughout when using the 2.1 mm i.d. column was 0.378 ml min^{-1}, the flow rate used with the experiment conducted on a 4.6 mm column was 1.0 ml min^{-1}. Indomethacin and flufenamic acid solutions, 250 ng ml^{-1}, were prepared in 0.02 M phosphate buffer (pH 7.0), mobile phase and a range of other methanol-aqueous buffer solutions indicated later in the text. Various volumes of these solutions were injected into the chromatographic system described above and peak area data were obtained.

Investigation of the effect of increasing the loop fill volume on the resulting peak areas of indomethacin

A Rheodyne injection valve was fitted with a Tefzel™ rotor seal and 20 μl PEEK ™ loop. A series of 20 μl injections were made using increasing volumes of indomethacin solution, 250 ng ml^{-1} in aqueous phosphate buffer 0.02 M, (pH 7.0)

to flush through the loop and injector during loading prior to injection. At least two injections were made for each loop flush volume. This was followed by a similar series of injections of indomethacin solution 250 ng ml^{-1} in mobile phase. The same experiments were carried out with a 20 µl stainless steel loop, a 20 µl fused silica loop and a commercially obtained (Whatman) 20 µl PEEK™ loop. The stainless steel loop had previously been connected to a Rheodyne valve and flushed with 20% aqueous nitric acid for 20 minutes with a flow rate of 1.0 ml min^{-1}. The experiments using the PEEK™ loop were repeated using flufenamic acid instead of indomethacin. The effect of the presence of naproxen on the peak areas of indomethacin was investigated by passing increasing loop flush volumes of a combined solution of naproxen 500 ng ml^{-1} and indomethacin 250 ng ml^{-1} in aqueous phosphate buffer through the PEEK™ loop.

Further studies of indomethacin interaction with valve components

A 20 µl PEEK™ loop was fitted to another injection valve and increasing loop flush volumes of indomethacin 250 ng ml^{-1} in aqueous phosphate buffer passed through the injection valve and sample loop . The loop was then washed free of indomethacin solution by passing 100 µl of blank aqueous buffer through the loop. The loop was then fitted onto the original valve, and flushed with a further 100 µl of buffer before injection.

Loop flush volumes of 100 µl, 250 µl and 500 µl of indomethacin, 250 ng ml^{-1} in a range of methanol - aqueous buffer ratios from 0% to 58% methanol were passed through the 20 µl PEEK™ loop prior to injection. A similar experiment was also conducted with a 20 µl stainless steel loop but in this instance a 4.6 mm i.d. analytical column was used.

RESULTS AND DISCUSSION

It is common when operating valve injectors to flush a volume of sample solution through the valve in excess of the loop volume to ensure that the sample loop is completely filled. It is recommended that at least five times the loop volume is used for this purpose to give an RSD of 0.1% on replicate injections (9).

However if analyte adsorption was taking place in the injection system, then the amount of analyte swept onto the column by the mobile phase would increase

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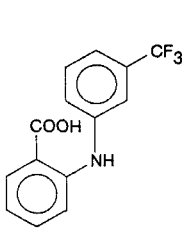
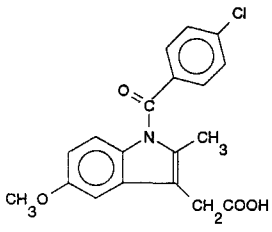
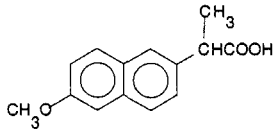


Flufenamic Acid



Indomethacin



Naproxen

FIGURE 1. Structures of NSAID.

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with increasing loop flush volume. This has already been clearly illustrated (5). The purpose of this study was to attempt to minimise adsorption in the injector sample loop in microbore LC of NSAID (Figure 1) by substituting a PEEK™ loop for a stainless steel loop. However it was found (Figure 2) that the peak areas of indomethacin obtained on injection of solutions of indomethacin in aqueous phosphate buffer using the PEEK™ loop increased more rapidly with increasing loop flush volume than occurred when using a stainless steel injection loop. Furthermore, this increase in area did not reach an upper limit followed by a plateau as would be expected if available adsorption sites were being progressively filled. In fact, on increasing the loop flush volume a maximum peak area was reached whereafter the peak areas were reduced. This effect was also noted using a commercially obtained PEEK™ sample loop (Figure 3) believed to be from a different batch of PEEK™.

Using a stainless steel loop of the same volume and i.d., a 13% rise in area for a 100 µl loop flush volume was observed on changing the injection solution from mobile phase to aqueous buffer. As has been demonstrated (5), this rise may be attributed to adsorption in the valve and sample injection loop. The

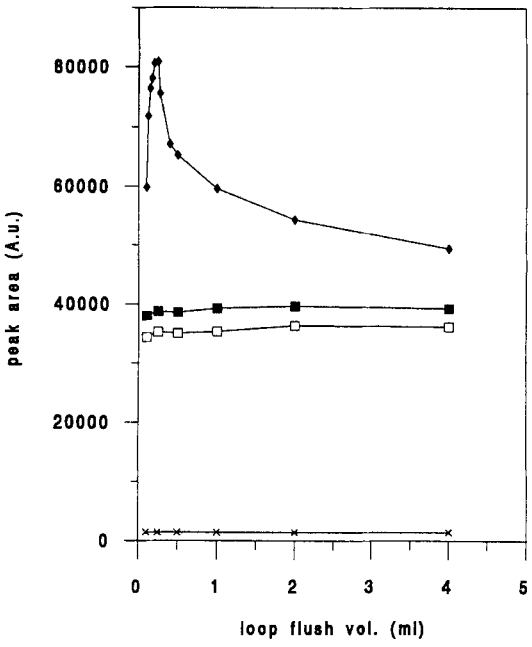

FIGURE 2. The effect on peak areas of indomethacin of increasing the loop flush volumes. ◆ injections of aqueous buffer solution with PEEK™ loop. ■ injections of aqueous buffer solution with stainless steel loop. □ injections of mobile phase solution with stainless steel loop. X injections with PEEK™ loop following blank aqueous buffer wash. Spherisorb ODS2 (5 μm), 12 cm x 2.1 mm i.d.column, mobile phase, methanol - 0.02 M phosphate buffer (pH 7.0) (58:42, v/v), flow rate 0.378 ml min^{-1}. Loop size 20 μl.

corresponding rise for the PEEK™ loop was +81% and this rose to a maximum of +137% when the loop flush volume was increased to 250 μl. The subsequent decrease in areas with higher loop flush volumes was unexpected. It is possible that this unusual behaviour may be due to some kind of aggregation phenomenon. There is a precedent that such a phenomenon may give rise to a sharp change in behaviour in that, for example, the formation of micelles does not take place until a critical concentration has been reached.

Subsequently an experiment was conducted whereby the PEEK™ loop was flushed with increasing quantities of indomethacin solution in aqueous buffer

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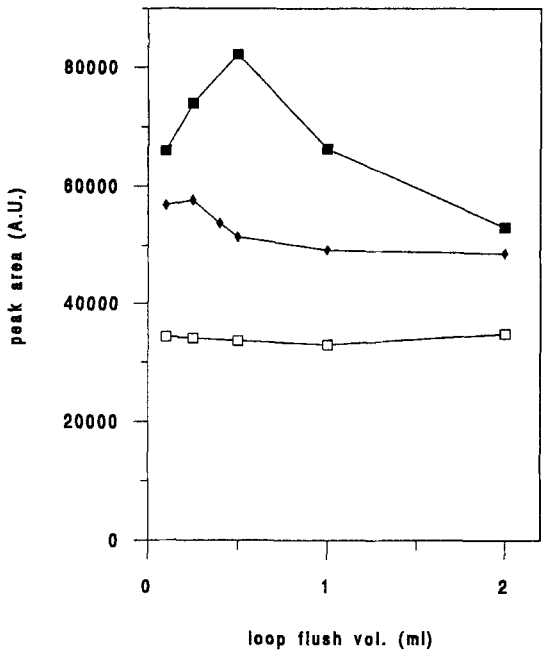

FIGURE. 3. The effect of the addition of naproxen on the peak areas of indomethacin with increasing loop flush volume. ■ injections of indomethacin solution in aqueous buffer, ♦ injections of indomethacin and naproxen solution in aqueous buffer, □ injections of indomethacin solution in mobile phase. A Whatman 20 μl PEEK™ sample loop was used, all other chromatographic conditions as Figure. 2.

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when fitted to another injection valve. The solution was then flushed from the loop with a 100 μl blank aqueous buffer wash and then transferred back to the original valve, washed again with 100 μl of blank aqueous buffer and then an injection made. The results Figure 2 show that although some indomethacin had remained adsorbed in the loop after the blank aqueous buffer wash, the quantity was much reduced and did not increase as the loop flush volume was increased up to 250 μl as had been found previously when the loop was filled in a conventional manner.

The areas obtained on the injection of 100 μl flufenamic acid solution in aqueous buffer were 48% greater than a 100 μl injection in mobile phase when

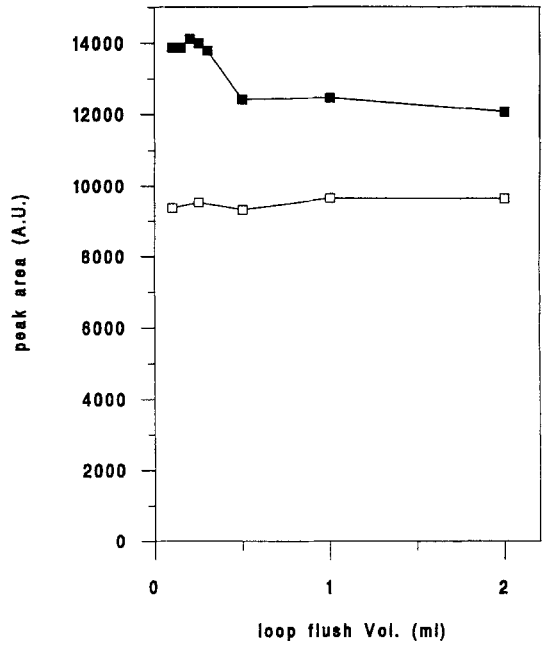

FIGURE 4. The effect on the peak areas of flufenamic acid of increasing loop flush volumes through PEEK™ loop. ■ injections of flufenamic acid solution in aqueous buffer, □ injections of flufenamic acid solution in mobile phase. Chromatographic conditions as Figure 2.

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using the PEEK™ loop but the areas did not show the same percentage rise with increasing loop flush volume as had been noted for indomethacin (Figure 4). This shows that whatever the mechanism of this anomalous effect, flufenamic acid is not affected to the same degree as indomethacin. However the areas obtained using loop flush volumes of solutions in aqueous buffer of more than 500 µl were less than those obtained for 100 µl loop flush volumes as had been noted with indomethacin. Again this is not consistent with what would be expected from an adsorption process eventually reaching a saturation limit as had been noted in previous work using stainless steel loops (5). The lower absolute values for flufenamic acid peak areas compared to indomethacin are due to the lower molar absorptivity of flufenamic acid at 254 nm.

If an absorption or aggregation process was occurring in the injector then the presence of a similar solute may be expected to alter the observed effects due to either interaction with the analyte or competition with the analyte for adsorbing surfaces within the loop. This was investigated by adding naproxen to a solution of indomethacin in aqueous buffer. On injection of this solution it was found that the rise in peak areas of indomethacin with increasing loop flush volume was much less than when naproxen was absent. This suggested that either naproxen interacts with indomethacin preventing the complete interaction of the indomethacin with PEEK™ or that naproxen competitively interacts with PEEK ™ to prevent complete interaction of the PEEK™ with indomethacin. The indomethacin peak areas were still 72% greater for the buffer solution than the solution in mobile phase for 100 µl loop flush volumes but this may be dependant on the concentration of naproxen or other competitive species in the injection solution.

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In the experiments described above NSAID injection solutions had been prepared in aqueous buffer i.e. the extreme conditions for obtaining peak compression. Further work was therefore carried out to determine whether similar behaviour took place in the presence of an organic modifier (methanol) in the injection solution, given that it would still be possible to obtain peak compression when using such methanol containing solutions. Varying the percentage of methanol in the injection solution followed by sample loading through the PEEK™ loop showed that even small reductions in the methanol concentration relative to that in the mobile phase produced a considerable rise in the observed indomethacin peak areas, indicating that adsorption was occurring (Figure 5). However, it was found that the unusual rise and fall in peak areas previously observed with increasing loop flush volumes did not occur for solutions of greater than 10% methanol concentration. This suggested that the interactions taking place in the aqueous buffer / PEEK™ system were probably hydrophobic in nature.

This study was completed by looking at other possible inert loop materials. It was found that, in part, the amount of sample adsorption which takes place within a sample loop depends on the condition of the internal surfaces of the loop. For example, a stainless steel loop which had been pre-flushed with 20% aqueous nitric acid gave negligible sample adsorption compared to that found when it was

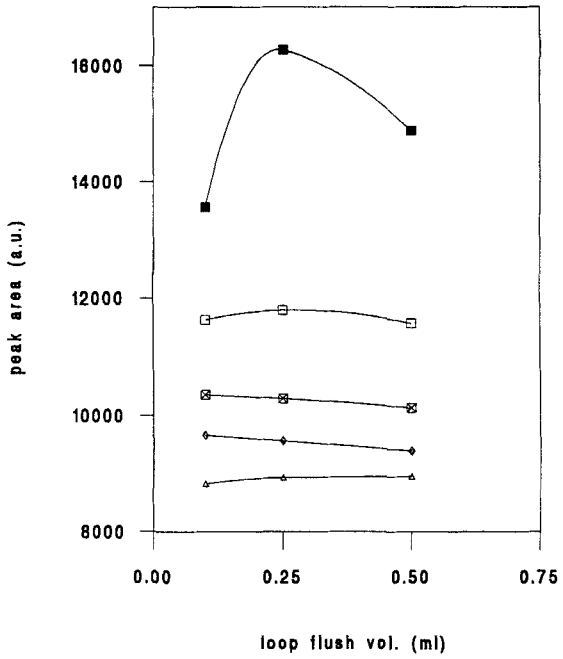

FIGURE 5. The effect on the peak areas of indomethacin on changing methanol concentration of the injection solvent and increasing loop flush volume through PEEK loop. ■ sample solvent, aqueous buffer, □ sample sovent, methanol-buffer, 10:90, ⊠ sample solvent, methanol -buffer 20:80, ♦ sample solvent, methanol-buffer 30:70, Δ sample solvent, mobile phase. Chromatographic conditions as Figure 2.

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untreated. A previously un-used fused silica loop also gave minimal adsorption of indomethacin. However, the degree of sample adsorption in both cases was not zero due to the contributing factors of the other internal valve surfaces. Also, although the percentage adsorption was reduced with the pre-treated stainless steel loops compared with that obtained with PEEK™, again adsorption occurred with only small reductions of the quantity of methanol present in the injection solvent (Figure 6). Furthermore the increase in peak area on increasing the loop flush volume from 100 μl to 500 μl became increasingly significant as the methanol concentration was reduced. This result highlighted the potential source

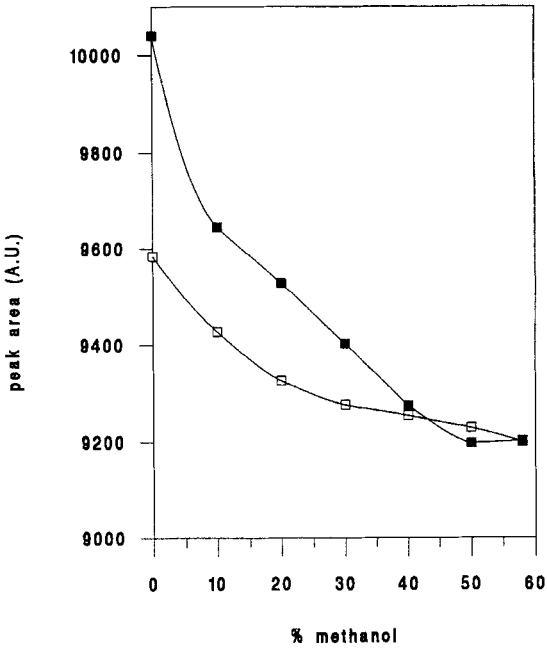

FIGURE 6. The effect on the peak areas of indomethacin on changing the methanol concentration of the injection solvent and increasing loop flush volume through a stainless steel loop. ■ 500 µl injections, □ 100 µl injections, Spherisorb ODS2 (5 µm), 12 cm x 4.6 mm i.d. column, mobile phase, methanol - 0.02 M phosphate buffer (pH 7.0) (58:42, v/v), 1.5 ml min^{-1}. Loop size 20 µl.

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of bias and imprecision which could be introduced into an analytical procedure if loop flush volumes were not consistent between one injection and another and an adsorption process was occurring.

CONCLUSIONS

Relative success in avoiding the adsorption of NSAID when injecting samples of low solvent strength was obtained by using fused silica and acid washed stainless steel sample loops. However the main conclusion from this study was that although steps may be taken to minimise sample adsorption, it may occur when it

is least expected. In addition, minimal adsorption or absence of absorption may be related to factors such as (i) the nature of components present in the sample other than the analyte - this may change and therefore the degree of adsorption may change from sample to sample; (ii) the regularity of acid treatment of stainless steel loops - this is inconvenient, may not always be effective and may have a long term adverse effect on the accuracy of the loops and the condition of other components in the injector. It is therefore recommended that, since analyte adsorption is very difficult to avoid with certainty when using weakly eluting solvents for sample solutions, it is best to proceed as though adsorption will occur. Consequently, in our ongoing work in the area of microbore LC with peak compression, the commonly used loop overfill method is being avoided and a half loop fill procedure is being used.

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