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Demonstration of the compatibility of narrow-bore packed column high-performance liquid chromatography with conventional detection systems

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Abstract: A number of commercially available high-performance liquid chromatography detectors fitted with conventionally sized flow cells (4.5 to 14 μl) have been evaluated for use with narrow-bore (1–3 mm i.d.) packed columns. Contrary to the popular misconception, small flow cell volume (and consequently short path length) does not appear to be a prerequisite for successful use of narrow-bore columns.

The 1 mm i.d. columns when used with certain detectors gave up to a 17-fold increase in sensitivity, compared with conventional 4.6 mm i.d. columns. This work, which was carried out using conventional injection volumes (50 μl), shows that narrow-bore HPLC columns can be used in most laboratories to significant advantage without costly conversion of existing equipment.

Keywords: *Narrow-bore HPLC, detectors, drug analysis.*

Introduction

The use of narrow-bore columns in high-performance liquid chromatography (HPLC) has been the subject of much interest in recent years [1–7]. Such columns can be defined as being conventionally-packed HPLC columns with reduced internal diameters (i.d.) of the order of 1–3 mm.

The advantages offered by the use of these columns are two-fold. Firstly, there are greatly reduced consumable costs, both in terms of packing materials and solvent consumption. Secondly, though more importantly, is the increase in mass sensitivity, this is on-column detectability, due to a reduction in peak dispersion and therefore an 'increase' in the concentration of a solute in a chromatographic peak. The maximum concentration of a solute at the apex of a chromatographic peak, C_{max}, is proportional to $N^{1/2}/D^2$, where N is the column plate count and D is the column internal diameter [8]. Consequently, reduction in internal diameter from 5 to 1 mm offers a 25-fold increase in mass sensitivity.

Despite these advantages there is still some confusion regarding the application of narrow-bore HPLC and the technique has not been widely applied to routine analysis. It has been reported [9, 10] that if injection volumes are scaled up in proportion to the square of the column internal diameter, then lower limits of detection with conventional (5 mm i.d.) columns will be comparable to those of narrow-bore columns. This view, however, ignores the fact that in bioanalysis it is rarely possible to increase the sample or injection volume 25-fold.

There is also a belief that reduced injection volumes and reduced detector flow cell volumes are necessary to realize the theoretical advantages. The use of small injection volumes has arisen from the noted inverse relationship between the dilution factor of a column and the injection volume [11]. Consequently, low or sub-microlite injections therefore have been considered necessary in narrow-bore HPLC. Since for practical reasons, 50 μl is generally considered to be the minimum dissolution volume for a dried extract [12], the increase in mass sensitivity with narrow-bore columns is negated by these reduced sample loadings. However, this problem can be overcome by making large injections in solvents of low eluotropic strength. This has been studied by Claessens and Kuyken [13].

It goes without saying that minimization of pre- and post-column tubing volumes and flow cell volumes is necessary to realize maximum plate counts with narrow-bore columns. In the

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area of narrow-bore HPLC this has led to the development of detectors with significantly reduced flow cell volumes. In the case of photometric detectors, this has been achieved by reducing the path length of the flow cell. But, as absorbance is proportional to the path length, this has had the unfortunate consequence of further reducing the sensitivity gains of the narrow-bore system.

Together these factors have led to the failure to convert mass sensitivity gains into corresponding reductions in limits of detection in bioanalysis, i.e. increased sample concentration sensitivity.

In a recent report, Taylor and co-workers [14] successfully used narrow-bore (1 mm) columns with a flow cell having a volume of 14 μl. Furthermore, other workers [15] have shown that certain purpose designed narrow-bore flow cells can give increased noise compared with conventional designs. This has led to the view that flow cell design and geometry may be as important as flow cell volume. Despite this evidence, there are still many reports which state or imply that the use of narrow-bore columns requires the use of detectors with small volume flow cells [e.g. 1, 10, 16–19]. The purpose of this work has been to clarify this situation with regard to narrow-bore columns.

In this work several commercial detectors fitted with conventional and micro-bore flow cells have been examined for compatibility with narrow-bore columns using conventional injection volumes of a mixture of non-steroidal anti-inflammatory drugs.

Experimental

Materials

Acetonitrile and methanol were obtained from FSA Laboratory Supplies (Loughborough, UK). Orthophosphoric acid was obtained from BDH (Poole, UK). Indomethacin was obtained from Sigma (Poole, UK). All other drugs came from the collection at ICI Pharmaceuticals. Water was distilled in glass in the laboratory.

Columns

All columns were 100 mm in length with internal diameters from 1.0 to 5.0 mm. Conventional 5 mm i.d. stainless steel columns and a similar 3 mm i.d. column were packed in the laboratory with Hypersil 5 μm ODS (Shandon Southern Products, Runcorn, UK) using familiar slurry procedures. A 2.1 mm i.d. column containing the same packing material was obtained from Hewlett Packard (Cheadle, UK). Several 1 mm i.d. columns were obtained from the following companies, HiChrom (Reading, UK), Shandon Southern Products and SGE (Milton Keynes, UK). Table 1 shows the measured plate counts (N) for the columns studied.

Chromatography

The chromatographic conditions employed were essentially those developed by Minkler and Hoppel [20] for the assay of ibuprofen. The eluent consisted of 4 ml of orthophosphoric acid diluted in 550 ml water and 450 ml acetonitrile. The flow rate was 1 ml min^{-1} for

Table 1
Theoretical plate counts (N) for the columns studied as measured using the Perkin Elmer LC-235 detector

Column i.d. (mm)	Manufacturer	N Dimethyl naproxen (k' = 6.7)	N Indomethacin (k' = 11.7)
5	Laboratory	5097	5607
3	Laboratory	6050	6640
2.1	Hewlett Packard	4622	5559
1	HiChrom	3593	4144
1	Shandon	4046	4951
1	SGE	5400	5708

Plate counts were measured from injections of 50 μl vol. into the chromatograph. N was calculated using the following equation:

$$N = 5.54 \times \left[\frac{R_v - I_v}{W_v^{1/2}}\right]^2,$$

where R_v = solute retention volume, I_v = injection volume, and $W_v^{1/2}$ = peak width of half height, expressed as a volume.

the 5 mm i.d. columns, and 0.36, 0.18 and 0.04 ml min^{-1} for columns of 3, 2.1 and 1 mm i.d., respectively. The eluent was monitored either at 230 nm, or where fluorescence detection was used, excitation was at 230 nm and emission at 470 nm.

The test compounds, aspirin, naproxen, the dimethyl analogue of naproxen and indomethacin were prepared in 0.4% (v/v) aqueous phosphoric acid containing not greater than 5% (v/v) methanol. It was found that by restricting the injection solvent methanol concentration to <5%, conventional injection volumes (50 μl) could be used without detriment to the chromatography on the narrow-bore columns.

Equipment

Chromatography was performed with either a LDC Milton Roy Constametric 3000 pump, modified by the manufacturer to give a flow rate range from 0.001 to 3.33 ml min^{-1}, or with a Waters 600 solvent delivery system. Injections were made using a Rheodyne 7125 injection valve fitted with a 50 μl sample loop. All post-injector connections were made with short lengths of 0.006 in i.d. stainless steel or PTFE tubing. Chromatograms were recorded with a Kipp and Zonen BD8 chart recorder or, where diode array detectors were employed, using the plotters supplied with the instrument. Table 2 shows the detectors studied and the volumes of the fitted flow cells.

Results and Discussion

When 3 mm i.d. columns were used in place of a 5 mm i.d. column all the detectors showed increases in response, i.e. mass sensitivity, for a given amount of injected drug. These were generally approaching the 2.78× gain that is theoretically possible. The observed gains were closer to this value for the later peaks in the chromatogram, i.e. those with greater k' values.

When the standard column was replaced with the 2.1 mm i.d. column all the detectors studied exhibited significant mass sensitivity gains with the exception of the LDC Milton Roy UVD (Table 3), with which the observed peak shape was very poor. The theoretical gain in mass sensitivity on reducing column diameter from 5 to 2.1 mm is 5.67×. As can be seen from Table 3 the observed increases appear to approach this value for the Waters 990 diode array detector and the Hewlett Packard 1046A fluorescence detector. The gains appear to be greater than theoretical for

Table 2
Detectors and flow cell volumes

Detector	Type	Flow cell volume (μl)	Path length (mm)
Hewlett Packard (HP) 1040A	UV diode array	4.5	6
Waters 990	UV diode array	8	10
Perkin Elmer (PE) LC 235	UV diode array	8	8
Applied Biosystems (ABS) 783	UV	12	8
		0.5	1
LDC Milton Roy UVD	UV	14	10
Hewlett Packard 1046A	Fluorescence	5	NA

NA = not applicable.

Table 3
Mean increases in detector response with various detectors for injections of equal quantities of the test mixture using a 100 × 2.1 mm i.d. column compared with a 100 × 5 mm i.d. column

Compound	k'*	Mean increases in detector response (n = 3) HP1040A	Waters 990	PE LC-235	ABS 783	HP1046
Aspirin	1.1	2.67×	2.29×	3.24×	2.76×	4.86×
Naproxen	4.6	4.00×	4.61×	5.53×	3.32×	5.19×
Dimethyl analogue of naproxen	6.7	4.53×	5.02×	6.04×	3.51×	5.53×
Indomethacin	11.7	4.49×	5.50×	6.50×	3.89×	NF

NF = not fluorescent under the conditions used.
* k' measured from data generated using a 5 mm i.d. column.

the Perkin Elmer LC-235 detector and this is attributed to the observed higher plate count (N) for this column during this phase of the studies.

Of the detectors tested with 1 mm i.d. columns, two produced chromatograms with good efficiency and significant mass sensitivity gains. These were the Hewlett Packard 1040A and the Perkin Elmer LC-235 diode array detectors. A typical chromatogram obtained using the Hewlett Packard instrument and the HiChrom 1 mm i.d. column is presented in Fig. 1.

The theoretical increase in mass sensitivity on switching from a 5 mm i.d. column to a 1 mm i.d. column is 25-fold. The observed increases, shown in Table 4 indicate that significant mass sensitivity gains are achieved under the conditions described and that these gains increase with increasing k' values, as expected. Capacity factors (k') were difficult to measure accurately when using the 1 mm i.d. columns, due to the chromatographic lag time resulting from the introduction of 50 μl of non-eluting solvent into a system with an approximate dead volume or hold-up time of only 40 μl. Despite this, it can be clearly seen that, for compounds with k' values approaching or greater than 7, narrow-bore packed columns can be used with some conventional detector flow cell systems. Furthermore, this is carried out using conventional injections and such systems can afford large gains in mass sensitivity.

The mass sensitivity gains observed with 1 mm i.d. columns fall short of the theoretical 25-fold. This may be due to the dilution effect of the connecting tubing and detector designs which do not always allow the use of extremely short column to flow cell connections and also to some loss in column efficiency. However, the observed increases in mass sensitivity are of sufficient magnitude to allow the application of the technique to the assay of drugs in biological fluids. In this area the benefits would be two-fold, allowing either similar gains in sample concentration sensitivity or a reduction in required sample volume. In the area of pharmaceutical development, for example, the latter, would make the following of concen-

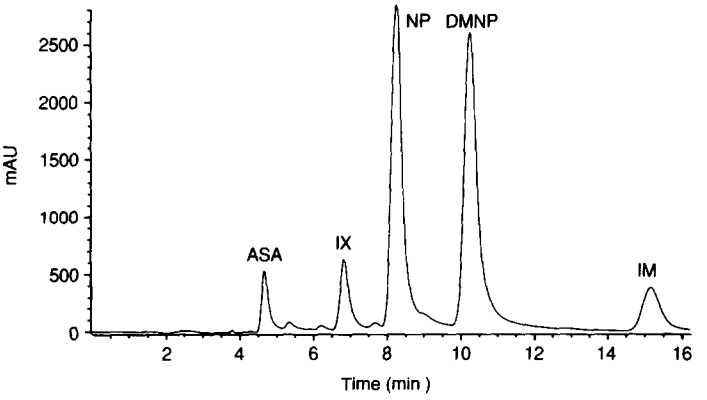

Figure 1
A typical injection of 250 mg of aspirin (ASA), isoxapac (IX, not used in calculations), naproxen (NP), the dimethyl analogue of naproxen (DMNP) and indomethacin (IM) in 5% methanol in 0.4% (v/v) aqueous orthophosphoric acid (50 μl) on a 100 × 1 mm i.d. HiChrom 5 μm Hypersil ODS column. The eluent was acetonitrile–water–orthophosphoric acid 45:55:0.4, v/v (40 μl min^{-1}) and was monitored at 230 nm using the Hewlett Packard HP1040 diode array detector.

Table 4
Mean increase in detector response with two detectors for injections of equal quantities of the test mixture using a 100 × 1 mm i.d. column compared with a 100 × 5 mm i.d. column

Compound*	Mean increase in detector response† (n = 3) Hewlett Packard 1040A	Perkin Elmer LC-235
Aspirin	6.52×	7.37×
Naproxen	10.80×	13.01×
Dimethyl analogue of naproxen	13.96×	15.38×
Indomethacin	17.85×	17.33×

*Compounds listed in order of increasing k'.
†The narrow-bore column was the HiChrom 100 × 1 mm i.d.

tration–time profiles in individual rodents a more practical and realistic proposition with resultant further savings in resources and reductions in observed inter-animal variations.

The problem observed with the other detectors studied including the Applied Biosystems 783 detector even with the purpose built narrow-bore flow cell of 0.5 μl volume was that of severe band broadening. This occurred with all the narrow-bore columns tested. The data suggest that it is the design of the flow cell and connecting tubing and hence factors affecting unswept volume, turbulent flow, etc. rather than absolute flow cell volume which is of fundamental importance in the application of narrow-bore column technology.

Conclusions

In this work the widely held belief that detectors with low volume flow cells are necessary for use with narrow-bore HPLC columns has been addressed. It has been shown that this is not necessarily correct and that considerable gains in mass sensitivity (up to 17-fold), and concentration sensitivity can be achieved using detectors with conventional flow cells and sample injection volumes which are common in the area of bioanalysis. This means that narrow-bore technology can be used in most HPLC laboratories without costly conversion of existing equipment.

Finally, it is suggested that manufacturers need to ensure when designing detectors that the flow cell is situated in a position such that column to detector connections can be made easily, with the minimum of connecting tubing. More importantly, however, to achieve maximum mass sensitivity in narrow-bore applications manufacturers need to give more consideration to design features other than flow cell path length or volume.

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