CHROMSYMP. 1087

ON THE POTENTIAL OF PACKED-COLUMN MICRO LIQUID CHROMATOGRAPHY WITH "IN-COLUMN" FLUORESCENCE DETECTION FOR TRACE ANALYSIS OF DRUGS

M. VERZELE and C. DEWAELE

Laboratory of Organic Chemistry, State University of Ghent, Krijgslaan 281 (S4), B-8900 Ghent (Belgium)

SUMMARY

Packed fused-silica columns of *e.g.* 0.32 mm I.D. provide a form of chromatography (micro liquid chromatography) which can be surprisingly simple and highly sensitive. Existing commercial instrumentation with fluorescence detection "in column" (in the packing through the transparent column wall) leads to the determination of sub-picogram amounts of compounds in biological or other fluids. A detailed analysis is described as an example. Some aspects of micro liquid chromatography are discussed.

INTRODUCTION

Of the various ways of miniaturizing high-performance liquid chromatography (HPLC), packed fused-silica columns (micro LC) appear to be the most promising, it was shown recently that below-pg detection limits are relatively easily attainable with this technique[1,2]. This is much better than conventional HPLC with UV detection, and even surpasses what is expected from a conventional column with fluorescence detection. Such sensitivity is obtained by "in-column" fluorescence detection. The excitation and emission light are measured (in the column, in the adsorbed state) through a "window" made in the polyimide coating of the fused-silica column, just above the end frit. In "on-column" detection, the fluorescence is measured conventionally for the eluted liquid through a window now made in a piece of empty tubing after the end frit. Yang[3] first proposed "on-column" detection for packed fused-silica capillary liquid chromatography. The same name was later used by Shelly *et al.*[4] and by Guthrie and Jorgenson[5] for measurements in the packing. These two ways of detection are obviously different and should not receive the same nomenclature. Therefore we suggest the terms "on-column" and "in-column".

The dilution effect of elution is well known. It is especially detrimental for peaks with (very) high k' values. Fluorescence measurement in the packing, in the column, avoids this dilution and involves the peaks in the adsorbed state at a concentration $k' + 1$ times higher than in the eluted state. With our experimental set-up, in which a 100-μm extension capillary in inserted in a 320-μm packed capillary column, "on-column" fluorescence detection is done on a surface only 100 μm wide,

while "in-column" detection is achieved on the 320 μm diameter capillary. These dimension differences should give a sensitivity difference of 3.2. The overall difference in sensitivity between "on"- and "in"-column detection with our experimental set-up would therefore be 3.2 $(k' + 1)$. This is what we observe for the drug analysis to be described in this paper. In some cases however, e.g., with polyaromatic hydrocarbon (PAH) samples, the sensitivity increase through "in-column" detection is much higher, even reaching a factor of 100. A possible explanation is that the quantum yield of fluorescence can be much higher in the adsorbed state than in solution.

The improved sensitivity, possible with "in-column" detection, enables the detection limit to be lowered to the pg range, as was demonstrated with pyrene[2]. Pyrene, of course, shows strong fluorescence. However, the point is that many compounds fluoresce, albeit at a wavelength where this cannot be observed visually. This then means that, e.g., drugs and pharmaceuticals, can be detected with pg sensitivity in, e.g., serum. The aim of the present paper is to show that this is readily feasible with the instrumentation available in many chromatography laboratories.

EXPERIMENTAL

Chromatographic pumps from different instrument manufacturers were used: Varian 5040 (Varian, Walnut Creek, CA, U.S.A.), Hewlett-Packard 1090 (Hewlett-Packard, Palo Alto, CA, U.S.A.) and Perkin-Elmer LC4 (Perkin-Elmer, Norwalk, CT, U.S.A.). When the pump could not deliver the microlitre flow-rates needed for micro LC, a conventional analytical column was mounted in parallel to generate the desired pressure at the head of the column (flow splitting).

An Hewlett-Packard diode-array detector, an Uvicon UV (Kontron, Zurich, Switzerland) or Varian 2050 and a Perkin-Elmer LS4 fluorescence detector were used. Slight alterations were necessasry to adapt these detectors to the fused-silica micro-LC columns. The injector was either a 60-nl or a 10-μl sample loop injector (Valco, Houston, TX, U.S.A.). The columns were of fused silica, 320 μm I.D., coated with a polymide layer as used in capillary gas chromatography. They were packed at the laboratory. The packing material was 5-μm ROSiL-C18-D (a spherical silica gel, octadecylated and end-capped; RSL/Alltech Europe, Eke, Belgium).

Other instrumentation (recorders, integrators, solvents, etc.) was conventional. The drug used was a beta blocker (tertiary aromatic amine). A detailed account of the instrumental aspects of our approach to micro LC will be published elsewhere.

RESULTS AND DISCUSSION

The chromatograms shown in Figs. 1–4 are self-explanatory. The conventional column chromatogram in Fig. 1 was used to establish the chromatographic conditions. Fig. 2 shows that the miniaturization alone has greatly increased the sensitivity of UV detection. Indeed, the amount injected in Fig. 1 was 3 μg while that injected in Fig. 2 was only 2 ng. These chromatograms were not optimized and are not strictly comparable. Much less than 3 μg could be chromatographed on the conventional column, but 2 ng would barely be seen.

This sensitivity problem of micro LC deserves further attention. It is not as trivial as it may seem. The ratio of the injected volumes (10 μl and 60 nl) is 166.
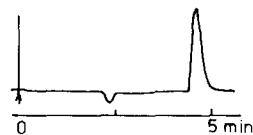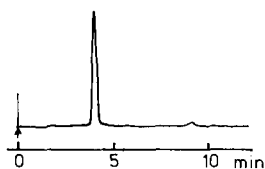
Fig. 1. Conventional HPLC with diode-array detection. Column: 5-μm ROSil-C18-D (25 cm × 0.46 cm). Mobile phase: acetonitrile–water–phosphoric acid, (60:40:0.5); flow-rate 1 ml/min. Detection: 280 nm. Sample: beta blocker at 3 mg/10 ml (10 μl or 3 μg injected).

Fig. 2. Micro LC with "on-column" UV detection at 280 nm. Column: fused silica, 150 mm × 0.320 mm. Packing and solvent as above. Flow-rate: 5 μl/min. Sample: beta blocker 3 mg/100 ml (60 nl or 2 ng injected). Detection is "through" a 0.1-mm capillary. The mass detectability of the beta blocker is below 1 ng.

Thus, for the same sample concentration the sample amount applied to the micro-LC column is 166 times smaller than on the conventional column. The path length of the detector cells is 10 and 0.1 mm (if detection occurs at the dead centre of the micro column) respectively. This leads to at least 100 times lower sensitivity for micro LC. However, the end result is that micro LC is much more sensitive. This apparent discrepancy is related to the fact that the dispersion of the sample during micro LC is 200 times less than on a conventional column. The dispersion depends on the column volume[6] and is equal to 4 $V_0/\sqrt{N}$, where V_0 is the void volume of the column and N is the plate number. The concentration in the detector cell is therefore 200 times higher for the micro-LC column with detection in a 0.1-mm cell. The overall gain factor for the micro-LC column, for the same sample size, is thus indeed about 2, in our experimental conditions.

In Fig. 3 the detection is by "on-column" fluorescence. The detection limit or limit mass detectability is now around 3 pg (twice the noise level). Finally, the detection limit in Fig. 4 with "in-column" fluorescence detection is about 100–200 fg.

It should be noted that no derivatization was required, and that performing this type of chromatography is exactly like traditional HPLC. There is nothing basically difficult experimentally, and the instrumentation is not unusual except for some
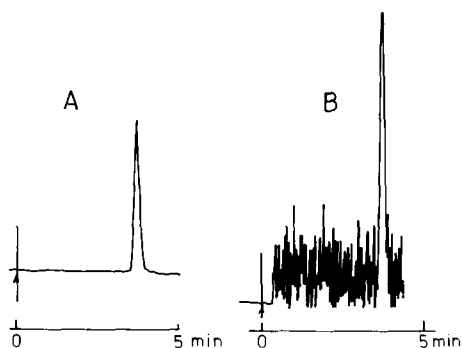

Fig. 3. Micro LC with "on-column" fluorescence detection. Column and solvent as in Fig. 2. Detector: Perkin-Elmer LS4; excitation at 290 nm, emission at 323 nm. Sample: (A), 1 mg/100 ml (60 nl or 0.6 ng injected); (B), 0.01 mg/100 ml (60 nl or 6 pg injected).
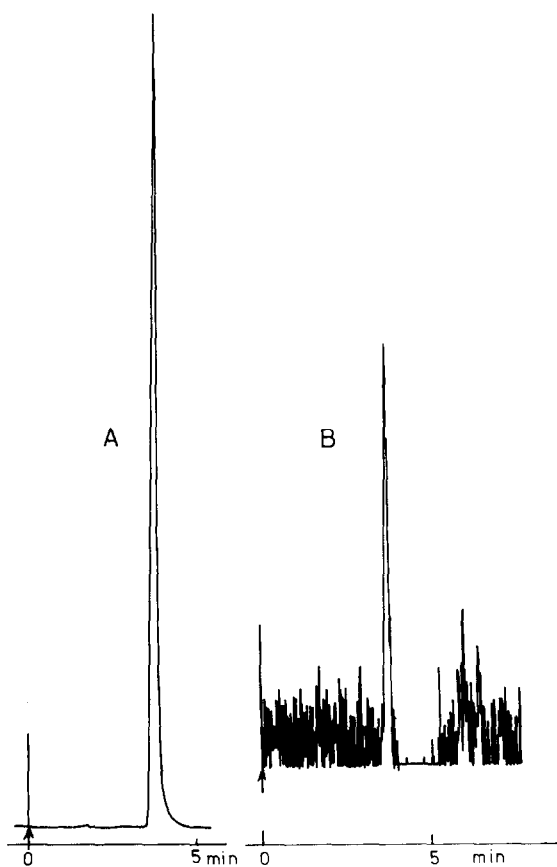
Fig. 4. Micro LC with "in-column" fluorescence detection through a 2-mm window. All other conditions as above. Sample: (A) 1 mg/100 ml (60 nl or 0.6 ng injected); (B) 0.01 mg/100 ml (60 nl or 0.6 pg injected).

small adaptation at the detector site. With 100 fg as the detection limit, it is relatively easy to detect sub-ng/ml of compounds in, *e.g.*, serum. When, for example, 10 pg of a drug are first isolated by liquid- or solid-phase extraction and, after evaporation, redissolved in 100 μl of a suitable solvent, it is necessary to inject at least 10% of this amount or 10 μl into the micro-LC column. Fig. 5 shows that this is easily possible. If the 10 μl of solvent has less eluting power than the isocratic solvent necessary for normal elution, the peak of interest will be concentrated at the column top, and no serious adverse effect will result from the injection of such a relatively very large volume of sample solution. The peak in Fig. 5 shows some tailing, but it also shows that, *e.g.*, 10 pg/ml serum would be easily detectable in the way described. Note that Fig. 5 was obtained without a gradient. The very large sample volume was simply injected with a solvent composition somewhat lower in eluting power than that of the isocratic solvent used for the actual chromatography; 10 μl is about the total amount of solvent in the micro-LC column. This then is to be compared with, *e.g.*, a 2-ml sample injected into a conventional column, as used in Fig. 1.
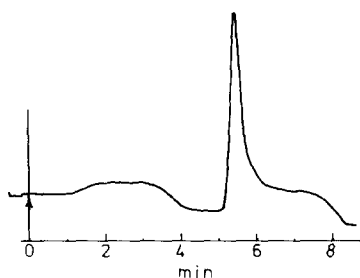
Fig. 5. Micro LC with "in-column" fluorescence detection. Column and other conditions as in Fig. 2. Injection: 10 μl of a solution of acetonitrile–water (2:3) containing 1 pg of the beta blocker.

REFERENCES

1 M. Novotny, in P. Kucera (Editor), *Microcolumn High Performance Liquid Chromatography*, Elsevier, Amsterdam, 1984, p. 246.
2 M. Verzele, M. De Weerdt, C. Dewaele, J. de Jong, N. Lammers and F. Spruit, *LC-GC Mag.*, 4 (1986) 1162.
3 F. Yang, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1981) 83.
4 D. Shelly, J. Gluckman and M. Novotny, *Anal. Chem.*, 56 (1984) 2990.
5 E. Guthrie and J. Jorgenson, *Anal. Chem.*, 56 (1984) 483.
6 M. Verzele, *J. Chromatogr.*, 295 (1984) 81.