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Note

Influence of sample solvent composition and volume on the efficiency of a 3-μm particle reversed-phase column in high-performance liquid chromatography

A. ROUCHOUSE, D. PAULOIN, J. T. BURKE and B. FERRANDES*

Department of Clinical Research, Laboratoires d'Etudes et de Recherches Synthélabo (L.E.R.S.), Meudon-La-Forêt (France)

The general trend in liquid chromatography in recent years has been towards the use of smaller particle packings in order to enhance chromatographic efficiency. The first use of 3-μm particles in reversed-phase high-performance liquid chromatography (RP-HPLC) was reported by Cooke and Olsen in 1980[1]. More recently, reports of applications to commercially available 3-μm reversed-phase columns have appeared[2–6]. The use of 3-μm particle columns with their inherently high efficiencies and low void-volumes imposes additional constraints on the chromatographic system. Extra-column band-broadening must be minimized[6–8]. In particular, equipment designers have recommended that the injected volume be maintained as small as possible ($\leqslant 6$ μl)[6,8]. However, since larger volumes (50–100 μl) are usually necessary to dissolve throughly dry residues following organic extraction, this limitation in the injection volume may lead to a loss in sensitivity for methods used to determine drugs in biological fluids.

Earlier authors, working with 5-μm particle columns[9,10] and microbore columns[11], have shown that the adverse effect of injecting larger sample volumes on the efficiency can be overcome by dissolving the solute in a non-eluting solvent. The aim of the present work is to evaluate whether large sample volumes can be injected into 3-μm columns without any loss in efficiency and whether this technique can be applied to routine analysis. To verify this possibility, increasing volumes (5–100 μl) of chemical products (benzophenone, *p*-nitroaniline) and drugs (carbamazepine, phenobarbital), dissolved in different solvents, were injected into a 3-μm particle column. The influence of the injection solvent composition, injection volume and number of injections were studied.

MATERIAL AND METHODS

Apparatus

All measurements were made with a Beckman Series 341 chromatograph (Gagny, France), consisting of a Model 112 pump and a Model 160 detector operated at 229 nm with a 5-μl (4.5-mm path) flow cell (time constant 0.2 s). A Beckman Model 210 injector, equipped with a 5-, 20- or 50-μl loop (0.5 mm I.D.), was used for single injections whereas a Perkin-Elmer Model ISS 100 autosampler (Bois d'Arcy, France) equipped with a 150-μl sample loop (0.5 mm I.D.) was used for repetitive

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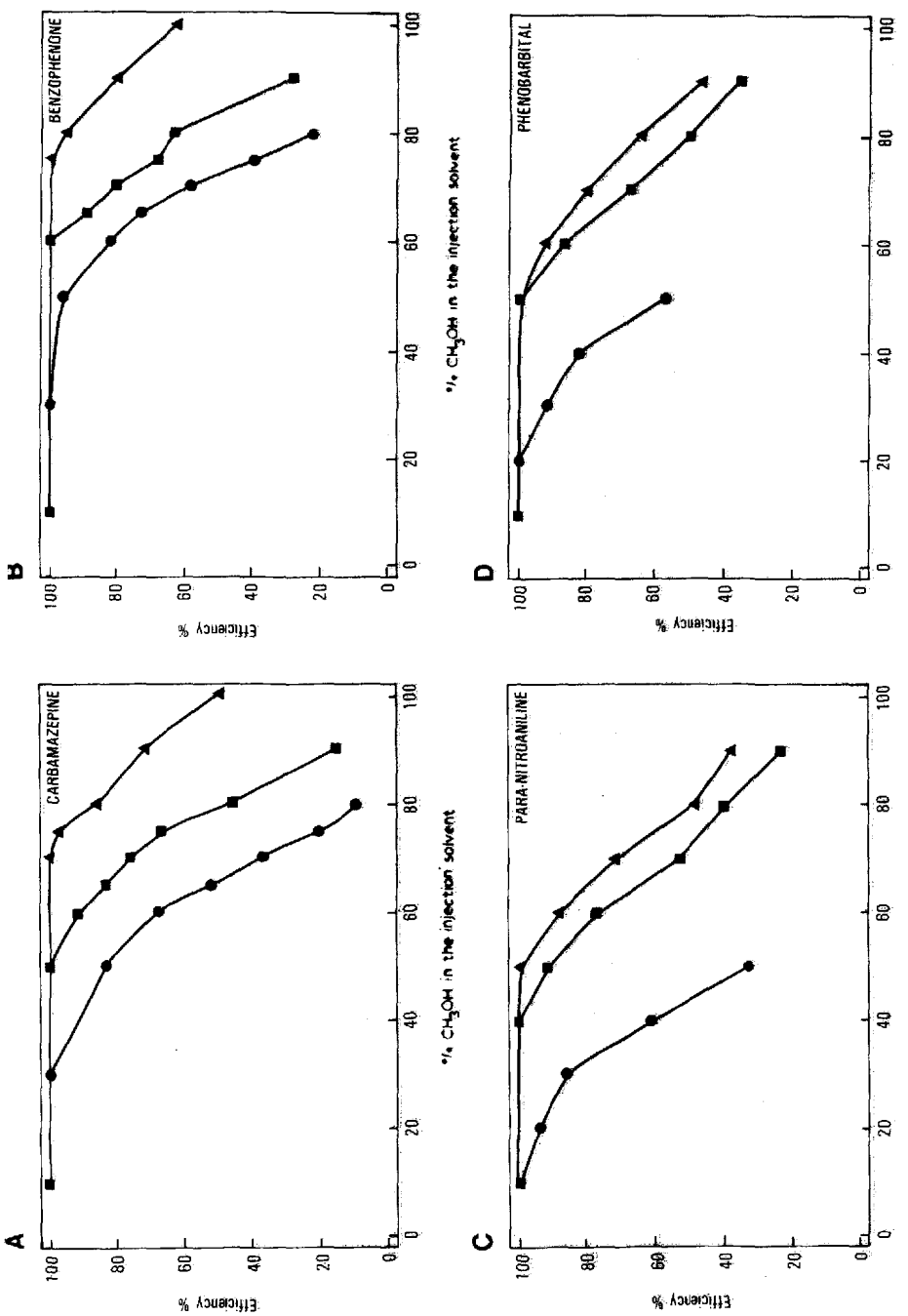

Fig. 1. Relative efficiency as a function of the sample solvent composition and volume. Eluent: methanol–water (70:30) for carbamazepine (A) and benzophenone (B); methanol–water (45:55) for *p*-nitroaniline (C) and phenobarbital (D). ▲, 5 *μl*; ■, 20 *μl*; ●, 50 *μl*.

injections. A total of 30 cm of stainless-steel tubing (0.18 mm I.D.) was used for the various connections. The detector signal was recorded with a Spectra-Physics SP 4270 computing integrator (Les Ulis, France).

Analytical conditions

A 3-μm Ultrasphere ODS column, maximum-coverage, fully end-capped packing (Altex, Berkeley, CA, U.S.A.), was used for all experiments. The column (75 × 4.6 mm I.D.) was operated at ambient temperature. Carbamazepine and benzophenone solutions were eluted with methanol–water (70:30). The mobile phase was methanol–water (45:55) for *p*-nitroaniline and phenobarbital solutions. The influence of repetitive injections on column efficiency was studied with benzophenone solutions eluted with acetonitrile–water (55:45). The flow-rate was maintained at 1.1 ml/min.

Compounds and solvents

Carbamazepine was obtained from Ciba-Geigy (Rueil Malmaison, France), phenobarbital from Sylatec (Tours, France), benzophenone from Sigma (St. Louis, MO, U.S.A.) and *p*-nitroaniline from Prolabo (Paris, France). Methanol (spectrophotometric quality) and acetonitrile (HPLC grade) were supplied by Carlo Erba (Paris, France). Water was purified with a Milli-Q system (Millipore, Vélizy, France).

Methods

The compounds were dissolved in solvents containing increasing percentages of organic modifiers (methanol or acetonitrile). Increasing volumes of samples (5–100 μl) were injected into the chromatographic column.

Efficiency (N) measurements were obtained by using the peak-width-at-half-height method:

$$N = 5.54\left(\frac{t_R}{\delta}\right)^2$$

where t_R is the retention time and δ is the peak width at half-height.

The maximum number of plates (N_{max}) was determined by injecting 5 μl of a methanol–water (10:90) solution containing the test compound. The relative efficiency (N_i), which is the number of plates after i injections divided by N_{max}, was calculated to determined the influence of the sample solvent composition and volume as well as the number of injections on the column efficiency. The N_{max} was verified at the beginning of each series of injections to assure that the column efficiency for the various test compounds was 100 000 plates/m (range 105 000–136 000 plates/m).

RESULTS AND DISCUSSION

The influence of the sample solvent composition and volume on the relative efficiency are displayed in Fig. 1A–1D for carbamazepine, benzophenone, *p*-nitroaniline and phenobarbital, respectively. Each point is a mean of three experiments. The retention times of carbamazepine and benzophenone were 1.3 and 2.6 min, respectively, with methanol–water (70:30) as the mobile phase. *p*-Nitroaniline and phenobarbital, which are more polar, were eluted with methanol–water (45:55) and their

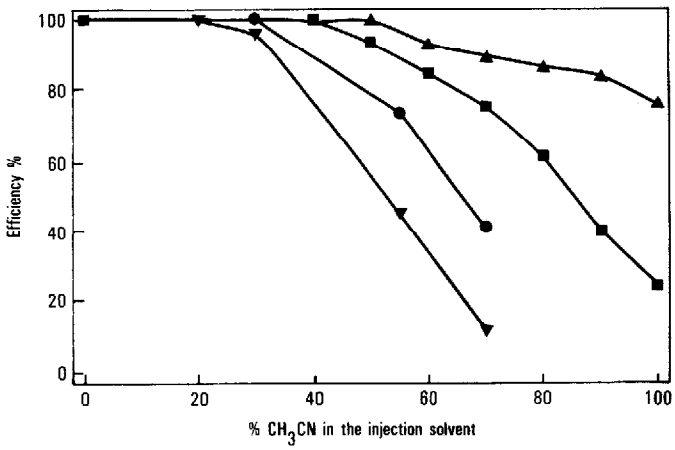

Fig. 2. Injection of large volumes (up to 100 μl) of benzophenone solutions onto a 3-μm ODS column: influence of the sample solvent composition and volume. Eluent: acetonitrile–water (55:45). ▲, 5 μl; ■, 20 μl; ●, 50 μl; ▼, 100 μl.

retention times were 1.6 and 2.6 min, respectively. These data show that the relative efficiency is a function of the injection solvent composition and volume. Sample volumes of 50 μl can be injected without any loss of efficiency if the injection solution is a non-eluting solvent (one which contains a smaller percentage of organic modifier than the mobile phase). Furthermore, the smaller the injection volume, the greater the percentage of organic modifier in the sample solvent can be. For example, 20 and 50 μl of carbamazepine solutions can be injected without any loss in efficiency if the injection solvent contains approximately 50 and 30% methanol, respectively. Losses in efficiency were observed, even with 5-μl injection volumes, when the sample solvent contained a higher percentage of organic modifier than the mobile phase. These losses were probably the result of a lack of peak compression.

As the k' increases, the loss in efficiency is less marked following injection of larger volumes. Fig. 1A and 1B shows that following injection of 50 μl containing 50% methanol in the sample solvent, the loss in efficiency for carbamazepine was 18% whereas the loss was only 5% for benzophenone. This phenomenon was also noted following injection of a mixture of *p*-nitroaniline and phenobarbital (see Fig. 1C and D).

TABLE I

RELATIVE EFFICIENCY N_i/N_1 (%) AFTER REPETITIVE INJECTIONS OF BENZOPHENONE DISSOLVED IN A NON-ELUTING SOLVENT (ACETONITRILE–WATER, 20:80): INFLUENCE OF THE NUMBER AND VOLUME OF INJECTIONS

Eluent: acetonitrile–water (55:45).

Injected volume (μl)	*No. of injections*			
	30	*40*	*50*	*60*
30	100	100	100	92
50	97	90	79	64

The injection of large volumes containing benzophenone dissolved in a fixed percentage of acetonitrile (Fig. 2) compared with the injection of benzophenone dissolved in the same percentage of methanol (Fig. 1A) demonstrates that the loss in efficiency will be smaller with methanol. This is in accordance with the relative solvent strengths of the two liquids in RP-HPLC and indicates that one can usually employ sample solvents with a larger percentage of methanol than of acetonitrile. Therefore, during the determination of poorly soluble drugs in biological fluids, it may be more advantageous to use methanol-containing solvents to dissolve the dry extracts. The dissolution properties of the solute in the sample solvent should, however, also be considered.

During a series of analyses, the column performance should not change if accurate measurements are desired. The relative efficiency after repetitive injections of benzophenone dissolved in a non-eluting solvent (acetonitrile–water, 20:80) is reported in Table I. These data indicate that it is possible to inject thirty 50-μl samples or fifty 30-μl samples without any loss in efficiency during routine analysis. Nevertheless, peak areas instead of peak height should be used for quantitative assays to reduce errors that may arise following abrupt changes in column efficiency. Column performance lost during repetitive injections of large volumes may be recovered by repacking the first millimetre of the stationary phase with a spatula.

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