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Performance evaluation of an aqueous–organic phase separator for post-column reactions in high-performance liquid chromatography, and its application to the enhanced detection of some basic drugs of abuse*

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Abstract: A phase separator is described that is suitable for post-column HPLC applications. It operates with commonly used HPLC eluents and immiscible organic solvents as long as the two phases remain immiscible. It is compatible with gradient elution systems. Separation efficiency is routinely better than 0.8, which ensures that analyte peak heights are about 95% of the maximum height under these conditions. An application for the detection of pethidine, cocaine, methadone, piritramide and dipipanone at 0.8–1.8 ng on-column loadings is described.

Keywords: *Aqueous–organic phase separator; post-column HPLC; basic drugs of abuse.*

Introduction

The extraction of compounds between water and immiscible organic solvents has been exploited in analytical procedures for many years, primarily as a batch extraction step, but more recently has been utilized as a continuous process in high-performance liquid chromatographic (HPLC) systems. For some compounds that possess weak UV absorptivity, HPLC detection can be considerably enhanced by the addition of a post-column reaction step with a chromophoric reagent. The minimization of analyte band spreading during this reaction step is a major consideration in the design of post-column systems. For reactions such as analyte plus chromophoric ion-pair reagent, that require an extraction step into an immiscible organic solvent, band spreading is minimized by segmentation of the HPLC aqueous eluent by the immiscible organic solvent, via a "T" connector. The segmented flow is then directed through a coil to enable liquid–liquid extraction to take place, and then a phase separator is required that is capable of continuously delivering a clean (water droplet-free) stream of organic phase to the detector.

In 1978, Karlberg and Thelander [1] published the first of many papers that developed the technique of flow-injection analysis (FIA), and described a simple modification of the glass Technicon (Tarrytown, NY, USA) A4 "T" — connector as a separating device. Teflon fibres in the connector guided the organic phase downwards to the detector and away from the upper aqueous phase. A similar approach was described by Lawrence *et al.* in 1979 [2] who used Teflon tubing to obtain the same effect. The availability of Teflon filter membranes and porous plugs has resulted in their exploitation in a number of designs [3–11] described between 1979–1988. They all depend upon the organic phase wetting and passing through the Teflon membrane or plug which is enclosed in a small volume chamber that rejects the aqueous phase together with some of the organic phase. None of these designs is commercially available. It has been reported [12], that biological fluids damage these membranes, reducing their useful working period.

In 1977, Copsey [13] described a "microcell on-line extractor for biological fluids" that exploited the dissimilar wetting properties of water and organic solvents towards hydrophilic

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(glass) and hydrophobic (PTFE) surfaces. This approach was adopted by Kinkel and Tomlinson who described a design in 1980 [14] that consisted of a single separating channel, and demonstrated its suitability for the rapid determination of drug partition coefficients and ion-pair extraction constants. No Teflon membrane or porous plug was needed in this design, so that its performance did not deteriorate with use. This design was made available to one of us (TMJ) for testing as an HPLC post-column phase separator, and found to be capable of delivering clean organic phase to the detector at aqueous/organic flow rates of 1 ml min^{-1} each, but subject to external leakage at these flow rates and also to the irregular break-through of water droplets into the organic phase. In order to overcome these difficulties a separator was designed by Jefferies that possessed three long channels instead of one short channel. The aqueous and organic phases were mainly separated in the central channel and then each phase could be "cleaned" a second time in separate channels, simultaneously. This was a novel approach and it increased the separation efficiency (fraction of organic phase sent to the detector) from about 0.6 to almost 1.0. Unfortunately, like the Kinkel and Tomlinson design, it leaked under pressure. In 1987 de Ruiter *et al.* [12] described the evaluation of several variations on a design that consisted of two stainless steel blocks with a thick PTFE gasket held together by four bolts. Each design possessed a single, short, separating channel with small (30–45 μl) internal volumes and provided separating efficiencies of 0.30–0.35 at 1 ml min^{-1}. A miniature design was also made with an internal volume of 8 μl that was suitable for narrow bore HPLC at 0.2 ml min^{-1}. These designs are commercially available from the Free University, Amsterdam. This report describes the performance of a dual channel design by Jefferies, that routinely provides separating efficiencies of at least 0.8, does not leak under typical HPLC conditions, and requires little attention during use.

Experimental

Apparatus

Figure 1 shows the design of the phase separator (Scientific Systems Inc., State College, PA, USA) which consists of two stainless steel blocks (A and C) and a central

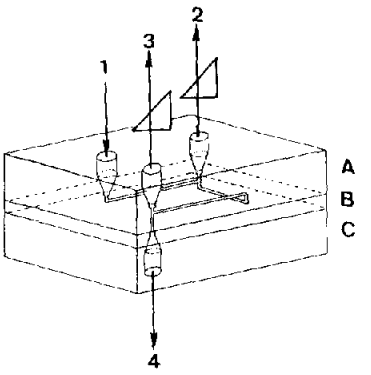

Figure 1
Phase separator design. A and C are stainless steel blocks, B is a Teflon block. 1, Input of HPLC eluent segmented by the immiscible organic phase; 2, microneedle valve to control the exit of aqueous phase plus droplets of organic phase; 3, microneedle valve to control the exit of droplets of aqueous phase in organic phase; and 4, exit of organic phase (water droplet free) to the detector.

PTFE block (B) held together by four bolts (not shown). Grooves were cut into both the steel and PTFE blocks to produce two channels having steel and PTFE surfaces, with a total internal volume of about 100 μl. Each channel has an exit controlled by a MCV-50 microneedle valve (Scientific Glass Engineering (UK) Ltd, Milton Keynes) for the controlled release of aqueous phase. All stainless steel tubing connections are made to the separator by 1/16 inch. SSI male fittings. The separator was configured with other components to produce an HPLC post-column reaction extraction unit (Fig. 2). A ternary gradient pump (model GS402, SSI) was used for the HPLC eluent, with an in-line Uptight pre-column (20 × 2 mm i.d.) packed with CPS-Hypersil to protect the analytical column from mobile phase effects. A Rheodyne 7125 injection valve with 10- or 20-μl loop was fitted into a model 505 LC column oven(SSI) maintained at 40°C. The analytical column was a 100 × 2 mm i.d. glass lined column packed with 5 μm Hypersil-CN (100GL2-CN-8/5,SGE). The two post-column pumps were model 350 (flow range 0.1–1.5 ml min^{-1}) pumps with either a model 210 Guardian pulse dampener or model LP-21 LO-pulse dampener(SSI). Additional pulse dampening was provided by 100 × 4.6 mm i.d. columns packed with 10 μm Partisil silica (aqueous phase) and 10 μm CPS-Hypersil (organic phase). The addition of the aqueous reagent to the HPLC eluent and segmentation of aqueous phase with organic phase was achieved using Tee-connectors, 1/16

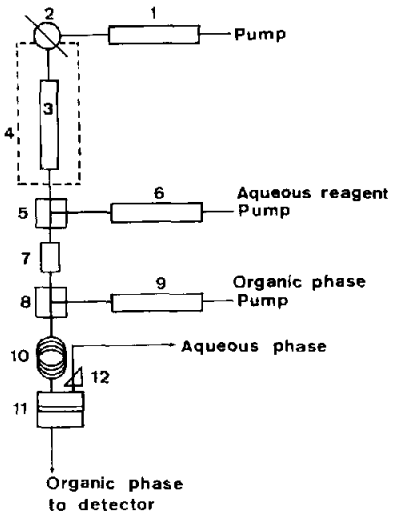

Figure 2
HPLC post-column reaction extraction unit. 1, In-line column; 2, injection valve; 3, analytical column; 4, column oven; 5, mixing Tee; 6, in-line column; 7, mixing column; 8, segmentation Tee; 9, in-line column; 10, extraction coil; 11, phase separator; 12, needle valves.

× 0.015 inch (SSI). An Uptight precolumn, 20 × 2 mm i.d., packed with 75 μm glass Ballotini beads, was used as a mixing chamber. The extraction coil was 0.8 mm i.d. stainless steel or PTFE tubing of various lengths and coil diameters. Detection was by a Jasco 820FP fluorimeter (LDC Analytical) connected to a Servogor 120 recorder.

Materials

Methanol, acetonitrile-far UV, isopropyl alcohol, heptane, dichloromethane, and 9,10-dimethoxyanthracene sulphonic acid, sodium salt were HPLC grade and obtained from Fisons (Loughborough, UK). Buffer components were analytical reagent grade and obtained from Fisons (Loughborough, UK). Water was double-distilled from a glass still.

Results and Discussion

Evaluation of the phase separator

Separation efficiency. The performance of the separator was evaluated in the configuration shown in Fig. 2 but without the HPLC column or detector. The aqueous phases tested were distilled water, and aqueous methanol or aqueous acetonitrile containing 10, 20, 40 or 60% (v/v) organic solvent. All the aqueous phases contained the green dye, Screened Methyl Orange; the organic phase was chloroform. Needle valve 2 was adjusted to permit the release of aqueous phase plus a small volume of organic phase, and then needle valve 3 was adjusted to permit the release of the remaining aqueous phase plus the minimum volume of organic phase. Measurements of clean (completely free from any green aqueous droplets) organic phase were then made for at least 1 h into a covered measuring cylinder. The range of aqueous/organic flow rates studied were: 1.0:1.0, 2.0:1.0, 1.2:0.6, 0.5:1.0 and 0.7:1.4. The separation efficiency (SE) of the separator was then defined as the ratio of the flow rate of organic phase from the separator divided by the flow rate of organic phase entering the separator.

It was found that SE values of 0.8 or better were readily obtained and, in the case of acetonitrile, often reached 1.10. This is possible because of extraction of the acetonitrile into the chloroform. Above 70% (v/v), both methanol and acetonitrile are completely miscible with chloroform, and so 60% (v/v) is the practical limit. The orientation of the separator had no effect upon its performance, in that it worked equally well upside-down, indicating that the separation effect was due to the wettability of the steel and PTFE surfaces towards the aqueous and organic phases, respectively. Density differences between the two phases were not necessary.

Post-column band broadening. A measure of the individual contributions to band broadening made by the segmentation Tee connector (8, Fig. 2) and phase separator when employed in the arrangement shown in Fig. 2 but without the components 3–7 was attempted. Using acetonitrile–water (20:80, v/v) as the aqueous phase with 1,2-dichloroethane as the organic phase, at aqueous/organic flow rates of 1.0:1.0 ml min^{-1}, the separation efficiency was adjusted to 0.8. Injections of a biphenyl solution (20 μl, $n = 10$) were then made and the peak widths measured at 10% peak height. This was repeated, firstly after removing the coil, and then the phase separator. With all the components in place, the mean initial band width was 11.4 s, of which the coil contributed 1.2 s, and the phase separator 3.5 s. This study was repeated at a separation efficiency of 0.99, and for a mean initial band width of 13.4 s, the coil contributed 2.2 s and the separator 4.1 s. When the study was repeated at an SE of 0.4, with heptane as the organic phase, the mean band width increased to 20.3 s, with coil and

separator contributions of 4.0 and 9.7 s, respectively.

Effect of separation efficiency on band width and peak height. In view of the observed effect of separation efficiency on band width, two additional aqueous/organic systems were studied to provide further data, namely methanol–water (70:30)/dichloroethane, and isopropyl alcohol–water (20:80)/heptane. The results for all the solvent systems examined showed that relative peak heights increased from about 70 to about 95% maximum peak height when the separation efficiency was raised from 0.4 to 0.8 (Fig. 3). Broadly similar results were obtained by De Ruiter *et al.* [12]. Band widths in the four systems decreased from 14–26 to 9–17 s, when the SE was raised from 0.4 to 0.8. For biphenyl as a test analyte, dichloroethane gave taller peak heights than heptane when acetonitrile–water (20:80, v/v) was the aqueous phase (Fig. 3). Choice of organic phase is also influenced by the solubility of the analyte in the organic phase. For post-column ion-pair reactions, the nature of the analyte–ion-pair complex will determine peak height in various organic solvents. Dichloroethane appears to be a good solvent for both aqueous methanol and aqueous acetonitrile systems.

The effect of SE on peak shape can be seen in Fig. 4. The decrease in peak band width at higher SE values is considered to be due to the increased linear velocity of the organic phase through the detector flow cell. It was possible to run the phase separator at a separation efficiency of about 0.9 without leakage of water droplets to the detector for as long as required. The same efficiency was obtained with acetonitrile–water (20:80, v/v)/pentanol–dichloroethane (10:90), and isopropyl alcohol–water (20:80, v/v)/pentanol–dichloroethane (10:90, v/v) solvent systems.

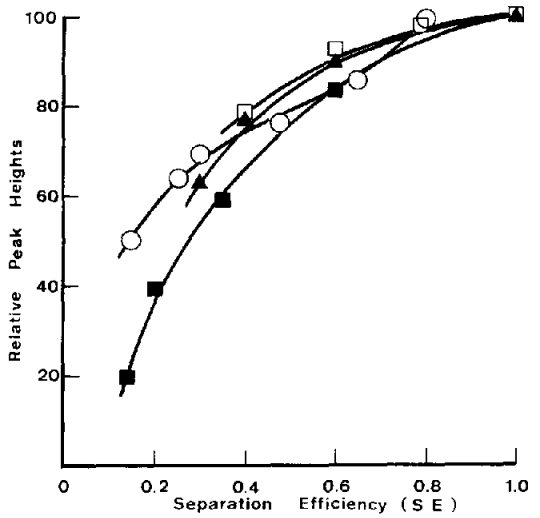

Figure 3
Effect of separation efficiency on peak height. ○, Acetonitrile–water (20:80, v/v) with heptane as organic phase; □, acetonitrile–water (20:80, v/v) with 1,2-dichloroethane; ▲, methanol–water (70:30, v/v) with 1,2-dichloroethane; ■, isopropyl alcohol–water (20:80, v/v) with heptane.

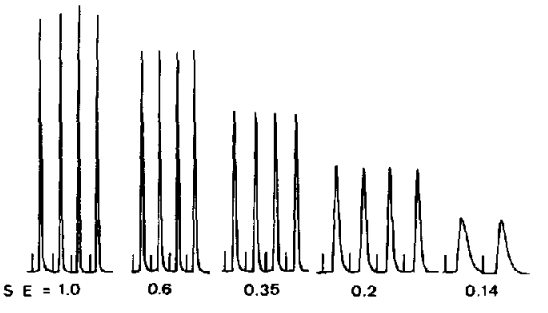

Figure 4
Effect of separation efficiency on peak shape. Isopropyl alcohol–water (20:80, v/v) with heptane.

Application to some drugs of abuse

Some drugs of abuse are difficult to detect because they possess weak UV absorptivity and also cannot be readily derivatized because they are tertiary amines. In a previous study [15] sensitivity was considerably enhanced to an on-column loading of about 8 ng for a range of compounds using UV detection at 205 nm. The availability of the phase separator enabled post-column ion-pair formation with 9,10-dimethoxyanthracene sulphonic acid, sodium salt (DAS) to be examined as an alternative approach. Pethidine, cocaine, methadone, piritramide and dipipanone were selected as typical of this type of analyte and, by using the components in Fig. 2, their operating conditions were optimized for maximum sensitivity. This will be reported elsewhere. Calibration solutions were prepared using normethadone as an internal standard, and linear regressions obtained ($r \sim 0.992$) down to 2 ng on-column (Fig. 5). This is a four-fold improvement in sensitivity over that obtained in the previous study [15] at 205 nm. Preliminary work indicates that the post-column system is less affected by interfering peaks extracted from plasma and urine, and extraction techniques are currently being optimized.

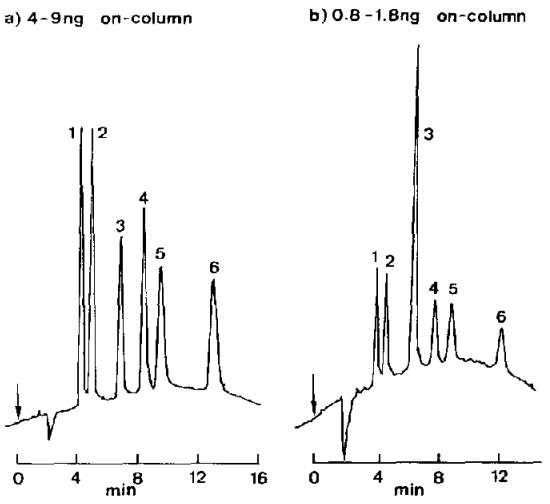

Figure 5
Enhanced detection of some drugs of abuse. Column, SGE 5 μm CPS-Hypersil (100 × 2.1 mm i.d.) with gradient elution at 0.2 ml min^{-1}. Solvent A, 0.025 M phosphate buffer (pH 4)–isopropyl alcohol–acetonitrile (94:3:3, v/v/v), Solvent B, as A but (50:25:25, v/v/v). Gradient, 0–5 min A:B (80:20, v/v), 5.1 min step gradient to A–B (55:45, v/v) until 20 min, 20.1–21.0 min linear gradient to A–B (80:20, v/v), 21.1–30 min A–B (80:20, v/v). Injection volume 10 μl containing in (a) 4–9 ng each solute and in (b) 0.8–1.8 ng each solute, dissolved in mobile phase. Post-column ion-pair formation with 9,10-dimethoxyanthracenesulphonic acid (8.8×10^{-5} M) at 0.4 ml min^{-1} followed by extraction into dichloromethane at 0.6 ml min^{-1}.

Conclusions

The phase separator routinely operates at separation efficiencies better than 0.8, which ensures maximum peak heights, and requires little attention during use. It can operate with HPLC eluents up to the point of their miscibility with the selected organic phase. Band broadening is minimal, about 3.5 s. Gradient elution systems can be used. The detection of some drugs of abuse has been further enhanced to about 2 ng on-column.

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