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Bypass high-performance liquid chromatography for purification of trace analytes

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Abstract

A technique that we have termed ''bypass HPLC'' is introduced as a noncontaminating way to define the retention time of a trace analyte for purification purposes on an HPLC column when the amount of the analyte in real samples is too low for on-line detection. The technique employs two HPLC columns (''calibration'' and ''purification'') of the same type that are connected in parallel, with appropriate valving, along with use of two accessory compounds. By injecting ordinary (on-line detectable) amounts of authentic analyte plus the two accessory compounds on the calibration column, and similarly the two accessory compounds onto the purification column, one can predict the retention of the analyte on the latter column without contaminating this column, as follows. The migration times of the first accessory compound provide a reference time on each column; the migration times of the second accessory compound are normalized on each column by subtracting the corresponding reference times; and then the retention time of analyte can be calculated on the second column since the ratio of its normalized retention times on the two columns equals the corresponding ratio for the second accessory compound.

Keywords: Bypass HPLC; Trace analysis

1. Introduction

Chromatographic steps are commonly employed in trace organic analysis for purification purposes. Probably solid-phase extraction (SPE) is most popular since it is a simple and convenient technique including the use of disposable, relatively low-cost extraction devices. However, the need to maintain a low production cost can compromise the performance of some SPE materials, especially for recovery of adsorption-prone analytes including batch-to-batch variations in this feature. In general, the performance is more likely to be poorer for SPE with less analyte and a more complex analyte matrix. This may largely be due to the difficulty in controlling active sites in a low-cost production process. Another general shortcoming of SPE is that it typically provides a low-resolution separation. Sometimes users will tune up the SPE conditions carefully (e.g. Ref. [1]), but this can be time consuming since detection after SPE is usually off-line, and additional steps also may be present in a method before detection is performed.

In our work on trace (sub-nanogram) detection with an emphasis on derivatization to enhance sensitivity, the above limitations of SPE are encountered frequently. As a consequence, we usually rely instead on HPLC for analyte purification. For example, we [2] along with others [3] have recommended HPLC for sample clean-up prior to

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detection of electrophore-derivatized analytes by gas chromatography electron-capture mass spectrometry (GC-EC-MS). Typically this gives a high-performance sample clean-up without much effort at tuning the separation conditions. Another motivation for such use of HPLC in our laboratory is multi-analyte detection in a given procedure. However, adsorption-prone analytes can readily contaminate an HPLC system [4].

''Satellite HPLC'' is one way to help control the contamination problem in sample purification by HPLC, at least where the problem arises due to injecting ≥ ng amounts of authentic analyte into a column in order to define the retention window for collecting much lower amounts of an invisible analyte (invisible to the on-line detector) in real samples [5]. This technique is based on the observation that most (e.g. 99.9%) of the carryover in HPLC can arise in the injector [6]. The satellite HPLC system is simply a secondary HPLC comprising a low-cost but reliable pump, injector, and thermostatted column. No detector is present since it plays no role, and its absence eliminates potential contamination sites. One first installs the given column in a conventional (parent) HPLC system fitted with a detector where the retention time of ordinary (on-line detectable) amounts of standard analyte are determined. Then, just the column and mobile phase are transferred to the satellite HPLC system for purification of real samples. Satellite HPLC systems have been in routine use in our laboratory for several years and work well for us.

Here we introduce ''bypass HPLC'' as a second way to avoid contamination of an HPLC system when ordinary amounts of analyte are injected to define a retention window.

2. Experimental

2.1. Reagents

Glycolic acid, triethylamine, α-hydroxy-γ-butyrolactone, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC), 2-(morpholino)ethanesulfonic acid (MES), and 1-hydroxybenzotriazole hydrate (HOBT) were purchased from Aldrich (Milwaukee, WI). AMACE1 and compound **3** were prepared as described [7]. All organic solvents (including those used for cleaning) were Optima Grade from Fisher (Pittsburgh, PA). All solutions were v/v unless indicated otherwise.

2.2. Synthesis of *N-{N-[3′,5′-(trifluoromethyl)benzyl]-N-[methyl]amidomethyl}-2,4-dihydroxy-butyramide* (**2**)

A solution of 24 mg (0.3 mmol) of α-hydroxy-γ-butyrolactone and 0.1 ml of triethylamine in 0.5 ml ethanol–water (1:1) was stirred at 40 °C for 2 h and then evaporated under N_2. Following the addition of 105 mg (0.3 mmol) of AMACE1, 81 mg (0.6 mmol) of HOBT and 77 mg (0.4 mmol) of EDAC in 4 ml of 0.1 MES (pH 7.0), the solution was stirred at room temperature overnight under N_2. It was then quenched with 10 ml of water and extracted with 30 ml of ethyl acetate. The upper phase was washed with ice-cold 0.5 N HCl, water, ice-cold 0.5 N NaOH, and brine followed by drying over $MgSO_4$, evaporation, redissolving in 0.5 ml of acetone, and then flash chromatography on 15 g of silica with elution by acetone/hexane (2:1) to give white plates (0.28 g, 68%), m.p. 105–106 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.19–7.89(d, 3H), 4.74(s, 2H), 4.15–4.09(d, 2H), 3.59–3.48(m, 1H), 3.02–2.98(s, 3H), 2.53–2.46(m, 2H).

2.3. Instrumentation

For HPLC purification of AMACE1 derivatives of glycolic acid, two Zorbax SB-C_{18} columns (4.6×150 mm, 5 μm) were used, one as a calibration column (6 months of prior use before assembly into our bypass HPLC system) and one as a purification column (no prior use), in a HP 1100 series HPLC system (Agilent, Wilmington, DE) contacted with a G1313A autosampler and a G1315A DAD detector. With the addition of a manual injector (we used a Rheodyne 7125 valve from Agilent), and modification of the plumbing, this HPLC system was converted into a bypass HPLC. Acetonitrile and exactly 0.1% trifluoroacetic acid (40:60, mixed dynamically by the HPLC system) at a flow-rate of 1.2 ml/min was used as the mobile phase with detection at 265 nm.

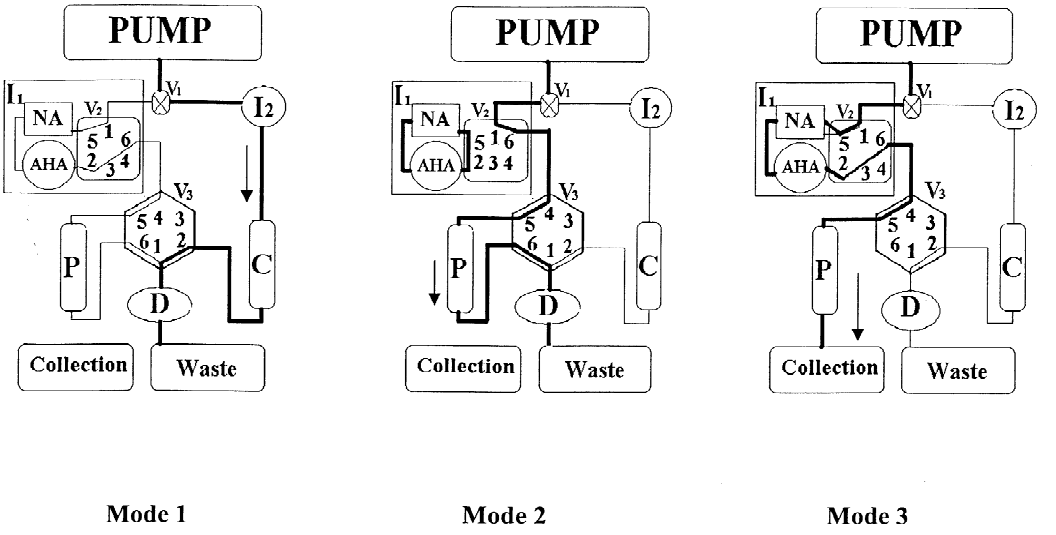

Fig. 1. Bypass HPLC system: P, purification column; C, calibration column; I_1, autoinjector; I_2, manual injector; NA, needle assembly; AHA, analysis head assembly; V_1, mobile phase mixer valve; V_2, injection-valve assembly; V_3, column switching valve assembly; D, detector. All components except the two columns and the manual injector are standard parts of the Agilent 1100 HPLC system. Modes 1 and 2 are for calibration and mode 3 is for purification of a trace analyte. Only the sample loading stage is shown for mode 2 (subsequent injection/separation in this mode takes place by activating V_2 as shown in mode 3). Similarly only the injection/separation stage is shown for mode 3. Thus, switching from mode 2 to 3 takes place by manually disconnecting the line after P at V_3 as shown.

2.4. Bypass HPLC procedure

Starting in mode 1 (Fig. 1), the calibration column was washed with mobile phase at 1.2 ml/min until a stable and flat baseline was obtained, followed by three injections of 20 μl acetonitrile. Injection of authentic compounds **1**, **2** and **3** (120, 60, 120 ng in 5 μl acetonitrile, respectively) was done, and their retention values were calculated (t_1, t_2, t_3, respectively). Switching to mode 2, the purification column was similarly washed and acetonitrile was injected as above. Then 5 μl of acetonitrile containing 40 ng of **1** and 20 ng of **2** was injected, and t_1' and t_2' were similarly determined. The retention time of **3** (t_3') on the purification column was calculated as $t_3'=t_1'+(t_3-t_1)(t_2'-t_1')/(t_2-t_1)$. The collection window for **3** on this latter column was then defined as $t_3'\pm0.25$ ml.

3. Results and discussion

In ''bypass HPLC,'' two columns of the same type are set up in parallel that we refer to as ''calibration'' and ''purification'' columns. Via appropriate injectors and valving, the trace analyte in the real samples does not contact the components of the HPLC that are exposed to the ordinary amounts of standard analyte.

A schematic of our bypass HPLC system is shown in Fig. 1, including its three modes of flow, where P is the purification column and C is the calibration column. To use bypass HPLC, two compounds in addition to the analyte (A) are needed, one of which is an analyte analog (AA) and the other of which is a selectivity marker (SM). Basically, one first injects a mixture of SM, AA and A onto the calibration column to observe their retention times in mode 1, and then one injects just SM and AA on the purification column in mode 2. The retention times for AA are normalized on each column, and A on the calibration column, by subtracting the corresponding retention times for SM. This allows one to calculate the retention time for A on the purification column, so that one can then purify A in real samples using mode 3. Thus one assumes that the ratio of normalized retention times for the A peaks on the two columns is the same as that for the AA peaks. The

procedure thereby takes into account any differences in retention of A on the two columns, including the fact that the manual injector in our system gives a 0.1-min shorter retention time than the autoinjector. In principle, one could omit SM and rely on the nonretained peak to normalize retention times (basing the calculation on retention factors), but in practice the nonretained peak is not always well defined, reducing the accuracy of predicting t'_3.

Previously, we reported a new electrophoric reagent, ''AMACE1'' (**1**, structure is shown in Fig. 2) for the derivatization of analytes possessing a carboxylic acid, lactone, ketone or aldehyde group in order to enhance their sensitivity for detection by

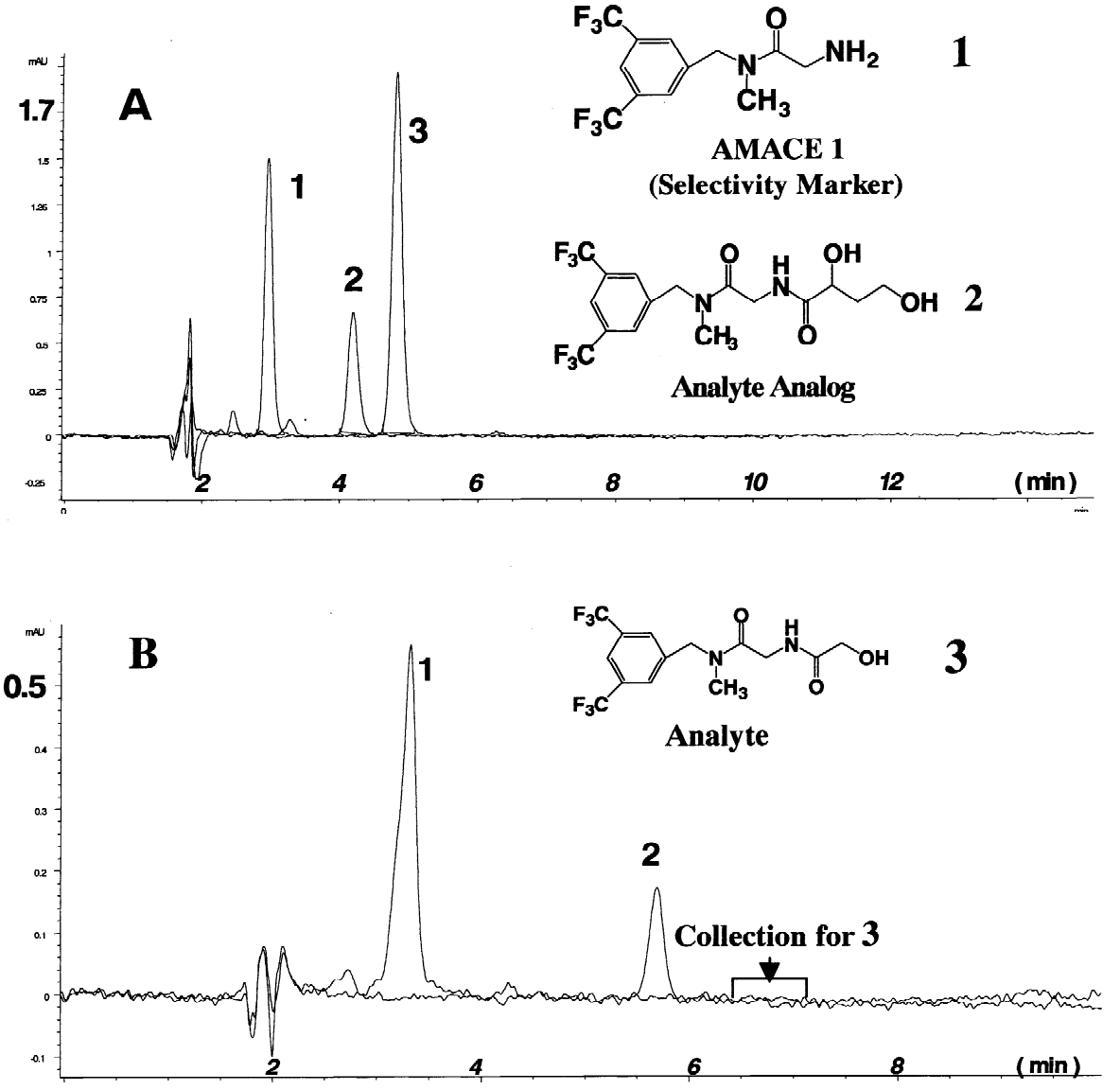

Fig. 2. Bypass HPLC separation of compounds on the calibration column (chromatogram A; mode 1 of Fig. 1), and on the purification column (chromatogram B; mode 2 of Fig. 1 after V_2 activation). Amounts injected: A, **1** (120 ng), **2** (60) **3** (120); B, **1** (40), **2** (20); mAU scales.

GC-EC-MS [7]. Currently, we are developing a method for glycolic acid (hydroxyacetic acid) based on this technique, and we have set up bypass HPLC for purification of the initial conjugation product, **3**. We are pursuing the measurement of trace amounts of glycolic acid since it is of interest as a metabolite in studies of enzymatic repair of sugar-oxidized DNA [8]. In the long term, the method is intended to measure a diversity of oxidized sugar products. The AMACE1 conjugate of α-hydroxy-γ-butyrolactone (**2**) has been selected to play the role of analyte analog (AA) in our bypass HPLC technique.

In Fig. 2A is shown a chromatogram where a mixture of **1** (as a SM compound), **2** and **3** have been injected into the bypass HPLC in mode 1. Fig. 2B similarly shows a chromatogram from mode 2, where **1** and **2** have been injected, and a retention window for **3** has been calculated. We find that this calculated window ($t_3' \pm 0.25$ ml), even though it only corresponds to a 0.5-ml volume from a 4.6 mm I.D. column, matches the actual elution window for **3** by >90%. At the outset of our study, the calibration column had been in use for 6 months, while the purification column was new, which may explain, in part, the differences between the two columns in absolute retention. During five runs spread out over 2 months, calculated t_3' varied (nonsystematically) from 6.3 to 6.9 min. One source for this variation may be the dynamic mixing of the two mobile phase solvents by the HPLC system. Thus we are continuing to employ bypass HPLC in our development of a method for trace detection of glycolate.

The disadvantages of bypass HPLC are that it: (1) employs a sophisticated HPLC system; (2) requires two accessory compounds (SM and AA); (3) does not guard against carryover that can take place among samples when some of them have much higher amounts of analytes than others; and (4) potentially may degrade in performance with use. The latter problem might arise since only column P receives real samples. This, in turn, could lead to a mismatch in the retention behavior between the two columns that makes the calculated collection window for analyte on column P inaccurate. Of course the remedy for this is to replace column P, or perhaps both columns. One can guard against this problem on a routine basis by periodically collecting and analyzing neighboring elution fractions relative to that calculated for A. Overall we consider that these disadvantages are minor or controllable, and outweighed by the convenience and usefulness of the technique.

4. Conclusion

Bypass HPLC is an attractive technique for purifying trace analytes by HPLC when the levels are too low for on-line detection, since it avoids contamination from injection of standards that may need to be injected periodically at higher levels in the absence of this technique.

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