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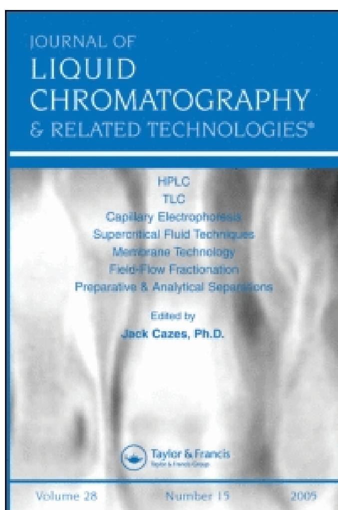

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SOLID PHASE DERIVATIZATIONS IN HPLC: BOROHYDRIDE/SILICA REDUCTIONS FOR CARBONYL COMPOUNDS

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ABSTRACT

Sodium borohydride adsorbed onto silica gel has now been utilized for on-line, pre- and post-analytical column chemical derivatizations via reductions of various organic carbonyl compounds. These on-line reactions have been performed using normal phase HPLC conditions, involving conventional silica gel packings, organic mobile phases, and commercially available HPLC equipment and instrumentation. This approach for on-line HPLC derivatizations has been evaluated for a large number of organic carbonyl compounds, at a variety of temperatures for aldehydes and ketones. The overall rates of such carbonyl reductions via sodium borohydride/silica are sufficiently different as a function of temperature of the reaction to permit for compound/class identifications. Analyte identification can be both qualitative and quantitative, even wherein an analyte co-elutes with a non-carbonyl compound. In-house prepared borohydride/silica gel reactors can be characterized via standard iodine titration procedures along with inductively coupled plasma (ICP) elemental boron analysis. These normal phase derivatization approaches have been applied to certain standard vitamins, and to cinnamaldehyde found in a commercial spice product. Such approaches to on-line HPLC derivatizations

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complement the use of polymeric borohydride reducing reagents for reversed phase HPLC type separations, as previously described.

INTRODUCTION (1)

In general, the development of derivatizations for high performance liquid chromatography (HPLC) has involved mostly off-line, homogeneous mixing of the sample solution and the derivatization solution(s), be this pre-injection or post-column (2-8). Although some progress has been made in the area of automated, post-column, on-line, homogeneous derivatizations, much less advancement has been realized in automated, pre-column, on-line, homogeneous derivatizations. The use of heterogeneous, on-line or off-line, solid phase derivatizations/reactions in HPLC has been described only a few times in the literature and reviewed, thus far, only twice (8, 9). Solid phase derivatization reactions in HPLC offer certain very attractive advantages over the more conventional and widely used homogeneous methods. In general, as described below, the solid phase approach does not require additional instrumentation, hardware, mixing chambers, or reaction chambers. Instead, all that is needed are the solid phase reactor, a dummy reactor column, and various HPLC fittings for connecting these reaction columns to the HPLC system, on-line. The solid phase reactor (SPR) should not introduce any additional extra-column dead volume, other than that normally introduced with any conventional guard column, as now used in HPLC. There may be a slight overall increase in the cost per analysis, but this is no more than that already encountered with homogeneous derivatizations. When compared with all off-line, homogeneous derivatizations, the SPR approach will not increase the overall time per analysis beyond that already needed for the direct analysis in the absence of derivatization. Contamination caused by the SPR should, in general, be much less than that already realized via conventional, homogeneous approaches. With the SPR, there is no derivatizing reagent being mixed with the mobile phase, and therefore detection limits are not adversely affected because of an increased baseline/background noise level. Thus, an SPR derivatization with the same reagents used in homogeneous derivatizations, assuming an equal degree of quantitative derivatization, should provide for better overall detection limits. The SPR method can also be used in either the pre- or post-analytical column mode, on-line, using differences in the overall chromatography (pre-) or differences in the detector responses with and without the SPR present (post-). Derivatizations with the SPR approach do not require any added sample handling or manipulation, but rather only require direct injection onto the HPLC system, with and without the SPR present on-line. Depending on the nature of the reagent support, viz., polymeric or silica gel, the SPR can be fully compatible with a wide variety of mobile phases, both normal phase and reversed phase. Since reactions occur on a solid support, most such reactions will occur at or slightly above room temperature, and generally in real-time,

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without analyte/sample hold-up on the SPR. Finally, in many instances described in the synthetic organic literature, SPR reactions for derivatization are more selective and specific than for the same reagent(s) in homogeneous, solution type reactions.

We have recently described in some detail the utilization of a certain polymeric borohydride SPR for performing on-line, real-time, aldehyde reductions with reversed phase columns and mobile phases (10, 11). This particular approach has been fully compatible with methanol/water (MeOH/HOH), ethanol/water (EtOH/HOH), and acetonitrile/water (ACN/HOH) reversed phase type solvents. It has not yet been possible to determine the compatibility of this polymeric borohydride SPR for normal phase HPLC interfacing. However, in view of the already rather extensive literature describing the use of borohydride(s) on silica gel, alumina, and related solid supports, it appeared advantageous to utilize these very SPRs with normal phase HPLC (12-20). Although the synthetic organic chemistry literature has described these supported borohydride reducing reagents for many years, they have apparently never been utilized or applied for on-line HPLC derivatizations, as described herein.

It has now been possible to prepare, in-house, various loadings of sodium borohydride on commercially available, HPLC grade silica gel, of varying particle size and surface area, and to characterize such SPR materials via in-house iodine titration procedures and external elemental boron analysis via inductively coupled plasma emission spectroscopy (ICP). These were the same approaches already described for the polymeric borohydride SPR (10, 11). These borohydride/silica SPRs have now been evaluated with regard to their stability, reproducibility, run-to-run precision, and chemical reactivity for a number of classes of organic compounds. In addition, a study has been made on the ability of borohydride/silica SPRs to differentiate between aldehydes and ketones, as a function of percent reductions vs temperature for members of each class as a function of the carbonyl group present. Indeed, it is now possible to differentiate between classes of carbonyl compounds via these methods. We have also determined the compatibility of this SPR for various ratios of iso-propanol (IPA) in hexane as the mobile phase for the HPLC separations. Finally, the borohydride/silica gel SPR has been applied to the analysis of cinnamaldehyde found in the spice product cinnamon. Also of interest is the fact that these approaches can be utilized for the analysis of various vitamins, such as the vitamin K series, as standards and presumably in real samples. This should provide for greatly improved specific identifications of these compounds in vitamin formulations, over-the-counter products, and in actual food or beverage samples or biological matrices. Although all of these studies have thus far utilized only ultraviolet (UV) detection in HPLC, it is clear that the SPR approach described here is fully compatible with all/any other commonly used HPLC detectors, such as fluorescence or electrochemical (EC). Studies are now in progress to demonstrate this compatibility, and to further

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elucidate and detail the applications possible *via* the on-line, real-time use of borohydride/silica based solid phase reactors and reactions in HPLC.

EXPERIMENTAL

Reagents

Most of the silica gel used for the in-house preparation of the borohydride/silica SPRs was obtained from Waters Associates (Milford, Mass.), as their 37-55 um particle size, HPLC grade silica. Other silica gel was obtained from E. Merck & Co. (Darmstadt, Germany) as their Perisorb A HPLC grade packing material, 30-40 um particle size. The sodium borohydride reducing reagent was obtained from Aldrich Chemical Co. (Milwaukee, Wisc.). A sample of sodium borohydride adsorbed onto silica gel was obtained from Alfa Products (Thiokol Corp., Danvers, Mass.), and this was used for some of the very early/preliminary studies. However, most of these studies have been done with in-house prepared and evaluated $NaBH_4$/silica gel materials.

All HPLC solvents were obtained from Waters Associates, as their HPLC grade mobile phase solvents, including isopropanol and hexane. The various chemicals and reagents utilized in evaluating the reactivities of the borohydride/silica SPR were obtained from a number of commercial suppliers, of the highest purity commercially available. These were all used as received, without further purification. They were obtained as follows: acetophenone (Aldrich); benzhydrol (Aldrich); 2-naphthaldehyde (Aldrich); 2-naphthol (Aldrich); 1-indanone (Aldrich); 1-indanol (Aldrich); salicylamide (Sigma Chemical Co., St. Louis, Mo.); cinnamoyl chloride (Aldrich); benzyl alcohol (Aldrich); *trans*-cinnamaldehyde (Alfa Products); cinnamyl alcohol (Aldrich); benzaldehyde (J.T. Baker Co., Phillipsburg, N.J.); benzophenone (Fisher Sci. Co., Medford, Mass.); p-nitrobenzaldehyde (Sigma); p-diethylaminobenzaldehyde (Aldrich); p-nitrobenzoyl chloride (Aldrich); cinnamon (R.T. French Co., Rochester, N.Y.); N-nitrosodiphenylamine (Aldrich); BCNU (Bristol Labs, Inc., Syracuse, N.Y.); benzanilide (Alfa Products); phenyl benzoate (Chem Service, West Chester, Pa.); methyl benzoate (Fisher Scientific); ethyl benzoate (MCB Chemicals, Inc., Cinc., Ohio); chlorobenzene (Aldrich); 3-cyclohexenone (Aldrich); 9-nitroanthracene (Aldrich); allyl iodide (ICN Pharmaceuticals, Inc., K&K Labs, Plainview, N.Y.); vitamin K_3 (Sigma).

Appratus

The HPLC system utilized for most of these studies consisted of a Waters U6K syringe loading injection valve (Waters Assocs.), a Waters Model 6000A solvent delivery system, a Waters Model 480 variable wavelength UV-VIS detector, and a Linear dual pen recorder (Linear Instruments, Inc., Irvine, Calif.). In most instances, a uPorasil, 10um, 30-cm x 3.9-mm i.d., stainless

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steel analytical HPLC column was used for the separations, but in some studies, a generic, 10um silica gel type analytical column was employed. The dummy and SPR columns were prepared using glass lined stainless steel tubing, 5-cm x 4.0-mm i.d., from Alltech Assocs., Inc. (Deerfield, Ill.). All dummy, SPR, and analytical end column fittings were zero dead volume type (Cambridge Valve & Fitting, Inc., Cambridge, Mass.).

Low, ambient, and elevated temperature solid phase reactions were performed using a Forma Scientific Model 2095 water bath and circulator (VWR Scientific, Inc., Boston, Mass.). At times, data from the HPLC-UV analyses were collected with a strip chart recorder together with an HP Model 3380A reporting integrator (Hewlett Packard Corp., Palo Alto, Calif.). This was done in order to improve both accuracy and precision when determining percent reductions as a function of analyte and SPR temperature.

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Methods

In all of these studies with the borohydride/silica gel SPR, standards of all organic compounds being analyzed were injected in the mobile phase or another mobile phase-compatible organic solvent. Such standard solutions, in known amounts, were generally injected in 10 ul aliquots, first onto a combination of the dummy column plus analytical column, and then onto the borohydride/ silica SPR and the same analytical column (silica gel). The dummy column consisted of the same silica gel packing used to prepare the borohydride/silica SPR, in the identical glass lined stainless steel tubing and end fittings. All retention times on both dummy and solid phase reactor columns, pre-analytical column, were determined by duplicate or triplicate injections of the analyte(s) of interest along with the expected reduction product, the alcohol. In almost all cases, with various mixtures of mobile phases, the retention times of the alcohols on both the dummy and SPR columns in-line with the same analytical column, agreed very well (±5% or less).

In the case of the analysis of cinnamaldehyde in cinnamon, this qualitative determination was done by first extracting the cinnamon, in a known amount, with a small volume of hexane (1-2 mls), filtering this extract and then immediately injecting the extract onto both the dummy and SPR columns, in the pre-analytical column mode. The identification of cinnamaldehyde and cinnamyl alcohol was based on measured retention times. Retention times for all compounds were based on HPLC chromatograms, an external automatic timer started at the point of injection and measured at the point of maximum peak height(s), and, at times, the Hewlett Packard retention time measurements on the reporting integrator.

Elemental analyses for elemental boron content were performed at Galbraith Laboratories (Knoxville, Tenn.). The active borohydride determinations were done in-house, using an iodine titration method described elsewhere (21).

The borohydride/silica gel reagent was prepared according to a somewhat modified literature procedure (16, 19). A known amount of fresh sodium borohydride (1g) was dissolved in about 2 ml of distilled water, and this final solution was made slightly basic with 2% sodium hydroxide. This was then added to about 10g of previously dried silica gel. After vigorous shaking, the homogenized material was heated at 39.8°C under 5-10 mm Hg, in a drying pistol containing phosphorous pentoxide, until water evolution appeared to be complete. This generally required overnight drying to remove all last traces of residual water. The final borohydride/silica reagent was stored in a vacuum dessicator over Drierite or a comparable dessicant. The actual SPR was packed in a glove bag under dry Helium, in order to prevent adsorption and/or reaction with any moisture in the air. All SPRs were dry packed using the tap-and-fill approach. For maximum activity and longevity, the dried borohydride/silica reagent must be protected from all moisture, both while being packed into the SPR and in actual use on-line. The mobile phases used were initially dried over molecular sieves, at least overnight, before being used for any on-line SPR studies. This has been now shown to greatly increase the overall lifetime and initial activity of the borohydride/silica reagent. In order to further improve lifetimes, the SPR can be freed of solvent, *in vacuo*, after HPLC involvement, and then stored in a vacuum dessicator over a suitable dessicant. A borohydride/silica SPR prepared as indicated and suitably protected from water, can be used for more than 50 separate analyses, even when used repeatedly with an IPA/hexane type mobile phase. The SPR can be given improved lifetimes by repeated borohydride coating of the same silica gel, before any utilization, in order to increase the percent loading of active borohydride on the same amount of silica gel substrate/support. For all of these studies, the SPR had a single coating of borohydride, and the precise percent borohydride or total boron content was determined before any utilization. It is important to know when the borohydride activity of the SPR is ineffective for on-line, real-time reductions of even simple aldehydes, *vide infra*. Elemental analyses and/or active borohydride titration determinations should be performed on each new batch of borohydride/silica, just to ensure the initial activity of the SPR before it is used on-line. In addition, effective reductions of a standard aldehyde in a fixed concentration injected should be demonstrated whenever the SPR is put back into use. This will ensure that no significant activity has been lost during storage, as above. Borohydride/silica reagents are much more susceptible to degradation *via* water than are the polymeric borohydride SPRs described by us previously (10).

RESULTS AND DISCUSSION

All of the HPLC-UV studies described here have used hexane or IPA/hexane as the mobile phase, with either isocratic or flow programming

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conditions, together with a commercial silica gel analytical column. Detection in all instances has been *via* a fixed or variable wavelength UV-VIS detector, but it is obvious that other commercial HPLC detectors could just as easily and readily been utilized, depending on the nature of the analytes of interest. That is, solid phase reactions are compatible with virtually all HPLC detectors, as long as the starting material and/or reaction product can be detected on the original detector. It could also be the case that dual detection approaches could be just as compatible with HPLC-SPR, wherein the starting analyte is detectible on one detector, while the product(s) are detectible on the other detector. It could also be the case that both starting analyte and reaction product(s) are visible on one or both of the dual detectors in operation. Clearly, this will depend on the initial chromophore, fluorophore, or electrophore present in the starting analyte, and whether or not it itself is modified in such a way as to increase or decrease detectibility on a particular HPLC detector. There can and will be situations wherein only the starting material will be visible or detectible, other cases where both starting material and product, and still others wherein only the product will be apparent. A judicious selection of the best detector to employ in a given HPLC-SPR application is required in order to fully realize the total analytical advantages possible for any given situation. Although we have not yet utilized gradient elution separations in any of this work, there is every reason to expect this method to be fully compatible with SPR derivatizations. The major requirement, clearly, is that the mobile phases being used for the overall separations be fully compatible with the SPR reagent(s) in use for the derivatization. With regard to the temperature required for these SPR derivatizations, we have mainly employed ambient or sub-ambient SPR conditions. It is indeed possible that elevated temperatures will lead to faster rates of reaction/derivatization, and that additional classes of organic compounds will undergo suitable reductions with this borohydride/silica SPR. To some extent, this is indicated by the results below. Elevated temperatures will be limited by the nature of the HPLC mobile phase, since volatilization of the solvent is not desirable. In all of this work, identification of the starting analyte is based on at least some degree of chemical reduction, leading to an alcohol or related product that has a different retention time under the given HPLC conditions. Wherein retention times for both starting material and product are identical, which would be unusual in normal phase HPLC for these compounds, differences in detector responses can also be utilized to denote a chemical reaction/reduction and compound identification. The use of a dummy column plus an analytical column together with the SPR plus analytical column yields two different HPLC-UV chromatograms. The differences between these two chromatograms, hence difference chromatography, provides the analyte identification needed for qualitative HPLC analysis today. The greater the selectivity of the SPR, the higher the assurance that the qualitative HPLC analysis is indeed specific for

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that analyte of interest. The less selective the SPR reaction, then the less specific will be that qualitative HPLC analysis. Although the vast majority of these studies have involved the SPR in the pre-analytical column mode, it is indeed possible and feasible to utilize the borohydride/silica reagent in the post-column mode. One example of this mode of operation is indicated below.

As an initial example of the use of HPLC-SPR and difference chromatography for the identification of a simple aldehyde, viz., benzaldehyde, Figure 1 indicates two different HPLC-UV chromatograms. One of these, Figure 1A, is the chromatogram for benzaldehyde itself, wherein a dummy column is in place before the analytical column. The second chromatogram, Figure 1B, indicates the use of the commercially available borohydride/silica SPR before the analytical column, demonstrating the complete reduction of the starting aldehyde and the concomitant formation of the expected product, benzyl alcohol. Specific HPLC-UV conditions are indicated in Figure 1. In this particular case, flow programming was used to elute the benzyl alcohol reduction product within 30 mins of injection. Gradient elution or step programming could also have been used here in order to have the alcohol elute in a shorter overall time frame. However, wherein gradient elution approaches are used with an SPR, it must be first demonstrated that more polar mobile phases will not react with and/or elute the inorganic or organic reagent supported on the silica gel. The current SPR is stable and compatible with as much as 15% IPA/hexane, as below.

Whereas all of the aldehydes thus far studied are completely reduced at ambient temperatures with this SPR, ketones are only partially reduced, as indicated in Figure 2A. Figure 2A indicates the HPLC-UV chromatogram for standard acetophenone, with the specific conditions and retention time given. Figure 2B is the analogous chromatogram for this same compound, but now using a commercially available borohydride/silica SPR before the analytical column, with somewhat different flow programming elution conditions for the expected reduction product, in this case, 1-phenylethanol. In all of the cases studied here, identification of the reduction product was based on the observed retention time of a standard alcohol, injected onto the SPR plus analytical column. In Figure 2, it is clear that the acetophenone has the same retention time on both the dummy plus analytical column as on the SPR plus analytical column arrangement. Thus, retention times are not being affected in going from the dummy case to the SPR arrangement, as is necessary for this type of difference chromatography to be reliable and useful. Since the acetophenone is not completely reduced under these particular HPLC-SPR conditions, some of the initial starting material remains apparent in Figure 2B. One could readily determine the percent reduction in this case, using either peak areas or peak heights, and this has now been done for all of those compounds which are not completely reduced at ambient temperatures. The percent reductions of most ketones is temperature dependent, vide infra.

Whereas the above two examples of the HPLC-SPR utilized commercially available borohydride/silica reagents, all of those examples that follow have

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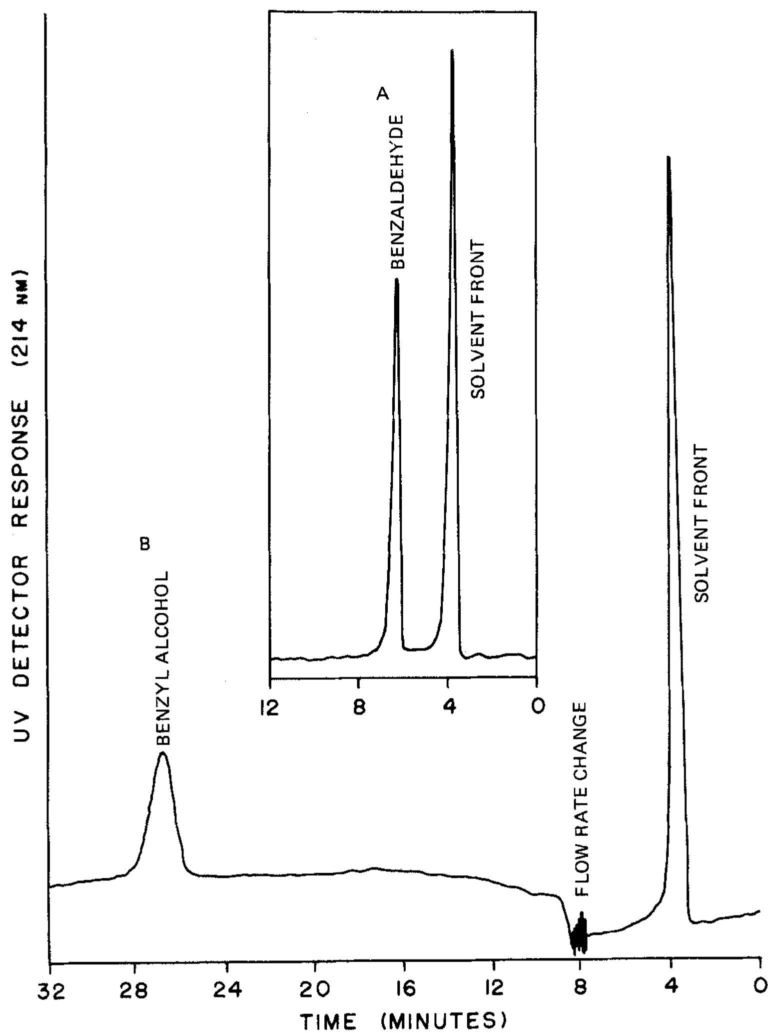

Figure 1. (A) HPLC-UV chromatogram of benzaldehyde using dummy column and silica gel analytical column with 0.3% IPA/hexane at 1 ml/min; (B) HPLC-UV chromatogram of benzaldehyde injected onto reduction borohydride/silica SPR and analytical column with 0.3% IPA/hexane at 1 ml/min to 8 mins and then flow rate of 4.3 ml/min to 32 mins.

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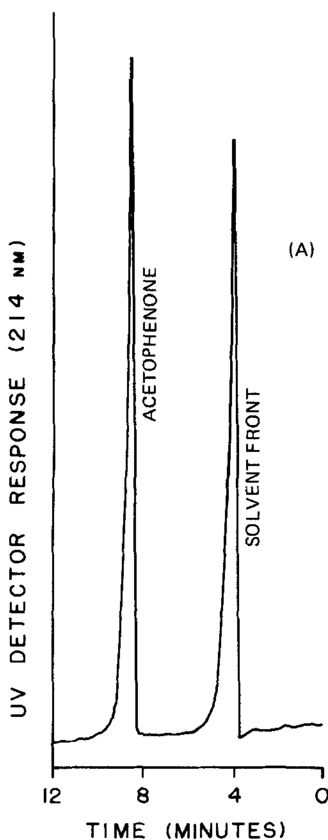

Figure 2. (A) HPLC-UV chromatogram of acetophenone using dummy column and silica gel analytical column with 0.3% IPA/hexane at 1.0 ml/min; (B) HPLC-UV chromatogram of acetophenone on borohydride/silica SPR plus analytical column with 0.3% IPA/hexane at 1.0 ml/min until 10 mins, then 4.3 ml/min to 24 mins, ambient temperatures with pre-column mode of SPR operation.

utilized in-house prepared SPRs. Although the first two examples utilized the pre-column mode of SPR operation, with detection at 214 nm, it is also possible to make use of the post-column arrangement, now with detection at 330 nm, Figure 3. In both of these chromatograms, the dummy or SPR are placed after the analytical column and before the UV detector. Thus, retention times are not affected by the SPR, even though some or all of the starting material is being reduced on-line. However, wherein the UV properties of the product differ from those of the starting material, which is indeed the situation in Figure 3, there can be either an increase or a decrease in the HPLC-SPR peak

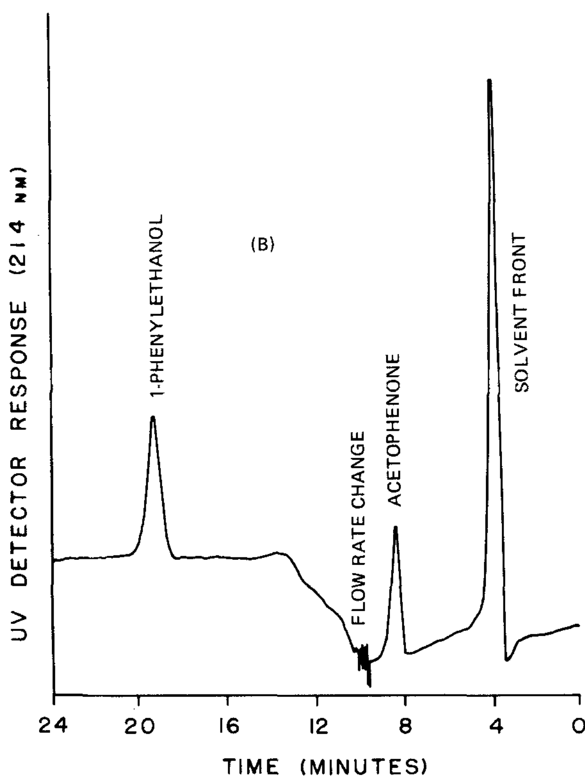

Figure 2B

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height or peak area for the reduction product. In most instances, since we are now reducing a conjugated enone system, the UV absorbance at 330 nm is reduced in comparison with the starting material. Hence, Figure 3B shows the resultant UV peak for the expected reduction product, cyclohexanone, which has a reduced absorbance at 330 nm when compared with 2-cyclohexenone. Based on a comparison of Figures 3A and 3B alone, it is not possible to determine just how much of a reduction of the starting material has indeed occurred. This could have been determined in a separate experiment, wherein the same SPR was used in the pre-analytical column mode, and the peak for the starting 2-cyclohexenone would partially or completely disappear under the same HPLC conditions as in Figure 3. It seems clear that the HPLC-SPR using the pre-column arrangement provides much more definitive qualitative and quantitative information than the post-column mode. For this very reason, we have tended to emphasize in these initial studies those applications which use a pre-column

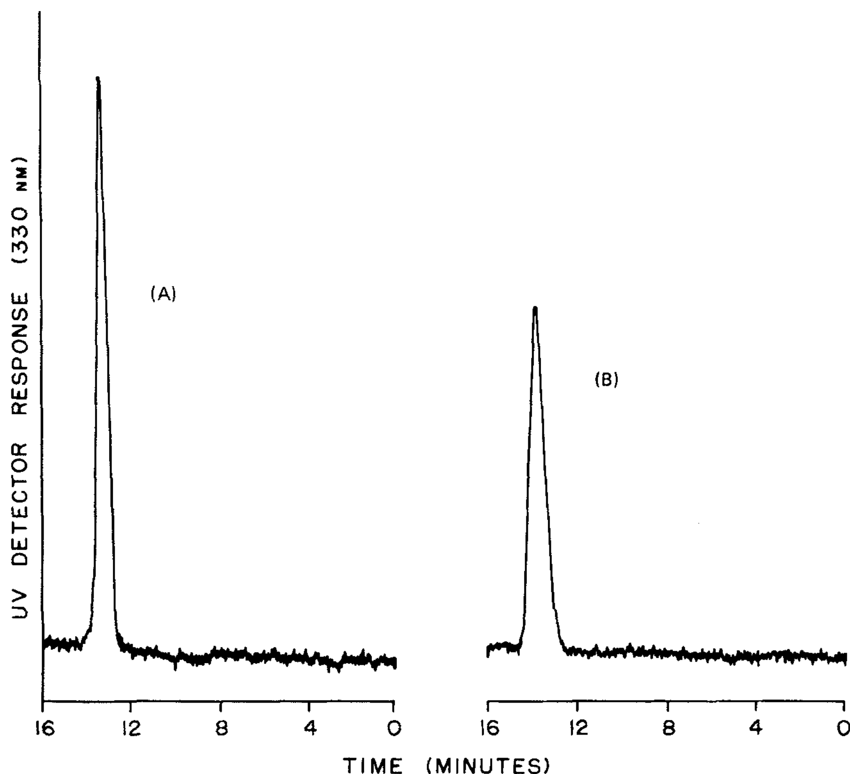

Figure 3. Post-column mode of operation for supported borohydride/silica reductions with UV detection at 330 nm: (A) 2-cyclohexenone injected onto dummy column after analytical column using hexane mobile phase at 2.6 ml/min; (B) 2-cyclohexenone injected onto SPR plus analytical column using hexane at 2.6 ml/min. Percent reduction by peak area 26%.

arrangement. Nevertheless, post-column utilization of HPLC-SPR should often be considered wherein a known amount of the correct standard can be analyzed under the exact same dummy and SPR conditions.

The above pre-column HPLC-SPR approach has now been applied to a large number of organic classes and individual members of each class, Table 1. The percent reductions indicated here were determined using the decrease or complete disappearance of the peak height for the starting material. In general, most aldehydes will be completely reduced at ambient temperatures under these SPR conditions, with the sole exception of p-diethylamino benzaldehyde, which is unreactive. The substituted benzaldehydes, and other compounds, had to be

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Table 1. Classes of compounds and individual compounds studied *via* HPLC-SPR with the borohydride/silica SPR.[a]

CLASS OF COMPOUND/INDIVIDUAL COMPOUNDS	PERCENT REDUCTION[b]
ESTERS	
Phenyl benzoate	NR[c]
Methyl benzoate	NR
Ethyl benzoate	NR
NITRO	
9-Nitroanthracene	NR
N-NITROSO	
N-nitrosodiphenylamine	NR
1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)	--[d]
ALKYL/ARYL HALIDES	
Chlorobenzene[e]	NR
Allyl iodide[e]	NR
AMIDES	
Salicylamide	--[d]
Benzanilide[h]	NR
ACID CHLORIDES	
Cinnamoyl chloride	NR
Cinnamoyl chloride[f]	NR
o-Nitrobenzoyl chloride	100
Benzoyl chloride[f]	90
ALDEHYDES	
Benzaldehyde	100
Cinnamaldehyde	100
2-Naphthaldehyde	100
p-Nitro benzaldehyde[g]	100
p-Diethylamino benzaldehyde[g]	NR
KETONES	
Benzophenone	71
Acetophenone	98
1-Indanone	91

a. HPLC-UV used a uPorasil analytical column with 0.3% IPA/hexane, 1 ml/min, UV at 214 nm, borohydride/silica in pre-column mode, 25°C temperature.
b. Determined by decrease in peak heights for dummy vs SPR chromatograms.
c. NR = no reaction observed.
d. Indicates that compound could not be eluted under normal phase HPLC.
e. HPLC used a mobile phase of 100% hexane at 0.65 ml/min.
f. HPLC used 0.3% IPA/hexane at flow rate of 0.39 ml/min.
g. HPLC used a mobile phase of 15% IPA/hexane at 1 ml/min.
h. HPLC used a mobile phase of 3% IPA/hexane at 1 ml/min.

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eluted from the analytical column in a reasinable time with 15% IPA/hexane. However, even under these more polar eluent conditions, the borohydride/silica SPR is still able to fully reduce p-nitrobenzaldehyde, but not the p-diethylaminobenzaldehyde. Thus, substituent effects for aromatic aldehydes can play a significant role in determining the degree of reduction possible via this particular SPR. We have observed this same substituent effect with these and analogous compounds using an entirely different, oxidizing SPR, to be described elsewhere (22). All three ketones studied here, viz., benzophenone, acetophenone, and 1-indanone, are partially or completely reduced using 0.3% IPA/hexane as the mobile phase at 1 ml/min. Changes in the flow rate will affect residence times within the SPR, which in turn should affect the degree of reduction possible with a given analyte and mobile phase composition. Clearly, increased percent reductions would be expected, if reduction is at all possible, for a given mobile phase at lower and lower flow rates. In general, this has been just what is observed for appropriate analytes. All of the other classes of compounds, except for the acid chlorides, are fully unreactive. Of the acid chlorides, there is, as yet, no clear picture of their collective reactivities. That is, cinnamoyl chloride is fully unreactive, whereas the other two acid chlorides studied, o-nitrobenzoyl chloride and benzoyl chloride, are reduced between 90-100%, Table 1. There is no clear reason for these vast differences in overall reactivities, especially that between cinnamoyl and benzoyl chlorides, two very similar compounds.

Table 1 has begun to suggest that there can be significant differences in the chemical reactivity of similar classes of compounds, such as aldehydes and ketones, via HPLC-SPR with the borohydride/silica SPR. It appeared that these reactivity differences might be accentuated and made more usable/useful, if the SPR were held at sub-ambient temperatures, with the other HPLC-UV conditions held constant. Indeed, Figure 4 summarizes all of these results, for three separate aldehydes and three separate ketones, as indicated, varying the SPR temperature from 1-25°C for the aldehydes, and from 25-45°C for the ketones. With this particular SPR, containing only 4.41% $NaBH_4$ by elemental boron analysis, all three ketones were less than 2% reduced at 25°C. This should be directly compared with these same ketones in Table 1, wherein another SPR, containing about 11.3% $NaBH_4$ by the same elemental boron analysis method, was found to reduce these compounds 71-98%. Clearly, borohydride content/loading plays a major role in the overall percent reductions possible under any given HPLC conditions, holding mobile phase and flow rate constant during such a comparison. Figure 4 demonstrates that it is indeed very easy to differentiate between all ketones and aldehydes, wherein the percent borohydride present on the SPR is low enough to discriminate between these two classes of carbonyl derivatives. As the percent borohydride content of the SPR, and hence its overall reactivity, increases, then the discriminating ability of the SPR decreases. The amounts of each aldehyde/ketone studied in Figure 4 are somewhat

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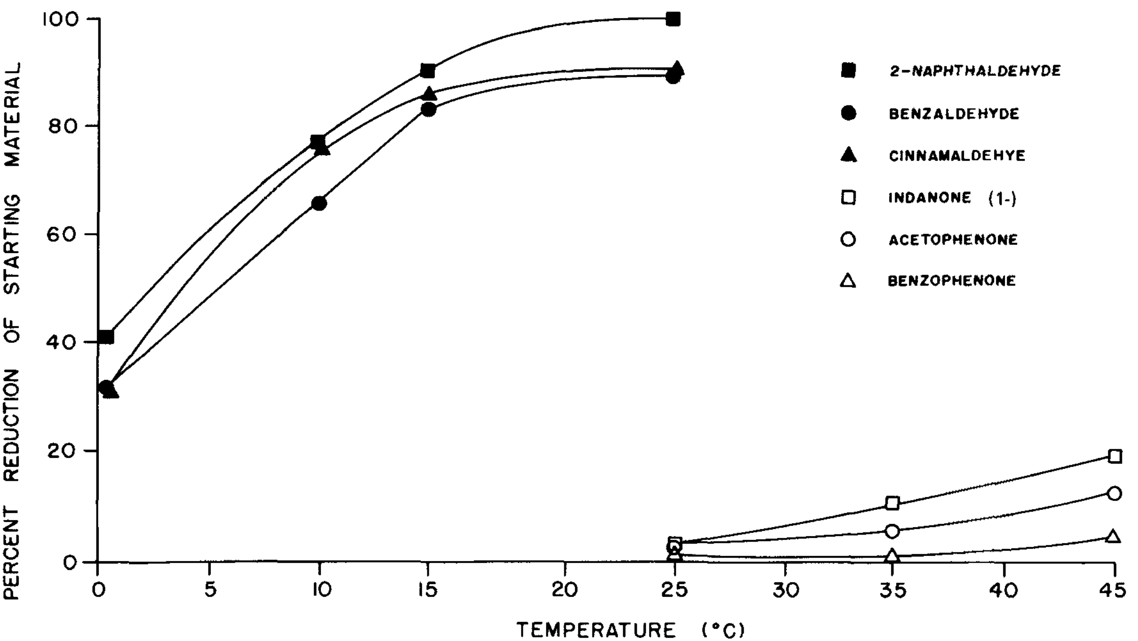

Figure 4. Percent reductions of various aldehydes and ketones as a function of the temperature of the SPR in the pre-column HPLC-SPR mode.

different, varying from 50 ng to about 450 ng, and in a separate study, we have shown that the amount of analyte present will affect the overall percent reduction realized _via_ HPLC-SPR. Thus, it is not yet possible to show that this particular SPR can indeed differentiate one aldehyde from another or one ketone from another, but we have reason to suspect that this will indeed be feasible and practical. At the moment, Figure 4 and other results already in hand, clearly demonstrate the ability of this particular HPLC-SPR approach to differentiate between classes of compounds, especially when such studies are done as a function of the SPR's temperature of operation.

Table 2 summarizes a study designed to demonstrate the effect of different absolute amounts (mass) of benzaldehyde on the percent reduction observed _via_ peak area changes (decreases). The specific analytical conditions are indicated in Table 2, herein using an SPR which contained 4.41% $NaBH_4$ by elemental boron analysis. It becomes immediately apparent that as the absolute amount of analyte entering the SPR is increased, from about 4 ng to over 10,000 ng, the percent reductions observed decrease from 100% to 58%. These percent reduction differences would be accentuated (increased) if the percent borohydride loading were lower than that used here. At the same time, with absolute amounts of analytes ranging from 4.0 to 400 ng, the observed percent reductions are all about 90% or better, at least with a 4.41% borohydride loading on the SPR. Since most trace analyses involve considerably less than 400 ng of any given analyte, one would expect with this borohydride loading not to observe drastic differences in percent reductions _via_ HPLC-SPR. Since any qualitative identification of an analyte in a complex sample matrix would have to rely on identical approaches with a standard at about the same mass or concentration level, these differences in reactivity as a function of the

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Table 2. Percent reductions of benzaldehyde as a function of absolute amount injected onto the HPLC-SPR system.[a]

AMOUNT OF BENZALDEHYDE (ng)	PERCENT REDUCTION OBSERVED[b]
4.15	100
41.5	95
415	88
1038	84
10,380	58

a. HPLC-SPR used a uPorasil analytical column with 0.3% IPA/hexane at 1 ml/min, SPR pre-column contained 4.41% $NaBH_4$ by weight of the silica gel reagent using Waters 37-55um material, SPR 5.0-cm x 4.0-mm i.d., UV detection at 214nm, SPR at ambient temperature (25.5^oC) with constant temperature bath. All injections were in 10 ul of solutions.

b. Determined by decrease in measured peak areas between dummy and SPR situations.

amount of analyte would disappear between the sample and standard. However, it then becomes terribly important for reliable qualitative analyte identifications to employ an external standard set of analyses, with dummy and SPR, that mimics as closely as possible the quantitative levels of that same analyte in the actual sample matrix.

Each in-house prepared batch of borohydride/silica was analyzed for percent boron and percent sodium borohydride per gram of reagent, after its preparation, using two different approaches. The first of these used a conventional boron elemental analysis via inductively coupled plasma emission spectroscopy at an outside analytical laboratory. The second method used a more classical iodine titration procedure, previously described in the literature (21). Table 3 summarizes such results for three of the many borohydride/silica reagents prepared in this study. It is clearly possible to prepare different batches of borohydride/silica which contain different loadings of active borohydride, whichever method of analysis is utilized, as already described above. Second, the value of percent borohydride determined via elemental boron analysis and that via iodine titration are quite different. Two explanations are immediately apparent, first that the ICP elemental boron analysis is not specific for borohydride, hence one obtains a much larger apparent value for borohydride present, Table 3. The second possible answer is that the ICP approach is indeed providing an accurate value of percent borohydride present, but some of this is buried within the silica gel matrix and is not available for titration with iodine. This would lead to a correct value for borohydride via the elemental analysis (boron) approach, but only surface/active borohydride is measured via the titration method. For the current purposes, it does not really matter which values are accurate, so long as one can qualitatively differentiate borohydride loadings from one batch to another by the same method, be this boron analysis or iodine titration.

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Table 3. Analysis of borohydride/silica solid phase reactors in HPLC.

SAMPLE OF SPR	% BORON[a]	% $NaBH_4$[a]	% BORON[b]	% $NaBH_4$[b]
$NaBH_4$/Waters silica gel #1	1.26	4.41	0.71	2.48
$NaBH_4$/Waters silica gel #2	3.24	11.34	1.33	4.68
$NaBH_4$/Perisorb A	4.73	16.55	2.26	7.90

a. These values were determined by elemental boron analysis via ICP at Galbraith Laboratories, Inc.
b. These values were determined via a $NaBH_4$ iodine titration method (21).

In order to demonstrate the potential usefulness of this particular HPLC-SPR approach for qualitative analyte identification in on-line HPLC reductions, we have applied the above approach in two situations. In a previous publication, we had demonstrated the ability of a polymeric borohydride SPR to selectively reduce aldehydes, and especially cinnamaldehyde found in spice cinnamon (10, 11). In order to extend this application to normal phase HPLC, we have therefore developed the analogous analysis for this same analyte in the same consumer food product/spice. Figure 5 illustrates the borohydride/silica identification of cinnamaldehyde in a hexane extract of cinnamon, using both the dummy plus analytical column, Figure 5A, and then the SPR plus analytical column, Figure 5B. In Figure 5, the single peak for cinnamaldehyde at about 10 mins is completely reduced in Figure 5B, with the expected formation of the known reduction product, cinnamyl alcohol. The retention times for both starting aldehyde and product alcohol were confirmed, as before, _via_ the analysis on both dummy and SPR of the standard compounds. Although the retention time for the cinnamyl alcohol is somewhat long in Figure 5B, due to the use of flow programming, this could have been greatly reduced by going to gradient elution or step programming. We have similarly applied this HPLC-SPR approach to the qualitative identification of cinnamaldehyde in a mouthwash, by initial hexane extraction of the aqueous solution and injection onto normal phase HPLC-SPR. These overall results were in excellent agreement with a related qualitative analysis of this same consumer product _via_ reversed phase HPLC-SPR with a polymeric borohydride reagent described earlier (10, 11).

Vitamin K_3 (menadione) is 2-methyl-1,4-naphthoquinone, having the physiologic properties of vitamin K. There is substantial interest in improved methods of trace analysis for all vitamin K analogs, especially wherein newer methods possess improved specificity for the individual compounds. Although borohydride/silica HPLC-SPR had not been evaluated with regard to its reactivity for benzoquinone or naphthoquinone derivatives, the reactivity for reduction already shown by cinnamaldehyde, suggested that this class of compounds, _viz_., quinones, should also be readily reduced/derivatized. Indeed, Figures 6A and 6B indicate the HPLC-UV chromatograms for vitamin K_3 analyzed first with a dummy plus analytical column (Figure 6A), and then with the borohydride/silica SPR before the analytical column (Figure 6B). The starting material is completely reduced under these conditions, in real time, on-line, using 1% IPA/hexane as the mobile phase. The reduction product of vitamin K_3 does not appear to be eluted under these particular HPLC conditions. It is possible that with more polar mobile phases, together with perhaps gradient elution approaches, this reduction product could be eluted and detected. However, just the disappearance of the starting material under these HPLC-SPR reaction conditions suffices to suggest the presence of this vitamin derivative. Improved specificity could rely on analysis for the product.

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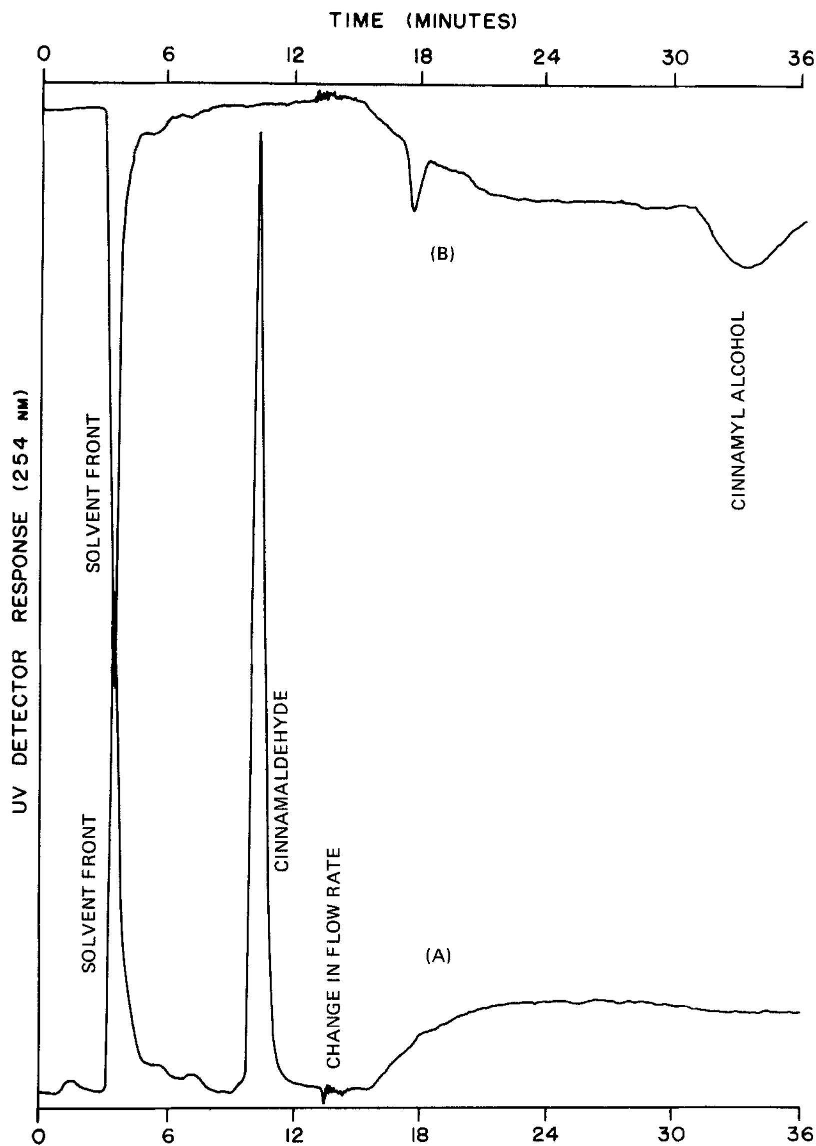

Figure 5. HPLC-UV analysis of cinnamon extract for presence of cinnamaldehyde using uPorasil analytical column with 0.3% IPA/hexane mobile phase at 1.0 ml/min (0-13 mins), then 4.3 ml/min. UV detection at 254 nm. (A) HPLC-UV chromatogram for dummy column before analytical column; (B) borohydride/silica SPR before analytical column.

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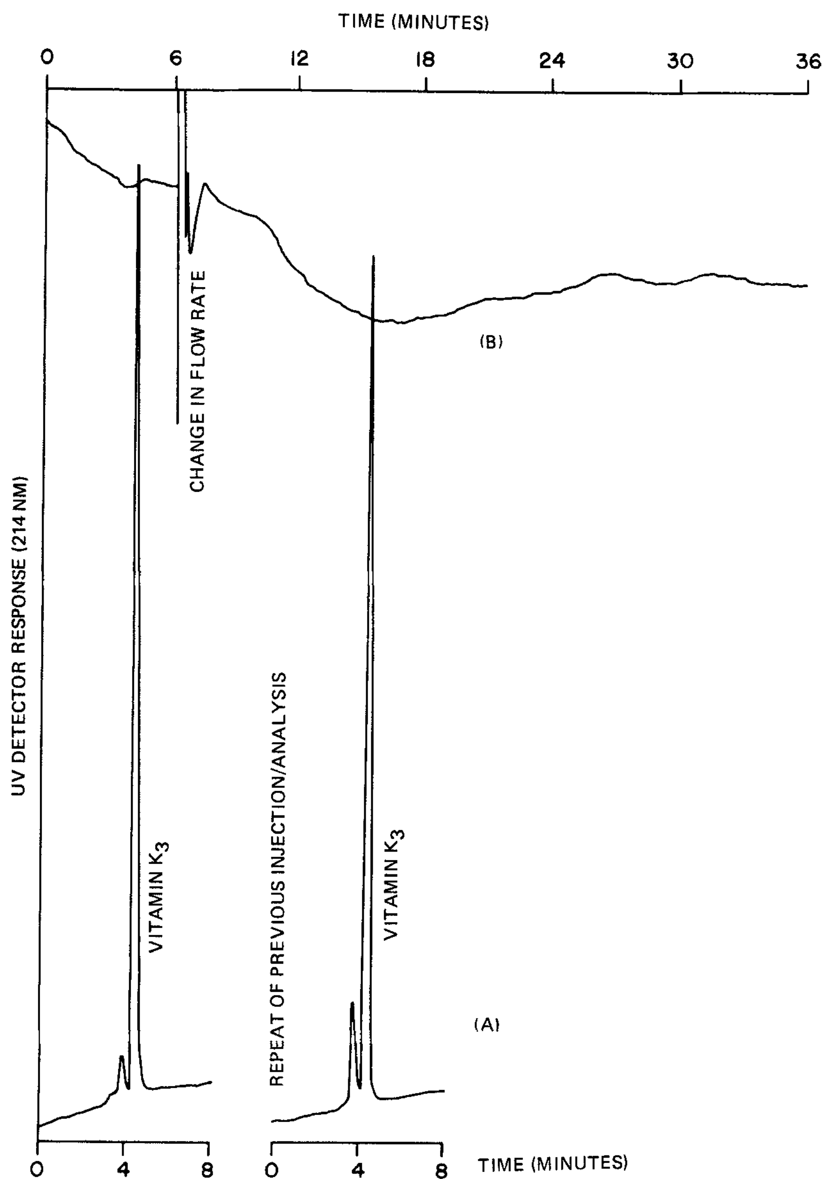

Figure 6. HPLC-UV analysis for standard Vitamin K_3 (menadione) using uPorasil analytical column with 1% IPA/hexane mobile phase at 1.0 ml/min (0-6 mins), then 4.3 ml/min. UV detection at 214 nm. (A) HPLC-UV chromatogram for dummy column before analytical column; (B) borohydride/silica SPR before analytical column.

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CONCLUSIONS

We have described here the preparation, elemental/borohydride analysis, derivatization properties in on-line, normal phase HPLC, scope of reactions, reactivities of various classes of compounds, and certain direct applications of a sodium borohydride on silica gel solid phase reactor. This particular SPR is ideal for normal phase HPLC separations, and has been shown compatible with various ratios of IPA/hexane, from 0-15% v/v. Specific qualitative identification of an individual analyte is based on its partial or complete reduction *via* this SPR, in either a pre- or post-analytical column orientation. Appearance of the expected, known reduction product, usually an alcohol, is additional confirmation for the presence of the initial starting material. The borohydride/silica SPR can also be used for organic class identification, especially in combination with high and low temperature studies for percent reductions. It may yet be possible to differentiate one particular ketone or aldehyde from related compounds, just as it is now possible to completely differentiate aldehydes from ketones from esters, etc. The in-house preparation and borohydride analysis of these SPRs is a simple matter, and repeated applications of borohydride to the same SPR batch leads to higher loadings/coatings with greater reactivities for individual compounds and longer lifetimes. Lifetime of an SPR is a function of initial loading on the silica, absence of water/moisture during packing of the SPR, dryness of the mobile phase solvents, and storage or care of each SPR when not in actual operation. It is indeed possible to have an SPR last for at least 50 individual analyses before any significant loss of reduction potential is observed. We envision the eventual development of several other silica or alumina supported reagents for on-line, real-time derivatizations in HPLC. These approaches are fully compatible with all commercially available HPLC equipment and approaches, and should find many more applications for real world samples.

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1. Abbreviations used: HPLC = high performance liquid chromatography; UV = ultraviolet detection; SPR = solid phase reactor/reaction; FL = fluorescence detection; IPA = iso-propanol (2-propanol); HEX = hexane; sodium borohydride = $NaBH_4$.

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