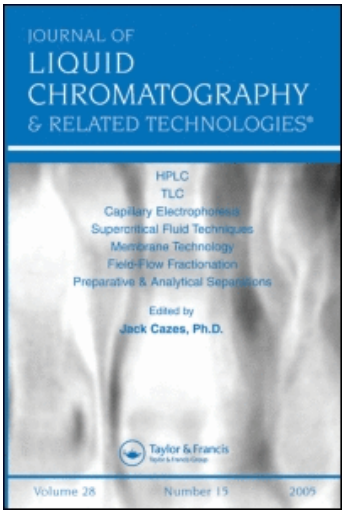

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Solid Phase Derivatizations in HPLC: Polymeric Permanganate Oxidations of Alcohols and Aldehydes in HPLC-SPR

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To cite this Article Xie, K-H. , Santasania, C. T. , Krull, I. S. , Neue, U. , Bidlingmeyer, B. and Newhart, A.(1983) 'Solid Phase Derivatizations in HPLC: Polymeric Permanganate Oxidations of Alcohols and Aldehydes in HPLC-SPR', Journal of Liquid Chromatography & Related Technologies, 6: 11, 2109 — 2127
To link to this Article: DOI: 10.1080/01483918308066564
URL: http://dx.doi.org/10.1080/01483918308066564

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(11), 2109-2127 (1983)

SOLID PHASE DERIVATIZATIONS IN HPLC: POLYMERIC PERMANGANATE OXIDATIONS OF ALCOHOLS AND ALDEHYDES IN HPLC-SPR

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ABSTRACT

On-line or off-line oxidations of various alcohols, aldehydes, and ketones can now be performed in conjunction with high performance liquid chromatography (HPLC), utilizing a newly developed polymeric permanganate solid phase reactor (SPR). These derivatization reactions are compatible with most reversed phase and normal phase solvents for HPLC separations, and many of these oxidations can be accomplished in real-time, on-line, at or above room temperature. Such HPLC-SPR approaches for chemical modifications and derivatizations of various oxidizable analytes provide a useful and quite practical newer approach for the HPLC-ultraviolet (UV) detection of appropriate analyte species. Difference chromatography, often with improved UV detection, can be used to confirm the suspected presence of a particular oxidizable analyte in a complex sample matrix. All of these solid phase derivatizations utilize conventional, commercially available HPLC instruments and accessories. These HPLC-SPR oxidation methods for chemical derivatization have also been applied to certain real world samples, in order to demonstrate the overall value and applicability of such analytical approaches.

INTRODUCTION (1)

Although a very large number of derivatization approaches have already been described for HPLC applications/utilization, perhaps more than 99% of these have utilized standard, homogeneous type approaches (2-10). Indeed, most conventional homogeneous derivatizations for HPLC are still done off-line, in the pre-injection/column mode, wherein the desired derivative(s) and excess derivatizing reagent(s) are injected together. More recently, automation of

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 0148-3919/83/0611-2109$3.50/0

both pre-column and post-column homogeneous derivatizations, off-line and on-line respectively, have become quite popular and widespread. Nevertheless, it is clear to us and others that homogeneous derivatizations have certain inherent, difficult to avoid/overcome, disadvantages in trace analysis. We have recently summarized many of these more serious disadvantages, and at the same time, we have discussed in depth what we believe are some of the more significant advantages of utilizing solid phase derivatizations in HPLC (2, 3, 11-15). In previous publications, we have described two distinct approaches to performing, on-line, in real-time, pre- or post-column chemical reductions of various aldehydes or ketones (11, 12). In one of these approaches, we used an in-house prepared polymeric borohydride solid phase reactor (SPR), which could then be utilized for the efficient reductions of aldehydes in reversed phase (RP) HPLC separations. In the other publication of ours in this area, we described the preparation and use of a silica supported borohydride reagent for performing similar reductions of aldehydes and ketones, now using normal phase HPLC solvents and separation conditions (11). At the same time, Frei's group in Amsterdam have just described similar approaches in HPLC-SPR, wherein the alumina support commonly used for HPLC separations has been utilized for certain catalytic reactions, pre-column, on-line, in real-time derivatizations (14). Our own interest in HPLC-SPR has now been extended to the development, optimization, and application of a newer polymeric oxidizing SPR for on-line and off-line derivatizations in HPLC.

We describe here the preparation, characterization, evaluation, optimization, and application of a polymeric permanganate resin for performing a wide variety of solid phase oxidations of suitable alcohols (primary/secondary), aldehydes, and some ketones. Such derivatization approaches have been developed for both on-line and off-line applications, with both reversed phase and normal phase HPLC conditions and separations. Percent oxidations have been determined as a function of the derivatization conditions and solvents, and where possible, these have been optimized with regard to time and temperature. At the same time, it has now been possible to evaluate what other classes of organic compounds do not undergo oxidations with this particular SPR. In all of these HPLC-SPR studies, difference chromatography has been utilized, wherein the analyte or sample matrix is first injected onto a dummy SPR plus the analytical column, and then in a separate injection, onto a combination of the oxidizing SPR plus the same analytical column. In both instances, the only change in the two chromatograms thus generated is due to the presence of the oxidizing reagent (permanganate) on the oxidizing SPR, but all other HPLC-SPR operating conditions remain constant. Although UV detection has been emphasized throughout these particular studies, often with enhanced UV detection following SPR oxidations, it is indicated that these approaches are totally amenable and applicable to virtually any known/commercial HPLC detector. This is true, as long as either the starting material and/or the product can be

detected with the particular HPLC detector of choice or availability (2). Indeed, a combination of HPLC detectors could be utilized with HPLC-SPR, and the overall analyte/product specificity could be considerably enhanced over the use of a single such detector.

Finally, these HPLC-SPR oxidation approaches have now been applied to two real world samples, resulting in the partial or complete oxidation of the starting alcohol present. In one such instance, it has been possible to oxidize benzyl alcohol present in a particular hair shampoo, and to observe the formation of the expected aldehyde and carboxylic acid following HPLC-SPR oxidation. In the second instance, riboflavin has been oxidized to a variety of products, perhaps due to the presence of a sugar moiety capable of undergoing oxidations of the various alcohol functionalities simultaneously. It is suggested that a large number of other possibly interesting applications for this particular oxidizing SPR will be developed in the very near future.

EXPERIMENTAL

Reagents and Chemicals

Certain preliminary studies on SPR oxidations were performed with a commercially available sample of chromic acid on Amberlyst A-26 (2.2 meq CrO_3/g reagent), obtained from Alfa Products, Ventron Division, Thiokol Corp. (Danvers, Mass.). This material was used directly in on-line and off-line work with various alcohols, but its oxidizing efficiency was less than adequate or desirable for most HPLC-SPR applications. The polymeric permanganate SPR was prepared using an analytical grade anion exchange resin, AG 1-X8, minus 400 mesh (Bio-Rad Laboratories, Richmond, California). This is a styrene-divinyl benzene based quaternary ammonium (Cl^- form) anion exchange resin which has proven useful in this and previous polymeric SPR studies (12).

HPLC solvents were obtained from Waters Associates, Inc. (Milford, Mass.), as their HPLC grade materials, distilled-in-glass. All such solvents were used as received, with de-gassing and filtering through a 0.45um solvent filtration kit/filter (Millipore Corp., Bedford, Mass.). At times, HPLC grade water was obtained from MCB Manufacturing Chemists, Inc. (Gibbstown, N.J.), as their Omnisolv brand HPLC solvent.

The various chemicals and reagents utilized here were obtained from a variety of commercial suppliers, of the highest purity available, and were used as received, without further purification. These were obtained as follows: potassium permanganate (Aldrich Chemical Co., Milwaukee, Wisc.); benzoquinone (Aldrich); acetophenone (Aldrich); benzhydrol (Aldrich); benzyl alcohol (Aldrich); trans-cinnamaldehyde (Aldrich); cinnamyl alcohol (Aldrich); benzaldehyde (J.T. Baker Chemical Co., Phillipsburg, N.J.); benzophenone (Fisher Sci. Co., Medford, Mass.); p-nitrobenzaldehyde (Sigma Chemical Co., St. Louis, Mo.); p-nitrobenzyl alcohol (Aldrich); Faberge Hair Shampoo (MediMart Drugs, Boston, Mass.); sec-phenethyl alcohol (Aldrich); salicylaldehyde (Aldrich); hydroquinone

(Aldrich); o-aminobenzyl alcohol (Aldrich); o-aminobenzaldehyde (Aldrich); benzoic acid (Aldrich); riboflavin (Vitamin B_2)(Sigma Chemical Co.). Lanthanum nitrate [$La(NO_3)_3$] was obtained from Matheson, Coleman, and Bell, Inc., (Norwood, Ohio).

The polymeric oxidizing resin (permanganate) was prepared by using the AG 1-X8 resin (5g), potassium permanganate (1.5g), and $La(NO_3)_3$ (0.5g) in 80ml of distilled water. This solution was then stirred for 1hr at room temperature, filtered, and washed extensively with water to remove excess, non-ionically attached MnO_4^-. It is essential to fully remove all excess, physically adsorbed permanganate by water washing/extraction, before this resin is used in any SPR studies/applications. Washing was done in a batch process, until the wash water was totally free of the violet color of MnO_4^-. At that point, the resin could be loaded into an SPR column.

Apparatus

The HPLC system utilized for most of these studies consisted of a Waters U6K syringe loading injection valve (Waters Associates, Inc., Milford, Mass.), a Waters 6000A solvent delivery system/pump, a Waters Model 480 variable wavelength UV-VIS detector, and a Houston Omniscribe, Model 5510 dual pen recorder (Houston Instruments, Inc., Austin, Texas). All HPLC separations were performed with uBondapak C_{18} reversed phase columns, 10um, 30-cm x 3.9-mm i.d. (Waters Assocs.). The dummy SPR column and the solid phase reactor (SPR) column were prepared using glass lined stainless steel tubing, 6-cm x 4.6-mm i.d., from Alltech Associates, Inc. (Deerfield, Ill.). All dummy, solid phase reactor, and analytical column end fittings were zero dead volume (Cambridge Valve & Fitting, Inc., Billerica, Mass.)(Waters Assocs., Inc.). Wherein both the dummy and SPR were both on-line simultaneously, individual injections were switched to either the dummy or SPR via a Rheodyne Model 7000 switching valve (Rheodyne Corp., Cotati, Calif.). The switching valve was located just after the HPLC injection valve and before the dummy and SPR columns, all of which was located just before the analytical column. A schematic diagram of the overall instrumentation arrangement has been published elsewhere (11, 12).

Methods

In all of these studies with the polymeric permanganate resin, standards of all organic compounds being analyzed were injected as solutions in either the mobile phase or neat acetonitrile. Such standard solutions, in known concentrations, were generally injected in 20ul aliquots, first onto a combination of the dummy column plus analytical column, and then onto the polymeric SPR oxidation column and the same analytical column. The dummy column consisted of the commercial anion exchange resin, usually the AG 1-X8, in its original chloride (Cl^-) form. The polymeric permanganate resin in the solid phase reactor and the dummy column were both slurry packed, at pressures of about

2000 psi. During normal operations, both the dummy and SPR columns were stable at pressures of at least 1500-2000 psi. It is probable that these polymeric packings are stable to higher HPLC back pressures, when used in the pre-column mode, but we have not had to determine maximum pressure stabilities. All retention times on both dummy and SPR columns, pre-analytical column mode, were determined by duplicate or triplicate injections of the analyte of interest along with the expected oxidation product(s), the aldehydes or ketones or carboxylic acids, wherever standards for these were known and/or available. In all cases, with various mixtures of mobile phases, the retention times of the alcohols, aldehydes, and ketones on both the dummy and SPR columns agreed very well (±5% or less). Retention times of all compounds were based on the HPLC chromatograms and an external automatic timer started at the point of injection and measured at the point of maximum peak height(s).

In the case of the commercial hair shampoo used for the determination of benzyl alcohol via its off-line oxidation, the initial sample of shampoo was first diluted 100-fold in the HPLC mobile phase of 50/50 (v/v) water/acetonitrile (HOH/ACN). The oxidation in this case was performed off-line, at room temperature, in 10 mins, by injecting 100ul of the initially diluted hair shampoo onto a column of the oxidizing resin. After oxidation of the sample, the entire material and oxidation products were eluted from the SPR with an excess of the HPLC mobile phase (10mls). This final, diluted solution was then injected directly onto the HPLC system, with only the analytical column on-line, since the SPR oxidation had taken place off-line here. In the application involving the off-line oxidation of riboflavin, a standard of this vitamin was dissolved in 200ul of 50/50 HOH/ACN, and this solution was then placed onto the oxidizing SPR, off-line, at room temperature, for 10 mins. At the end of this time period, the oxidized products were eluted with 5mls of 50/50 HOH/ACN. An aliquot (20-25ul) of this eluted solution was then injected directly onto the analytical HPLC system, in order to determine remaining riboflavin and its oxidation products.

SPR oxidations of various standard alcohols, aldehydes, or ketones were performed in a number of possible manners, including: 1) off-line, 10 mins or less, at room temperature or 46°C (elevated temperature), followed by elution of the reaction mixture with mobile phase, then HPLC injection; 2) on-line, pre-analytical column mode, room temperature or above, real-time or extended time in SPR, followed by HPLC elution of the oxidized products onto RP analytical column. SPR reactions performed at elevated temperatures were done using a constant temperature water bath, Precision Scientific/GCA Corp. (VWR Scientific, Inc., Boston, Mass.). Although SPR oxidations, in principle, could be performed on-line, real-time, post-column, we have not made use of such approaches in these studies. Previous publications have described and discussed how post-column, on-line SPR derivatizations can be performed, and what sort of qualitative/quantitative results might be expected (*2*, *11*, *12*).

Elemental analyses for manganese (Mn) content, and therefore an indication of permanganate loading on the final polymeric SPR, were performed at Galbraith Laboratories, Inc. (Knoxville, Tenn.). Further characterization of the final SPR was done in-house, using a permanganate titration method developed independently. This involved weighing a certain amount of the permanganate resin into a 250ml titration flask with 20ml distilled, deionized water. To this was added an excess of a ferrous sulfate ($FeSO_4.7HOH$, 0.05M) solution, the entire mixture was stirred, with a final pale yellow color developing. The excess ferrous ion (Fe^{+2}) was back titrated with a standard permanganate solution ($KMnO_4$, 0.05M) until the final solution changed color from pale yellow to pale violet. In general, results of these titrations indicated an average percent loading of MnO_4^- on the polymeric SPR of about 9.8% (n=4), which translates into about 100-125mg permanganate in one typical loaded SPR column. A typical permanganate loaded SPR would require about 1.2g of polymeric material to completely load the empty, glass-lined, stainless steel tubing. The elemental analyses for manganese (Mn) performed at Galbraith Labs involved inductively coupled plasma (ICP) emission spectroscopic methods for total Mn. These results, on the same batch of SPR resin used for the above titration determinations, indicated a somewhat higher loading of total Mn/MnO_4^-. This may be due to the titration method measuring only surface available/loaded permanganate, while the elemental analysis, ICP method, measures total Mn, surface and internally loaded. Internally loaded MnO_4^- may not be titratable using the procedure described above.

RESULTS AND DISCUSSION

In general, on-line, real-time, ambient temperature solid phase oxidations/derivatizations are to be preferred over off-line, delayed/stopped-time, elevated temperature approaches with the same polymeric permanganate SPR. That is, on-line approaches permit overall analyses in just two simple injections, one with the dummy column plus analytical column in-line, and the second with the polymeric SPR plus analytical column in-line. The two overall chromatograms obtained *via* these two separate injections of the same sample solution or solution of standards, then leads to the difference chromatography useful for indicating the presence or absence of the suspected analyte capable of undergoing oxidation with this particular SPR under HPLC conditions. Such on-line approaches do not require any sample manipulation prior to SPR oxidation, other than that normally used/required for HPLC injections, and there is a minimal chance of sample loss or contamination. Thus, on-line approaches have always been the desired goal in this and previous HPLC-SPR research and development work (2, 11, 12). However, at times, it has not been possible to utilize on-line, real-time approaches with this type of SPR, and thus it has been necessary to go to off-line, delayed time, above room temperature oxidation conditions, at times. This is a completely feasible

and practical approach, and still possesses some significant advantages over conventional, homogeneous derivatizations now performed off-line, as well. Since polymeric oxidizing reagents can be stored in the SPR column almost indefinitely, they represent a ready source of an efficient oxidizing system for many organic substrates/analytes. This then avoids the necessity of separately preparing the oxidizing solution each time that an oxidation is needed, and in principle, this and other SPRs could be stored in a readily available and usable bank of derivatizing reagents. Because of the relatively high loading of permanganate on this particular oxidizing SPR, often in the range of 100-125mg/SPR, we have found the final oxidizing columns to be stable and usable/practical for extended periods of time, often months. Clearly, they cannot be used on-line or off-line with an HPLC mobile phase or solvent that could itself undergo oxidation, since that would rapidly and inefficiently consume the permanganate loading on the SPR. At the same time, impurities in the HPLC mobile phase, if these are oxidizable, could also quickly consume and expire the SPR. Also, since these polymeric oxidizing resins are derived from an anion exchange support material, the permanganate reagent is only ionically bound to the polymeric support. Thus, wherein inorganic or organic anionic salts are included in the HPLC mobile phase, it is to be expected that these may/will displace the permanganate loading, and that eventually the final SPR will be ineffective as an oxidizing reagent. This is often evident by an inability to stabilize the UV detector, since MnO_4^- has some absorbance at many/most UV-VIS wavelengths of interest in HPLC. Also, the final mobile phase eluent exiting from the SPR and/or detector has a slight violet tinge to it if the permanganate is being released/exchanged from the SPR. Not only can inorganic/organic anions in the mobile phase cause this displacement of the reagent from the SPR, but also certain ionic analytes can have the same effect. Thus, in general, we have not realized much success in the attempted oxidations of ionic or zwitterionic analytes, such as catecholamines, catechols, or phenols. It is not that such compounds cannot be oxidized by permanganate in solution, just that they also will displace the reagent from the SPR into the mobile phase, and then the UV detector becomes unusable and unmanageable for normal HPLC operations and detection of the expected products or analytes.

HPLC-SPR oxidations or any such derivatizations are most useful wherein there is a change in the peak area/peak height of the starting analyte, and a concomitant appearance of one or more expected/known products of such an oxidation reaction. At the same time, the percent disappearance of the starting analyte should closely match the percent appearance of the expected product, in a quantitative sense, in order to truly optimize the selectivity and overall specificity of the analyte identification. However, often qualitative disappearance/appearance results suffice to confirm a suspected analyte, in addition to the conventional use of retention times vs a known external standard. In all of this work, we have used changes in both peak areas and/or peak

heights in order to determine percent oxidations of starting material/analyte. It is not always necessary to have 100% oxidations of all analytes, often 50% derivatizations will suffice for confirmation purposes, but such less than 100% results must be highly reproducible in order to be useful and reliable. In using this approach to derivatization on a particular analyte that is not fully oxidized in the sample, it can be most helpful for confirmatory purposes to demonstrate that the analyte in the sample and the external standard of that same analyte are oxidized to the same approximate degree in separate HPLC-SPR experiments. It is also possible that a particular alcohol analyte will undergo an initial oxidation to the aldehyde or ketone, and that in either on-line or off-line approaches, this product will/could then undergo a second stage oxidation to a carboxylic acid. Indeed, we have already seen this type of dual stage oxidation occurring with standards and actual sample matrices. Such multiple oxidations can provide additional confirmation of the nature/structure of the original analyte under investigation *via* HPLC-SPR.

In all of the work that follows, it must be emphasized that the nature of the HPLC mobile phase used must meet at least two criteria, at least for on-line derivatizations. First, it must be compatible with the required oxidation reaction and polymeric reagents, and it must not react or dissolve the reagent present. Second, the mobile phase must be suitable for the desired separations of the starting analyte from its expected/known oxidation products and/or other materials present in the sample matrix/solution. If these two criteria can be met with a single mobile phase composition, then it is quite likely, all other things being equal, that the desired oxidation can be performed on-line, in either real- or extended-time, at room or above temperatures. In certain cases, it may be worthwhile to determine which mobile phase composition may/will provide compatibility with the above suggested requirements for on-line HPLC-SPR work.

To demonstrate the overall approaches utilized here for oxidations in HPLC-SPR, Figure 1 contains two separate chromatograms with analytical column plus reagent SPR on-line. In Figure 1A, a standard solution of only p-nitrobenzyl alcohol has been injected, in real-time, with the SPR maintained at 46^{o}C, on-line. The retention time of the starting alcohol and its known oxidation product, *viz.*, p-nitrobenzaldehyde, were confirmed using a dummy column plus analytical column set-up with separate injections of each standard compound. In Figure 1B, the same HPLC-SPR conditions prevail as in Figure 1A, but now only a standard solution of the oxidation product, p-nitrobenzaldehyde has been injected. Under these conditions, 1B, some of the aldehyde injected has been oxidized further, and this has been demonstrated in a separate set of experiments. However, for purely qualitative purposes, Figure 1B indicates the retention time of the initial oxidation product of the starting alcohol under actual HPLC-SPR conditions. Other HPLC-SPR conditions used here are indicated in Figure 1. Percent oxidations of both alcohol and aldehyde are presented below, Table 1.

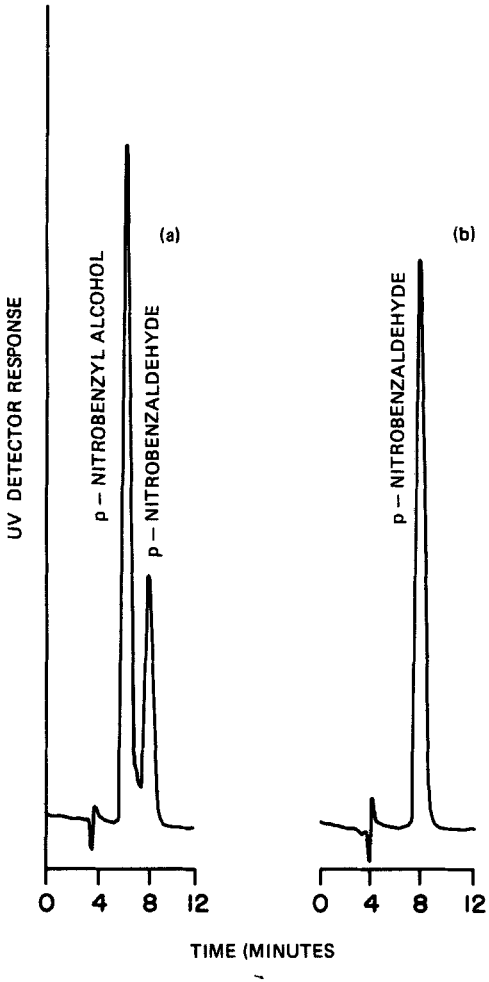

Figure 1. HPLC-SPR chromatograms for the oxidation of p-nitrobenzyl alcohol to p-nitrobenzaldehyde using uBondapak C_{18} analytical column with 50/50 HOH/ACN mobile phase at 0.8 ml/min flow rate: (A) only alcohol was injected with SPR on-line before analytical column, SPR at 46°C; (B) only aldehyde was injected, with HPLC-SPR conditions as in (A).

Whereas p-nitrobenzyl alcohol is only partly/partially oxidized in HPLC-SPR on-line approaches, Figure 1 and Table 1, a similar aromatic alcohol, o-aminobenzyl alcohol, is almost fully oxidized even at room temperature to the expected o-aminobenzaldehyde, Figure 2. Thus, Figure 2 illustrates the exact same HPLC-SPR oxidation of the same starting alcohol, on-line, using the same permanganate SPR, same mobile phase in the HPLC separations, but now varying the temperature of the SPR, pre-column, from room temperature (A) to

TABLE 1.

SUMMARY OF VARIOUS ALCOHOLS/ALDEHYDES STUDIED IN HPLC-SPR WITH POLYMERIC PERMANGANATE PRE-COLUMN ON-LINE IN REAL TIME[a]

COMPOUND NAME	PERCENT OXIDATION[b]
o-AMINOBENZYL ALCOHOL	100%
SALICYLALDEHYDE	100%[c]
p-METHOXYPHENOL	100%[c]
p-METHOXYNAPHTHOL	100%
HYDROQUINONE	42%
BENZYL ALCOHOL	11%
BENZALDEHYDE	15%
p-NITROBENZYL ALCOHOL	52%
p-NITROBENZALDEHYDE	53%

a. Oxidations were performed on-line, in pre-column mode, in real-time, using HPLC-UV with a Waters uBondapak C-18 column with mobile phase of HOH/ACN (50/50) at 0.8 ml/min flow rate, UV at 254nm. Polymeric permanganate SPR operated at 46°C.
b. Percent oxidations determined by changes in peak area or peak heights for dummy vs oxidizing difference chromatograms.
c. In these two analyses, the peak of starting material disappeared, but no new peak appeared for the oxidation product(s). Such products may have remained on ion-exchange support of SPR or not eluted under HPLC mobile phase conditions used here.

46°C (B). In Figure 2A, some of the starting o-aminobenzyl alcohol is still visible at the correct retention time of about 6 mins, but in Figure 2B, with the SPR kept at a higher temperature, there is no remaining alcohol visible, concomitant with an increased peak height for the product aldehyde. The same amounts of alcohol were injected in both (A) and (B), Figure 2, and thus it can be safely assumed that at the higher SPR working temperature used in (B), complete oxidation of the alcohol has now been realized, with an additional observed formation of the known oxidation product, the aldehyde indicated. Clearly, HPLC-SPR oxidations are or can be greatly affected as a function of the temperature of the SPR on-line, in real-time.

We have attempted to demonstrate the linearity of these oxidations over a wide range of concentrations of at least two alcohols injected under HPLC-SPR conditions, as above. Figure 3 illustrates a log-log plot of the amount (ng) of o-aminobenzaldehyde derived/formed from the on-line, real-time, 46°C SPR oxidation of the o-aminobenzyl alcohol already described above. We have here plotted the amount of oxidation product expected vs peak height, rather than amount of alcohol injected, since in this example, even at room temperature, all of the starting alcohol is completely oxidized at all levels injected. Thus, it was only possible to utilize amount of product expected vs peak heights, in order to demonstrate the linearity of the oxidation reaction over

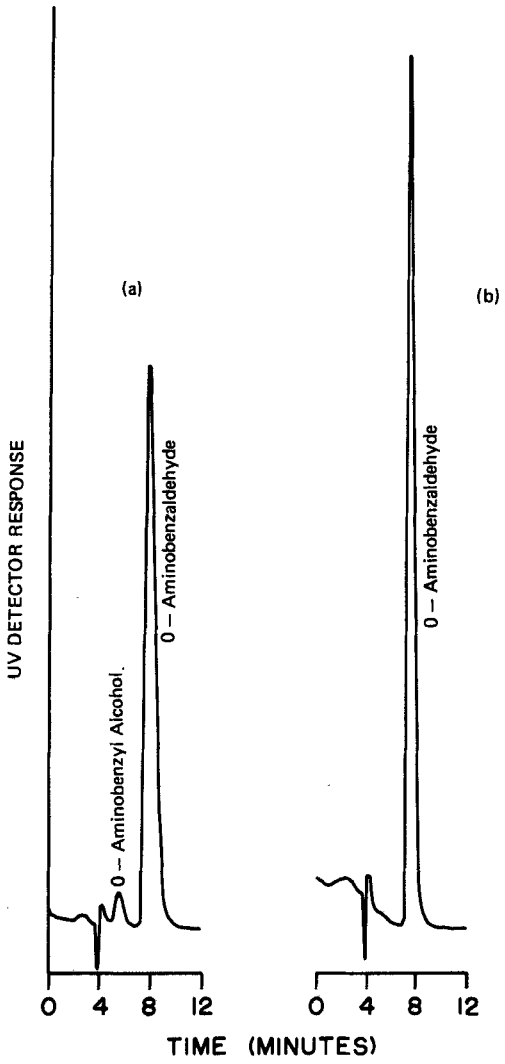

Figure 2. HPLC-SPR chromatograms for the oxidation of o-aminobenzyl alcohol to o-aminobenzaldehyde using analytical column of uBondapak C_{18} with 50/50 HOH/ACN mobile phase at 0.8 ml/min flow rate with SPR on-line, just before analytical column: (A) only alcohol was injected with SPR kept at room temperature; (B) only alcohol was injected with SPR at 46°C throughout.

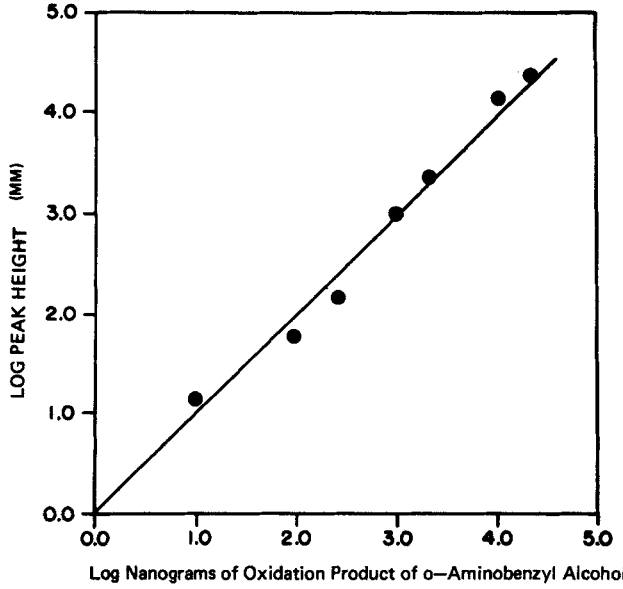

Figure 3. Plot of amount (ng) of expected oxidation product formed as a function of varying amounts of starting alcohol injected onto HPLC-SPR, on-line, real-time, at 46°C, starting alcohol was 100% oxidized at all levels injected onto HPLC-SPR to the expected o-aminobenzaldehyde.

at least 4-5 orders of magnitude of amount (ng) injected via HPLC-SPR. This might have been expected based on the known/demonstrated absolute amount (mass) of permanganate loaded onto a typical SPR oxidizing column (100-125mg/column). The correlation coefficient for linearity of the plot in Figure 3 is 0.995. A similar study is described/summarized in Figure 4, which is another log-log plot of peak heights observed at various levels/amounts (ng) of the oxidation product expected from the HPLC-SPR derivatization of p-nitrobenzyl alcohol. In this instance, the starting alcohol is not fully oxidized at room temperature, Table 1, and thus we have also been able to use peak heights for the alcohol injected at various levels together with peak heights for the oxidation product expected/observed. Again, using either peak heights for the alcohol or aldehyde at various amounts/levels injected, the calibration plot is linear over about 2-3 orders of magnitude. The correlation coefficient of linearity for this plot of Figure 4 is in excess of 0.900. However, in this example, there is observed some degree of non-linearity at the higher ranges/levels of alcohol injected. This is probably due to the fact that this alcohol is incompletely oxidized at lower levels, and thus requires a longer residence time or higher SPR reaction temperature to provide linearity at the higher levels injected. As in all types of derivatizations, it is desirable to utilize

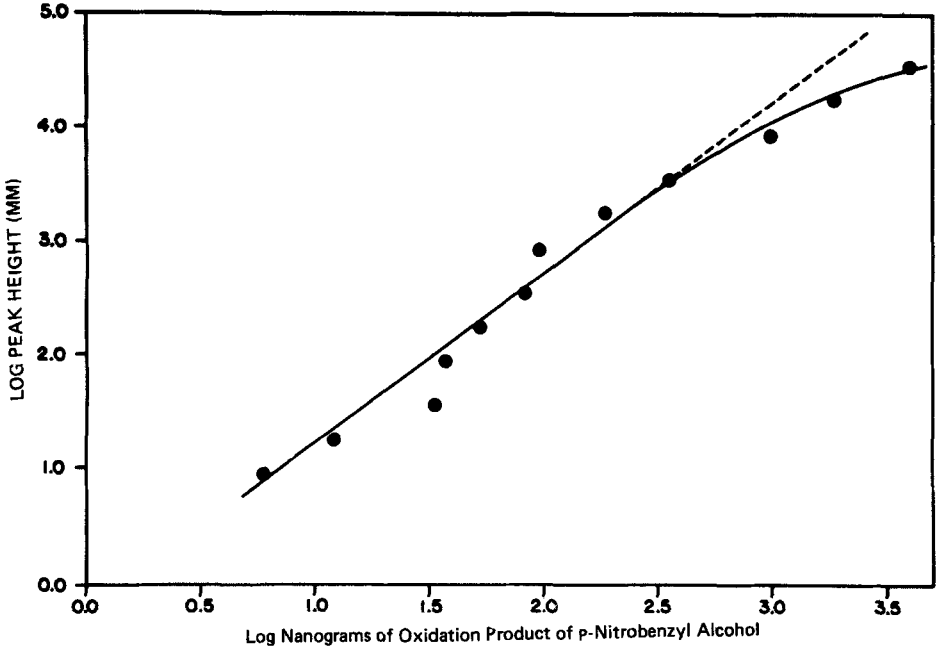

Figure 4. Plot of amount (ng) of expected oxidation product formed, p-nitrobenzaldehyde, as a function of varying amounts of starting alcohol injected onto HPLC-SPR, on-line, real-time, room temperature. Starting alcohol was not 100% oxidized at all levels initially injected in these studies.

ratios of reagent/substrate that will provide for maximum possible conversion/derivatization at all times/levels. Hence, in this particular case, it might be advantageous to inject amounts of the starting alcohol that would fall on the linear portion of the plot in Figure 4.

We have now studied a fairly large number of alcohols and aldehydes in oxidative HPLC-SPR approaches, and these are summarized in Table 1, along with the percent oxidations determined for each starting compound. The percent oxidations were calculated/determined by measuring changes in either peak areas or peak heights, or both, for the same starting material injected onto the dummy column plus analytical column and the SPR plus the same analytical column. In most of these studies, disappearance of the starting material peak was concomitant with appearance of the expected/known oxidation product. However, in two cases, salicylaldehyde and p-methoxyphenol, there was no evidence of any oxidation product formed. This may have been due to retention of the oxidation product on the anion exchange support of the SPR, because on the dummy column, each of these two starting materials were unretained and eluted

TABLE 2.
COMPARISON OF ON-LINE AND OFF-LINE HPLC-SPR OXIDATIONS OF VARIOUS COMPOUNDS USING NORMAL PHASE SOLVENT CONDITIONS IN HPLC FOR OXIDATION/SEPARATION

COMPOUND STUDIED	PERCENT OXIDATION	
	ON-LINE[a]	OFF-LINE[b]
BENZYL ALCOHOL	4.3%	66%
BENZALDEHYDE	3.4%	46%
CINNAMYL ALCOHOL	3.1%	71%
CINNAMALDEHYDE	6.7%	83%

a. HPLC conditions used a uPorasil column (10um) with 5% THF/HEXANE as mobile phase at flow rate of 1.1 ml/min at room temperature, with polymeric permanganate SPR in pre-column mode of operation. Injections made on-line, in real-time, at room temperature, with percent oxidations indicated.
b. Oxidations performed off-line, preparing THF solution of compound studied, placed onto oxidizing column for 10 mins at room temperature, analyte eluted from SPR off-line, injected directly onto HPLC-UV, as above.

in reproducible retention times. Hence, we suspect that it is the products of the SPR oxidation that are being held-up on the polymeric anion exchange support in the SPR only. In Table 1, these analyses were all done with the SPR on-line, real-time, with the SPR maintained at 46°C, with other conditions as indicated. Percent oxidations have varied from a low of 11% for benzyl alcohol to a high of 100% for several of these starting materials/analytes.

Whereas the above studies, Table 1, involved the use of reversed phase solvents, viz., HOH/ACN, it was also important to determine if normal phase organic solvents could be used with this particular oxidizing SPR in HPLC. These results, Table 2, suggest that, at least in the on-line mode, at room temperature, the percent oxidations observed with 5% tetrahydrofuran (THF)/ hexane as the mobile phase, were less than adequate or satisfactory for most applications. However, these same oxidations could be adequately performed in the off-line manner, Table 2, using THF alone as the reaction solvent, and then analyzing for the product and starting material by injecting onto an analytical HPLC system, using again 5% THF/HEX as the mobile phase. In the off-line mode of SPR derivatization, the exact same polymeric permanganate material was used for the oxidation reactions, but the oxidation was allowed to proceed for 10 mins at room temperature. The extended period of time that the analyte/ alcohol(aldehyde) was allowed to remain in contact with the polymeric SPR was presumably responsible for the increased percent oxidations observed, Table 2. It would therefore appear feasible/possible to utilize this particular SPR in an off-line mode/approach, even with normal phase solvents such as THF for the derivatization step, followed by normal phase HPLC separations/methods.

In all of the above examples of HPLC-SPR with suitable substrates, we have not yet described the possibility of oxidizing secondary alcohols. Table

TABLE 3.
SOLID PHASE OXIDATIONS OF SECONDARY ALCOHOLS WITH HPLC-SPR OFF-LINE OR ON-LINE

COMPOUNDS STUDIED	PERCENT OXIDATION	
	OFF-LINE[a]	ON-LINE[b]
BENZHYDROL	83.8%	30%
sec-PHENETHYL ALCOHOL	83.0%	16%

a. Oxidations performed off-line by preparing acetonitrile solution of analyte (secondary alcohol), injecting this onto polymeric oxidizing SPR, holding for 10 mins at 46°C, eluting and injecting onto HPLC. HPLC conditions used a uBondapak C_{18} column with mobile phase of ACN/HOH (65/35) at 0.9 ml/min flow rate, UV detection at 254nm.
b. On-line oxidations performed at 46°C in real-time, using HPLC-UV conditions as above with ACN/HOH was mobile phase at 0.9 ml/min flow rate.

3 indicates the solid phase oxidations of two such secondary alcohols, again comparing off-line with on-line approaches. And again, it is clear that the on-line derivatizations are less effective/complete overall than the off-line approaches, even when now both approaches use an elevated temperature of 46°C. In each of these two examples of secondary alcohol SPR oxidations, the expected ketone derivatives were observed at the end of each reaction, off-line or on-line, and thus we are fairly certain that these are indeed SPR caused oxidation type reactions of the substrates indicated. Additional experimental conditions for these particular studies are indicated in Table 3.

With regard to specific applications of the use of this oxidizing SPR in HPLC, Figure 5 indicates the analysis of benzyl alcohol present in a commercial hair shampoo. In Figure 5A, the original, diluted shampoo sample has been analyzed *via* reversed phase HPLC, with a peak at the correct retention time for standard benzyl alcohol. However, use of just chromatogram 5A to confirm the presence or amount of benzyl alcohol in this particular sample would be difficult, since there are so many other UV absorbing compounds/peaks present close to the peak of interest. Improved HPLC resolution conditions could be developed in order to further resolve the suspected benzyl alcohol peak from other materials in this sample. However, Figure 5B indicates the considerably simpler overall chromatogram for the same sample injected now onto the permanganate SPR, off-line, with oxidation at room temperature for 10 mins. There is now no longer a peak at the correct retention time for benzyl alcohol, but there are peaks now present for the expected oxidation products, *viz.*, benzaldehyde and benzoic acid. The benzaldehyde is the expected initial oxidation product of the alcohol, while the benzoic acid is the expected/known oxidation product of this aldehyde. The retention times for these two oxidation products were confirmed by injecting separate standards of each compound onto the HPLC system alone.

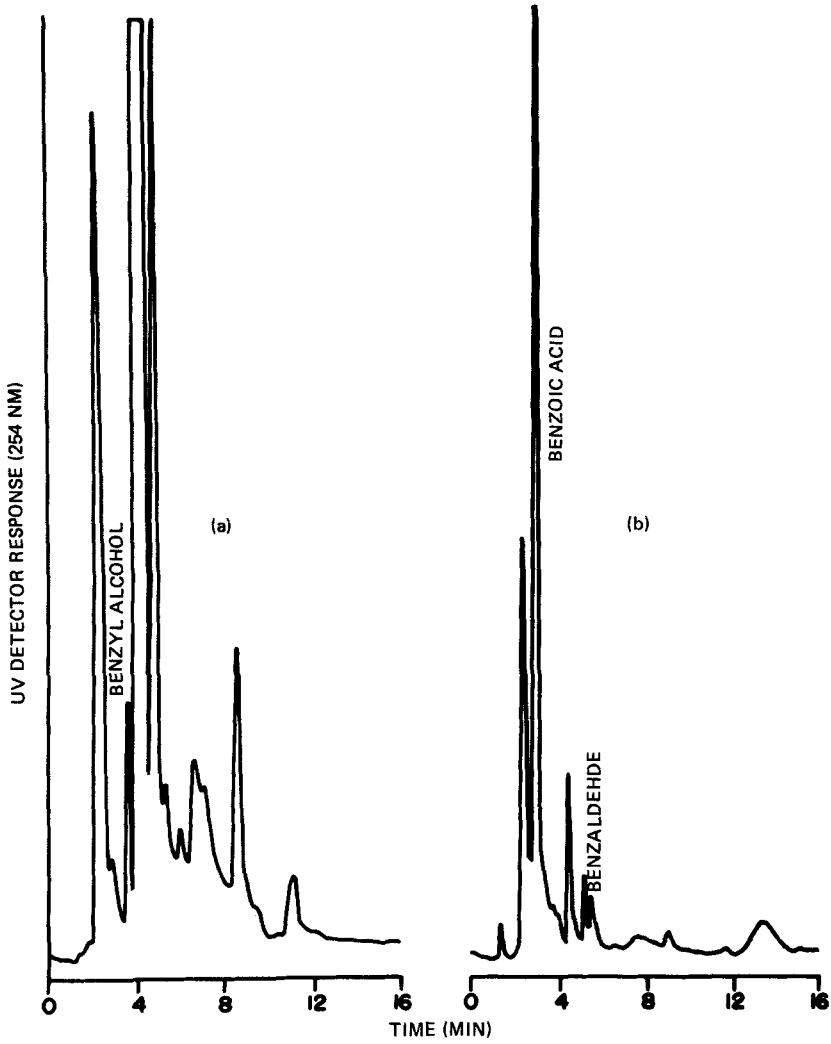

Figure 5. HPLC-UV chromatograms using Waters uBondapak C_{18} analytical column with mobile phase of HOH/ACN (50/50) at a flow rate of 1.0 ml/min, UV at 254nm, for off-line polymeric permanganate oxidation of benzyl alcohol in a commercial hair shampoo: (A) direct analysis of the diluted shampoo sample before oxidation; (B) after off-line oxidation at room temperature for 10 mins, showing disappearance of the alcohol.

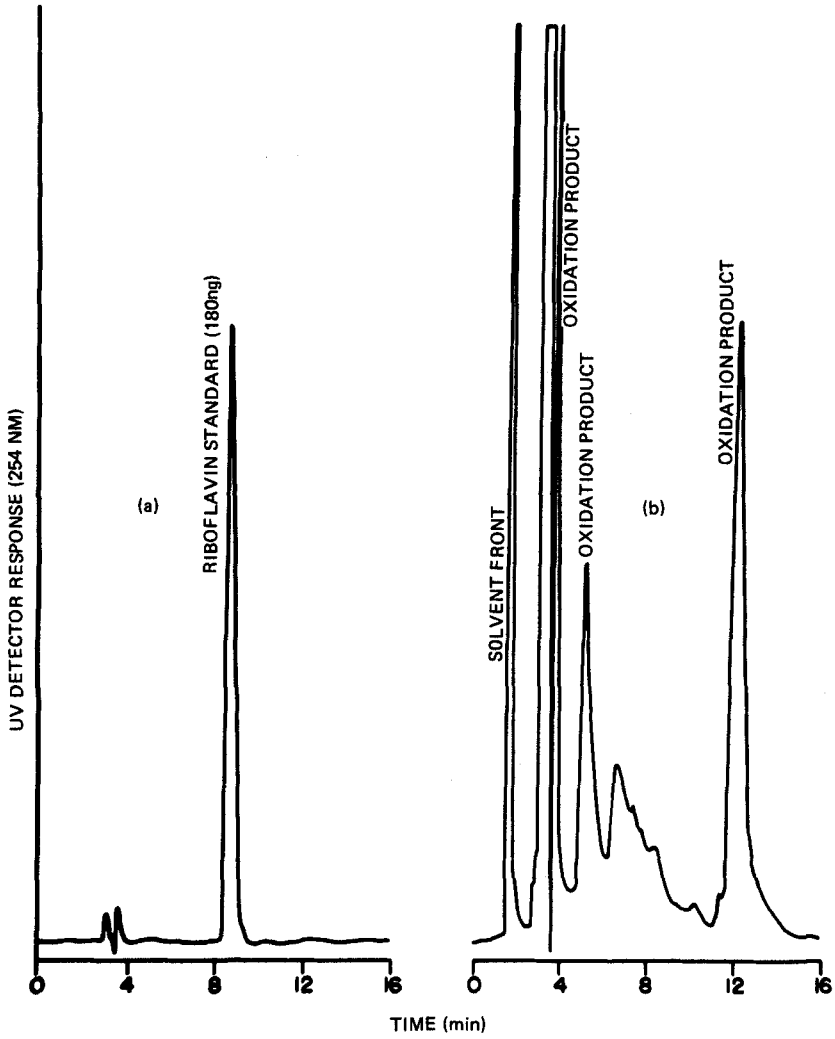

Figure 6. HPLC-UV chromatograms using Waters uBondapak C_{18} analytical column with mobile phase of HOH/ACN (85/15) at flow rate of 1.0 ml/min, UV detection at 254nm, for off-line polymeric permanganate oxidation of riboflavin: (A) standard of riboflavin before SPR oxidation off-line; (B) HPLC analysis of oxidation products of riboflavin oxidized off-line at room temperature for 10 mins in ACN/HOH (50/50).

A second application of HPLC-SPR techniques is indicated in Figure 6, which is the off-line SPR oxidation of a standard of riboflavin, vitamin B_2. This particular substrate was chromatographed before oxidation, Figure 6A, and then following off-line oxidation, Figure 6B, with the specific conditions as indicated. In this case, since riboflavin has a sugar side-chain on the parent molecule, there are several sites for oxidation, and thus several possible oxidation products. Indeed, the chromatogram of Figure 6B indicates the formation of a number of oxidation products, with essentially no starting riboflavin present. Thus, the formation of multiple oxidation products, Figures 5 and 6, provides additional confirmation for the presence of the original analyte, as long as a standard of this compound is available to demonstrate in a separate set of experiments the nature/complexity of the reaction products formed via HPLC-SPR. We have not, as yet, attempted to analyze for riboflavin in an actual vitamin supplement sample or a biological/food sample, but such applications are being planned and should be practical/successful in the future.

CONCLUSIONS

We have now demonstrated the preparation of a polymeric permanganate oxidizing reagent that is useful in various HPLC-SPR derivatizations for alcohols and aldehydes. Although we have not fully delineated what other classes of organic compounds might be suitable for these off-line or on-line reactions, it should be possible to at least oxidize various ketones, based on certain preliminary results. These approaches are compatible with both reversed and normal phase solvents, in either on-line or off-line modes, at room temperature or above. Percent oxidations of various substrates are influenced by a number of factors, including: solvent, analyte structure, residence time within the SPR, temperature of SPR, nature of HPLC mobile phase, and ratio of reagent/substrate injected. It is suggested that these derivatization approaches should find wide acceptance and application in the area of HPLC derivatizations, especially when the ease and convenience of solid phase reactions/reagents becomes better recognized and appreciated. Additional applications of this particular HPLC-SPR approach are now under investigation and development.

ACKNOWLEDGEMENTS

We acknowledge the interest and encouragement provided by a number of our colleagues within both Northeastern University and Waters Associates, especially that provided by B.L. Karger, B.C. Giessen, R. McNeil, S. Colgan, R. Shansky, and Wm. LaCourse. This work would not have been possible without the assistance and continued interest provided by K. Weiss at NU and C. Rausch at Waters Associates. Financial assistance and technical support for this joint research and development program was provided to NU by Waters Assocs., Millipore Corporation. We very gratefully acknowledge both the financial and technical contributions provided to NU in support of this joint R&D program.

Mr. K-H. Xie is a Visiting Chinese Scientist at Northeastern University, coming from the Analytical Institute of the Chinese Academy of Sciences, Beijing, China. We are grateful to the Government of the People's Republic of China and the Chinese Academy of Sciences for allowing Mr. Xie to undertake studies and research as a Visiting Chinese Scientist at NU.

Additional financial support was provided, in part, by a NIH Biomedical Research Support Grant, No. RR07143, Department of Health and Human Services, to Northeastern University.

This is contribution number 169 from the Institute of Chemical Analysis at Northeastern University.

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1. Abbreviations used: HPLC = high performance liquid chromatography; SPR = solid phase reactor/reactions; MnO_4^- = permanganate; UV = ultraviolet detection; HOH = water; ACN = acetonitrile; THF = tetrahydrofuran; HEX = *n-hexane*; *mins* = *minutes*; *ml/min* = milliliters per minute; nm = nanometer.

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