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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SUBSTITUTED *p*-BENZOQUINONES AND *p*-HYDROQUINONES

I. INTERPLAY OF ON-COLUMN REDOX REACTION AND THE CHROMATOGRAPHIC RETENTION PROCESS

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SUMMARY

Chromatograms exhibiting unusual elution profiles were obtained in the reversed-phase chromatography of methoxy-substituted hydroquinones with hydro-organic mobile phases. The dependence of the elution profiles on the flow-rate and the mass of injected sample indicated that peaks were caused by the interplay of the chromatographic retention process and the concomitant oxidation of hydroquinones in the chromatographic column. The reaction was first investigated in free solution and the first-order rate constants and equilibrium constants were estimated for the oxidation of 2-methoxy-, 2,5-dimethoxy-, 2,6-dimethoxy-, 2,3,5-trimethoxy- and 2,3,5,6-tetramethoxyhydroquinones. The effect of Fe^{3+} and Cu^{2+} on the oxidation of 2,6-dimethoxyhydroquinone was also examined and it was found that Fe^{3+} caused a ten-fold increase in the reaction rate. The oxidation of 2-methoxy- and 2,6-dimethoxyhydroquinones in the chromatographic column showed first-order and zeroth-order kinetic behavior at low and high sample loads, respectively.

Such change in reaction order is typical for heterogenous reactions that follow Langmuir–Hinshelwood kinetics and suggests the existence of catalytically active sites on the silica. Upon purging the columns with EDTA, on-column oxidation of the hydroquinones was attenuated so that no reaction zones appeared on the chromatogram. Subsequent loading of the column with Fe^{3+}, however, gave rise to broad reaction zones and possibly to total conversion of the hydroquinones to benzoquinones in the column. In all cases investigated the rate of oxidation was much higher in the column than in free solution. On the basis of the results obtained here, heavy metal-catalyzed reaction at the stationary phase surface is proposed to be the dominant mechanism for the oxidation reaction examined here.

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INTRODUCTION

The reversed-phase chromatography of *p*-hydroquinones requires special precautions in order to suppress their oxidation in the column to the corresponding *p*-benzoquinones, caused by the relatively low redox potential of such solute pairs[1,2] and the ubiquitous presence of oxygen. Unless precautionary measures are taken, a substantial fraction of the *p*-hydroquinone injected may be converted to *p*-benzoquinone while in the column. If significant on-column oxidation occurs, unusually-shaped elution profiles are obtained upon injection of even a single, chemically pure substance. The simultaneous effect of on-column chemical transformation of the eluite and the chromatographic retention process has recently been investigated both theoretically and experimentally[3]. The results obtained with *cis*- and *trans*-isomerization of dipeptides in reversed-phase chromatography also showed elution profiles similar to those reported in this study.

Some of the methoxy-substituted *p*-benzoquinone and *p*-hydroquinone redox systems studied here are physiologically significant[4], and the simultaneous assay of both species of the redox pairs may be required to explain the mechanisms of metabolic oxidation. The development of an appropriate analytical method, however, necessitates a greater knowledge of the redox properties of such substances than that available from the literature. Therefore, the goal of the present investigation was to elucidate the oxidation reaction which interferes with the assay of methoxy-substituted hydroquinones in reversed-phase chromatography. We have found that high-performance liquid chromatography (HPLC) is an eminently suitable tool for the study of the oxidation of hydroquinones to benzoquinones both in free solution and on the surface of the stationary phase.

EXPERIMENTAL

Apparatus

Three liquid chromatographs were used in the course of these studies. One comprised two Beckman (Berkeley, CA, U.S.A.) Model 100A pumps with system controller. The second consisted of a Model 870 pump module (DuPont, Wilmington, DE, U.S.A.) with a series 880 gradient controller. Both chromatographs were equipped with a Model 7120 (Rheodyne, Cotati, CA, U.S.A.) injection valve. The effluent was monitored by a Model 770 UV spectrometer (Kratos, Ramsey, NJ, U.S.A.) or a Model No. 852 UV detector (DuPont), and the signal was traced on a strip-chart recorder, Model No. 56 (Perkin-Elmer, Norwalk, CT, U.S.A.) or by a Model CI-10 recording integrator (LDC/Milton Roy, Riviera Beach, FL, U.S.A.). The detector was set at 285 or 290 nm, except for the 2-methoxybenzoquinone which was detected at 254 nm. The third chromatographic system was from IBM Instruments (Danbury, CT, U.S.A.) and consisted of a Model LC/9533 ternary gradient liquid chromatograph with micro-computer-controlled operator station, a Model 9522 UV detector with fixed wavelength of 254 nm and a Model 9540 integrator. This system was equipped with a Model 7125 injector (Rheodyne) and a Model 500 Recordall strip-chart recorder (Fisher, Fair Lawn, NJ, U.S.A.).

Chemicals

2-Methoxyhydroquinone (2-MHQ), 2,6-dimethoxyhydroquinone (2,6-DMHQ), 2,3-dimethoxyhydroquinone (2,3-DMHQ), 2,5-dimethoxyhydroquinone (2,5-DMHQ), 2,3,5-trimethoxyhydroquinone (2,3,5-TMHQ) and 2,3,5,6-tetramethoxyhydroquinone (2,3,5,6-TMHQ), and the corresponding benzoquinones were gifts of G. Fodor (University of West Virgina). 1,4-hydroquinone (HQ) and 1,4-benzoquinone (BQ) were obtained from Sigma (St. Louis, MO, U.S.A.) and Matheson Coleman Bell (Cincinnati, OH, U.S.A.), respectively. Methanol, acetonitrile and tetrahydrofuran (THF) were HPLC-grade from Baker Chemical (Phillipsburg, NY, U.S.A.) or Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Distilled water was prepared with a Barnstead unit available in both of our laboratories.

Columns

The stationary phases used were octyl-Zorbax, phenethyl-Zorbax (DuPont), Supelcosil LC-1, Supelcosil LC-18 (Supelco, Bellefonte, PA, U.S.A.), IBM-C_8, IBM-C_{18} (IBM Instruments). The column dimensions were 250 × 4.6 mm or 4.5 mm. A laboratory-packed column containing Supelcosil C_{18} (150 × 4.6 mm) was also used.

Operating conditions

Unless otherwise stated, a flow-rate of 1.0 ml/min was used under isocratic condition. In most cases, the sample volume was 20 μl. The columns were operated at ambient temperature in the range 22–26°C.

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms obtained upon injection of pure HQ and 2-MHQ into octylsilica and octadecylsilica columns with the use of methanol–water (10:90, v/v) as mobile phase. All chromatograms show several local maxima of the elution profile. The absence of well-defined peaks is not due to low column efficiency, as the injection of BQ and 2-MBQ yielded over 11,000 theoretical plates under the same conditions. Chromatography of these substances on other types of reversed-phase columns, *e.g.*, octyl-Zorbax, phenethyl-Zorbax, Supelcosil LC-18, and Supelcosil LC-1, resulted in similar elution profiles having multiple peaks of anomalous band widths including a "reaction zone". It was found that the nature and concentration of the organic cosolvent in the hydro-organic eluent also had a significant effect on the shape of the elution profiles. Rechromatography of certain fractions resulted in elution profiles similar to that obtained originally upon injection of pure hydroquinone samples into the same column. The center of gravity of the elution profile, however, shifted toward the retention time of benzoquinone upon subsequent rechromatography, suggesting that oxidation of hydroquinone took place in the column.

Oxidation in free solution

In order to assess the effect of the silica-bound hydrocarbonaceous stationary phase on the reaction believed to occur in the column, first the rates of hydroquinone oxidation in only the mobile phase proper were estimated. 2-MHQ, 2,3-DMHQ,

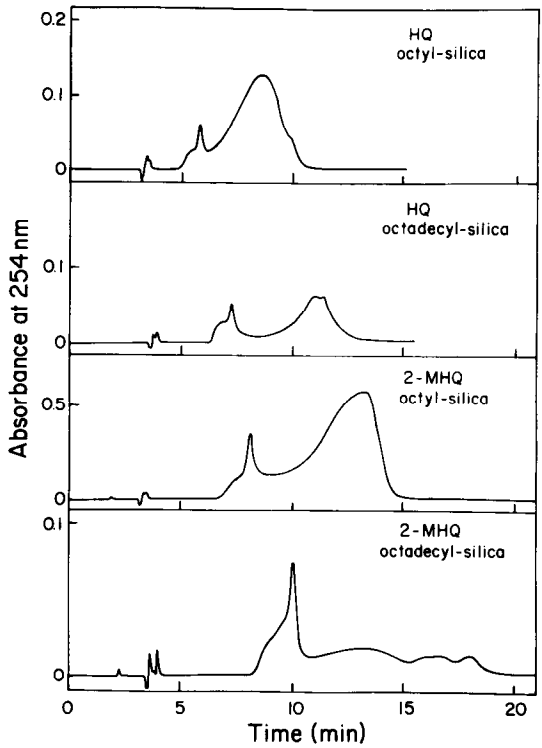

Fig. 1. Chromatograms of hydroquinone (HQ) and 2-methoxyhydroquinone (2-MHQ) on octyl- and octadecylsilica columns. The data were obtained with methanol–water (10:90) as mobile phase at 1.0 ml/min flow-rate. The columns (250 × 4.5 mm) were packed with 5-μm endcapped octyl- and endcapped octadecylsilica (IBM Instruments). The temperature was 25°C. Sample size: 11–17 μg in 20 μl.

2,6-DMHQ, 2,5-DMHQ, 2,3,5-TMHQ and 2,3,5,6-TMHQ were incubated in 25 m*M* sodium acetate buffer, pH 4.75, at room temperature for various lengths of time. Thereafter, aliquots of the reaction mixture were chromatographed with a mobile phase containing 20 percent (v/v) of acetonitrile in 50 m*M* aqueous $(NH_3OH)_3PO_4$ containing 25 m*M* Na_2EDTA. These reducing and complexing agents had been found to attenuate the on-column oxidation. Salicylic acid was used as the internal standard. For each reaction mixture, the fraction of hydroquinone remaining was evaluated and plotted *versus* time, as shown in Fig. 2. The apparent rate constant was evaluated by using the integrated rate equation for reversible first-order reaction, given as

$$[H] = ([H]_0 - [H]_{eq}) \{\exp [-(k_f + k_r)t]\} + [H]_{eq} \quad (1)$$

where [H], $[H]_0$ and $[H]_{eq}$ are the instantaneous, initial, and equilibrium concentrations of hydroquinone, and k_f and k_r are the rate constants for oxidation and reduction, respectively. The quality of the fit can be evaluated from the solid theoretical lines which have been calculated by use of the parameters $[H]_{eq}$ and $k_f + k_r$ that were obtained from non-linear least-squares analysis. The apparent equilibrium con-

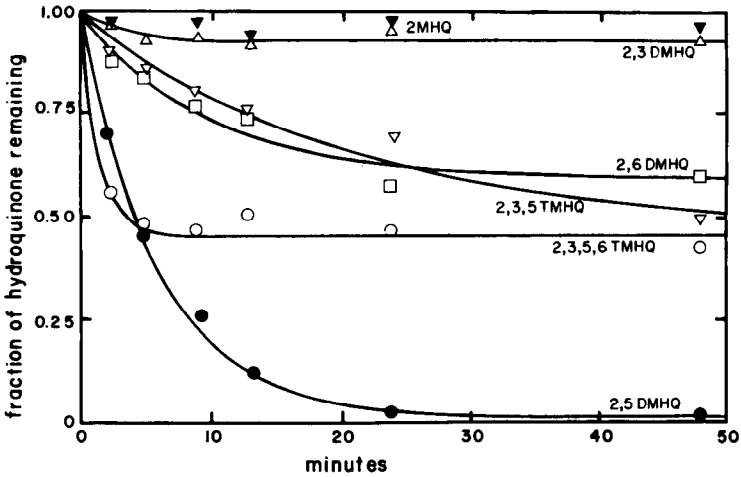

Fig. 2. Time course of hydroquinone oxidation in free solution. The reaction was carried out in 25 m*M* sodium acetate buffer (pH 4.75) at room temperature. The concentration of the various hydroquinones in the column effluent was evaluated by measuring their peak height relative to that of salicylic acid used as the internal standard.

stants, K_{eq}, have been estimated from $[H]_{eq}$ and $[H]_0$. With the values of K_{eq} and the parameter $k_f + k_r$ the rate constants for the oxidation reaction were calculated by using the relationship

$$k_f + k_r = k_f (1 + 1/K_{eq}) \quad (2)$$

which applies to reversible first-order reactions. The values of K_{eq} and k_f are given in Table I. The logarithmic equilibrium constants are roughly colinear with the oxidation–reduction potentials at pH 7.4, as reported in the literature[2]. The relationship should be exact, and failure to be so reflects experimental uncertainties in the values of $[H]_{eq}$, estimated by non-linear regression analysis. On the other hand, the logarithmic rate constants for the oxidation reaction are more nearly colinear with the oxidation potential.

TABLE I

EQUILIBRIUM CONSTANTS AND RATE CONSTANTS FOR THE OXIDATION OF *p*-HYDROQUINONES AT 25°C IN 25 m*M* SODIUM ACETATE BUFFER (pH 4.75) AND IN THE PRESENCE OF 10^{-2} *M* IRON(III) SULFATE AND 10^{-2} *M* COPPER(II) SULFATE

Compound	K_{eq}	k_f (h^{-1})
2-MHQ	0.072	0.0256
2,6-DMHQ	0.62	0.0554
2,5-DMHQ	146	0.164
2,3,5-TMHQ	1.22	0.025
2,3,5,6-TMHQ	1.14	0.411
2,6-DMHQ + Fe^{3+}	5.31	0.547
2,6-DMHQ + Cu^{2+}	–	0.321

The effect of reducible heavy metal ions on the reaction rate in free solution was also investigated. Solutions of 2,6-DMHQ, containing 10^{-2} *M* Fe^{3+} or 10^{-2} *M* Cu^{2+}, were incubated and aliquots were periodically chromatographed to determine the increase in the concentration of 2,6-DMBQ with time. The equilibrium and rate constants were evaluated by non-linear least-squares regression of the data to fit the equation for the rate of product formation by a reversible first-order reaction given by

$$[B] = [B]_{eq} \{1 - \exp [-(k_r + k_f)t]\} \quad (3)$$

where [B] and $[B]_{eq}$ are the benzoquinone concentrations at time t and equilibrium, respectively. The forward rate constants and equilibrium constants thus obtained are listed in Table I. It is seen that the reaction rate increased about 6- and 10-fold in the presence of Cu^{2+} and Fe^{3+}, respectively. Nevertheless, in solution, the rates of both the uncatalyzed and the catalyzed reactions are significantly lower than the rate of the oxidation on the chromatographic column, as seen in the next section.

On-column oxidation reaction

Qualitative evidence for on-column reaction is obtained by examination of the chromatogram obtained at different flow velocities. We shall use the previously developed theory[5,6] for the interplay of on-column reaction and chromatographic retention to provide a semi-quantitative interpretation of the results obtained with certain hydroquinones in reversed-phase chromatography.

The second moment and shape of the elution profile that comprises both the reactant and product is governed by the dimensionless chromatographic Damkoehler number, Da. If the reaction is first order, Da is given by:

$$Da = kL/u_0 \quad (4)$$

where k, u_0, and L are the first-order (or pseudo-first-order) reaction rate constant, the mobile phase velocity, and the column length, respectively. In the limit of no reaction Da equals zero and the hydroquinone injected traverses the column without undergoing on-column oxidation and is eluted as a single peak. With increasing Da the elution profile reflects the dynamic interplay between the oxidation reaction and chromatographic retention, both occurring simultaneously in the column. Depending on the number of intermediates, the retention factors of the species, and the pertinent equilibrium constants, the elution profile may show several maxima. At sufficiently high value of Da, all of the hydroquinone is oxidized to benzoquinone in the column, and the hydroquinone injected appears as a single benzoquinone peak in the effluent. According to the theory, the elution profile rapidly changes with Da in the domain of $10 > Da > 0.1$, and therefore is strongly affected by changes in column length or in flow velocity. The elution profiles of 2,6-DMHQ shown in Fig. 3 were obtained at three different flow velocities with octyl-Zorbax as stationary phase and water–acetonitrile (90:10, v/v) as the mobile phase. It is seen that with decreasing flow-rate, *i.e.* increasing Da, the region between the limiting "peaks", representing the pure hydroquinone injected and the benzoquinone formed in the elution profile, also increases. This part of the elution profile is characteristic for Da in the critical domain and is termed "reaction zone". At a flow-rate of 1 ml/min, the profile is nearly rec-

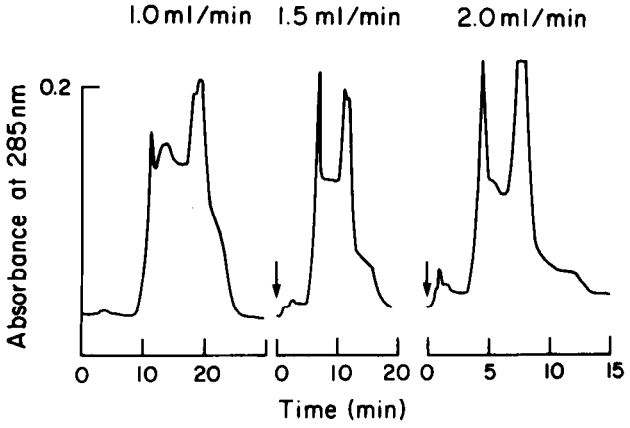

Fig. 3. Chromatograms illustrating the effect of the flow-rate on the elution profiles of 2,6-dimethoxyhydroquinone on octylsilica column. The data were obtained with acetonitrile–water (10:90) as the mobile phase and a column (250 × 4.6 mm), packed with 10-μm octyl-Zorbax. The temperature was 25°C. Sample size: 10–20 μg in 20 μl.

tangular in shape and with increasing flow velocity the maxima corresponding to 2,6-DMHQ and 2,6-DMBQ become more pronounced.

According to the theory[5], the shape of the elution profile is independent of sample size when the reaction is first-order in hydroquinone concentration but changes with the sample size when the order of reaction is different. In order to shed light on the apparent reaction order, elution profiles of 2,6-DMHQ were obtained with sample loads ranging from 0.2 to 20 μg. The results are shown in Fig. 4. The elution profiles are essentially the same for samples containing 0.2 and 2.0 μg of hydroquinone but change when the sample size is increased to 20 μg. In view of the

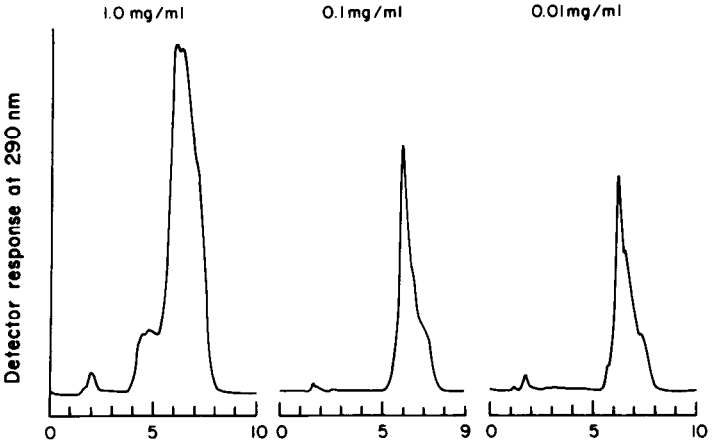

Fig. 4. The effect of sample size on the elution profile of 2,6-dimethoxyhydroquinone. The concentrations of hydroquinone in the sample solutions (mg/ml) are indicated. In each case, the sample volume was 20 μl. The data were obtained with acetonitrile–water (30:70) as mobile phase at 1.0 ml/min flow-rate and with a column (250 × 4.6 mm) packed with 10-μm phenethyl-Zorbax.

theoretical predictions, the result suggests that the reaction is nearly first-order at low sample loads, but the order of reaction is different at high sample load. It is known that alkyl-silica stationary phases bind iron and probably other heavy metals[7,8], and thus can act as heterogeneous catalysts for such oxidation reaction. Heterogeneous catalytic reactions frequently obey Langmuir–Hinshelwood kinetics[9], which in the limit of low and high reactant concentrations exhibit first- and zeroth-order rate behavior. It is likely that the system under investigation is subject to a similar rate law. Indeed, when the oxidizing agent and/or catalytic sites required for the reaction in the column are in relatively short supply, at sufficiently high concentration of hydroquinone, the rate is limited by the local availability of that agent or sites, and in the limit becomes zeroth-order in hydroquinone concentration. At low concentrations of hydroquinone, however, the oxidizing agent is in adequate supply and/or abundant catalytic sites are available and the rate is first-order in hydroquinone concentration.

In the case of first-order reaction behavior the integrated rate equation is given by

$$\log ([H]/[H]_0) = -\log (1 + ([B]/[H]) = -kt \tag{5}$$

$$\log ([B]/[H]) = \log [\exp (kt) - 1] \tag{6}$$

where k is the first-order rate constant, t is time, $[H]_0$ is the initial concentration of hydroquinone, and [H] and [B] are the respective concentrations of hydroquinone and benzoquinone at time t. Eqn. 5 shows that the conversion and the ratio [B]/[H] depends on time but not the initial concentration of hydroquinone.

On the other hand, with zeroth-order reaction the instantaneous concentration of hydroquinone is given by

$$[H] = [H]_0 - k_0 t \tag{7}$$

where k_0 is the zeroth-order rate constant. If initially no benzoquinone is present then

$$[B] = k_0 t \tag{8}$$

and from eqns. 7 and 8 it follows that

$$\log ([B]/[H]) = -\log \{([H]_0/k_0 t) - 1\} \tag{9}$$

Thus, with zeroth-order reaction the ratio [B]/[H] is dependent on the starting concentration of the hydroquinone according to eqn. 9. Eqns. 5–9 are valid only if no other routes for appearance or disappearance of hydroquinone and benzoquinone exist, a condition which does not apply to the transient reaction in the chromatographic system. In order to avoid the complex mathematical treatment[9] required for a complete description of the reaction events in such a "pulse reactor", only a crude semi-quantitative approach will be taken to assess the limiting kinetic behavior in view of eqns. 5 and 8.

For the pairs 2,6-DMHQ and 2,6-DMBQ as well as 2-MHQ and 2-MBQ, the elution profiles on the chromatograms obtained at different sample loads of the corresponding hydroquinone were evaluated to obtain the ratios of the peak heights (maxima) representing the unoxidized and oxidized forms. The logarithms of the ratios, assumed to represent [2,6-DMBQ]/[2,6-DMHQ] and [2-MBQ]/[2-MHQ], were plotted against the logarithm of the initial concentration of the appropriate hydroquinone $[2,6\text{-DMHQ}]_0$ and $[2\text{-MHQ}]_0$, respectively. The results shown in Fig. 5 demonstrate that at low sample load, *i.e.* low $[H]_0$ values, the ratio approaches a constant value, whereas at high sample load, *i.e.* when more than 2 μg of hydroquinone is injected, the ratio is a function of the sample load. As suggested by eqn. 9, the slope is negative and close to unity. Even such a simple representation of the data shows that the kinetics of the on-column oxidation of the hydroquinones are first- and zeroth-order in the limits of low and high concentrations of the hydroquinone.

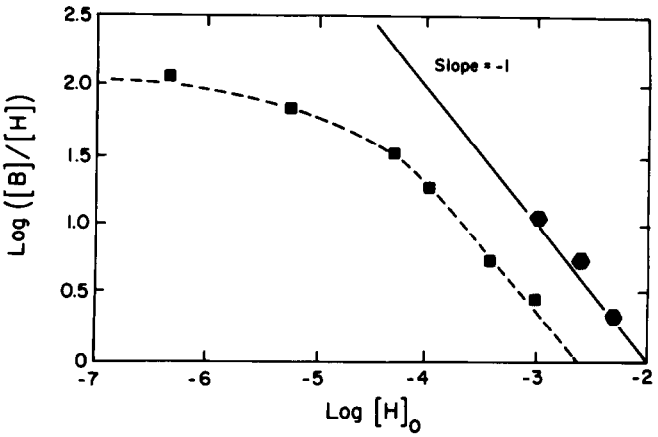

Fig. 5. Dependence of the concentration of benzoquinone relative to that of hydroquinone at the column outlet on the concentration of 2-methoxy- and 2,6-dimethoxyhydroquinones injected. In view of eqns. 5 and 8, the graph illustrates that at low- and high-input concentrations the oxidation of hydroquinone in the column follows first- and zeroth-order kinetics. The chromatographic conditions were identical to those given in Fig. 4, ■, 2-MHQ; ●, 2,6-DMHQ.

The theory of chromatography with first-order reaction in the column predicts that elution profiles depend not only on the Damkoehler number but also on the equilibrium constant for the reaction and the retention factors of the reactant and product[5,6]. The effect of the former in the present case of a redox equilibrium is such that a single peak of the hydroquinone is found if the value of Da is less than 0.1 and practically no conversion occurs, and two clearly distinguished peaks representing the hydroquinone and benzoquinone are found if Da lies between 1 and 10 (ref. 6). Of course, with irreversible reactions, only a single peak, that of the product, would be obtained at sufficiently high Damkoehler numbers. For intermediate values of Da the elution profile is dominated by the reaction zone. From inspection of the elution profiles obtained at different flow-rates, we estimate the rate constant for on-column oxidation of 2,6-DMHQ to be about 6 h^{-1}. A similar value was found for the same reaction with 2,5-DMHQ, another hydroquinone studied.

Comparison of the results obtained for the oxidation of hydroquinone in free solution and in the chromatographic column shows that the rate of on-column oxidation is greater than that in free solution, even at relatively high metal concentrations. This further corroborates the conclusion that the reaction takes place on the surface of the silica-bound hydrocarbonaceous stationary phase which binds adventitious metal ions from mobile phase and thus forms catalytic sites for the oxidation of hydroquinones[10,11]. In a recent study, about 150 μg of iron was removed from an alkyl-silica column of usual dimensions[7]. Bound iron is proposed to form the catalytic sites for three reasons: (i) trace iron in reagents and iron leached from fittings, pumps and lines of the chromatograph could be adsorbed on the stationary phase, (ii) as shown above, iron markedly increases the oxidation rate in free solution, and (iii) silica-supported iron is known to oxidize chemically similar species[11].

In order to investigate whether silica-bound iron is responsible for the on-column oxidation of hydroquinones, 2-MHQ and 2,6-DMHQ were chromato-

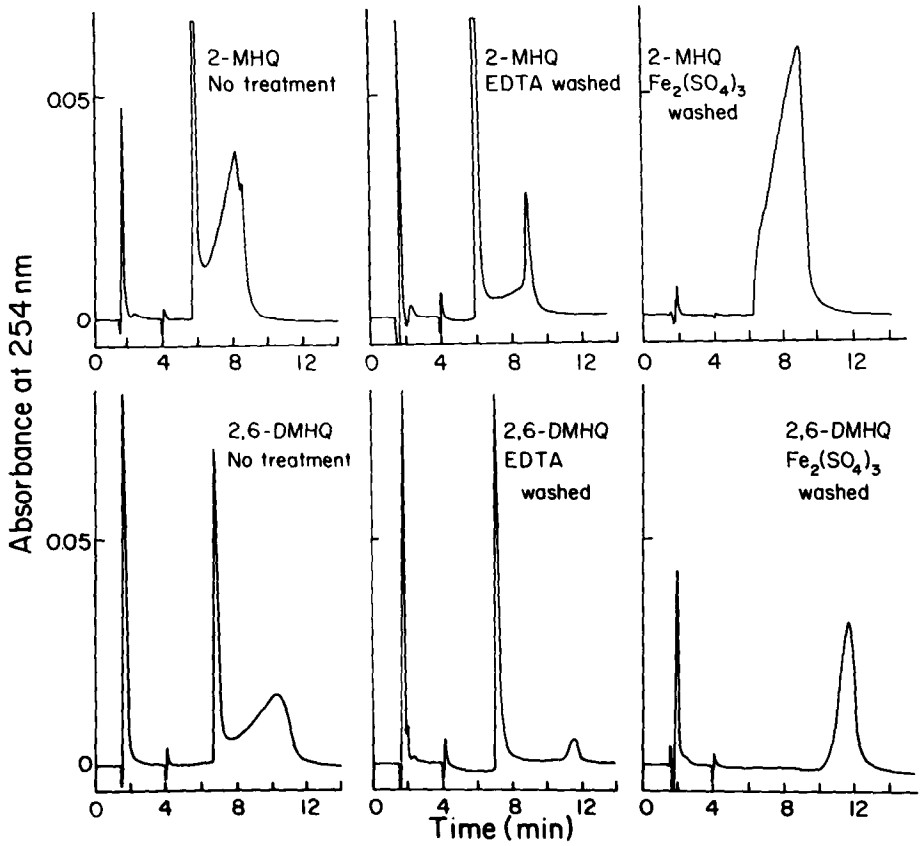

Fig. 6. Effect of pretreatment of the column by EDTA and Fe^{3+} on the elution profiles of 2-methoxy- and 2,6-dimethoxyhydroquinones. The panels on the left show chromatograms prior to any treatment of the column. The center panels are chromatograms obtained after the column had been washed with EDTA and the panels on the right show chromatograms following treatment of the column with ferric sulfate. Column, octyl-Zorbax (250 × 4.6 mm), mobile phase, acetonitrile–water (10:90), flow-rate, 1.0 ml/min. Sample size, 10–20 μg in 20 μl.

graphed on octyl-silica column with an aqueous mobile phase containing 10% (v/v) of acetonitrile under conditions usually employed in reversed-phase chromatography. In each case, the elution profile exhibited the characteristic reaction zone as shown in Fig. 6. Then the column was washed with 40 column volumes of 95 m*M* EDTA solution in order to remove trace metals. The chromatograms obtained subsequently with the same samples showed significantly reduced reaction zones, as can also be seen in Fig. 6. Thereafter, the column was perfused with 40 column volumes of 25 m*M* $Fe_2(SO_4)_3$ and washed with water for removal of excess iron. The chromatograms of hydroquinones obtained with the column after loading it with iron are also depicted in Fig. 6. They all exhibit broad reaction zones in excess of those observed originally, and the chromatograms show no peaks corresponding to remaining hydroquinone. Whereas these results do not indisputably demonstrate the participation of iron in the reaction, they do strongly suggest that iron or another reducible metal ion adsorbed to the stationary phase is involved in the oxidation of hydroquinones in the chromatographic columns.

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