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HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF ANILINE AND ITS METABOLITES

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SUMMARY

A high-pressure liquid chromatographic method has been developed for the determination of nanomole quantities of aniline; its metabolites *o*- and *p*-aminophenol, phenylhydroxylamine, nitrosobenzene and nitrobenzene; and azobenzene and azoxybenzene which form non-enzymatically by condensation of reactive metabolites. These compounds were separated by reverse-phase chromatography (μ-Bondapak C_{18} column) and detected spectrophotometrically. The first four components were eluted using methanol–water (15:85) containing 0.26 *M* ammonium acetate and 0.015 *M* nickel acetate as mobile phase. The remaining compounds were eluted with methanol–water (50:50). The stabilities of the metabolites were studied electrochemically and results were used in the development of the chromatographic system.

INTRODUCTION

Cancer induction by specific aromatic amines and amides is believed to involve their interaction in a metabolically modified form with nucleic acids and protein[1]. N-Hydroxylated metabolites of these amines have been cited as proximal carcinogens[1,2]. These amines are alternatively metabolized to isomeric aminophenols and nitroso derivatives[3–6]. The low levels at which these compounds are present in biological samples and their ease of oxidation have hindered development of a reliable method for their simultaneous analysis. Specific arylhydroxylamine analysis has been a major problem. Analytical methods currently available for detection and quantitation of primary arylhydroxylamines require multiple extractions, often at alkaline pH, and subsequent chemical conversion to a more spectrophotometrically sensitive derivative[7,8]. Under the conditions of reported assays, primary arylhydroxylamines are susceptible to oxidation, rearrangement or disproportionation. In addition, most assays utilize chemical reactions which convert the hydroxylamine metabolite to a derivative that is indistinguishable from alternate metabolic products. For example, under the acidic conditions of the Bratton–Marshall[9,10] procedure, primary arylhydroxylamines isomerize, at least in part, to the corresponding aminophenol, which is sub-

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sequently derivatized. Similarly, oxidative assay procedures[11] generate C-nitroso compounds, which are also metabolites of amine and nitro biotransformation. Final readout has involved spectrophotometry[8,12], fluorimetry[13], and isotopic methods[14]. Electrochemical methods, although sensitive[15-17], are unable to distinguish the hydroxylamine from the aminophenols, or the isomeric aminophenols from each other, since all of these compounds have similar oxidation potentials. Furthermore, reactions occurring at the electrode surface produce materials which condense with amino and hydroxylamino compounds, thus introducing additional errors in measurement. Hence, prior separation is required for analysis.

Separation of the isomeric aminophenols from aniline and each other has recently been reported using cation exchange high-pressure liquid chromatography (HPLC)[18]. Our experience has been, however, that cation exchange is unsuitable when the hydroxylamine is present.

A method has been developed to quantitate aniline, the corresponding isomeric aminophenols, phenylhydroxylamine, nitrosobenzene, nitrobenzene and azobenzene at micromolar levels. The compounds were separated by HPLC using reverse-phase (C_{18} column) partition chromatography with an aqueous mobile phase containing nickel ion. Components were detected spectrophotometrically in the eluent. The method, with only minor modifications, should be applicable to a number of arylamine–hydroxylamine systems.

EXPERIMENTAL

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000-A solvent delivery system, Model U-6K septumless injector and Model 440 dual-channel absorbance detector operated at 254 nm. The separation of aniline, *o*-aminophenol, *p*-aminophenol and phenylhydroxylamine utilized a 30 cm × 4 mm O.D. μBondapak C_{18} column (Waters Assoc.) operating at 2.0 ml/min with methanol–water (15:85) containing 0.26 *M* ammonium acetate and 0.015 *M* nickel acetate as mobile phase. Nitrosobenzene, nitrobenzene and azobenzene were separated with the same system using methanol–water (50:50) as mobile phase. Periodic purging (after *ca.* 50 injections) of the chromatographic system with 100% methanol is recommended for removal of condensation products formed from components.

Electrochemical measurements were made with a standard potentiostat[19] and electrode configuration[20] that have been previously described. All electrochemical measurements were made in glass cells at stationary carbon paste [graphite–silicone oil (3:1)] electrodes (geometric surface area 1.77 mm^2) relative to a saturated calomel electrode with a brass rod as auxiliary electrode.

Reagents

Reagent grade aniline was purified by vacuum distillation. J. T. Baker (Philipsburg, N.J., U.S.A.) practical grade *p*-aminophenol was purified by three successive recrystallizations from ethanol–petroleum ether mixture. Practical grade *o*-aminophenol (Eastman-Kodak, Rochester, N.Y., U.S.A.) was purified by sublimation. Reagent grade nitrobenzene was used without further purification. Phenylhydroxyl-

amine was synthesized by reduction of nitrobenzene with zinc and ammonium chloride[21]. Nitrosobenzene was synthesized by subsequent oxidation of the phenylhydroxylamine with dichromate[22]. Methanol was "Distilled-in-Glass" grade from Burdick & Jackson (Muskegon, Mich., U.S.A.).

Procedures

For quantitative chromatography, 1×10^{-3} *M* stock solutions of aniline, *p*-aminophenol, nitrobenzene, nitrosobenzene and azobenzene were prepared in methanol and diluted as required with mobile phase to give standard solutions in the range from 1×10^{-3} to 1×10^{-6} *M*. Phenylhydroxylamine and *o*-aminophenol were quantitatively analyzed by preparing fresh 1×10^{-3} *M* stock solutions in deoxygenated (argon) methanol at 0°, followed by immediate dilution and chromatographic analysis. All quantitative measurements were made relative to a constant amount (5 nmoles) of internal standard, 4-fluorophenol. Standard curves were prepared for each compound by plotting concentration *vs.* peak height for 6–7 concentrations (1×10^{-6} *M*, 5×10^{-6} *M*, 1×10^{-5} *M*, 5×10^{-4} *M*, 1×10^{-4} *M*, 5×10^{-3} *M* and 1×10^{-3} *M*) of material. All measurements were made in triplicate and subjected to linear regression analysis.

Electrochemical (chronoamperometric) measurements were made on 5×10^{-4} *M* solutions of *o*-aminophenol and phenylhydroxylamine as previously described[15]. Currents were measured after 9 sec controlled electrolysis at +0.4 V *vs.* a saturated calomel electrode, and plotted *vs.* incubation time.

RESULTS

Mixtures of aniline, *o*- and *p*-aminophenol and phenylhydroxylamine were separated by HPLC using reverse-phase partition chromatography with isocratic elution of components using methanol–water (15:85) containing 0.26 *M* ammonium acetate (to maintain pH at 7) and 0.015 *M* nickel acetate as mobile phase. All components were separated with resolution greater than 1.1 and total analysis time was approximately 15 min (Fig. 1a). Components were quantitated by measuring peak heights relative to an internal standard (4-fluorophenol; V_R = 26.0 ml). A linear relationship was found between peak height ratio and concentration for all components in the concentration range of 5×10^{-6} to 1×10^{-3} *M* (Table I). Analyses gave reproducibility of ±5% or better. All analytes except *p*-aminophenol could be readily quantitated at the sub-nanomole level (minimum amount injected *ca.* 250 picomoles). Due to interference from the solvent front, *p*-aminophenol could not be quantitatively determined at concentrations less than 1×10^{-5} *M*. Qualitative detection of *p*-aminophenol was possible, however, to *ca.* 1×10^{-6} *M*.

Difficulty was encountered in achieving adequate chromatographic resolution of the mixture with concomitant maximization of stability of the components. Cation-exchange HPLC proved unsuitable when the hydroxylamine was present, although the aminophenols and aniline could be separated[18]. The hydroxylamine rapidly decomposed when in contact with the exchange resin, making quantitation impossible. Reverse-phase HPLC, however, proved suitable for the analysis in that none of the components appears to decompose on contact with the ODS stationary phase over the analysis period. However, resolution of the *o*-aminophenol and the hydroxylamine was

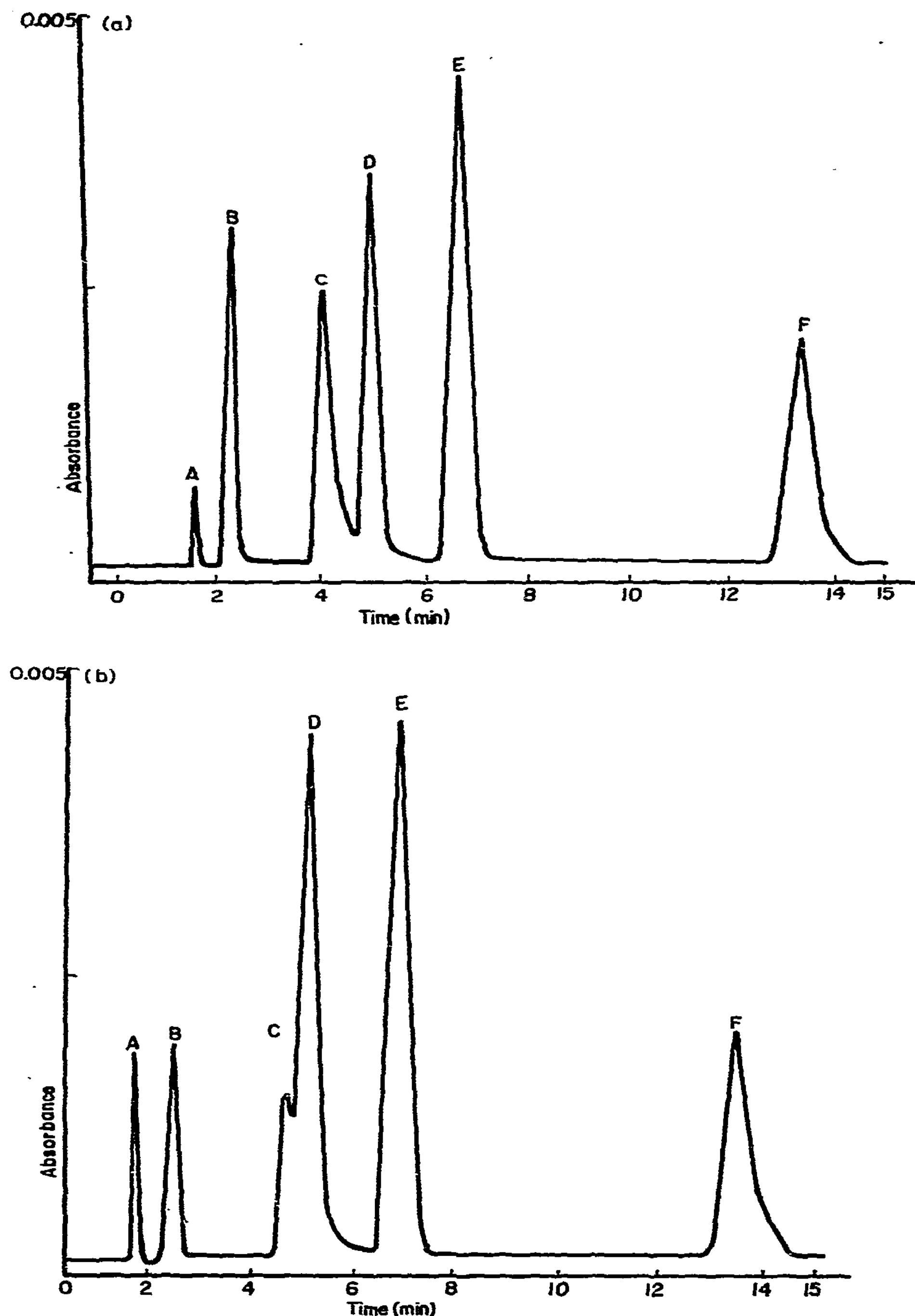

Fig. 1. Chromatogram of a mixture of *p*-aminophenol (B), *o*-aminophenol (C), phenylhydroxylamine (D), aniiine (E), and 4-fluorophenol (F; internal standard) separated by reverse-phase chromatography using methanol–water (15:85) containing 0.26 *M* ammonium acetate as mobile phase, (a) containing 0.015 *M* nickel acetate and (b) in which nickel ion is absent. A is solvent peak.

TABLE I

STANDARD CURVES FOR HPLC ANALYSIS OF AMINE METABOLITES

Each curve represents triplicate analyses of each compound at 6 or 7 concentrations in the range 5×10^{-6} to 1×10^{-3} *M*. The analysis was performed as described under Experimental. Flow-rate 2.0 ml/min. The amount of each compound was determined as ratio of peak height of compound relative to internal standard (4-fluorophenol). Azoxybenzene was not quantitated due to lengthy analysis times and baseline instabilities.

Compound	V_R *(ml)*	*Sensitivity limit (M)*	*Linear regression parameters*		
			Slope	*Intercept*	*Correl. coeff.*
p-Aminophenol	4.2*	1×10^{-5}	21382	−0.265	0.995
o-Aminophenol	7.8*	5×10^{-6}	882	0.002	0.994
Phenylhydroxylamine	9.6*	5×10^{-6}	8680	−0.172	0.997
Aniline	13.0*	5×10^{-6}	4806	0.012	0.999
Nitrobenzene	5.8**	5×10^{-7}	11462	0.003	0.999
Nitrosobenzene	6.4**	5×10^{-6}	1674	−0.003	0.999
Azobenzene	11.1**	5×10^{-6}	1475	0.007	0.999
Azoxybenzene	104.0**				
Azoxybenzene	38.0***	1×10^{-7}			

* Mobile phase: methanol–water (15:85) containing 0.26 *M* ammonium acetate and 0.015 *M* nickel acetate (V_R of 4-fluorophenol = 26.0 ml).

** Mobile phase: methanol–water (50:50) (V_R of 4-fluorophenol = 6.5 ml).

*** Mobile phase: methanol–water (50:50) stepped to methanol–water (70:30) 11 min after initiation of the chromatogram.

inadequate even with a 100% aqueous mobile phase, which gave maximum separation of all other components. This system also resulted in unacceptably long analysis times (> 30 min).

o-Aminophenol forms stable chelates with transition metal ions, particularly Ni(II) ion; *e.g.*, in dioxan–water (50:50), log $K_1 = 6.1$ (ref. 23). Nickel ion cannot effectively chelate with N-hydroxylamines. Advantage was taken of this difference in reactivity to affect separation of *o*-aminophenol from phenylhydroxylamine, by incorporating Ni(II) ion in the mobile phase. Fig. 1a shows the separation achieved using mobile phase containing 0.015 *M* nickel acetate. The resolution of the *o*-aminophenol and the phenylhydroxylamine was sufficient (R_s *ca.* 1.1) to permit accurate quantitation of both compounds. Chelation of *o*-aminophenol increases its hydrophilicity resulting in a decrease in its capacity factor and retention volume. In the same mobile phase containing no Ni(II) ion, the retention volume of the *o*-aminophenol was increased such that the resolution of these two components was poor ($R_s < 0.5$) (Fig. 1b). Retention volumes of *p*-aminophenol, aniline and phenylhydroxylamine were not affected by addition of Ni(II) ion to the mobile phase.

The chromatographic elution of nitrosobenzene and nitrobenzene, which are the "metabolites" of higher oxidation state, was accomplished using methanol–water (50:50) as solvent system (Fig. 2). This system could also be used to elute azobenzene which forms non-enzymatically by condensation of aniline or phenylhydroxylamine with nitrosobenzene. Azoxybenzene forms by condensation of nitrosobenzene and phenylhydroxylamine; however, its retention volume (104 ml) using this mobile phase was too large to make the system of practical use. However, if a step gradient to methanol–water (70:30) is introduced after elution of azobenzene, azoxybenzene is sub-

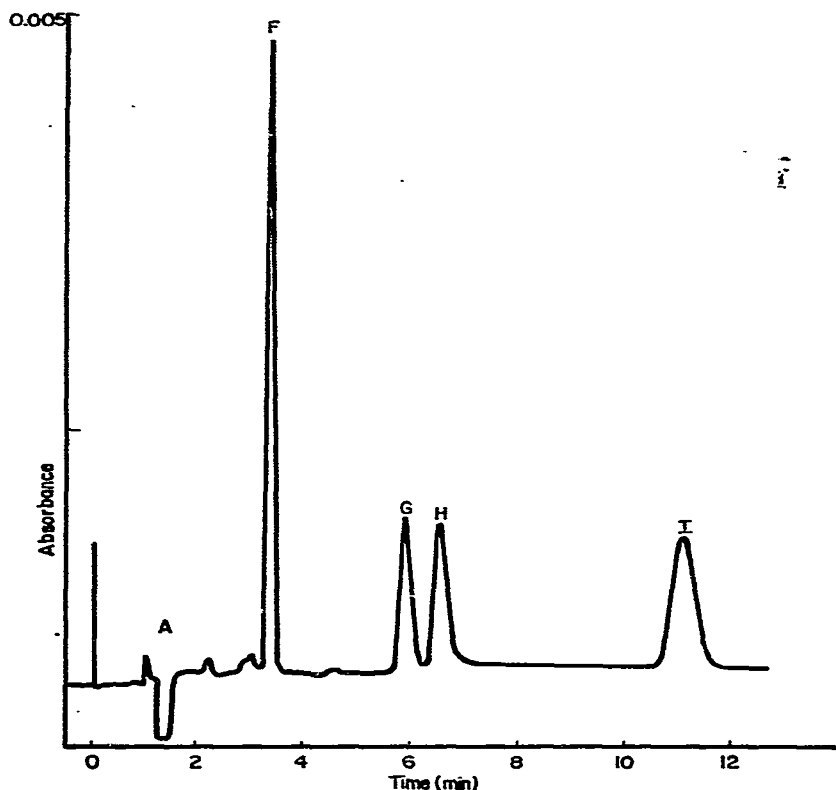

Fig. 2. Chromatogram of a mixture of 4-fluorophenol (F), nitrobenzene (G), nitrosobenzene (H), and azobenzene (I) separated by reverse-phase chromatography using methanol–water (50:50) mobile phase. A is solvent peak.

sequently eluted with a retention volume of an additional 16 ml. Standard curves were also constructed for this system, using the same approach as previously stated, with 4-fluorophenol as the internal standard. A linear relationship was again found to exist between analyte concentration and peak height relative to internal standard (Table I). The radical difference in the polarity of the components in these mixtures precluded an isocratic elution of all components.

o-Aminophenol and phenylhydroxylamine are very susceptible to air oxidation in solution, and to photooxidation; the hydroxylamine also undergoes rearrangement at low pH. To evaluate the stability of these two compounds, chronoamperometric analyses[15,17] were carried out on 5×10^{-4} *M* solutions of each compound individually in aqueous 0.26 *M* ammonium acetate, aqueous 0.26 *M* ammonium acetate deoxygenated with argon, deoxygenated mobile phase without nickel, and deoxygenated mobile phase with nickel. Fig. 3 shows the decomposition of phenylhydroxylamine in non-deoxygenated aqueous 0.26 *M* ammonium acetate solution. The decomposition exhibits apparent triphasic kinetic behavior, with the rate of disappearance of phenylhydroxylamine increasing with time. Solutions of the hydroxylamine prepared in the same solvent which was deoxygenated with argon and protected from

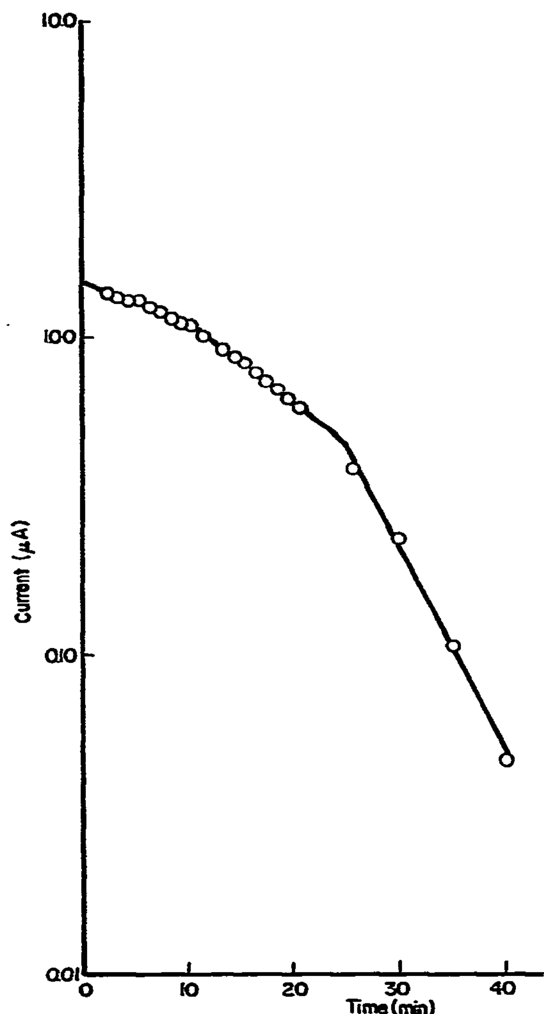

Fig. 3. Decomposition of phenylhydroxylamine (4.98×10^{-4} *M*) in aqueous 0.26 *M* ammonium acetate solution as a function of time, followed chronoamperometrically. Current levels were measured after controlled electrolysis for 9 sec at +0.4 V (*vs.* SCE) and are plotted *vs.* incubation time.

contact with the atmosphere did not exhibit significant decomposition, even after two hours. Addition of methanol and Ni(II) ion to the solution did not cause any decomposition of the phenylhydroxylamine. Therefore, mobile phase for chromatographic analysis of the hydroxylamine was routinely deoxygenated with argon and subsequently protected from the atmosphere.

o-Aminophenol showed different behavior. As seen in Fig. 4, the decomposition initially occurs rapidly, then abruptly slows. This kind of behavior was observed in all cases. This may indicate the presence of an easily decomposed electroactive impurity in the aminophenol solution which effectively disappears after 10–20 min. The stability of *o*-aminophenol was enhanced by the addition of Ni(II) ion to the solution;

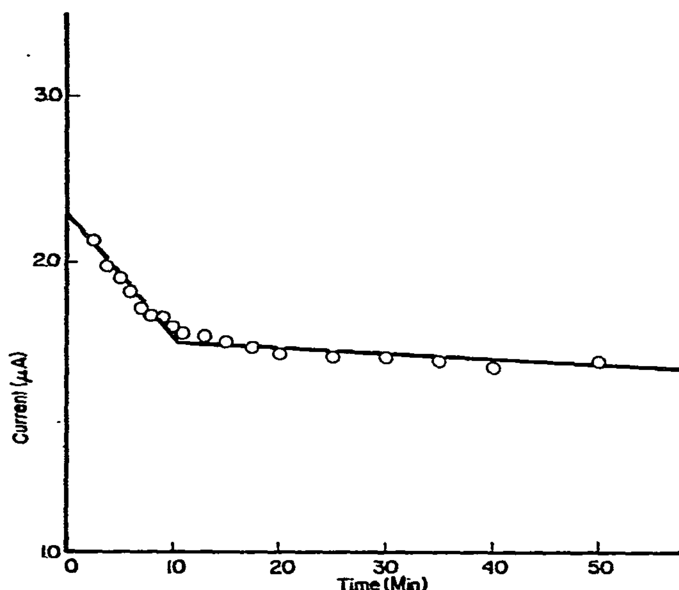

Fig. 4. Decomposition of *o*-aminophenol (5.18×10^{-4} *M*) in methanol–water (15:85) containing 0.26 *M* ammonium acetate solution as a function of time, as studied chronoamperometrically. Current levels were measured after controlled electrolysis for 9 sec at +0.4 V (*vs.* SCE) and are plotted (on logarithmic scale) *vs.* incubation time.

its half-life without nickel is *ca.* 19 min without prior deoxygenation, *ca.* 27 min with prior deoxygenation, and *ca.* 135 min with both deoxygenation and the addition of nickel.

The stability of both of the aminophenols and of aniline are enhanced by addition of a small amount of bisulfite (0.008 *M*) to the mobile phase, where it acts as an anti-oxidant. However, bisulfite rapidly decomposes the hydroxylamine ($t_{\frac{1}{2}} \leq 2$ min). HPLC analysis indicates that bisulfite is a sufficiently strong reducing agent to convert the hydroxylamine to aniline. Therefore, bisulfite-containing mobile phase cannot be used when detection of arylhydroxylamine is desired, but may prove useful when this type of compound is absent. The stabilities of *o*-aminophenol and phenylhydroxylamine are sufficient (even in the absence of antioxidant) to permit their analysis, if care is taken to protect samples from air and light, and if mobile phase is deoxygenated and kept under an argon atmosphere.

DISCUSSION

A sensitive analytical method has been described for the simultaneous quantitative determination of aniline and its metabolites including the reactive N-hydroxylamine. The inherent instabilities of the compounds do not present serious analysis problems if the precautions previously described are taken.

Previously reported methods all suffer from serious disadvantages. For example, phenylhydroxylamine has been determined spectrophotometrically after conversion to nitrosobenzene[7]. However, nitrosobenzene is also a metabolite of aniline

and may form *via* several alternative enzymatic and non-enzymatic routes. Therefore, this method cannot accurately indicate hydroxylamine levels.

Electrochemical methods have proven quite valuable in analyzing aminophenols and arylhydroxylamines[15–17]. However, all the hydroxylated compounds have similar oxidation potentials and, therefore, can only be determined as the total amount of oxidizable species present. Individual quantitation thus requires prior separation of the isomeric species. Furthermore, the electrochemistry of these compounds is very complex. The electrolysis products couple with unreacted amine, aminophenol or hydroxylamine, generating a wide variety of products which can themselves be either electrochemically active or inactive[24]. This complication gives rise to inaccurate quantitative analysis, and also results in coating of electrode surfaces, requiring frequent regeneration of the electrodes. Separation of the compounds from each other prior to analysis would minimize this problem. Coupling appears to involve a second order reaction of hydroxylamine or amine with the hydroxylamine oxidation product nitrosobenzene (formed either electrochemically[25] or by contact with air[26]), forming azoxybenzene[27,28] or azobenzene[28,29], respectively. These products may react further with the hydroxylamine or amine to form polymeric azoxy or azo compounds.

This behavior would also be expected of non-electrolyzed aqueous solutions of arylhydroxylamines in contact with air and may account for the triphasic decomposition kinetics observed with phenylhydroxylamine. The first phase represents loss due to air oxidation yielding nitrosobenzene. The second, more rapid phase may involve both the oxidation pathway as well as disappearance of phenylhydroxylamine by its reaction with nitrosobenzene (as nitroso concentration builds up) to form azoxybenzene. The "apparent" first order rate constant for the third phase is greater than the two earlier phases. This further acceleration in hydroxylamine disappearance may occur as a third alternative pathway for breakdown of the parent compound becomes available, *i.e.*, reaction with azoxybenzene to form polymeric derivatives. The details of the kinetics will be published separately.

The instability of *o*-aminophenol can be circumvented by addition of Ni(II) ion to the mobile phase, which greatly increases its stability, as well as improving chromatographic resolution of the components in the analytical mixture. Selective complexation with metal ions should also be applicable to other systems (*e.g.*, naphthalene and fluorene analogs), although different pH and/or metal ions may prove more suitable. Other metal ions that may prove useful include Co(II), Zn(II), Mn(II), and Fe(II). Metal ion candidates must not only complex strongly with *o*-aminophenols but must also not be oxidizing agents.

The stability of the system can be improved by the addition of an antioxidant to the mobile phase. However, there are stringent requirements that must be met by any such compound. The compound must not react or precipitate (sulfhydryl compounds) with nickel ion, nor can it absorb strongly in the UV spectral region (*e.g.*, ascorbate). Bisulfite ion meets these requirements (its UV absorbance is not too strong for use in the mobile phase), and it was used to stabilize systems not containing phenylhydroxylamine. However, HSO_3^- ion rapidly reduces the hydroxylamine to aniline, and, therefore, is unsuitable for systems containing this compound. A good antioxidant must also not react in any way with the analytes in the samples. Although a rigorous search was not conducted, all antioxidants tested in this investigation were

unsuitable because of high UV absorbance, reaction with Ni(II) ion or reduction of phenylhydroxylamine.

In conclusion, an HPLC method is described for the quantitative analysis of aniline, *o*- and *p*-aminophenol, phenylhydroxylamine, nitrosobenzene, nitrobenzene and azobenzene, involving the novel use of selective chelation and component stabilization through mobile phase modification.

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