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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF LOW LEVELS OF PRIMARY AND SECONDARY AMINES IN AQUEOUS MEDIA VIA DERIVATISATION WITH 1,2-NAPHTHOQUINONE-4-SULPHONATE

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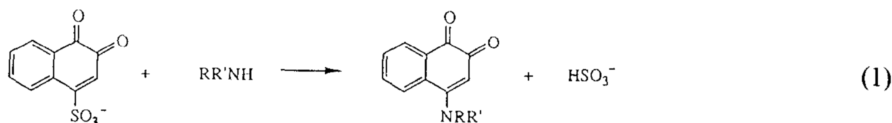
SUMMARY

1,2-Naphthoquinone-4-sulphonate has been used as a derivatising agent for a variety of primary and secondary aliphatic amines in dilute aqueous solutions prior to separation by reversed-phase high-performance liquid chromatography. Although the method is generally reliable, it does not work with some amines and this was explained in terms of steric hindrance during derivatisation and failure to transfer derivatives of hydrophilic amines to the organic phase during the extraction/concentration stage.

INTRODUCTION

Displacement of sulphonate in an activated aromatic reagent can be the basis of convenient derivatisation procedures for determining low levels of amine. Trinitrobenzene sulphonate (TNBS)[1] and related nitrophenyls[2] have been used for estimating and modifying primary amines in biological materials[3], and TNBS itself has also been employed as a spray reagent for locating amino compounds on paper[4]. When considering precolumn derivatisation methods for high-performance liquid chromatographic (HPLC) analysis of a large number of very dilute aqueous amine solutions, we rejected TNBS because hindered secondary amines react very slowly[5], and we found no report of its use in conjunction with HPLC. In addition the sodium salt induces contact hypersensitivity[6], the amine derivatives are known to be photosensitive[5], and the possibility of forming explosive residues[7] encouraged us to seek a more desirable reagent. 1,2-Naphthoquinone-4-sulphonate (NQS) appeared ideal, being readily obtained from β-naphthol[8–10], commercially available, and having a long history associated with amine analysis. It is susceptible[11] to nucleophilic attack by amine as shown in eqn. 1, and this causes a colour change from pale orange to dark red.



As little as 1 ppm of aniline can be detected[12], and Folin[13] first described its use for determining amino acids. Since then a range of amine analysis applications have been described, including simple colorimetric[14–16], spectrophotometric[17] and fluorimetric[18] methods and a wide variety of chromatography-related applications such as a visualising agent for paper chromatography[19], thin-layer chromatography (TLC)[20] and column chromatography[21]. Rcently NQS has been employed as a derivatising agent prior to normal-phase TLC[22] and HPLC[23] separation of stimulant amine drugs[22,23]. It therefore appeared that the fundamental methodology for the NQS determination of amines was well understood. However, we found instances where an NQS-based HPLC method did not work well; the reasons for this were examined and the results are reported here.

EXPERIMENTAL

Reagents

HPLC-grade acetonitrile was obtained from Rathburn (Walkerburn, U.K.) and HPLC-grade chloroform and isoamyl alcohol from Aldrich (Dorset, U.K.). NQS as the sodium salt was supplied by Aldrich and sodium hydrogencarbonate by May and Baker (Poole, U.K.). Amines were from Aldrich and Fluka.

Apparatus

The HPLC equipment was a Waters 840 system with two 510 pumps, a WISP 710B autosampler, a 490 programmable UV–visible detector and a temperature-controlled column oven. The HPLC column was a Partisil ODS, 5 μm particle size (250 mm × 4.6 mm I.D.). UV–visible spectra were measured on a Shimadzu UV-260 spectrometer. Mass spectra were determined on Kratos MS3074 and Finnigan 4021 mass spectrometers. ^{1}H NMR spectra were measured on Bruker MSL (300 MHz) and JEOL Model FX 90Q (90 MHz) spectrometers.

Derivatisation procedure for HPLC analysis

The aqueous sample (20 cm^3) containing amines was treated with 8% sodium hydrogencarbonate solution (1 cm^3) and 0.5% aqueous NQS sodium salt (1 cm^3). The mixture was heated, in the dark, at 70°C for 30 min sealed in a screw-top vial. After cooling, the solution was extracted with chloroform (2 cm^3), and this extract (0.5 cm^3) was diluted with acetonitrile (2 cm^3). These solutions were stored in the dark. Quantification was performed by external standard calibration using 1, 2, 5, 10 and 20 ppm of standard amine solutions.

Chromatography

Direct injection of chloroform solutions gave poor peak shapes probably due to phase separation on contact with the eluent. Rather than evaporate and redissolve samples in eluent it was found more convenient to dilute the chloroform solutions

with acetonitrile as noted above. Derivatised samples in chloroform–acetonitrile were filtered and injected (10 μl) onto the column via the WISP autosampler. The amine derivatives were eluted with acetonitrile-water (70:30) at a flow-rate of 1.3 cm^3 min^{-1}. The eluting peaks were monitored at 280 and 460 nm simultaneously.

Measurement of amine NQS derivative partition coefficients

Ethanolamine and 2-amino-2-methylpropanol derivatives partition coefficients for isoamyl alcohol and water, and for water and chloroform. Aqueous ethanolamine (100 ppm, 25 cm^3), 8% sodium hydrogencarbonate (2.5 cm^3) and 0.5% NQS solution (2.5 cm^3) were heated in a stoppered round-bottomed flask at 70°C for 30 min. The resulting solution was extracted once with isoamyl alcohol (10 cm^3), and 5 cm^3 of this were further diluted by a factor of 5.

A portion (5 cm^3) of the dilute solution was shaken with water (5 cm^3), and the emulsion separated by centrifugation. The UV–visible spectra (700–190 nm) of the final organic and aqueous layers were measured. The aqueous layer (4 cm^3) was shaken with chloroform (4 cm^3). The emulsion was separated, and the UV-visible spectra of both layers were recorded. Absorbance values at the λ_{max} close to 460 nm were used in the calculation of the apparent partition coefficient. The whole process was repeated for the 2-amino-2-methylpropanol NQS derivative, but initial dilution of the organic layer was not necessary.

Morpholine- and piperidine-NQS derivatives partition coefficients for water and chloroform. Aqueous solutions of the amines (100 ppm) were derivatised as described above for ethanolamine. Chloroform was used in place of isoamyl alcohol and the extract diluted by a factor of 20 with chloroform. The UV–visible spectrum of this solution (700–190 nm) was measured. The chloroform layer (5 cm^3) was shaken with water (5 cm^3), and the UV-visible spectra of the two resulting phases were recorded.

Sensitivity ratios

Morpholine and tert.-butylamine using chloroform. The aqueous amine solution (100 ppm, 10 cm^3), 8% sodium hydrogencarbonate (1 cm^3) and 0.5% NQS solution (1 cm^3) were reacted at 70°C in sealed screw-top vials for 30 min. The solutions were then extracted into chloroform (4 cm^3). In order to obtain a sufficiently dilute solution for absorbance measurements the morpholine derivative extract was diluted by a factor of 20 with chloroform. The UV-visible spectra of the two solutions were measured over the range 700–190 nm. The absorbance values at 460 nm (the HPLC monitoring wavelength) were used to calculate the relative sensitivity of the method for the two amines.

Ethanolamine and 2-amino-2-methylpropanol using isoamyl alcohol. The amines were derivatised and spectra measured as above; isoamyl alcohol (4 cm^3) was used instead of chloroform.

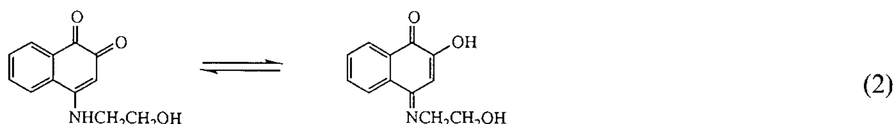
Isolation and characterisation of NQS derivatives

Piperidine derivative. Piperidine (100 ppm, 100 cm^3), 0.5% NQS solution (20 cm^3) and 8% sodium hydrogencarbonate (20 cm^3) were heated at 70°C for 30 min in a stoppered round-bottomed flask. The resulting solution was extracted twice into chloroform (20 cm^3). The extracts were combined, washed with 2% sodium hydrogencarbonate (2 × 50 cm^3) and water (2 × 50 cm^3). The extract was dried (sodium

carbonate), and the solvent removed under vacuum. The structure of the resulting red solid compound was confirmed by ^1H NMR (60 MHz) and mass spectrometry.

Ethanolamine derivative. Ethanolamine in water (0.25 g in 100 cm^3), 0.5% NQS (50 cm^3) and 8% sodium hydrogencarbonate (50 cm^3) were heated as above, and the resulting brown solution was evaporated to dryness under vacuum. The residue (unreacted NQS, base and the derivatised amine) was extracted with 100 cm^3 of hot isoamyl alcohol to give a red–brown solution which was filtered and concentrated to dryness on a rotary evaporator. The deep red solid showed three fractions by reversed-phase TLC in a mixture of methanol–water (70:30). This solid was purified by chromatography on water-deactivated alumina. The sample was introduced as an aqueous solution and eluted with methanol to remove the very mobile pale yellow fraction. Methanol–water (50:50) eluted two bands with similar R_F values, one orange and the other red.

The major part of the orange band, which eluted first, was collected and the remainder, with the red material, was re-chromatographed. However, a pure sample of the red material was not obtained. The very similar R_F values of these bands, together with there being relatively less of the red material (and a corresponding increase of orange material) on the second separation suggests the materials may be isomers that are interconverting on the column as shown in eqn. 2. After removal of solvent on a rotary evaporator, the orange solid was dried (vacuum), and its high-resolution ^1H NMR (300 MHz) and accurate mass spectra were recorded.



RESULTS AND DISCUSSION

An HPLC method for the determination[23] of amphetamines via derivatisation with NQS was modified for the estimation of low levels of aliphatic amines in water. To improve reliability and reproducibility, reversed-phase HPLC was employed, and this became a routine method for the analysis of mixtures of a range of primary and secondary amines. Simple amines that are relatively uncrowded around the nitrogen atom reacted smoothly to give easily characterised NQS derivatives. Thus piperidine, morpholine and isopropylamine were readily determined with comparable molar sensitivities. The UV-visible spectra of their NSQ derivatives were similar, and the ^1H NMR and mass spectra (electron impact and chemical isonisation) of the piperidine derivative confirmed the displacement of the sulphonate group by the amine according to eqn. 1. A chromatogram of a mixture containing the NQS derivatives of two typical amines is shown in Fig. 1, and displayed in Fig. 2 is a typical HPLC calibration curve. The achieved sensivity is such that less than 1 ppm of amine can be detected, permitting routine analysis of concentrations in the range 1–50 ppm. This has proven to be a very reliable technique for determining many amines. We were therefore very surprised when the method failed to detect 2-amino-2-methylpropanol (I Fig. 3), although the closely related norephedrine (II, Fig. 3) has been determined

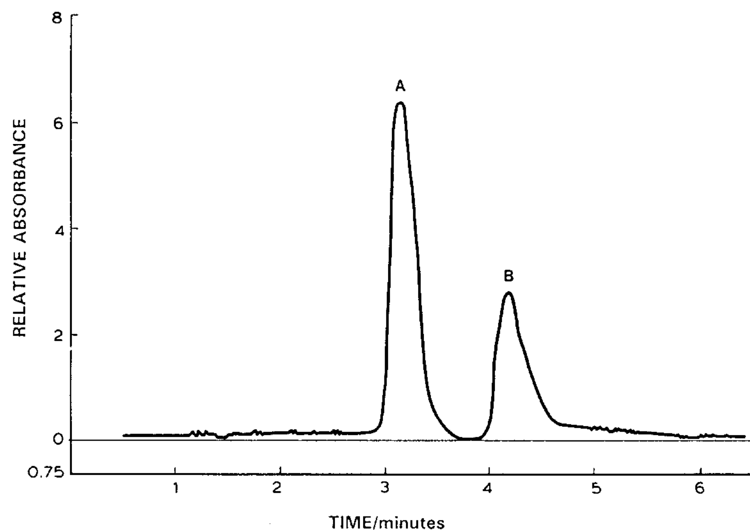


Fig. 1. Typical HPLC profile showing the separation of NQS derivatives of (A) morpholine (2.0 ppm) and (B) hexamethyleneimine (2.0 ppm). Chromatographic conditions: mobile phase, acetonitrile–water (70:30); flow-rate, 1.3 cm^3 min^{-1}; injection volume, 10 μl; monitoring wavelength, 460 nm.

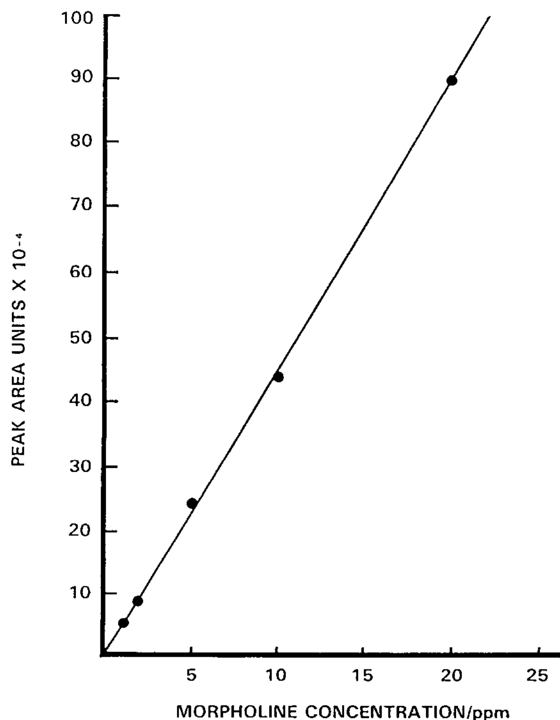


Fig. 2. Typical HPLC calibration graph for determination of morpholine via NQS derivatisation over the concentration range 0–20 ppm with a monitoring wavelength of 460 nm.

using similar methodology[23]. In order to ascertain the reasons for this we examined the reactivity of some simple model amines. The most important of these compounds were ethanolamine (III) and *tert.*-butylamine (IV) (Fig. 3), selected to probe the roles of the major structural moieties present in 2-amino-2-methylpropanol. Ethanolamine was chosen because it has amino and hydroxyl groups on adjacent carbon atoms and is the least sterically hindered *β*-amino alcohol available. *tert.*-Butylamine and 2-amino-2-methylpropanol are homomorphous making the former a suitable model for the steric environment of the nucleophilic nitrogen centre in 2-amino-2-methylpropanol, in the absence of any electronic effect.

Using UV–visible spectroscopy to monitor the presence of the reaction product, after extraction into chloroform, it was found that with ethanolamine no derivative was present in the organic phase, whereas under the same conditions morpholine, piperidine and other simple amines gave intensely coloured solutions. However, it has been reported previously[17] that ethanolamine can be estimated spectrophotometrically after extraction of the NQS derivative into isoamyl alcohol. We also ob-

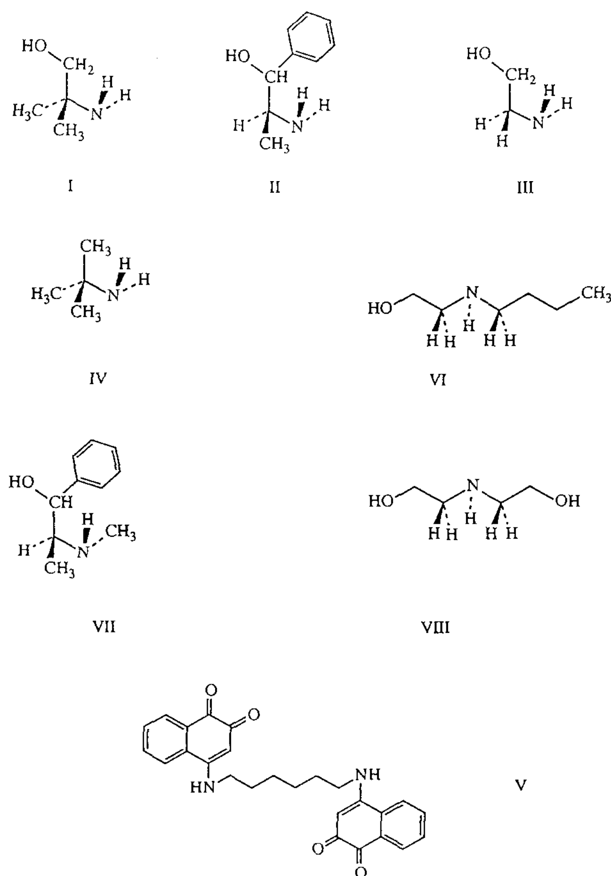


Fig. 3. Structures of 2-amino-2-methylpropanol (I), norephedrine (II), ethanolamine (III), *tert.*-butylamine (IV), the bis-NQS derivative of 1,6-hexanediamine (V), N-butylethanolamine (VI), ephedrine (VII) and diethanolamine (VIII).

tained a coloured solution when extracting into this solvent, and accurate mass spectrometry (MW 217.0739 predicted, 217.0759 ± 0.002 measured) and the ^{1}H NMR spectrum of the product were consistent with it being the ethanolamine-NQS derivative. Thus the method failed with ethanolamine because the NQS derivative was not extracted into chloroform. The partition of some representative amine-NQS derivatives between organic and aqueous phases was therefore investigated by measuring the absorbance of equilibrated aqueous and organic phases at the appropriate wavelength. The results are summarised in Table I. With chloroform the degree of partitioning decreases dramatically as the hydrophilicity of the amine substituent increases. This is so marked with the hydroxylic ethanolamine derivative that no partition is observed. With the more hydrophilic solvent isoamyl alcohol, it is possible to extract this derivative, although the efficiency is so poor that the overall sensitivity of the method is much reduced compared with that for other amines in the procedure using chloroform extraction. Similar behaviour is observed for 2-amino-2-methylpropanol, which has slightly less hydrophilic character than ethanolamine and hence gives higher partition coefficients.

Steric effects can be very important in nucleophilic displacement reactions, and to examine their importance in the derivatisation by NQS the relative efficiencies of the method were investigated using two pairs of amines (morpholine and *tert.*-butylamine, and ethanolamine and 2-amino-2-methylpropanol). With chloroform as the extractant, morpholine was determined with a sensitivity some eighteen times greater than that for *tert.*-butylamine; this is a minimum estimate of the difference in reactivities since the morpholine-NQS derivative should have a lower partition coefficient than the analogous *tert.*-butylamine derivative. With isoamyl alcohol as extractant ethanolamine was determined with a sensitivity some nine times greater than that for 2-amino-2-methylpropanol. These results show that steric effects are significant in retarding the derivatisation reaction.

A further problem was encountered when attempting to derivatise 1,6-hexanediamine which appeared to react with NQS, but gave no colour in the chloroform extract. Instead a red precipitate was formed, which was insoluble in common organic solvents. This is believed to be the bis-NQS derivative (V, Fig. 3) which, although very slightly water-soluble in the presence of a base, is not soluble in common organic solvents.

TABLE I

PARTITION COEFFICIENTS FOR AMINE-NQS DERIVATIVES BETWEEN ORGANIC AND AQUEOUS PHASES

Amine-NQS Derivative	*Partition coefficient*[a]	
	Isoamyl alcohol–water	*Chloroform–water*
Morpholine	–	60
Piperidine	–	>100
Ethanolamine	2.7	0
2-Amino-2-methylpropanol	3.7	<1

[a] Absorbance (organic)/absorbance (aqueous) measured at λ_{max} close to 460 nm.

CONCLUSIONS

Steric effects can be significant in retarding amine derivatisation with NQS, but extraction of hydrophilic derivatives can be a major problem. NQS derivatives of N-butylethanolamine (VI) and ephedrine (VII) (Fig. 3) can be estimated[17, 23] using chloroform to extract them because they contain strongly hydrophobic groups which counter-act the effect of the hydroxyl group. In contrast, the NQS derivatives of diethanolamine (VIII, Fig. 3) and ethanolamine are too hydrophilic to be extracted into chloroform and only partially extracted into isoamyl alcohol[17]. Similarly the hydroxyl in 2-amino-2-methylpropanol (although not the ether linkage in morpholine) prevents extraction of the NQS derivative into chloroform. Isoamyl alcohol can be used as extractant, but the resulting efficiency is low. NQS derivatives of diamines and polyamines may not be sufficiently soluble in organic solvents to permit their extraction, so making the method unsuitable for their determination.

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