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Note

o-Phthalaldehyde–sulfite derivatization of primary amines for liquid chromatography–electrochemistry

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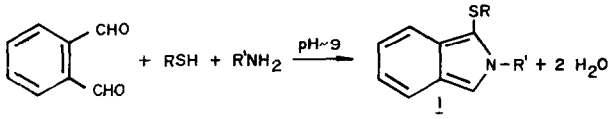
The reaction between primary alkylamines and *o*-phthalaldehyde (OPA) in the presence of an alkylthiol (Scheme 1) is a widely used derivatization technique for determination of amines and amino acids by liquid chromatography (LC). The normal products of this reaction, 1-alkylthio-N-alkylisoindoles, are formed rapidly in high yield, and can be detected with good sensitivity using either fluorescence or electrochemical detection (ED). A considerable problem associated with this chemistry stems from the poor stability of the isoindole derivatives, due to further reaction with excess OPA in the derivatization matrix[1–3].



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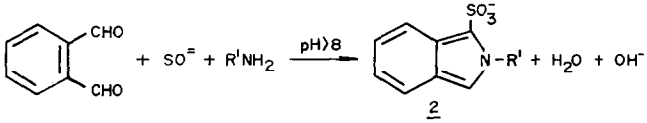
Scheme 1. Reaction between primary alkylamines and OPA in the presence of an alkylthiol.

If sulfite is substituted for the usual thiol coreagent, rapid and clean formation of different derivatives takes place. These appear to be N-alkyl-1-isoindole sulfonates (2 in Scheme 2). The OPA–sulfite derivatives are relatively inert toward excess OPA and exhibit good *in situ* stability. They are easily oxidized at carbon electrodes making them useful for determination of amines by LC–ED.



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Scheme 2. Reaction showing the appearance of N-alkyl-1-isoindole sulfonates.

EXPERIMENTAL

Chemicals

o-Phthalaldehyde (Aldrich) was recrystalized three times from hexane. Alkylamines (Aldrich), amino acids (Sigma), 2-mercaptoethanol (Pierce), and anhydrous sodium sulfite (Mallinckrodt) were used without further purification. Solvents and buffer salts were all reagent grade or better (J.T. Baker).

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Liquid chromatography

Isocratic chromatography was carried out using an LC-154 (Bioanalytical Systems) liquid chromatograph fitted with a Biophase 6017 ODS column (5 μm particle size, 25 × 0.46 cm I.D.) or a Phase II 6213 ODS column (3 μm particle size, 10 × 0.30 cm I.D.). The detector for this system was an LC-4B/17 operated at an applied potential of +0.85 V *vs.* Ag/AgCl. In some cases an LC-6 fixed-wavelength (254 nm) detector was used in series. For gradient runs, an SP-8700 (Spectra Physics) was used.

Mobile phases were buffered to pH 6.5 with 0.025 *M* maleic acid. Methanol and acetonitrile were used as organic modifiers.

OPA reagents

The derivatizing reagents were prepared using equimolar quantities of OPA and sodium sulfite (or 2-mercaptoethanol) in methanol–water buffered to the desired pH. The usual reagent solvent was a mixture of 10% (v/v) 1.0 *M* sodium carbonate pH 9.5 and 25% methanol. The carbonate buffer was replaced with borate or phosphate as needed for pH studies.

Synthesis of N-isopropyl-1-isoindole sulfonic acid, sodium salt

An amount of 2.0 g OPA was dissolved in 25 ml methanol and mixed with a solution of 1.5 g sodium bisulfite in 25 ml water. This mixture was stirred under nitrogen for 5 min. A volume of 1.4 ml of isopropylamine was then added all at once. The colorless solution turned pale yellow immediately. Chromatography of aliquots showed the presence of a single product. After stirring at room temperature for 30 min, the mixture was concentrated *in vacuo* to a heavy yellow oil. Benzene (150 ml) was added and the mixture heated to reflux under nitrogen using a Dean-Stark trap for azeotropic removal of residual water. A thick white precipitate formed after removal of about 1 ml water. After 1–2 h when a clear distillate was obtained, the mixture was filtered and the solid washed with a little benzene. The product was dried 24 h under vacuum at 50°C yielding 3.21 g of fluffy white powder (crude yield 82%). Attempts at further purification were unseccessful, resulting in substantial decomposition. The crude product decomposed at 240°C. NMR ($C^2H_3O^2H$): δ 1.51, D 6; δ 5.26, M 1; δ 7.5, M 5. NMR also indicated a significant quantity of residual water.

RESULTS AND DISCUSSION

Although the assignment of an unequivocal structure to the products of the reaction between OPA, sulfite, and primary amines is not possible based on present results, NMR data on the crude isopropylamine derivative support the assignment of the 1-isoindole sulfonate structure. The formation of these derivatives is consistent with recent studies on the mechanism of derivative formation when thiols are used as coreagents[2,4]. These studies showed that the role of thiol is that of nucleophile, acting to capture the initial product of condensation between amine and OPA and leading ultimately to formation of the isoindole product. It has subsequently been shown, as well, that cyanide can participate in this reaction sequence, resulting in formation of 1-cyanoisoindoles[5].

An initial examination of the redox behavior of the crude isopropylamine de-

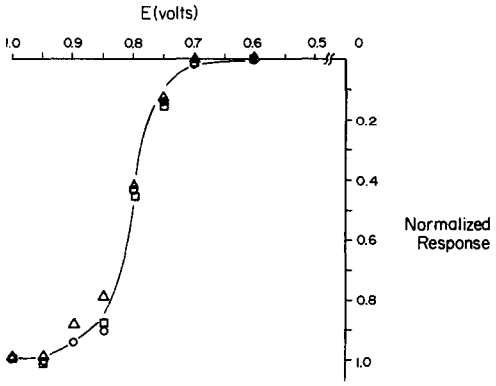


Fig. 1. Hydrodynamic voltammograms of OPA–sulfite derivatives. Mobile phase: 0.025 *M* maleic acid, pH 6.5, containing 15% (v/v) acetonitrile. Flow-rate: 1.0 ml/min. Key to symbols: ○ = methylamine; □ = ethylamine; △ = 2-propylamine.

rivative by cyclic voltammetry showed the presence of a single irreversible oxidation, with a peak potential of +0.71 V *vs.* Ag/AgCl (pH 7.0, phosphate buffer). Using methylhydroquinone as a reference, an *n* value of 1.8 was calculated, suggesting that the oxidation is a two-electron process, as is the case for the usual thiol based derivatives[6]. Hydrodynamic voltammetry on derivatives formed *in situ* (Fig. 1), under actual chromatographic conditions, yielded half-wave potentials of approximately +0.8 V *vs.* Ag/AgCl. For thiol derived isoindoles this is usually in the range of +0.5 to 0.6 V (ref. 6). Such a shift in the oxidation potential is not unexpected based on replacement of the alkylthio moiety by the sulfonate. An applied potential of +0.85

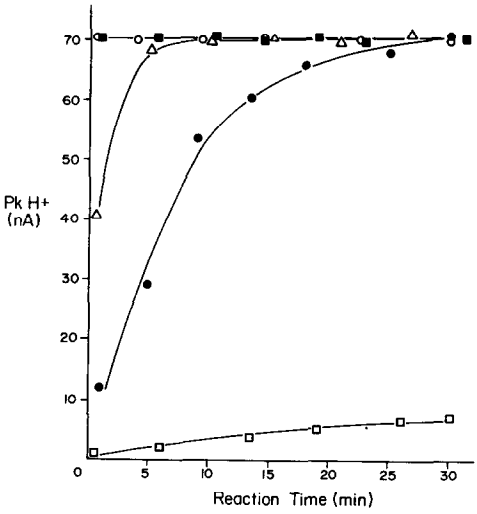


Fig. 2. pH Dependence of OPA–sulfite derivatization. $2.5 \cdot 10^{-3}$ *M* OPA–sulfite mixed 1:1 with $2.0 \cdot 10^{-5}$ *M* 2-propylamine. All reagents contained 25% (v/v) methanol. Key to symbols: ■ = 0.1 *M* cabonate, pH 10.5; ○ = 0.1 *M* carbonate, pH 9.5; △ = 0.1 *M* borate, pH 8.5; ● = 0.1 *M* phosphate, pH 7.5; □ = 0.1 *M* phosphate, pH 6.5.

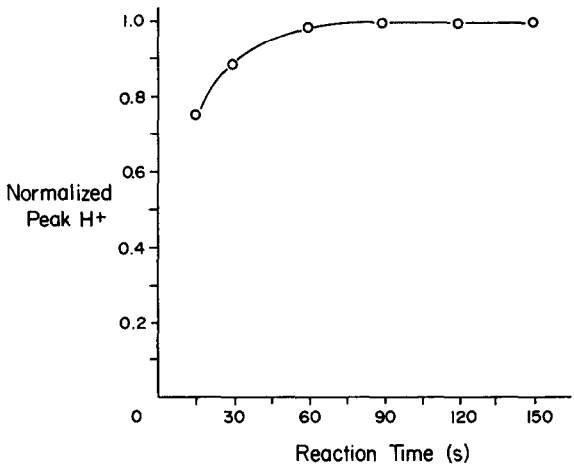


Fig. 3. Time course of 2-propylamine derivatization at pH 9.5. Conditions as in Fig. 2.

V *vs.* Ag/AgCl was chosen for routine operation, as this provided a reasonable trade-off between maximal sensitivity and minimum background current.

Derivative formation using OPA–sulfite was found to closely parallel that using OPA–thiol reagents. The rate of derivatization is strongly pH dependent (Fig. 2), requiring a mildly alkaline matrix for facile reaction. Fig. 3 follows the reaction at pH 9.5 up to 2.5 min, and shows that derivatization is complete after 1 min. The actual reagent concentration in the reaction matrix was $1.25 \cdot 10^{-3}$ *M* and conditions were pseudo-first order with respect to amine at $9.75 \cdot 10^{-6}$ *M*. As illustrated in Fig. 4, however, this reagent concentration is adequate for substrate concentrations up

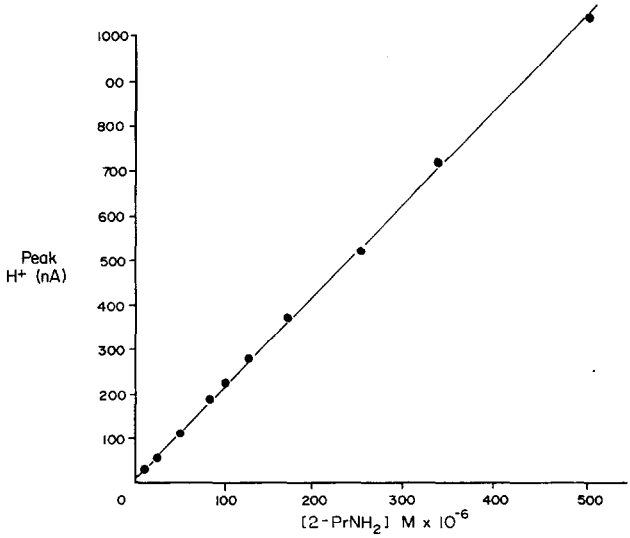


Fig. 4. Linearity of 2-propylamine (2-$PrNH_2$) derivatization. Conditions as in Fig. 2 at pH 9.5.

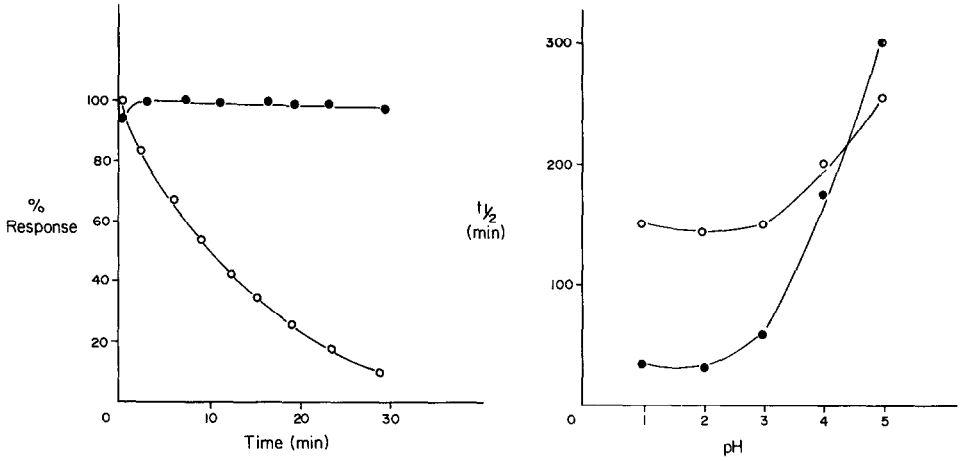


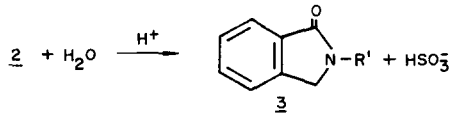
Fig. 5. Relative *in situ* stability of sulfite (●) and 2-mercaptoethanol (○) derived isoindoles. Reactions as in Fig. 2 with methylamine as substrate.

Fig. 6. Partial pH dependence of isoindole sulfonate hydrolysis. pH adjusted with appropriate buffers after preforming derivatives in dilute carbonate. Key to symbols: ● = methylamine; ○ = 2-propylamine.

to at least $2.50 \cdot 10^{-4}$ *M* where pseudo-first order conditions no longer exist. The range of linear derivatization can be extended simply by using a more concentrated reagent.

A significant problem associated with conventional OPA–thiol derivatization is relatively poor derivative stability. Instability *in situ* is due primarily to reaction with excess OPA in the matrix[1–3] involving electrophilic attack by OPA at C-3 of the isoindole[7]. This problem can be minimized in a number of ways, most notably by changes in thiol structure[2,3,7] or reaction solvent[3]. In contrast, the OPA–sulfite derivatives are relatively inert to reaction with excess OPA. The *in situ* stability of methylamine derivatives based on sulfite and 2-mercaptoethanol are compared in Fig. 5. Separate experiments involving OPA spikes to preformed derivatives yielded second order rate constants of (0.0859 ± 0.0006) M^{-1} min^{-1} and (17.8 ± 0.05) M^{-1} min^{-1} for the sulfite and 2-mercaptoethanol derivatives respectively. Thus, the OPA–sulfite derivatives are roughly 2-fold less susceptible to this complication.

We have found, however, that the OPA–sulfite derivaties are susceptible to decomposition at low pH (Fig. 6). This appears to be a simple hydrolytic process, resulting in formation of the phthalimidine (3 in Scheme 3). Incubation of the methylamine derivative at pH 1 resulted in the formation of a UV active material that co-chromatographed with an authentic sample of N-methylphthalimidine prepared by a literature procedure[8]. As outlined in Table I, the relative tendency toward hydrolysis is structure dependent, with bulky amine substituents providing some pro-



Scheme 3. Phthalimidine formation.

TABLE I

APPARENT FIRST-ORDER RATE CONSTANTS FOR HYDROLYSIS OF OPA–SULFITE DERIVATIVES AT pH 2.0 IN 0.1 *M* DICHLOROACETATE

$R–NH_2$	K_{app} (min^{-1})
$-CH_3$	0.0253
$-CH_2CH_3$	0.0122
$-CH_2CH_2CH_3$	0.0119
$-CH_2CH_2CH_2CH_3$	0.0118
$-CH(CH_3)_2$	0.006
$-CH_2CO_2H$	0.250
$-CH(CH)_3CO_2H$	0.116
$-CH(CO_2H)CH(CH_3)_2$	0.078
$-CH(CO_2H)CH_2Ar$	0.085
$-CH(CO_2H)CH_2CH_2CH_3$	0.152
$-CH_2CH_2CO_2H$	0.0538
$-CH_2CH_2CH_2CO_2H$	0.0318

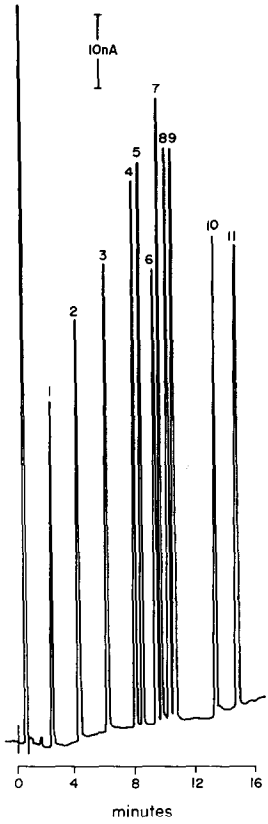


Fig. 7. Chromatogram of OPA–sulfite derivatives of alkylamines. Solvent A: 0.025 *M* maleic acid, pH 6.5, containing 5% (v/v) acetonitrile. Solvent B: 0.025 *M* maleic acid, pH 6.5 containing 50% (v/v) acetonitrile. Linear gradient from 15% B to 100% B over 15 min at 1.0 ml/min. Column; Phase II ODS, 10 × 0.31 cm I.D. Amine derivatives (approximately 100 pmol each) in sequence: methyl, ethyl, isopropyl, *n*-butyl, isobutyl, *n*-anyl, neopentyl, heptyl, octyl, benzyl, and phenethyl.

tection. Of particular interest is the observation that amino acids are significantly more susceptible to this decay than sterically comparable amines, suggesting that the reaction may be susceptible to intramolecular catalysis. Because of the low pH instability, mobile phases need to be buffered above pH 5 or 6. Otherwise, on-column hydrolysis can reduce sensitivity. Hydrolysis does not, however, contribute background peaks because the N-alkylphthalimidines are not oxidizable.

Despite their relative hydrophilicity (the isopropylamine derivative is readily soluble in water), OPA–sulfite derivatives of alkylamines retain well on reversed-phase columns and can be efficiently separated (Fig. 7). Most amino acid derivatives display adequate retentivity as well. Very hydrophilic amino acids, especially aspartic acid and glutamic acid, are difficult to retain, however. This problem is exacerbated by the inability to buffer mobile phases below the pK_a values of the carboxylate moieties. It should be possible to circumvent this limitation by the use of appropriate ion-pairing agents.

CONCLUSIONS

The OPA–sulfite derivatization represents a viable alternative to the conventional OPA–thiol chemistry for determination of primary alkylamines by LC–ED. The derivatives form rapidly and cleanly, and demonstrate good *in situ* stability. In addition, the reagents are easy to prepare, without undesirable thiol odors. Premixed reagents are stable for at least two weeks at room temperature, showing no apparent loss in activity. The major disadvantage associated with this class of derivative is the tendency toward hydrolysis at low pH, which limits the range of useable chromatographic conditions. While this is not a problem for alkylamines, adequate retention of polar amino acid derivatives on reversed-phase materials is not possible.

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