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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY DETECTION OF MORPHINE BY FLUORESCENCE AFTER POST-COLUMN DERIVATISATION

II. THE EFFECT OF MICELLE FORMATION

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SUMMARY

The phenolic oxidative coupling of morphine and related opiates to yield fluorescent products, used for post-column high-performance liquid chromatography detection, has been studied in micellar solutions. The nonionic surfactant, Triton X-100, was found to enhance the detection limit. This enhancement was a result of increased coupling rather than increased fluorescent response of the morphine dimer, pseudomorphine. Ionic surfactants either reduce the sensitivity of detection, or were not applicable to the reaction system used.

INTRODUCTION

Surfactants form aggregates of molecules or ions when their concentration in the bulk solution exceeds a limiting value. These aggregates are termed micelles, and the concentration at which they form is termed the critical micelle concentration (CMC) of the surfactant. Once formed, a micelle can enclose an analyte, and thus drastically modify its environment compared to that in a bulk phase when micelles are absent. This can have significant effects in analytical chemistry, and the effect is well known in what has been termed ion-pair and soap chromatography. Less well known to chromatographers is the potential effect of micelle formation upon enhancing fluorescent properties of analytes, or of catalysing reactions in chromatographic derivatisation procedures. For example, large alterations in fluorescent responses of phenols[1], cyanine dyes[2], dansyl glycine[3] and fluorescent derivatives of amino acids[4] have been observed. Another effect of analytical significance is micellar catalysis, for example, electrophilic oxidative coupling[5], azo dye formation[6] and the reaction between cyanide ion and 5,5′-dithio-bis(2-nitrobenzoic acid) used in a spectrophotometric determination of cyanide[7]. These effects arise from the micellar modification of the environment of the analyte, compared to the environment in a bulk phase where surfactants are absent. Properties such as fluorescence can be influenced

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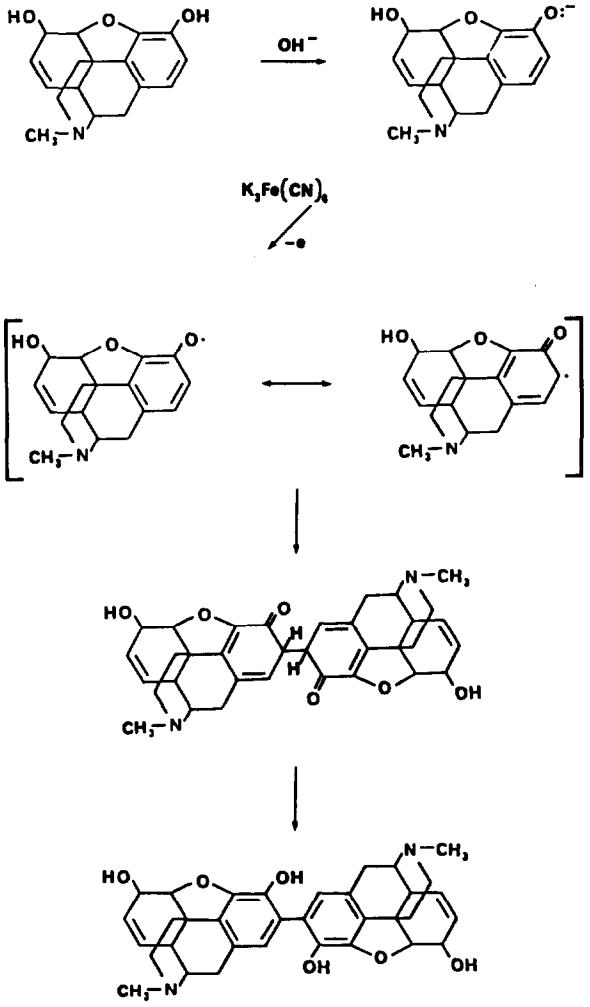
by micellar effects on inter- and intra-molecular interactions, and the effect upon ionization of acids and bases can influence the reactivity of analytes.

The present study explores the effect of micelle formation upon the sensitivity of a post-column derivatisation system developed for fluorescence detection in high-performance liquid chromatography (HPLC)[8]. The model system used involves the oxidative coupling of morphine to its fluorescent dimer, pseudomorphine using alkaline potassium ferricyanide. This oxidation (Scheme I) involves a free radical mechanism similar to that described for the oxidative coupling between disubstituted *p*-phenylene diamine and phenols[9], a reaction whose rate has been reported to be substantially increased by micellar catalysis[5]. The effect of micelles upon this reaction, and their effect upon the fluorescent response of pseudomorphine were examined to determine their influence on the detection limits of the post-column derivatisation HPLC system.



Scheme 1.

METHODS

Reagents and drug standards

All general reagents were analytical reagent grade. Triton X-100 (TX-100) was reagent grade (BDH), and sodium laurylsulphate (SLS), was puriss (Fluka). The surfactants were used without further purification. Morphine sulphate and nalorphine hydrobromide were BP grade. Pseudomorphine, normorphine, dihydromorphine and 6-monoacetylmorphine were synthesised in this laboratory.

Chromatography

The chromatographic system was used as previously described[8] with the exceptions that an Aminco Bowman spectrophotofluorometer equipped with a flow cell, ratio photometer, and a Xenon source was used in conjunction with the Aminco Bowman filter fluorometer previously described, and that the reaction coil was deformed by 'crochet' knotting to reduce band spreading[10,11]. The spectrophotofluorometer was used with an excitation wavelength of 324 nm and an emission wavelength of 430 nm. For conventional HPLC a 100 × 4 mm I.D. Zorbax ODS (8 μm) column, prepared by slurry packing, was used. The eluent was methanol–0.1 *M* potassium bromide (12.5:87.5) adjusted to pH 3 with phosphoric acid[8].

The post-column derivatising reagent comprised 50 mg of potassium ferricyanide [$K_3Fe(CN)_6$] in 250 ml of 4 *M* ammonium hydroxide. To determine any effect of the surfactants examined, they were added to this reagent at concentrations greater than their CMC. The CMC of the surfactants used are given in Table I[4].

TABLE I

CRITICAL MICELLE CONCENTRATIONS OF SURFACTANTS STUDIED

Surfactant	*CMC (mM)*
Triton X-100	0.24–0.42
Sodium lauryl sulphate	8.1
Cetyltrimethylammonium bromide	1.3

Calibration curves for each of the opiates were prepared by injection of quantities ranging from 100 ng to 2 μg, with fluorescent response being measured by peak heights. For rapid collection of reaction-rate data, the column was omitted from the chromatographic system. Linear regression analyses were made of the fluorescence response obtained for the amount of opiate examined.

RESULTS AND DISCUSSION

The effect of TX-100 on the post-column oxidation of morphine and nalorphine, used to standardize the system, is shown in the chromatogram in Fig. 1. The correlation coefficient, *r*, the intercept, *a* (peak height) and slope, *b* (peak height/μg), of the calibration curves obtained for five opiates converted to fluorescent products under the conditions described are shown in Table II. Part A lists the results obtained in the absence of surfactant, and parts B and C list the results obtained with SLS

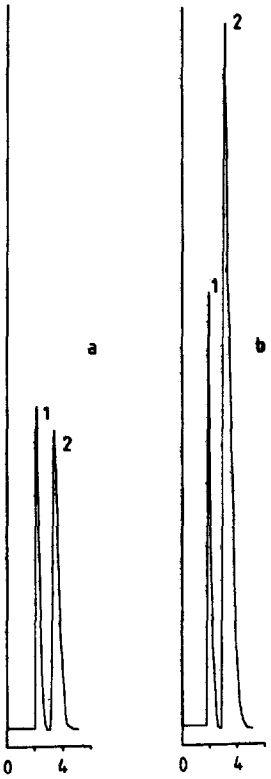


Fig. 1. The chromatographic response after post-column oxidation of 500 ng morphine (1) and 920 ng nalorphine (2), in the absence of TX-100 (a) and in the presence of TX-100 (4%, w/v) (b).

and TX-100 respectively present at concentrations greater than their CMC in the derivatising reagent. The slopes of the calibration curves, represented by *b* in these tables indicates the fluorescent response obtained. The calibration curves obtained for nalorphine hydrobromide with no surfactant present, and in micelles of TX-100 and SLS, are depicted in Fig. 2. The effect of the surfactants upon the derivatisation reaction can thus be compared as shown in Table III. The effect of the nonionic surfactant, TX-100 upon the derivatisation reaction enhanced the fluorescent response obtained over the range 100 ng–2 μg for each of the five opiates examined. The effect of SLS, an anionic surfactant, decreased or had no effect on the fluorescent response for all but dihydromorphine, which showed an increase. Any effect of the cationic surfactant, CETAB, could not be measured, as addition of this to the alkaline $K_3Fe(CN)_6$ resulted in the formation of a precipitate which prevented pumping of the derivatisation reagent into the system.

In a separate experiment, shown in Table IV, the ratio of the slopes of the linear regression lines of morphine to pseudomorphine indicated a 30.5% conversion of morphine to pseudomorphine when TX-100 was omitted from the oxidizing reagent, and a 42.5% conversion in the presence of TX-100 (4%, w/v). The slopes obtained for pseudomorphine in the presence of TX-100 was only 3.5% greater than

TABLE II

LINEAR REGRESSION ANALYSES OF FLUORESCENT RESPONSE AFTER OXIDATION OF OPIATES

The range of these analyses was from 100 ng–2 μg. The response was measured in chart divisions (2.54 mm) of the strip chart recorder used. The intercept of the calibration curve is *a*, the slope (peak height/μg) is *b* and the correlation coefficient is *r*.

	Opiate	*r*	*a*	*b*
(A) No surfactant	Morphine	0.999	−2.0	200
	Nalorphine	0.999	−3.2	170
	Dihydromorphine	1.00	2.5	220
	Normorphine	0.996	−4.2	220
	6-Monoacetylmorphine	1.00	−6.3	180
(B) 0.04 *M* SLS	Morphine	1.00	−1.6	170
	Nalorphine	0.999	−0.9	112
	Dihydromorphine	0.999	−2.0	320
	Normorphine	0.998	−7.5	220
	6-Monoacetylmorphine	0.999	−5.5	140
(C) TX-100 (4%, w/v)	Morphine	1.000	−7.6	250
	Nalorphine	0.980	−8.6	280
	Dihydromorphine	0.996	−7.2	450
	Normorphine	0.995	−9.8	280
	6-Monoacetylmorphine	1.000	13.00	260

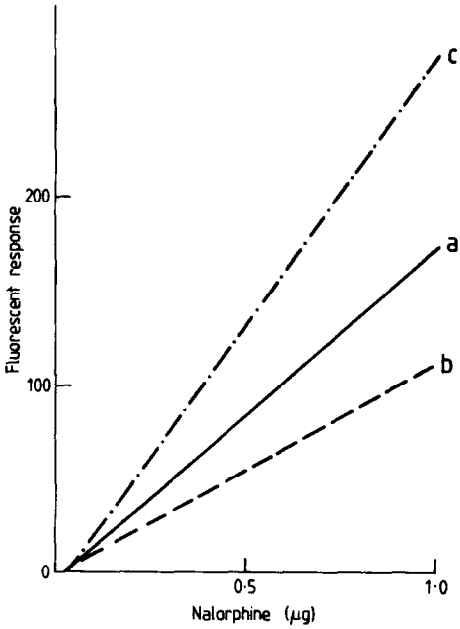


Fig. 2. The calibration curves, plotted from regressions given in Table II, of nalorphine following post-column oxidation in the absence of surfactant (a), in the presence of 0.04 *M* SLS (b) and TX-100 (4%, w/v) (c).

TABLE III

COMPARATIVE FLUORESCENT RESPONSE AFTER OXIDATION OF OPIATES IN PRESENCE OF SURFACTANTS

Opiate	*No Surfactant*	*SLS (0.04 M)*	*TX-100 (4%, w/v)*
Morphine	1	0.85	1.25
Nalorphine	1	0.65	1.65
Dihydromorphine	1	1.45	2.04
Normorphine	1	1.0	1.27
6-Monoacetylmorphine	1	0.78	1.44

that obtained in the of TX-100, indicating fluorescent enhancement of the pseudomorphine by micelle formation is not a factor in the observed results.

The mechanism for phenolic oxidative coupling involves the oxidation of the phenolate anion to a free phenoxy radical, which then couples rapidly and irreversibly to form dimeric and polymeric products (reaction Scheme I). In phenolic oxidations by $K_3Fe(CN)_6$, electron transfer to the metal ion occurs via the cyano ligands, *i.e.*, the transfer is a non bonded process. The phenolate anion is better able to transfer an electron in such a process than free phenol, thus ferricyanide oxidations generally require an alkaline medium[12]. The inclusion of the oxidant and the substrate in the micelle is unlikely to enhance such an oxidation, and it has been observed that micelle formation suppressed the ionization of phenols[5,13,14] which could inhibit oxidation. However, it is highly likely that inclusion of the free radicals resulting from the oxidation into a non polar micelle could influence the situation. The uncharged free radicals could be concentrated in micelles of TX-100, increasing the rate of coupling and reducing unwanted side reactions. Once formed within the micelle, the dimer would be protected both from further oxidation and from the polar reaction media. This effect is analogous to the use of a two-phase reaction system that has been found very effective for $K_3Fe(CN)_6$ oxidation[12].

TABLE IV

LINEAR REGRESSION ANALYSES OF FLUORESCENT RESPONSE AFTER OXIDATION OF MORPHINE COMPARED TO PSEUDOMORPHINE.

The ranges of these analyses were 19–80 ng for pseudomorphine, and 80–320 ng for morphine (n = 8–10). The response was measured as peak height using a HP 3390A reporting integrator, and ranged from 13,000–50,000. The intercept of the calibration curve is a, the slope (peak height/ng) is b and the correlation coefficient is r.

	r	a	b
A: No surfactant			
Pseudomorphine	0.990	5609	563
Morphine	0.986	3506	172
B: TX-100 (4%, w/v)			
Pseudomorphine	0.991	5009	583
Morphine	0.996	341	248

Some support for this explanation of the results observed with TX-100 arose from observations using the anionic surfactant, SLS. Ionic surfactants are expected to have a much greater effect upon the reaction of charged species than nonionic surfactants[6,15]. The results obtained using SLS were equivocal, with the reaction observed for three opiates being reduced, one remaining unchanged and one increased, compared to an increased reaction for all five opiates in the presence of TX-100. This indicated that ionic interactions, that is the oxidation of the substrate to form a free radical, was not influenced to any extent by the presence of micelles of SLS.

CONCLUSIONS

The nonionic surfactant, TX-100, was found to improve the fluorescent response of a post-column HPLC reaction detector for the determination of opiates as their pseudomorphine analogues. The improvement probably resulted from micellar catalysis of the coupling of the phenoxy free radicals, and the protection of the product from further oxidation. The procedure is simple, and should be applicable to other post-column HPLC reaction systems involving chemical and photolytic oxidation reactions. The procedure should also be suitable for the study of micellar fluorescent enhancement as a means of improving HPLC detection limits for fluorescent compounds.

REFERENCES

1 B. K. Selinger and A. Weller, *Aust. J. Chem.*, 30 (1977) 2377.
2 R. Humphry-Baker, M. Gratzel and R. Steiger, J. Amer. Chem. Soc., 102 (1980) 847.
3 G. A. Davis, *J. Amer. Chem. Soc.*, 94 (1972) 5089.
4 H. N. Singh and W. L. Hinze, *Analyst (London)*, 107 (1982) 1073.
5 L. K. J. Tong and M. C. Glesmann, *J. Amer. Chem. Soc.*, 79 (1957) 4310. 4310–4313.
6 M. Poindexter and B. McKay, *J. Org. Chem.*, 37 (1972) 1674.
7 S. Spurlin, W. Hinze and D. W. Armstrong, *Anal. Lett.*, 10 (1977) 997.
8 P. E. Nelson, S. L. Nolan and K. R. Bedford, *J. Chromatogr.*, 234 (1982) 407.
9 J. H. Fendler and E. J. Fendler, *Catalysis in Micellar and Macromolecular Systems*, Academic Press, New York, 1975, p. 247.
10 K. Hofmann and I. Halász, *J. Chromatogr.*, 199 (1980) 3.
11 M. Uihlein and E. Schwab, *Chromatographia*, 15 (1982) 140.
12 H. Musso, in W. I. Taylor and A. R. Battersby (Editors), *Oxidative Coupling of Phenols*, Marcel Dekker, New York, 1967, p. 1.
13 L. K. J. Tong and M. C. Glesmann, *J. Amer. Chem. Soc.*, 79 (1957) 4305.
14 H. Hoshino, T. Saitoh, H. Taketomi, T. Yotsuyanagi, H. Watanabe and K. Tachikawa, *Anal. Chim. Acta*, 147 (1983) 339.
15 U. Khuanga, R. McDonald and B. K. Selinger, *Z. Phys. Chem. (Wiesbaden)*, 101 (1976) 209.