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Naphthalene- and anthracene-2,3-dialdehyde as precolumn labelling reagents for primary amines using reversed- and normal-phase liquid chromatography with peroxyoxalate chemiluminescence detection

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ABSTRACT

Naphthalene-2,3-dialdehyde (NDA) and anthracene-2,3-dialdehyde (ADA) were applied as pre-column labelling reagents for the peroxyoxalate chemiluminescence detection of primary amines. The advantages of these labels are the selective derivatization reaction with primary amines and the good chemiluminescence properties. A serious disadvantage is the formation of cyanide-induced side-products which are major interferences in reversed-phase chromatography. For normal-phase chromatography, the excess of reagent was removed by adding a polar amine after derivatization, with subsequent extraction of the labelled analyte with an apolar solvent. The detection limit for NDA-labelled fluvoxamine, an anti-depressant, was in the low femtomole range in standard solutions and in urine samples. For ADA-labelled analytes difficulties were obtained with linearity in peroxyoxalate chemiluminescence detection, probably owing to oxidation of the derivative by hydrogen peroxide.

INTRODUCTION

In high-performance liquid chromatography (HPLC), the detection of analytes containing a primary amine functional group is routinely carried out in many laboratories with detection limits in the low nanogram range. Well known fluorescence derivatization reagents such as *o*-phthalaldehyde (OPA), fluorescamine and 5-dimethylaminonaphthalene-1-sulphonyl chloride (dansyl chloride) can be used for the sensitive detection of these types of compounds[1–3]. In order to obtain lower detection limits, a new label was recently synthesized, naphthalene-2,3-dialdehyde (NDA); the reaction is based on the selective isoindole formation of primary amines similar to the reaction with OPA[4,5]. The fluorescence quantum yield of these benzisoindole derivatives is about 0.5–0.6 in aqueous solvents[6] and very low detection limits (0.2–1 fmol) can be obtained using an argon-ion laser as light source[7]. The same principle

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was applied by Beale and co-workers[8,9], who used 3-benzoyl-2-quinolinecarboxaldehyde, a fluorogenic reagent yielding derivatives with absorption maxima compatible with the 442-nm line of the He–Cd laser.

Peroxyoxalate chemiluminescence (CL) has been shown to be a highly sensitive detection principle for HPLC, yielding detection limits in the low femtomole and even attomole range[10–13]. Therefore, CL may be regarded as a serious competitor for laser-induced fluorescence detection in HPLC. Recently it has been reported that NDA derivatives can conveniently be detected by the peroxyoxalate CL detection system[14,15]. Hayakawa *et al.*[15] compared dansyl, 4-fluoro-7-nitrobenzoxadiazole (NBD-F) and NDA derivatization with peroxyoxalate CL detection, and found that NDA derivatives could be detected ten times more sensitively than dansyl derivatives and more than 50 times better than NBD-F derivatives.

The aim of this study was to investigate the applicability of NDA and anthracene-2,3-dialdehyde (ADA) as labels for reversed- and normal-phase HPLC with peroxyoxalate CL detection. The emission wavelengths of ADA derivatives are expected to be above 550 nm. As published in a previous paper dealing with a rhodamine label, long-wavelength emitters are favourable for peroxyoxalate CL detection[16], because a 550-nm emission cut-off filter will reduce the noise caused by the CL background. Further, the fluorescence and CL characteristics of ADA and NDA derivatives were compared. The determination of the anti-depressant fluvoxamine in urine is shown as an example.

EXPERIMENTAL

Chemicals

HPLC-grade solvents were purchased from Baker (Deventer, The Netherlands) and bis(2,4,6-trichlorophenyl) oxalate (TCPO) and bis(2,4-dinitrophenyl) oxalate (DNPO) from Fluka (Buchs, Switzerland). Bis(2-nitrophenyl) oxalate (2-NPO) was synthesized as described[17]. NDA and ADA were obtained from Molecular Probes (Eugene, OR, U.S.A.). Fluvoxamine {5-methoxy-1-[4-(trifluoromethyl)phenyl]-1-pentanone(*E*)-O-(2-aminoethyl)oxime} was supplied by Duphar (Weesp, The Netherlands) and amphetamine by Aldrich (Brussels, Belgium); Fig. 1 shows the structures of both analytes. Lissamine Rhodamine B sulphonyl chloride was purchased from Kodak (Weesp, The Netherlands) and 5-dimethylaminonaphthalene-1-sulphonyl chloride from Aldrich. Perylene-3-sulphonate was synthesized by P. de Wit (Department of Organic Chemistry, University of Amsterdam, The Netherlands[18]). All other chemicals were of analytical-reagent grade.

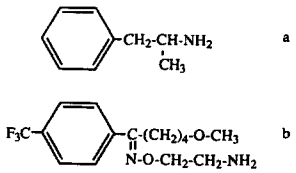

Fig. 1. Structural formulae of (a) amphetamine and (b) fluvoxamine.

High-performance liquid chromatography

In the reversed-phase systen, the mobile phase was delivered by a Gilson Model 302 pump equipped with a Gilson Model 308 manometric module (Gilson, Villiers-le-Bel, France). A laboratory-made six-port injection valve with a 25-μl loop was used for the introduction of samples onto a 150 × 3.1 mm I.D. analytical column packed by a slurry technique with 5-μm LiChrosorb RP-18 (Merck, Darmstadt, F.R.G.). Reversed-phase HPLC was carried out with acetonitrile–imidazole buffer (2.5 m*M*, pH 7.0) (75:25, v/v) as eluent at a flow-rate of 0.5 ml/min.

In the normal-phase system, a Gilson 302 pump with a laboratory-made membrane-type pulse damper was used to deliver the HPLC mobile phase. A laboratory-made six-port injection valve with a 35-μl loop was used for injection onto a 250 × 3.1 mm I.D. 5-μm LiChrosorb Si 60 (Merck) silica column. Chromatography of standard solutions was carried out using a mobile phase of dichloromethane containing 0.3% methanol at a flow-rate of 0.5 ml/min. For urine samples 0.2% methanol was used in the mobile phase.

Detection system

The reversed-phase system was essentially the same as that described in a previous paper[16]. Hydrogen peroxide and 2-NPO dissolved in acetonitrile at final concentrations of 50 and 5 m*M*, respectively, were mixed just before use and added to the column effluent with a pulseless Isco (Lincoln, NE, U.S.A.) μLC-500 syringe pump. The mobile phase (0.5 ml/min) and the reagent stream (0.2 ml/min) were mixed with a standard Valco T-piece immediately before the detector. A Kratos (Ramsey, NJ, U.S.A.) FS 970 fluorescence detector (with the lamp turned off) equipped with a 2π steradian mirror, a laboratory-made 50-μl flow cell and a 418-nm emission cut-off filter (NDA) or a 550-nm filter (ADA) was used for detection.

The set-up for the normal-phase system is shown in Fig. 2. A laboratory-made pulseless syringe pump delivered a mixture of 50 m*M* hydrogen peroxide and 0.5 m*M* triethylamine in acetonitrile–dichloromethane (1:1, v/v) at a flow-rate of 0.1 ml/min. A second laboratory-made syringe pump delivered 5 m*M* TCPO in dichloromethane, also at a flow-rate of 0.1 ml/min. A Kratos FS 980 fluorescence detector, with the lamp

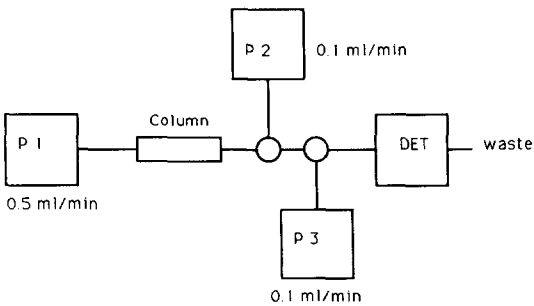

Fig. 2. Experimental set-up for normal-phase HPLC with peroxyoxalate CL detection. P1 = Dichloromethane containing 0.2–0.3% methanol, flow-rate 0.5 ml/min; P2 = 50 m*M* hydrogen peroxide + 0.5 m*M* triethylamine in acetonitrile–dichloromethane (1:1, v/v), flow-rate 0.1 ml/min; P3 = 5 m*M* TCPO in dichloromethane, flow-rate 0.1 ml/min; DET = Kratos FS 980 fluorescence detector with the lamp turned off.

turned off, equipped with a standard 25-μl flow cell and a 418-nm (NDA) or a 550-nm (ADA) emission cut-off filter was used for detection.

Derivatization of primary amines with NDA or ADA

The derivatization procedure is a modification of that described by de Montigny *et al.*[4]. Standard solutions of amines were prepared in 0.02 *M* borate buffer (pH 9.5). To 500 μl of amine solution, 100 μl of a 10 m*M* sodium cyanide solution in borate buffer (pH 9.5) were added and, subsequently, 500 μl of a 0.1 m*M* NDA or ADA solution in methanol. After reaction for 20 min at ambient temperature, a 25-μl aliquot was injected onto the reversed-phase HPLC system. For the normal-phase HPLC system, after derivatization 100 μl of 0.1 *M* glycine in borate buffer (pH 9.5) were added to the reaction mixture and allowed to react for 10 min. The relatively apolar derivative was extracted with 500 μl of toluene–hexane (1:1, v/v); 400 μl of the toluene–hexane layer were diluted with 800 μl of dichloromethane and a 35-μl aliquot was injected onto the silica column.

Analysis of urine samples

To 8 ml of urine, 2 ml of a borate buffer (0.1 *M*, pH 9.5) were added. After filtration through a 0.2-μm disposable filter, derivatization was carried out as described in the previous section, the only exception being the concentration of NDA (1 m*M* instead of 0.1 m*M*). For fluvoxamine analysis, 7 ml of urine (+ 2 ml of borate buffer) were spiked with 1 ml of a fluvoxamine solution and treated in the same way.

RESULTS AND DISCUSSION

Derivatization reaction

Derivatization conditions. In their derivatization procedures for primary amines with NDA, de Montigny *et al.*[4] used 50 μl of sample solution and a total amount of 450 μl of reagent solution (borate buffer, cyanide and NDA). In this study, a distinctly larger volume of sample was used (500 μl). The conversion was still quantitative with a reaction time of only 20 min at room temperature for both NDA and ADA and for the model compound amphetamine (see reaction scheme in Fig. 3). In agreement with the data reported by de Montigny *et al.*[4], we found that the NDA derivatives are very stable. After extraction into toluene–hexane (1:1) no decrease in signal intensity was found even after 8 h. ADA derivatives, however, were found to be relatively unstable, probably owing to oxidation of one of the aromatic rings. About a 50% loss in signal was observed after 4 h. Therefore, ADA derivatives had to be injected within 30 min after derivatization (3–4% loss). The stability of the ADA derivatives in the aqueous solvent used for reversed-phase HPLC was found to be similar.

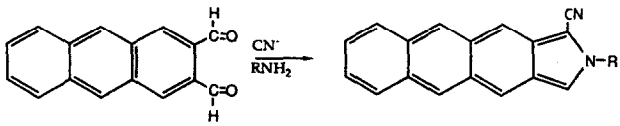

Fig. 3. Derivatization reaction of ADA with primary amines.

Side-product formation. In both reversed- and normal-phase HPLC various unknown peaks appeared in the chromatograms when analysing blank and standard amphetamine solutions after derivatization. These interfering peaks present severe problems at low analyte concentrations (10^{-8}–10^{-9} *M*), as illustrated in Fig. 4. Surprisingly, injection of the reagent (NDA or ADA) itself did not cause any interferences; these only occurred after mixing the reagent with borate buffer and cyanide. Roach and Harmony[7] mentioned the possible formation of benzoin condensation side-products. It is known that cyanide can induce the condensation of two aromatic aldehydes to form an α-hydroxyketone[19]. As two neighbouring aldehyde groups are present in NDA and ADA, many condensation products are, in principle, possible.

The formation of interferences in derivatization procedures seems to present a general problem in trace-level analysis. Many workers have only demonstrated the high sensitivity of their CL detection systems with commercially available derivatives, *e.g.*, with dansyl derivatives of amino acids[20,21]. Others probably carried out the derivatization at high analyte concentration and then prepared a series of diluted test solutions. In some instances, such as in the derivatization of catcholamines with fluorescamine[22] and thiols with N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]male-

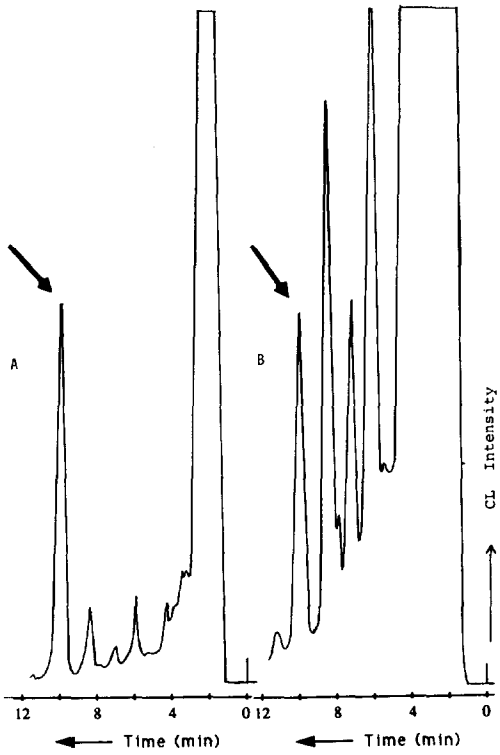

Fig. 4. Reversed-phase HPLC of (A) 10^{-7} *M* and (B) 10^{-8} *M* amphetamine derivatized with ADA and detected by peroxyoxalate CL. The amphetamine ADA derivative is indicated by an arrow. Mobile phase: acetonitrile–water (70:30, v/v), containing 2.5 m*M* imidazole. For other conditions, see Experimental.

imide (DBPM)[23], the final sample solution could be injected directly after derivatization onto an HPLC column without dilution. In actual practice, however, the problem often is that the reagent, at a concentration of 1.0 or 0.1 m*M*, is *ca.* 10^5–10^6 times in excess when derivatizing analyte concentrations of 10^{-8} or 10^{-9} *M*. This means that 0.1% of reagent impurities and side-products can cause severe interferences in trace-level studies. Dilution of the reagent generally is not possible as it will cause a large increase in reaction time. For a reaction that is carried out in, *e.g.*, 10 min the reaction rate will probably decrease 10-fold or more on diluting the analyte 10^3–10^4-fold and the reagent 10-fold[24].

In the case of peroxyoxalate CL detection of NDA and ADA derivatives, the selectivity of the chemical excitation process can be used to reduce the interferences caused by side-products. This is illustrated in Fig. 5 in which the same derivatization reaction, after reversed-phase HPLC, is monitored by fluorescence and CL detection. It can be clearly seen that the sensitivity of CL detection is higher and that the selectivity also is much better in the case of CL detection (see, *e.g.*, ref. 25). Unfortunately, for both NDA and ADA derivatives of amphetamine in reversed-phase HPLC the interfering peaks still start to dominate the chromatogram at the 10^{-8} *M* level even with standard solutions, so that real trace-level analysis cannot be carried out.

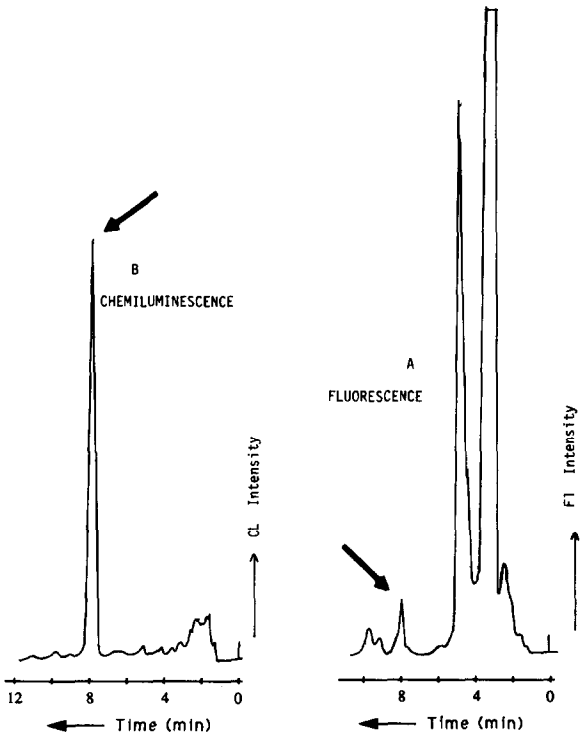

Fig. 5. Reversed-phase HPLC of a 10^{-6} *M* ADA-labelled amphetamine solution detected by (A) fluorescence and (B) chemiluminescence. The amphetamine ADA derivative is indicated by an arrow. Mobile phase: acetonitrile–water (75:25, v/v), containing 2.5 m*M* imidazole. For other conditions, see Experimental.

For normal-phase HPLC studies, the excess of reagent, and part of the interfering products, were removed by means of a simple liquid–liquid extraction. The excess of reagent can be separated from the analyte by adding a small amount (100 μl of a 0.1 *M* solution) of a very polar amine, *e.g.*, glycine, with subsequent extraction of the derivative of the analyte into an apolar organic phase [toluene–hexane (1:1, v/v)]. After extraction with toluene only, various interfering peaks were still visible in the chromatogram; an extraction with hexane only resulted in relatively clean chromatograms with, however, a slight decrease in recovery. A 1:1 mixture of both solvents gave the best results. After adding dichloromethane, the organic layer can be injected directly onto a normal-phase column. In this way a much cleaner chromatogram can be obtained and low-level derivatization of primary amines of medium polarity with peroxyoxalate CL detection becomes possible (see Fig. 6). If the same procedure is carried out with fluorescence detection, with excitation at 260 nm, the chromatogram still contains a large interfering band from t_0 to about 16 min. Obviously, the naphthalene-type side-products fluoresce relatively well, but seem to have poor CL characteristics. This again demonstrates well the superiority of peroxyoxalate CL over fluorescence detection.

In principle, a similar purification method can be carried out for a reversed-phase system. After the derivatization, addition of a very apolar amine, such as

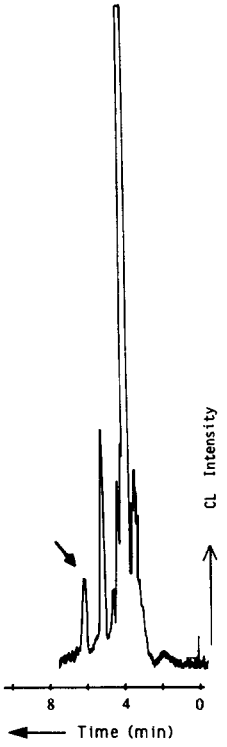

Fig. 6. Normal-phase HPLC of $1 \cdot 10^{-9}$ *M* fluvoxamine derivatized with NDA (indicated by an arrow). Mobile phase: dichloromethane containing 0.3% methanol. For other experimental conditions, see Fig. 2.

n-heptylamine, will allow the extraction of the excess of reagent into an apolar solvent. This was not useful for a fluvoxamine NDA derivative as it was co-extracted with the *n*-heptylamine NDA derivative into the organic solvent. However, for more polar amines, such as amino acids, this approach seems very promising, but was not investigated here.

Optimization of CL conditions

Reversed-phase HPLC. The CL detection system was the same as described in previous paper[16]. The oxalate, 2-NPO, and hydrogen peroxide were mixed in acetonitrile just before use and were added to the column eluate with a pulseless syringe pump. The CL reaction rate is then mainly determined by the pH of the mobile phase and the presence of a catalyst, often imidazole or triethylamine. Surprisingly, we found that the imidazole concentration (10 m*M*) used with rhodamine-labelled chlorophenols[16] was too high for the NDA and ADA derivatives. Varying the imidazole concentration from 10 to 0.5 m*M* resulted in an optimum at 2.5 m*M*, with a gain in signal-to-noise (S/N) ratio of about one order of magnitude. On carrying out further optimization studies, we found that the half-life of the CL signal for the NDA and ADA derivatives differed significantly from that of other well known chemiluminophores[26]. The results are summarized in Table I.

It is surprising that the three chemiluminophores rhodamine sulphonate, perylene sulphonate and dansylated ethanolamine all have comparable CL half-lives of 10–15 s, which are substantially different from the 1.5-s half-lives found for the NDA and ADA derivatives of ethanolamine (the latter amine was selected as a model compound at this stage because of the short retention time of its NDA and ADA derivatives). Interestingly, under the present conditions the half-life of the CL background (measured in the stopped-flow mode) was found to be over ten times higher than that of the latter two compounds. This means that with an appropriate volume of the flow cell a large fraction of the signal produced by the NDA and ADA derivatives can be measured while only a relatively low background signal is measured. The residence time in the flow cell should then be of the same order of magnitude as the half-life of the derivative. In the present system with a volume of *ca.* 2 μl between the T-piece and the flow cell and a total flow-rate of 700 μl/min, it takes 0.15 s from the T-piece to the detector and, subsequently, the CL signal is measured during *ca.* 4 s in

TABLE I

CL HALF-LIVES OF CHEMILUMINOPHORES IN REVERSED-PHASE HPLC

Conditions for all chemiluminophores: 2.5 m*M* imidazole in mobile phase, acetonitrile–water (75:25, v/v); 5 m*M* 2-NPO and 50 m*M* hydrogen peroxide added in acetonitrile at a flow-rate of 0.2 ml/min. The half-lives were measured by switching a valve, inserted just before the detector, realizing an immediate stopped-flow; the decay time was then recorded from the maximum to 50% intensity. In order to minimize injection artifacts, all experiments were carried out under reversed-phase HPLC conditions as described above. For further details, see Experimental.

Chemiluminophore	*Half-life (s)*	*Chemiluminophore*	*Half-life (s)*
Rhodamine sulphonate	13	Ethanolamine NDA	1.5
Perylene sulphonate	10	Ethanolamine ADA	1.5
Dansylated ethanolamine	11	CL background	19

the 50-μl flow cell. This means that these conditions are suitable for the efficient discrimination between the analyte signal and the background signal, clearly illustrating that the CL half-life plays an important role in the optimization of the S/N ratio. Another aspect is that a comparison of the sensitivity of two different chemiluminophores with the same HPLC system is not possible if their CL half-lives differ too much. In practice this will not often be a real problem as labelling procedures are carried out with one label at a time.

Normal-phase HPLC. In a previous study dealing with normal-phase HPLC and peroxyoxalate CL detection, relatively large amounts of acetonitrile and methanol were present in the mobile phase[16]. Therefore, the addition of hydrogen peroxide could be performed with a so-called perhydrit reactor, which contains hydrogen peroxide held on a urea support. With NDA and ADA derivatives of fluvoxamine, however, mobile phases consisting of chloroform or dichloromethane with a low content of methanol (0.2–0.3%) as modifier were used. In these solvents the solubility of hydrogen peroxide of the perhydrit is much too low; therefore, other methods of hydrogen peroxide addition had to be developed. A solution of aqueous hydrogen peroxide in acetonitrile (100 m*M*) was mixed with the same volume of triethylamine in dichloromethane (1 m*M*) and added to the column eluate with a pulseless syringe pump (flow-rate 0.1 ml/min). Final concentrations of hydrogen peroxide higher than 50 m*M* did not increase the S/N ratio and therefore this concentration was used for all further experiments. The concentration of triethylamine was varied between 20 and 0.1 m*M*; 0.5 m*M* was found to be the optimum, with a gain in S/N ratio by a factor of about 30. This triethylamine concentration is in sharp contrast with the 100 m*M* utilized in the normal-phase system described by Nozaki *et al.*[27]. This can probably be explained by the faster CL kinetics of NDA derivatives compared with dansyl derivatives (see above) and perhaps also by the use of different solvents. The oxalate, TCPO, was dissolved in dichloromethane and added with a second syringe pump (see Fig. 2), at a flow-rate of 0.1 ml/min. Varying the TCPO concentration between 1 and 10 m*M* showed 5 m*M* to be the optimum concentration.

In normal-phase HPLC all initial studies were carried out with both ADA and NDA derivatives of fluvoxamine. However, we found a strange non-linear behaviour for the ADA derivatives in flow-injection analysis and in normal-phase HPLC. This was observed with all possible combinations of the oxalates DNPO, 2-NPO and TCPO and the catalysts imidazole and triethylamine. Fluorescence batch experiments showed that the ADA derivatives, in contrast with NDA derivatives, are unstable in the presence of hydrogen peroxide (*ca.* 90% loss in 30 min). Obviously, the ADA derivatives are rapidly oxidized during the CL reaction, which essentially agrees with the observations made during optimization of the reaction conditions (see above). NDA derivatives, however, are more stable in the presence of hydrogen peroxide and, therefore, in all further experiments NDA was used. With NDA derivatives, good linearity was found with TCPO and triethylamine.

Analytical data and application

Comparison of CL and fluorescence detection. The Kratos FS 980 fluorescence detector is equipped with a deuterium lamp and, based on the excitation spectra of the present derivatives, excitation wavelengths of 260 nm (NDA; 252 nm in ref. 4) and 280 nm (ADA) were selected. These wavelengths should be used if a real comparison

has to be made between fluorescence and CL sensitivity with the Kratos detector. However, in fluorescence analysis better selectivity will be achieved if excitation is carried out in the visible region, *viz.*, at about 420 nm (maxima at about 414 and 438 nm for NDA derivatives of amino acids in ref. 4). A xenon lamp with high intensity in the visible region may well offer the best compromise with regard to sensitivity and selectivity.

The results are summarized in Table II. It should be emphasized that in three out of the four cases tested (for the exception, see below), it was not possible to derivatize low analyte concentrations (10^{-9} *M*) because of the presence of large interfering peaks or the instability of the derivatives as discussed in the previous sections. Therefore, relatively concentrated (*ca.* 10^{-6} *M*) solutions were diluted 10–1000-fold to measure the S/N ratios. From the data in Table II one can conclude that, under ideal conditions, CL detection is at least an order of magnitude more sensitive than fluorescence detection, but there is little difference between normal- and reversed-phase HPLC and an ADA or an NDA derivative. The better sensitivity of CL compared with fluorescence detection is in agreement with the results of Hayakawa *et al*[15]. Their better detection limits may be ascribed to the different detector optics, a larger flow cell volume and different composition of the final CL solvent. However, the aspect of overriding importance is that only in the case of NDA derivatives analysed by normal-phase HPLC with CL detection was direct derivatization of a 10^{-9} *M* solution possible (see Fig. 6). In other words, this is the only technique which is applicable to real-sample trace-level analyses.

Linearity and application. Using normal-phase HPLC with CL detection the linearity of the total procedure was measurd in the range $5 \cdot 10^{-7}$–$5 \cdot 10^{-10}$ *M* fluvoxamine. Good linearity was obtained over the whole range ($n = 8$, $r = 0.998$). As an application, Fig. 7 shows the analysis of a urine sample spiked with $5 \cdot 10^{-9}$ *M* fluvoxamine and the corresponding blank. The urine was filtered and, after adjustment of the pH with a borate buffer, was analysed without further treatment. Therapeutic

TABLE II

CL AND FLUORESCENCE DETECTION LIMITS (S/N = 3) FOR NDA AND ADA DERIVATIVES

For reversed-phase HPLC amphetamine was used as a model compound (see Table I); for normal-phase HPLC fluvoxamine was used (see Fig. 2).

Mode of operation	*Detection limit (fmol)*	
	NDA	*ADA*
Chemiluminescence		
Reversed-phase	4	2
Normal-phase	5	—[a]
Fluoresence		
Reversed-phase	100[b]	100[c]
Normal-phase	40[b]	80[c]

[a] Not determined because of non-linear behaviour (see text).
[b] Emission cut-off filter of 418 nm, $\lambda_{exc.}$ = 260 nm.
[c] Emission cut-off filter of 550 nm, $\lambda_{exc.}$ = 280 nm.

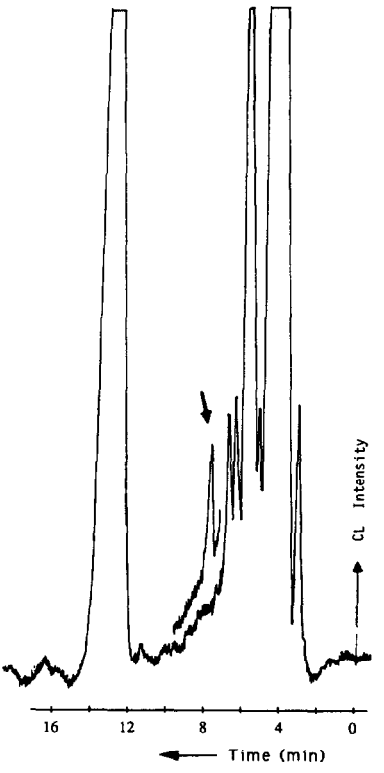

Fig. 7. Normal-phase HPLC of blank urine and a urine spiked with $5 \cdot 10^{-9}$ *M* fluvoxamine (indicated by an arrow), both derivatized with NDA. Mobile phase: dichloromethane containing 0.2% methanol. For other conditions, see Fig. 2 and Experimental.

levels of fluvoxamine in plasma are in the range 10^{-7}–10^{-8} *M*, that is, the sensitivity of the present system is obviously satisfactory for bioanalysis. As there are many primary amine-type compounds in urine, the concentration of NDA had to be increased from 0.1 to 1.0 m*M* and even with this reagent concentration the recovery was not quantitative, as can be seen by comparing peak heights (and concentrations) in Figs. 6 and 7. However, the fluvoxamine NDA derivative could still be linearly detected over at least two orders of magnitude (10^{-7}–10^{-9} *M*). Further optimization of the method is necessary for the routine analysis of real samples.

CONCLUSIONS

In normal-phase HPLC with peroxyoxalate CL detection, NDA can be used as a sensitive label for the detection of primary amines if the excess of reagent is removed from the mixture by adding a concentrated glycine solution after the derivatization step. With the anti-depressant fluvoxamine as analyte, a detection limit of 5 fmol is obtained, with good linearity in the range 10^{-6}–10^{-9} *M*. The practicability of the procedure is shown by the determination of $5 \cdot 10^{-9}$ *M* fluvoxamine in urine. In

contrast to NDA, ADA is not a promising label. ADA derivatives decompose fairly rapidly, especially in the presence of hydrogen peroxide.

In this study, CL detection was shown to be 10–50-fold more sensitive than fluorescence detection, which is in agreement with data reported by Hayakawa *et al.*[15]. In addition, the selectivity is distinctly better with the CL technique. Although other workers[7] using laser fluorescence detection have reported better detection limits (0.2–1 fmol) than those achieved in this work, the use of peroxyoxalate CL detection will often be preferable because of the relatively low cost of the detection system and the higher selectivity of the chemical excitation process.

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