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TRACE DETERMINATION OF ALIPHATIC AMINES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH CHEMILUMINESCENCE EXCITATION AND PHOTON COUNTING

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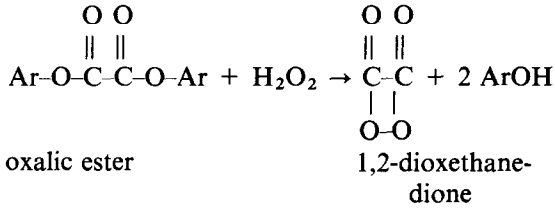
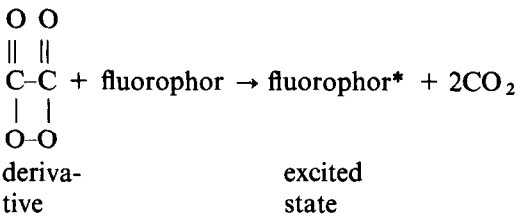
SUMMARY

Samples containing primary alkylamines ranging from *n*-butylamine to *n*-decylamine have been derivatized with 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dns-Cl), 4-chloro-7-nitrobenzo-1,2,5-oxadiazole (NBD-Cl) and *o*-phthaldialdehyde (OPT) to produce fluorescent derivatives. The products were analysed with a reversed-phase high-performance liquid chromatographic system in combination with a chemiluminescence detector. The Dns derivatives gave the lowest limits of detection, ranging from 0.8 fmol for *n*-butylamine to 14 fmol for *n*-decylamine. The corresponding values for the NBD derivatives were 19 and 270 fmol, respectively, and for the OPT derivatives 94 and 580 fmol. The generation and detection of chemiluminescence was performed with a novel, specially designed system using photon counting for measurement of the emitted light, which gives a far higher sensitivity than conventional fluorescence measurements.

INTRODUCTION

Chemiluminescence has recently been introduced as a new sensitive detection method for liquid chromatography[1–3]. It is similar to fluorescence detection in many respects but it is induced by a completely different mechanism. The method used in this work for chemical excitation is of the peroxylate type with the proposed mechanism, where Ar is 2,4,6-trichlorophenyl:

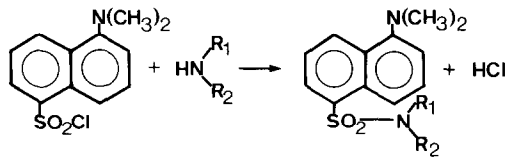




In the first step of the reaction, 1,2-dioxethanedione is generated and in the second, energy is transferred from it to the fluorophor. The sensitivity of the method is related to the energy required to excite the fluorophor, which determines the quantum yield. Important factors in this connection are the stereochemistry of the fluorophor and the position of its electronic energy levels.

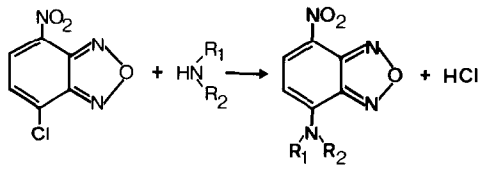
The chemiluminescence method has previously been successfully applied to 5-dimethylaminonaphthalene-1-sulphonyl (Dns) derivatives of catecholamines[1] and amino acids[1,2,4], and fluorescamine derivatives of catecholamines have been used[3] with similar results. An attempt by one of the present authors to analyse urea derivatives formed on reaction of 9-(N-methylaminomethyl)anthracene with aromatic isocyanates, however, gave very poor results and high detection limits. It is obvious that some fluorescent derivatives are better suited to chemiluminescence detection than others, and that it still remains to be investigated which these derivatives are.

In this work comparison is made between derivatives obtained with some widely used methods for derivatization of amines, including the reaction with Dns chloride (Dns-Cl), 4-chloro-7-nitrobenzo-1,2,5-oxadiazole (NBD-Cl) and *o*-phthaldialdehyde (OPT). The reaction of Dns-Cl with amines occurs according to the equation:



reagent Dns-Cl | reactant amine | derivative

The resulting product shows a green to yellow fluorescense with an emission maximum of 500–530 nm in organic solvents. The NBD-Cl reacts in a similar manner, forming a stable derivative:

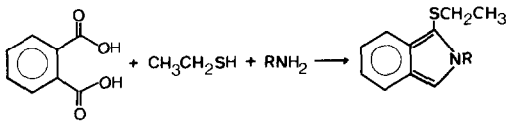


reagent NBD-Cl | reactant amine | derivative

In this case the emission maximum is shifted somewhat towards longer wavelengths

compared with the Dns derivatives, the maximum being at 530–550 nm. On visual inspection, the fluorescence appears to be brightly yellow. The excitation of NBD derivatives affords less energy than the other two kinds of derivative and the excitation maximum is at wavelengths as long as 460–480 nm. Unfortunately for conventional work the excitation and emission spectra overlap, which may cause a high background level.

OPT is somewhat special as it requires a second substance to give a fluorescent derivative:



reagent	co-reagent	reactant	derivative
OPT	ethanethiol	amine	

The reaction is very fast and is complete within 1 min at room temperature. Unfortunately the stability is poor, making it necessary to analyse the samples immediately after derivatization. The fluorescence shows an emission maximum at 400–450 nm.

EXPERIMENTAL

Apparatus

The apparatus used has been described in detail previously[1]. High-performance liquid chromatography (HPLC) was performed with a 200 × 5 mm I.D. column packed with Nucleosil C_{18} (5 μm) (Macherey, Nagel & Co., Düren, F.R.G.). The pump used was a LDC Model Constametric III HPLC pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) and the injector was a Rheodyne 7120 (Rheodyne, Berkeley, CA, U.S.A.), equipped with a 20-μl sample loop. The photon counter was developed and constructed by Auratronic Electronic Consultant (Stockholm, Sweden). It utilizes a Hammamatsu type R268UH-HA-P photomultiplier (PM) tube (Hamamatsu TV, Hamamatsu-City, Japan). Filters were manufactured by Carl Zeiss (Oberkochen/Würft, F.R.G.). The fluorescence detector was a Kontron labotron FFM 32 from Messtechnik (Gelting, F.R.G.). The system for light measurement was constructed at this laboratory[1].

Chemicals

n-Alkylamines were obtained from Fluka (Buchs, Switzerland), and dansyl chloride was from Sigma (St. Louis, MO, U.S.A.). Ethyl acetate, acetone, hydrogen peroxide, tris(hydroxymethyl)aminomethane (Tris) and OPT were obtained from E. Merck (Darmstadt, F.R.G.) and toluene of HPLC grade was supplied from the same source. Acetonitrile for HPLC and ethanethiol were from BDH (Parkstone, U.K.). NBD-Cl was obtained from Janssen Chimica (Beerse, Belgium). Bis-(2,4,6-trichlorophenyl)oxalate (TCPO) was prepared by the method of Mohan and Turro[5]. All water was doubly distilled.

Purification of chemicals

Analytical grade ethyl acetate and acetone were distilled using a 1-m filled column. The TCPO was recrystallized from HPLC-grade toluene, the solution being

treated with charcoal, and Tris was recrystallized from aqueous ethanol[6]. All reagents, including the TCPO and H_2O_2 solutions, were degassed carefully by means of evacuation, while being immersed in the bath of an ultrasonic cleaner.

Analytical conditions

In all determinations the flow-rate of the mobile phase was maintained at 1 ml/min. For the NBD and Dns derivatives a mobile phase of acetonitrile–0.05 *M* Tris · Hcl buffer, pH 7.7 (75:25, v/v) was found to be suitable. To achieve a reasonable time of elution, the acetonitrile contents were increased to 85% when the OPT derivatives were analysed. The TCPO was dissolved in ethyl acetate at a concentration of 5 m*M* and 30% (v/v) of aqueous H_2O_2 was diluted with acetone to a final concentration of 0.5 *M*. The flow-rates were 0.3 ml/min for the TCPO solution and 0.5 ml/min for the H_2O_2 reagent.

Sample preparation

Dns derivatives were prepared in the usual way using a weakly alkaline buffer to control the pH. A 1-ml volume of the sample and 1 ml of $NaHCO_3$ buffer (pH 8.5) were mixed with 5 ml of 0.1% (w/v) Dns-Cl in acetone. The reaction was complete after 10 min at 40°C, whereafter the samples were diluted to the appropiate concentration.

The NBD derivatives were prepared by mixing 500 μl of a 10 *M* solution of the amines with 100 μl of 0.1 *M* $NaHCO_3$ and 2 ml of 0.05% (w/v) NBD-Cl in ethanol. After 60 min at 55°C the derivatives were diluted to the desired concentration. A special syringe containing no teflon parts but only stainless steel and glass had to be used with the NBD derivatives, since PTFE seemed to adsorb the sample giving unexpected peaks in the chromatograms.

The OPT derivatives were prepared as follows. A 1-ml sample containing the amines of interest was mixed with 3 ml of a freshly prepared reagent consisting of 30 ml of 0.05 *M* borate buffer (pH 9.5), 500 μl of 1% (w/v) OPT-Cl in ethanol and 500 μl of 0.1% (v/v) ethanethiol in ethanol. The mixture was shaken vigorously for 1 min and then immediately injected on to the column.

Pure standards were prepared by preparative chromatography on a 500 × 8.3 mm I.D. column packed with 5 μm C_{18} material. The yield in the reaction with Dns–Cl was determined to 50%, and the reaction with NBD-Cl had an overall yield of 27%. The low stability of the OPT derivatives prevents their purification with this method; however, the yield was estimated to be quantitative. Light was excluded during all derivatization procedures.

Procedure

The system outline is shown in Fig. 1. It consists mainly of four parts, a conventional chromatographic system, equipment for delivery of TCPO and H_2O_2 reagents, a mixing device and a photon counter for light measurement. The TCPO and H_2O_2 solutions are pumped by forcing them through narrow capillary tubing with pressurized helium. This gives a pulse-free delivery, which is essential in order to achieve a low noise level. Then the solutions are mixed and 1,2-dioxethanedione is formed.

After the column, the eluate is mixed with the 1,2-dioxethanedione-containing

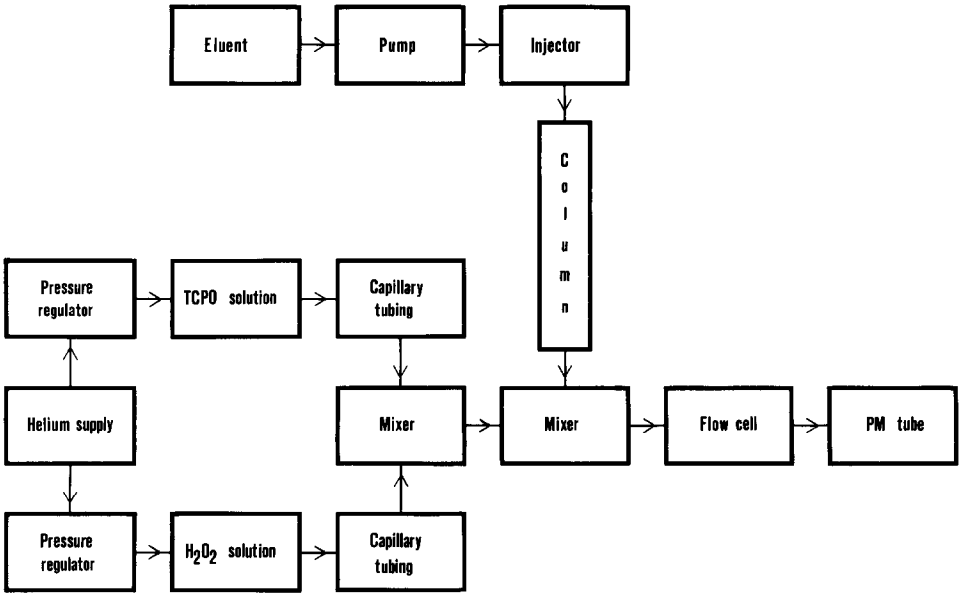


Fig. 1. Block diagram of the complete analytical system.

solution, and the flow is directed into a spiral-shaped flowcell mounted directely in front of the PM tube. Between the flowcell and the PM tube is an optical filter, which can be adapted to the kind of samples being analysed. Flowcell, filter, PM tube and a pre-amplifier are mounted in a carefully sealed stainless-steel box. Light is measured as soon as possible after the mixing, since the intensity declines very rapidly. By using photon counting, the weak light emitted can be accurately measured without any interference from dark current fluctuations. The result is registered on a recorder in a semi-continuous manner with a new reading every second.

Different optical filters were tested in order to achieve as good signal-to-noise ratios as possible. For the DNS derivatives a GG8 filter turned out to be the best choice, and for the NBD derivatives a GG10 filter was chosen. Finally a BG28 filter was used when analysing the OPT derivatives.

Experiments have also been performed using a conventional fluorescence detector instead of the chemiluminescence system, in order to make a comparison between the two methods. The chemiluminescence system was replaced with the fluorescence detector, but the rest of the system was the same. Excitation and emission wavelengths were chosen by the use of cut-off filters placed in the light path so that the lowest possible detection limits were reached.

RESULTS AND DISCUSSION

Detection limits for amines

It can be seen from Table I that the three kinds of derivative differ considerably in the detection limits. There is also a change in detection limit with the molecular weight of the amine. The detection limits were determined by extrapolating the calibration curve to twice the noise level.

TABLE I

DETECTION LIMITS OF ALIPHATIC AMINES USING DIFFERENT DERIVATIVES

C = Chemiluminiscence measurement; F = fluorescence measurement. Except for OPT, the detection limits are the true values corrected according to the yield of the reaction. The yield for OPT is assumed to be quantitative.

Amine	*Dns (fmol)*		*NBD (fmol)*		*OPT (fmol)*	
	C	*F*	*C*	*F*	*C*	*F*
n-Butylamine	0.8	140	19	170	94	2110
n-Pentylamine	1.4	180	19	220	58	3750
n-Hexylamine	3.8	220	19	220	400	4310
n-Heptylamine	6.5	250	30	200	220	4900
n-Octylamine	12	430	57	300	280	9260
n-Nonylamine	12	510	124	320	320	12,500
n-Decylamine	14	650	270	340	580	17,900

It was expected that the OPT derivatives would show a relatively weak chemiluminescence, since they require a higher excitation energy than the other derivatives. However, it is somewhat surprising to find that the NBD derivatives also give relatively poor detection limits, indeicating that other factors than the excitation energy are important in the chemiluminescence process.

A comparison between the detection limits for the chemiluminescence and a conventional fluorescence detector shows that the present method is between 50 and 175 times more sensitive than the conventional method when using the Dns derivatives. The difference is much smaller for the NBD derivatives (1.2–11 times more sensitive), as a consequence of the relatively low sensitivity for the NBD derivatives

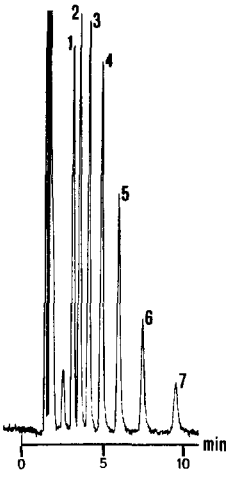


Fig. 2. Chromatogram of NBD-amine derivatives. Injected volume, 20 μl; sample concentration: $1.9 \cdot 10^{-7}$ *M*. The peaks are derivatives of (1) *n*-butyl-, (2) *n*-pentyl-, (3) *n*-hexyl-, (4) *n*-heptyl-, (5) *n*-oxtyl-, (6) *n*-nonyl- and (7) *n*-decylamine. The chromatographic conditions are given in the text.

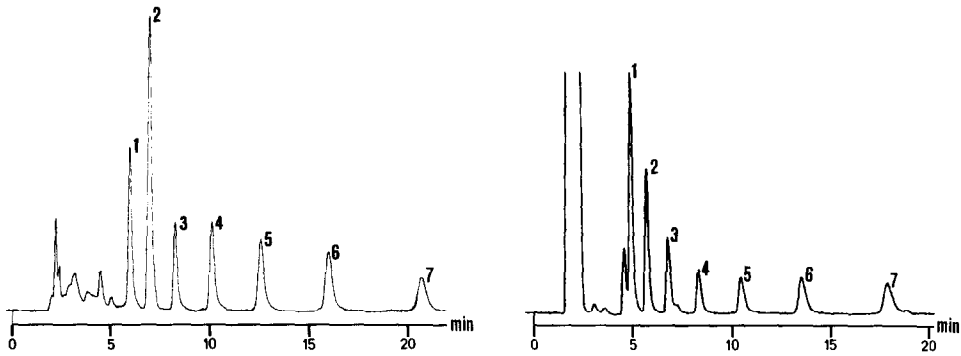


Fig. 3. Chromatogram of OPT-amine derivatives. Injected volume, 20 μl; sample concentration, $7.5 \cdot 10^{-7}$ *M*. Peaks as in Fig. 1. The chromatographic conditions are given in the text.

Fig. 4. Chromatogram of Dns-amine derivatives. Injected volume, 20 μl; sample concentration, $1.4 \cdot 10^{-8}$ *M*. Peaks as in Fig. 1. The chromatographic conditions are given in the text.

in the chemiluminescence system. For the OPT derivatives again, the difference is greater, the chemiluminescence method being between 20 and 40 times more sensitive.

Chromatographic parameters

From Figs. 2–4 it can be seen that the NBD derivatives offer the fastest separation of the test substances, whereas DNS and OPT derivatives take longer. A plot of log capacity factor (k') *versus* the number of carbon atoms in the alkyl chain (Fig. 5) shows a linear relationship as would be expected. This indicates a normal retention behaviour in spite of the low concentrations used, and a low adsorption to the packing material. Of the tested derivatives, Dns derivatives seem to be the best alternative especially from a sensitivity point of view. However, a direct derivatization of a very

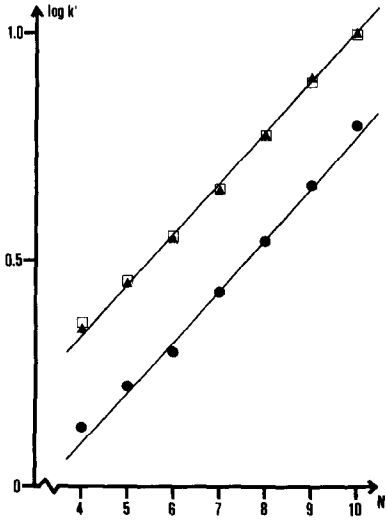


Fig. 5. Correlation between k' and the number of carbon atoms (N). ●, NBD derivatives; ▲, Dns derivatives; □, OPT derivatives.

dilute amine solution with Dns requires thorough attention to the removal of excess reagent.

OPT gives very pure derivatives which can be injected directly without removing the regent even at extremely low concentrations. A disadvantage is, however, the comparatively low sensitivy for the derivatives and the unpleasant odour of the ethanethiol reagent.

During the work with the chemiluminescence method, it was observed that the background has a tendency to rise slowly until it finally affects the performance of the system. Rinsing of the column with pure ethanol followed by a re-equilibration with the mobile phase cures this phenomenon and restores the baseline. The procedure has to be repeated about once every week.

CONCLUSIONS AND FURTHER WORK

The present peroxylate chemiluminescence method seems to be applicable to the assay of many different kinds of fluorescent substances. The sensitivity of the method appears to rely heavily on the choice of regent for forming the fluorescent derivative, which choice therefore is of highest importance. On that account, it is intended to study the applicability of the present method to the detection of other kinds of organic compound, *viz.* to carbonyl compounds after conversion into fluorescent derivatives with 4-bromomethyl-7-methoxycoumarin and to reducing sugars after reaction with Dns-hydrazine.

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