Vol. 170 No. 1, April 1, 1985 ISSN 0003-2670

International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences, Gent, Belgium, September 3–6, 1984

ANALYTICA CHIMICA ACTA

International journal devoted to all branches of analytical chemistry

ELSEVIER

ANALYTICA CHIMICA ACTA

International journal devoted to all branches of analytical chemistry
Revue internationale consacrée à tous les domaines de la chimie analytique
Internationale Zeitschrift für alle Gebiete der analytischen Chemie

PUBLICATION SCHEDULE FOR 1985

	J	F	M	A	M	J	J	A	S	O	N	D
Analytica Chimica Acta	167	168	169	170/1 170/2	171	172	173	174	175	176	177	178

Scope. *Analytica Chimica Acta* publishes original papers, short communications, and reviews dealing with eve aspect of modern chemical analysis, both fundamental and applied.

Submission of Papers. Manuscripts (three copies) should be submitted as designated below for rapid and efficie handling:

Papers from the Americas to: Professor Harry L. Pardue, Department of Chemistry, Purdue University, West Lafayet IN 47907, U.S.A.

Papers from all other countries to: Dr. A. M. G. Macdonald, Department of Chemistry, The University, P.O. Box 3(Birmingham B15 2TT, England. Papers dealing particularly with computer techniques to: Professor J. T. Cle Universität Bern, Pharmazeutisches Institut, Baltzerstrasse 5, CH-3012 Bern, Switzerland.

Submission of an article is understood to imply that the article is original and unpublished and is not being consider for publication elsewhere. Upon acceptance of an article by the journal, authors will be asked to transfer the copyrig of the article to the publisher. This transfer will ensure the widest possible dissemination of information.

Information for Authors. Papers in English, French and German are published. There are no page charg Manuscripts should conform in layout and style to the papers published in this Volume. Authors should cons Vol. 160 for detailed information. Reprints of this information are available from the Editors or from: Elsevier Editor Services Ltd., Mayfield House, 256 Banbury Road, Oxford OX2 7DH (Great Britain).

Reprints. Fifty reprints will be supplied free of charge. Additional reprints (minimum 100) can be ordered. An orc form containing price quotations will be sent to the authors together with the proofs of their article.

Advertisements. Advertisement rates are available from the publisher.

Subscriptions. Subscriptions should be sent to: Elsevier Science Publishers B.V., Journals Department, P.O. B 211, 1000 AE Amsterdam, The Netherlands. Tel: 5803 911, Telex: 18582.

Publication. *Analytica Chimica Acta* appears in 12 volumes in 1985. The subscription for 1985 (Vols. 167–178) Dfl. 2400.00 plus Dfl. 264.00 (p.p.h.) (total approx. US $986.70). All earlier volumes (Vols. 1–166) except Vols. and 28 are available at Dfl. 215.00 (US $79.60), plus Dfl. 15.00 (US $5.60) p.p.h., per volume.

Our p.p.h. (postage, packing and handling) charge includes surface delivery of all issues, except to subscribers in t U.S.A, Canada, Japan, Australia, New Zealand, China, India, Israel, South Africa, Malaysia, Singapore, South Kor Taiwan, Pakistan, Hong Kong and Brazil who receive all issues by air delivery (S.A.L. — Surface Air Lifted) at no ex cost. For the rest of the world, airmail and S.A.L. charges are available upon request.

Claims for issues not received should be made within three months of publication of the issues. If not they cannot honoured free of charge.

For further information, or a free sample copy of this or any other Elsevier Science Publishers journal, readers in t U.S.A. and Canada can contact the following address: Elsevier Science Publishing Co. Inc., Journal Informati Center, 52 Vanderbilt Avenue, New York, NY 10017, U.S.A., Tel: (212) 916-1250.

ANALYTICA CHIMICA ACTA
VOL. 170 (1985)

ANALYTICA CHIMICA ACTA

International journal devoted to all branches of analytical chemistry

ELSEVIER Amsterdam–Oxford–New York–Tokyo

0003-2670/85/$03.30

Printed in The Netherlands

SPECIAL ISSUE

Proceedings of the International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences, Gent, Belgium, September 3–6, 1984

Analytica Chimica Acta, 170 (1985) 1
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

Foreword

The first International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences was held at the Faculty of Pharmaceutical Sciences, State University of Ghent, Belgium, on September 3—6, 1984. Research into luminescence techniques has shown tremendous growth in recent years; such techniques clearly offer great potential for rapid quantitative work. The Symposium enabled the current state of methodology to be seen in perspective and the many problems and challenges to be identified for further research.

This issue of *Analytica Chimica Acta* contains a selection of the papers that were presented at the Symposium; plenary lectures will be published separately in *Pure and Applied Chemistry.* It is to be hoped that these publications will stimulate interest in both fundamental and analytically applied luminescence spectrometry.

The Organizing Committee take this opportunity to thank all those who contributed to the success of the Symposium.

Willy R. G. Baeyens,
Symposium Chairman.

0003-2670/85/$03.30

Analytica Chimica Acta, 170 (1985) 3—12
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

ROOM-TEMPERATURE PHOSPHORESCENCE, SENSITIZED PHOSPHORESCENCE AND FLUORESCENCE OF LICIT AND ILLICIT DRUGS ENHANCED BY ORGANIZED MEDIA

L. J. CLINE LOVE*, MARY LYNN GRAYESKI and JOYCE NOROSKI
Seton Hall University, Department of Chemistry, South Orange, NJ 07079 (U.S.A.)
ROBERT WEINBERGER
Kratos Analytical Instruments, Ramsey, NJ 07446 (U.S.A.)
(Received 29th October 1984)

SUMMARY

The ability of microscopically organized media, in the form of surfactant micelles and α- and β-cyclodextrins, to enhance the luminescence phenomena of several licit and illicit drugs is discussed. Because physiological samples are not often amenable to direct spectrometric measurements without pretreatment, the applicability of these organized media to liquid chromatography is also considered. Fluorescence enhancements for certain hallucinogenic drugs such as *N,N*-dimethyltryptamine, mescaline and ibogaine are seen in cyclodextrin media compared to conventional, homogeneous solutions. Heavy-atom substituted sodium dodecyl sulfate micelles induce phosphorescence from cationic and/or hydrophobic drugs at room temperature in fluid solution; drugs such as propranolol, diflunisal, naphalozine, and selected quinoline derivatives can be determined conveniently. Sensitized phosphorescence is observed for several drugs including brethine, cocaine, didrate, estradiol, meprobarbital, methaqualone, phenobarbital, and sulfanilamide; it can be enhanced markedly when micellar solutions are used as the solvent. The energy-transfer step is facilitated by the organizing ability of the micelle; limits of detection can be decreased by over two orders of magnitude compared to homogeneous solvents. Sensitized phosphorescence can also be measured in cyclodextrin solutions, but the detectability is inferior to that in micellar media. Which form of organized medium is superior for determinations of drugs is discussed.

As the analytical limits of conventional luminescence instrumentation are approached, chemical manipulation of analytes is being attempted in order to improve sensitivity and/or selectivity of the analysis. In liquid chromatography (l.c.), pre- or post-column derivatization of the analyte can be used [1, 2], and more recently chemiluminescence detection has been shown to be valuable [3]. These procedures can be complicated instrumentally or time-consuming, and alternative, simpler means of enhancement are often preferred. Chemical species capable of organizing lumiphors on a molecular level have been employed in recent years to produce enhanced intensities or drastically altered excited-state lifetimes, and to induce remarkably intense triplet-state emission in fluid solution at ambient temperature [4—7]. Surfactant molecules such as sodium dodecyl sulfate (SDS) form

0003-2670/85/$03.30

micellar aggregates in solution which selectively solubilize many hydrophobic lumiphors with a resultant increase in fluorescence intensities [8, 9]. In the presence of added, external heavy atoms, triplet-state emission has been observed for several phosphors. Cyclodextrins form inclusion complexes with numerous lumiphors, producing enhanced fluorescence intensities, and phosphorescence if an external heavy atom is also included [10—12]. Recent studies have shown that both micellar and cyclodextrin media not only enhance the phosphor—heavy atom interaction, but can enhance triplet—triplet energy transfer from an analyte donor to acceptor emitter with considerable improvements in limits of detection [13].

This paper describes the present status of the use of various organized media applied to the determination of several licit and illicit drugs. The discussion focuses on only those methods employed at room temperature in fluid solution, and gives a perspective on the ultimate utility of these approaches for various types of drugs. The ultimate goal of this work is to define techniques that utilize organized media to provide both chromatographic separations and enhanced luminescence detection [14, 15]. The merits and limitations of each of these combined techniques for determinations of drugs are outlined.

EXPERIMENTAL

Reagents and apparatus

Sodium dodecyl sulfate (Aldrich) was used as received. The α- and β-cyclodextrins were recrystallized once from boiling water, and the γ-cyclodextrin was used as received (all from Aldrich). The 1,2-dibromoethane (Aldrich) degraded upon distillation, so was used as received. Biacetyl (Merck) was used as received. Most of the drugs were used without further purification. All hallucinogens were obtained from Sigma Chemical Co. All solvents were spectroscopic grade (Fisher), and all solutions were prepared with twice-distilled deionized water.

All luminescence spectra were obtained with a Fluorolog 2 + 2 spectrofluorimeter (SPEX Industries, Metuchen, NJ) equipped with double excitation and emission monochromators. The light source was a 450-W xenon continuous lamp, and detection was accomplished with a Peltier-cooled Hammamatsu R928 photomultiplier (PMT). The SPEX data computer corrected all spectra for variations in lamp intensity and PMT response. Print-outs of spectra were obtained on a Houston Instruments x-y plotter. A Varian Model 2200 spectrophotometer was used for all absorbance measurements.

The l.c. system consisted of a high-pressure pump (Technicon FAST.LC), and a sample injector with a 20-μl loop (Rheodyne, Model 7120). A fluorimetric detector (Kratos Instruments, Model FS-970) was used for both fluorescence and phosphorescence detection of eluates. The experimental conditions are given in the figure captions. In order to minimize dissolution

of the analytical column packing, a precolumn (12.5 × 4.6 mm i.d.; Whatman) packed with silica gel (25—40 μm) was placed between the pump and the sample injector to saturate the mobile phase with silica. Chromatograms were recorded on a Fisher Recordall Model 5000 strip-chart recorder (Fisher Scientific, Springfield, NJ).

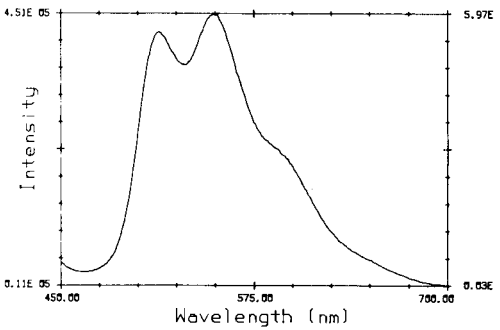
Procedure

All glassware was rinsed with methanol and baked in an oven prior to use. For phosphorescence measurements, solutions were deaerated for 20 min with high-purity nitrogen passed through an indicating oxygen trap, and sealed with a teflon stopper. Samples were not deaerated for phosphorescence-detected l.c. because the oxygen separates from the phosphor and elutes at the solvent front. The mobile phase was routinely deaerated. Exact procedures for preparation of analyte solutions have been described previously [6, 11, 15].

RESULTS AND DISCUSSION

Micelle-stabilized luminescence

Heavy-atom substituted SDS micelles induce phosphorescence at room temperature in aqueous solution from drugs with little solubility in water, and are a convenient means for determinations of drugs in body fluids. Drugs such as diflunisal (with time gating), propranolol, naphalozine, and selected quinoline derivatives exhibit micelle-stabilized room-temperature phosphorescence (r.t.p.) that is useful for therapeutic drug monitoring for certain species. The micelle-stabilized r.t.p. spectrum of naproxen, a non-steroidal anti-inflammatory drug, is shown in Fig. 1. Because micelle-stabilized r.t.p. permits the use of fluid solutions at ambient temperature, phosphorescence can be used with conventional fluorimeters for detection



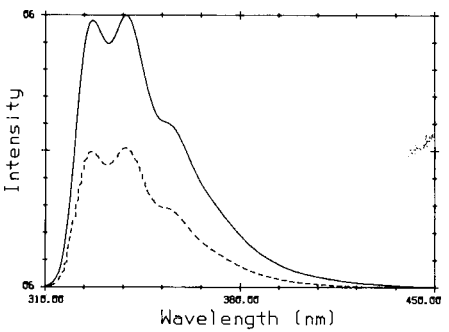


Fig. 1. Micelle-stabilized r.t.p. spectrum of 3 × 10^{-5} M naproxen in 0.1 M Tl/NaDS (30:70). Excitation wavelength, 331 nm; slits, 3.6 nm.

Fig. 2. Fluorescence enhancement of 1 × 10^{-5} M propranolol: (—) in 0.1 M SDS; (---) in methanol. Excitation wavelength, 295 nm.

of phosphors in liquid chromatography [16]. The advantage of this is increased selectivity, because not all drugs that fluoresce will phosphoresce, and the emission is red-shifted to a less-crowded spectral region. This latter point is very useful in the analysis of body fluids where the sample matrix produces fluorescence emission background that can severely reduce the signal-to-noise ratio and limit sensitivities. Micellar chromatography with phosphorescence detection also aids the spectroscopy by separating quenchers from the phosphor (including oxygen present in the sample), thus simplifying sample preparation.

Micelles also can increase fluorescence intensities of drugs by solubilizing them in a more favorable micro-environment. This can produce changes in both the molar absorptivity of the molecules and the quantum efficiency, with concomitant improvement in limits of detection. Figure 2 shows the fluorescence enhancement observed for propranolol in both SDS anionic micellar solution and in methanol, illustrating a 10-fold increase in intensity.

Fluorescence detection has been used for the determination of drugs such as quinidine, morphine, propanolol, and quinine in body fluids with relative standard deviations generally less than 10% [17]. Figure 3 contains chromatograms of 200 ng ml^{-1} of quinine and quinidine added to urine, and the blank, with micelles as the mobile phase for the separation. Detection limits with fluorescence detection are well within the range used in routine therapeutic drug monitoring (0.01—0.4 μg ml^{-1}), and retention times are typically <10 min. The use of micelles in high-performance l.c. with fluorescence or phosphorescence detection, serving as both the chromatographic mobile phase and the spectroscopic signal enhancer, appears to be the deciding factor influencing the choice of which organized media (cyclodextrin or micelles) should be used for drug determinations at this time.

Because of the hydrophobic character of the micellar assembly, most drugs that are highly soluble in water do not undergo favorable partitioning into or onto the micelle, and reside primarily in the bulk aqueous solvent unless favorable electrostatic effects are present (e.g., cationic drug attracted by anionic micelle). Some drugs are actually repelled by similarly charged micelles, e.g., phenobarbital and cocaine in SDS. This usually precludes observation of phosphorescence for most nitrogen heterocycles, and far fewer cases of fluorescence enhancements have been reported. One recent report on micelle-stabilized r.t.p. of nitrogen heterocycles demonstrated that phenazine and acridine do phosphoresce but only in the presence of external silver(I) ions [18]. It was found that, generally, silver serves two roles in inducing r.t.p. from nitrogen-containing aromatics; in one, silver serves as a heavy atom promoting spin-orbit coupling, and in the second, it serves to complex the pyridinic nitrogen, causing it to reside near the micellar surface. However, silver-containing micelles cannot be used as mobile phases because of deposition problems.

The low binding constants of hydrophilic drugs with normal micelles that prevent positive micellar enhancements of luminescence can be overcome

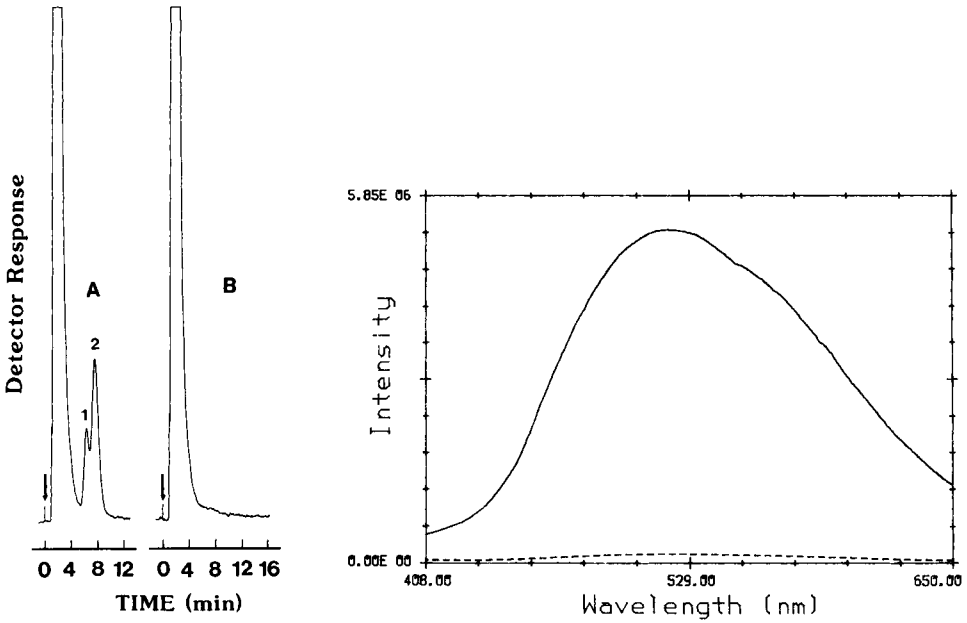


Fig. 3. Fluorescence-detected chromatograms: (A) 200 ng ml^{-1} quinine (1) and quinidine (2); (B) the blank. Chromatographic conditions: column, Supelcosil-CN; mobile phase, 0.05 M SDS; flow rate, 1 ml min^{-1}; detector voltage, 700 V; sensitivity range, 0.05 μA; excitation wavelength, 260 nm; emission cut-off filter, 300 nm.

Fig. 4. Fluorescence enhancement for 5×10^{-5} M 5-aminothiabendazole: (—) spectrum in 0.12 M AOT with $[H_2O]/[AOT] = 9.0$ (pH 6.5 for added water); (---) spectrum in pH 6.5 water. Excitation wavelength, 300 nm; slits, 8 nm excitation, 4 nm emission.

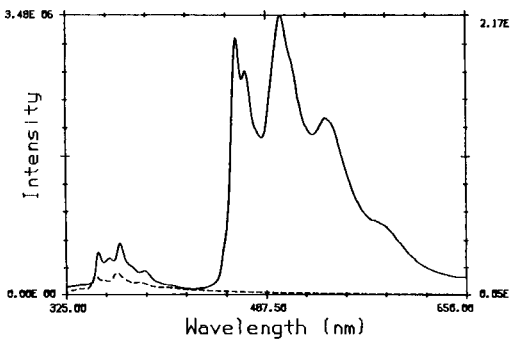
by using reversed micellar systems. These surfactants, e.g., sodium bis(2-ethylhexyl)sulfosuccinate (Aerosol-OT or AOT), aggregate in apolar solvents with their charged headgroup orientated inwards into a small water pool, and their hydrophobic tails extending outwards in the bulk solvent. Thus, polar molecules partition favorably into the water core/interfacial region and pronounced changes in the luminescence characteristics can be observed. A good example of these effects is seen for benzimidazole analogs, in particular thiabendazole, a human and veterinary anthelmintic drug and food fungicide, and its major metabolite, 5-hydroxythiabendazole. This family of analogs is interesting spectroscopically because the type and strength of transitions observed (π-π* of free base and dication, and charge transfer of monocation) depend on the immediate micro-environment of the lumiphor in terms of pH, polarity and viscosity [19]. The properties of the water core vary greatly depending on the amount of water, and this can be used to induce the desired transition instead of pH or polarity adjustments. For example, at low water content in AOT micelles, 5-aminothiabendazole undergoes a charge-transfer transition at 341 nm characteristic of the

dication, but at higher water content undergoes a charge-transfer transition at 520 nm characteristic of the monocation. The intensity of the latter transition is enhanced 40-fold over that in water by incorporating the molecule into an inverted micelle (Fig. 4). The ability of the micellar microenvironment to mimic more stringent chemical conditions without detrimental effects on the analyte could be a decided advantage in many analyses.

Cyclodextrins

When drug molecules are included into a cyclodextrin cavity, water is expelled and strong hydrophobic interactions within the cavity produce stable inclusion complexes. The three major cyclodextrins (α, β and γ) differ in the diameter of their cavities, thus allowing some selectivity based on the molecular size of the drug. Only molecules at least partially included within the cavity can be induced to phosphoresce. Numerous carbocyclic and heterocyclic compounds form stable complexes with cyclodextrins and, in the presence of a heavy atom-containing species such as 1,2-dibromoethane, emit intense phosphorescence at ambient temperatures. Molecules too large to fit into the β-cyclodextrin cavity (e.g., 1-phenylnaphthalene) will not emit, whereas naphthalene fits easily into the cavity and emits strongly. Figure 5 shows an example of the effect of cavity size, where phenanthrene phosphoresces in β-cyclodextrin, but does not in the smaller α-cyclodextrin which cannot include the molecule.

In addition to size, the hydrophobicity of the drug establishes the strength of the complex; more hydrophilic species form weaker complexes producing faint emission signals. The phosphorescence spectrum of naproxen in β-cyclodextrin is shown in Fig. 6. Peaks appear on the tail of the fluorescence,



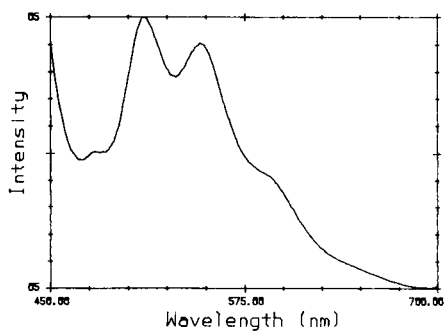


Fig. 5. Corrected luminescence spectra of 5×10^{-5} M phenanthrene illustrating greatly enhanced phosphorescence in β-cyclodextrin at the longer wavelengths: (—) in 0.01 M β-cyclodextrin; (---) in 0.01 M α-cyclodextrin. Excitation wavelength, 300 nm; slits, 14.4 nm excitation, 3.5 nm emission; scan rate, 1 nm s^{-1}; [1,2-dibromoethane] = 0.58 M.

Fig. 6. Phosphorescence of 3×10^{-5} M naproxen in 0.01 M β-cyclodextrin with 0.58 M 1,2-dibromoethane added. Excitation wavelength, 331 nm; slits, 8 nm excitation, 2 nm emission.

with poorer resolution and lower phosphorescence/fluorescence intensity ratio compared to that seen in micellar media because of the weak inclusion in cyclodextrin (cf. Fig. 1). The size constraint and required hydrophobic match will limit the applicability of cyclodextrin media in drug determinations. In the absence of an external heavy atom, the fluorescence intensity of certain drugs can be enhanced [10]. The fluorescence enhancements observed for selected hallucinogens are given in Table 1. Although these molecules are polar, enhancements of 1.2—4.0 were observed in aqueous β-cyclodextrin solutions, and the mescaline derivatives, which are extremely weak fluorophors in homogeneous solution, gave limits of detections of 0.8—1.4 $\mu g\ ml^{-1}$ by this technique.

Sensitized phosphorescence in organized media

By exciting a donor analyte molecule that undergoes appreciable intersystem crossing to the triplet state, and in the presence of an appropriate acceptor species (e.g., biacetyl), phosphorescence can be observed that is characteristic of the acceptor, with an intensity proportional to the concentration of the donor drug species. Examples of drugs that sensitize biacetyl emission are brethine, cocaine, didrate, estradiol, meprobarbital, methaqualone, phenobarbital, purine and sulfanilamide.

The prerequisites for sensitized phosphorescence can be summarized as follows: (1) the donor triplet radiative rate must be less than the energy transfer rate; (2) E_T (donor) $> E_T$ (acceptor); (3) the donor triplet state must be appreciably populated, and the donor must not be excited at a wavelength where the acceptor absorbs; (4) donor and/or acceptor triplet lifetimes must be less than the residence time in the micelle or cyclodextrin; and (5) the analyte and acceptor must associate with the cyclodextrin or micelle within approximately the same time frame. By dissolving the drug donor and biacetyl acceptor in micellar or cyclodextrin media, pronounced enhancement of sensitized phosphorescence intensity occurs for carbocyclic species [13]. This is illustrated for naphthalene in Fig. 7 showing sensitized phosphorescence in homogeneous acetonitrile, β-cyclodextrin and SDS micelles. For example, the limit of detection for naphthalene decreases over three

TABLE 1

Ratios of integrated areas of fluorescence emission in cyclodextrin[a]/water and limits of detection for selected hallucinogens

Compound	Emission ratios		Detection limit[b] ($ng\ ml^{-1}$)
	α-CD/water	β-CD/water	
N,N-Dimethyltrytamine	1.2	1.4	13
Mescaline hemisulfate	2.2	2.5	1419
Ibogaine hydrochloride	2.6	4.0	6
Mescaline hydrochloride	1.2	1.8	832

[a]CD = cyclodextrin. [b]Signal = 3× noise.

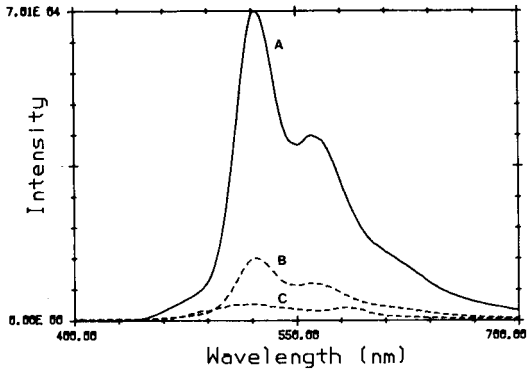


Fig. 7. Corrected phosphorescence spectra of 1.0×10^{-4} M biacetyl sensitized by 1.2×10^{-5} M naphthalene in (A) SDS; (B) β-cyclodextrin; (C) acetonitrile. Excitation wavelength, 276 nm; slits, 14.4 nm excitation, emission; 470 cutoff filter; scan rate, 1 nm s^{-1}.

orders of magnitude in SDS micelles compared to that found in homogeneous acetonitrile solution. The ability of micelles to bring the donor and acceptor molecules into close proximity for efficient energy transfer and partial protection of the excited phosphor provides evidence of the power of organized media in analytical schemes. Less dramatic results are found for heterocyclic species such as quinoline, lepidine, etc., because of the decreased partitioning of more water-soluble species into the micelle. The barbiturates would not be expected to exhibit intensity enhancement in micellar medium because of electrostatic repulsion.

Several carbocyclic and heterocyclic species can be included into cyclodextrin cavities, where complexation produces a decrease of about an order of magnitude in the sensitized phosphorescence limits of detection compared to homogeneous medium. The solubility of the donor in water appears to be less important in the formation of inclusion complexes of drug and biacetyl, in contrast to micellar media. However, in view of the lower enhancement by β-cyclodextrin, its use as an organized medium for determination of drugs appears to be of limited value at present.

Perspective on drug determinations via organized media

From the requirements listed in Table 2 for room-temperature luminescence emission from drugs, one can formulate criteria for probable success and eventual usefulness of micelles and cyclodextrins in the analysis of body fluids for drugs. Hydrophobic molecules will generally strongly associate with both micelles and cyclodextrins, but an additional constraint on physical size of the lumiphor in the cyclodextrin will limit its applicability to small to moderately sized drugs. However, this aspect can offer selectivity in some applications. Although the γ-cyclodextrin extends the technique to larger species because of its large interior cavity, its cost will probably be prohibitive for many spectroscopic applications, and will certainly be

TABLE 2

Advantages and limitations of organized media for drug analysis

Cyclodextrins
For molecules which are complexed, markedly enhanced fluorescence and phosphorescence intensities are observed.
Chromatographic properties are not well established.
Relative aqueous solubility of the cyclodextrin is often limiting.
Steric problems prevent inclusion of large drugs.
Extreme hydrophobic effects exclude even moderately water-soluble molecules.
Drug and heavy-atom species must occupy the cyclodextrin cavity during the same time frame.
Excited drug must emit before it leaves the cavity and is quenched.
Micelles
Hydrophobic effect is less constraining than for cyclodextrin.
Size effect is unimportant.
Charge selection is possible to offer better selectivity.
Chromatography is well established.
Drug must associate appreciably with the micellar aggregate.
Excited drug must emit before it leaves the micelle.

prohibitive for chromatographic methods. Both of these media require that drug—substrate association be strong enough for emission to occur before the analyte and associated reagents (heavy atom species, biacetyl, etc.) partition out of the aggregate and the emission is quenched.

At present, cyclodextrin-enhanced spectroscopy and chromatography are less well understood than micelle-mediated events, and have more constraints and fewer decided advantages than micelles for drug determinations. Although initial reports on cyclodextrin-enhanced r.t.p. pointed out several unique characteristics of cyclodextrins, such as partial insensitivity to oxygen quenching and excellent sensitivity, these can be taken advantage of only for those molecules meeting the criteria listed in Table 2. For drugs which meet these criteria, one would expect better sensitivities and selectivities than those obtained from homogeneous solution.

For drug determinations, micelles offer a more versatile and generally more applicable medium for both enhanced spectroscopy and chromatography and, although not size-selective like the cyclodextrins, they offer charge selection through the choice of anionic, cationic, neutral, or zwitterionic surfactants, and polarity selection through the choice of normal (aqueous) micelles or reversed (apolar) micelles. Micellar chromatography is emerging as a competitive alternative to conventional h.p.l.c., with some interesting advantages such as speed in the analysis of complex mixtures. Although both media have been used for phosphorescence detection in h.p.l.c., only micelle-sensitized r.t.p. detection will be practical until the problems of low efficiency with cyclodextrin chromatography are solved [20]. The micellar medium offers definite advantages over cyclodextrin medium when coupled with sensitized phosphorescence, which suggests that

it will generally be better in organizing other types of reactants for enhanced luminescence spectroscopy.

The support of the National Science Foundation grant No. CHE-8216878 to LJCL is gratefully acknowledged.

REFERENCES

1 R. W. Frei, in R. W. Frei and J. F. Lawrence (Eds.), Chemical Derivatization in Analytical Chemistry, Vol. 1, Plenum Press, New York, 1981, p. 211.
2 L. A. Sternson, in R. W. Frei and J. F. Lawrence (Eds.), Chemical Derivatization in Analytical Chemistry, Vol. 1, Plenum Press, New York, 1981, p. 127.
3 I. Kobayashi and K. Imai, Anal. Chem., 52 (1980) 424.
4 J. R. Escabi-Perez, F. Nome and J. H. Fendler, J. Am. Chem. Soc., 99 (1977) 7749.
5 K. Kalyanasundaram, F. Grieser and J. K. Thomas, Chem. Phys. Lett., 51 (1977) 501.
6 L. J. Cline Love, M. Skrilec and J. G. Habarta, Anal. Chem., 52 (1980) 754.
7 L. J. Cline Love, J. G. Habarta and M. Skrilec, Anal. Chem., 53 (1981) 437.
8 W. L. Hinze, in M. L. Mittal (Ed.), Solution Chemistry of Surfactants, Vol. 1, Plenum Press, New York, 1979, p. 79.
9 L. J. Cline Love, J. G. Habarta and J. G. Dorsey, Anal. Chem., 56 (1984) 1132A.
10 O. Jules, S. Scypinski and L. J. Cline Love, Anal. Chim. Acta, 169 (1985) 355.
11 S. Scypinski and L. J. Cline Love, Anal. Chem., 56 (1984) 322.
12 S. Scypinski and L. J. Cline Love, Anal. Chem., 56 (1984) 331.
13 F. DeLuccia and L. J. Cline Love, Anal. Chem., 56 (1984) 2811.
14 W. L. Hinze, Sep. Purif. Methods, 10(2) (1981) 159.
15 M. Arunyanart and L. J. Cline Love, Anal. Chem., 56 (1984) 1557.
16 R. Weinberger, P. Yarmchuk and L. J. Cline Love, Anal. Chem., 54 (1982) 1552.
17 B. Gerson and J. P. Anhalt, High-Pressure Liquid Chromatography and Therapeutic Drug Monitoring, American Society of Clinical Pathologists, Chicago, IL, 1980.
18 R. J. Woods and L. J. Cline Love, Spectrochim. Acta, Part A, 40 (1984) 643.
19 P. C. Tway and L. J. Cline Love, J. Phys. Chem., 86 (1982) 5223, 5227.
20 S. Scypinski, Ph.D. dissertation, Seton Hall University, South Orange, NJ, 1984.

Analytica Chimica Acta, 170 (1985) 13—20
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

DETERMINATION OF 6-ACETYLMORPHINE IN URINE AS A SPECIFIC MARKER FOR HEROIN ABUSE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

H. J. G. M. DERKS*, K. VAN TWILLERT and G. ZOMER

Rijksinstituut voor Volksgezondheid en Milieuhygiene, Postbus 1, 3720 BA Bilthoven (The Netherlands)

(Received 5th September 1984)

SUMMARY

A sensitive method is described for the determination of 6-acetylmorphine in urine, to detect the use of heroin by drug addicts participating in a morphine-dispensing experiment. The method is based on an extraction procedure, reaction of the isolated 6-acetylmorphine with excess of morphine to give a highly fluorescent mixed dimer, and determination by normal-phase high-performance liquid chromatography (h.p.l.c.) with fluorescence detection. The calibration graph was linear at low 6-acetylmorphine concentrations and the absolute detection limit was 0.4 ng. In urine samples, 6 $\mu g\ l^{-1}$ of 6-acetylmorphine could be detected. A reversed-phase system is also outlined.

In 1983, the Municipal Health Department (GG & GD) of the City of Amsterdam started an experiment in which extremely problematic drug addicts were provided with injectable morphine on a daily basis. As part of a concomitant evaluation study, done in cooperation with the local health authorities, it was decided to investigate whether the participating addicts continued the use of other drugs such as heroin, metaqualone and benzodiazepines. In addition, the morphine ampoules supplied contained a marker substance which permitted their proper use to be checked by urine analysis.

As no suitable method for the selective detection of heroin usage via urine analysis was available, such a method had to be developed. In order to discriminate between heroin abuse and the use of dispensed morphine, it was necessary to develop a method that would not rely on the detection of morphine, which is the principal metabolite of heroin. Several contaminants of street heroin or their metabolites were considered, but the only safe marker for heroin appeared to be 6-acetylmorphine. This compound is formed in vivo very rapidly after heroin administration, but is almost entirely converted into morphine subsequently; 1—2% of the dose is excreted as 6-acetylmorphine during the 40.5 h following intravenous heroin administration [1], either as the free compound or as its glucuronic acid conjugate [2].

In order to have a reasonable chance of detecting heroin abuse under these conditions, the method to be developed had to be extremely sensitive.

0003-2670/85/$03.30

Therefore, it was decided to devise a procedure based on high-performance liquid chromatography (h.p.l.c.) with fluorescence detection. To prepare a fluorescent derivative of 6-acetylmorphine, the oxidation method developed by Takemori [3] for morphine was used. In this method, the opiate is converted to a highly fluorescent dimer by phenolic coupling induced by a mild oxidant. The same principle was used by Jane and Taylor [4] in an h.p.l.c. method for morphine.

EXPERIMENTAL

Chemicals and equipment

All reagents were analytical grade. Water was distilled twice from pyrex glass.

6-Acetylmorphine was prepared from morphine as follows. Morphine hydrochloride (190 mg) was dissolved in a mixture of 20 ml of dry pyridine and 10 ml of acetic anhydride. After reaction for 48 h at room temperature, the reagents were evaporated under reduced pressure. Trituration of the residue with diethyl ether yielded a light yellow product, which was dissolved in 40 ml of a saturated sodium hydrogencarbonate solution. This solution was extracted with one 40-ml and three 20-ml portions of chloroform. The combined organic phase was dried with anhydrous sodium sulphate and after evaporation of the chloroform, 160 mg of heroin base was obtained [n.m.r. δ(ppm): 2.12 (s,3H), 2.25 (s,3H), 2.42 (s,3H), 3.3 (m,1H), 5.1 (bs,2H), 5.5 (AB,2H), 6.67 (AB,2H)].

Heroin (70 mg) was dissolved in 5 ml of ethanol and 200 mg of hydroxylammonium chloride was added. After 18 h at room temperature, the reaction mixture was evaporated under reduced pressure and the residue was dissolved in 10 ml of saturated sodium hydrogencarbonate solution. This solution was extracted four times with 10 ml of chloroform and, after the combined organic phase had been dried and evaporated as above, 50 mg of 6-acetylmorphine was obtained [n.m.r. δ(ppm): 2.1 (s,3H), 2.42 (s,3H), 3.4 (m,1H), 5.05 (bs,2H), 5.47 (AB,2H), 5.9 (bs,1H), 6.5 (AB,2H)].

H.p.l.c.-columns (150 × 4.6 mm) were packed with either Hypersil (5 μm) or Hypersil ODS (5 μm) (Shandon, Runcorn, G.B.) using a Shandon slurry packer as recommended by the manufacturer. The chromatographic equipment consisted of a Kipp Analytica (Emmen, Netherlands) model 9208 pump, a WISP 710B (Waters Associates) automatic injector, a home-made column thermostat and a Perkin-Elmer model 650-10LC fluorescence spectrometer.

Procedures

Extraction. Urine samples were collected when drug addicts visited the morphine dispensing post and were frozen at −20°C as soon as possible. They were kept frozen until the day of analysis. After thawing, ca. 10 ml was taken from each urine sample and centrifuged at 3000 rpm for 5 min.

An 8-ml portion was taken from the supernatant fluid and 12 ml of a 1 mol l^{-1} ammonium chloride buffer solution (pH 9.0) was added. After mixing, the solution was applied to an Extrelut (Merck) extraction column (27 × 85 mm) and allowed to drain into the column bed for 10 min. The Extrelut column was placed over a 50-ml separating funnel and eluted with 40 ml of 15% (v/v) 2-propanol in dichloromethane. The eluate (ca. 25 ml) was back-extracted with 10 ml of 0.05 mol l^{-1} sulphuric acid and the organic phase was discarded. A 10-ml portion of an ammonium chloride buffer (1.12 mol l^{-1}, pH 9.34) was added and the resulting solution was applied to a fresh Extrelut column, which was eluted as described above. The final eluate was divided into halves, one of which was stored as a back-up sample. The other was evaporated under nitrogen at 40°C and the residue was transferred to a 1-ml reaction vial using the Extrelut eluent as solvent. Finally, the solvent was evaporated under nitrogen at 40°C.

Derivatization. The dry residue was dissolved in 20 μl of 0.015 mol l^{-1} hydrochloric acid which contained 25 μg of morphine hydrochloride per 20 μl of solution. The mixture was briefly agitated on a vortex mixer to enhance dissolution; 10 μl of 0.3 mol l^{-1} Tris buffer (pH 8.5) and, after brief mixing, 10 μl of aqueous 0.015 mol l^{-1} potassium hexacyanoferrate(III) were then added. After 2 min, the reaction was stopped by adding 10 μl of 0.8 mol l^{-1} hydrochloric acid and 10 μl of acetonitrile. An aliquot (10 μl) of the resulting solution was injected for h.p.l.c. within 15 h.

High-performance liquid chromatography. Two different columns and solvent systems were used. At first, a Hypersil (5 μm) silica gel column was used with a solvent mixture (system I) consisting of acetonitrile, 2 mol l^{-1} ammonia and aqueous 1 mol l^{-1} ammonium nitrate (85:10:5 by volume). The flow rate was 2 ml min^{-1}. At a later stage, a Hypersil ODS (5 μm) column (C18 reversed phase) was used with the solvent mixture (system II) acetonitrile/water/triethylamine (15:85:0.1 by volume). The flow rate was 1.5 ml min^{-1}. In both cases, the column temperature was maintained at 30°C. The monochromators of the fluorescence spectrometer were set at 320 nm for excitation and 436 nm for emission, both with a 10-nm bandwidth.

RESULTS AND DISCUSSION

An obvious solution to the analytical problem dealt with in this investigation would have been to apply the oxidative coupling reaction in a post-column reaction system. In that case, the different opiates expected in the urine of the drug addicts would have been separated first on an h.p.l.c. column before being converted into fluorescent dimers. Simultaneous determination of all opiates yielding fluorescent products would have been possible. Unfortunately, when post-column reaction was attempted, the sensitivity of the method proved to be unacceptably low. The absolute detection limit was ca. 100 ng eluted from the column compared to 16 ng when the reaction was completed before injection. Recently, Nelson et al. [5] reported a similar

method for morphine, and from their results a detection limit of 133 ng of 6-acetylmorphine eluted from the column can be inferred. The difference in detection limit between pre- and post-column reaction might be partly attributed to quenching effects [5] caused by the reagent in the post-column reactor, but the main reason probably was the lower concentration of 6-acetylmorphine in the post-column reaction mixture resulting from chromatographic dilution. A similar effect was reported for morphine by Takemori [3], who found that decreasing the reaction volume enhanced both calibration linearity and detection limit.

If the coupling reaction is the rate-limiting step, the reaction rate must be second order with respect to opiate concentration. It can be shown, therefore, that the relative yield of dimer is not independent of the opiate concentration but decreases with decreasing initial concentration, except when the reaction is allowed to proceed to equilibrium. The latter is usually impractical in a post-column reaction system and might even be impossible, because, for example, an increase in oxidant concentration to speed up the reaction would also favour degradative reactions.

At first sight, a pre-column reaction method seemed impractical, too, because of the presence of a number of different opiates in the urine sample. However, it was realized that in all instances morphine would be present in a large excess, and assuming roughly equal rates for all relevant coupling reactions, the main products would be the morphine dimer itself and mixed "dimers" of morphine with each of the minor metabolites. Because the morphine concentration was of no interest in this study, it was even possible to increase the morphine concentration purposely to a level that made coupling reactions in which no morphine was involved highly unlikely. Another advantage of this concept was that because one of the reactants would be present in a large excess, the reaction rate would be first order in the other. Obviously, the reaction rate would be dependent also on the amount of free morphine present in the sample, but its influence could easily be made negligible by adding enough extra morphine.

To test these assumptions reaction mixtures containing only morphine, a mixture of morphine and 6-acetylmorphine and only 6-acetylmorphine were chromatographed in h.p.l.c. system I (Fig. 1). As expected the mixture yielded, apart from the peaks of the morphine and 6-acetylmorphine dimers, a peak with an intermediate retention time. When various amounts (0—160 ng) of 6-acetylmorphine were oxidized in the presence of a large excess of morphine (2 μg) and injected subsequently, the peak height (y, in mm) of this mixed "dimer" was linear with the amount of 6-acetylmorphine (x, in ng) in the reaction mixture; the regression line was $y = 1.046x - 0.375$ for 7 data points, with standard error of estimate 2.023, standard deviation (s.d.) of the slope 0.027, and s.d. of the intercept 1.122. No peak of the 6-acetylmorphine dimer was observed in any instance. The absolute detection limit was ca. 0.4 ng, but it was felt that further optimization could yield an even lower detection limit.

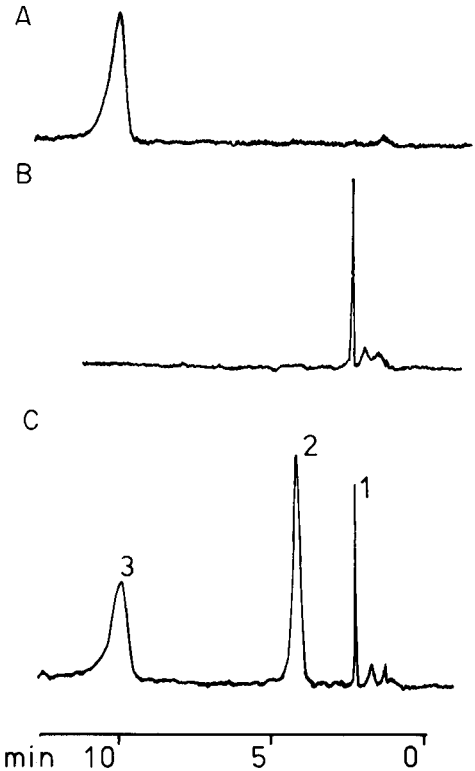


Fig. 1. Chromatograms obtained after oxidative coupling: (A) morphine alone; (B) 6-acetylmorphine alone; (C) a 4:1 mixture of morphine and 6-acetylmorphine. Peaks: (1) 6-acetylmorphine dimer; (2) mixed morphine—6-acetylmorphine "dimer"; (3) morphine dimer. (H.p.l.c. system I.)

Urine samples of six laboratory staff members were processed as described above and examined by h.p.l.c. system I. In all chromatograms, a small interfering peak with a slightly smaller retention time than the mixed dimer was found to be present. The response of the interfering compound was, on average, equivalent to 2.5 $\mu g\ l^{-1}$ (s.d. = 1.2 $\mu g\ l^{-1}$) of 6-acetylmorphine in urine. Quantitation was based on the response obtained from a separate urine sample enriched with 40 $\mu g\ l^{-1}$ 6-acetylmorphine.

In order to establish the recovery of the procedure, control urine samples were enriched with 10 or 50 $\mu g\ l^{-1}$ 6-acetylmorphine. After the samples had been processed as described under Experimental, the peak heights obtained were compared to those of pure standards. This procedure was repeated on five different days. The mean recoveries were 69.2% (s.d. = 12.9%) and 81.6% (s.d. = 9.4%) for the 10- and 50-$\mu g\ l^{-1}$ samples, respectively.

The preferred way to assess the performance of the method in detecting heroin abuse would have been to administer heroin to several volunteers followed by analysis of urine samples voided at different times after adminis-

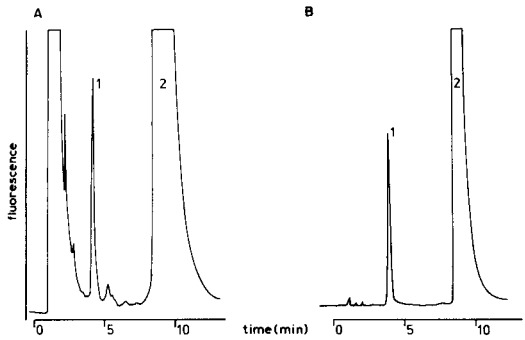


Fig. 2. Chromatograms obtained from (A) a urine sample and (B) a standard solution (h.p.l.c. system I). In (B), the peaks represent: (1) 50 ng of 6-acetylmorphine; (2) 2 μg of morphine.

tration. However, such an experiment is obviously impossible. Therefore, 50 urine samples which had been found to be positive in an enzyme-mediated immunotechnique (e.m.i.t.) for total morphine (detection limit 0.4 mg l^{-1}) were selected. These urine samples were obtained from drug addicts who did not participate in the morphine program, so that heroin abuse could safely be assumed. On analysis (see Fig. 2), 20 samples were found to contain large amounts of 6-acetylmorphine (group A, >60 μg l^{-1}). Another 16 samples contained at least 6 μg l^{-1} (group B), 12 samples at least 2.5 μg l^{-1} (group C) and the remaining two samples <2.5 μg l^{-1} (group D). Groups A and B were considered to be positive on the basis that the 6-acetylmorphine response differed by at least three standard deviations from the average response of the interfering compound in control urine samples (see above). In group C, 9 samples showed responses which differed at least one standard deviation from the average response of controls, suggesting that a considerable number of samples in this group contained 6-acetylmorphine. However, in connection with the quantitative uncertainties inherent in the method of sampling practiced, it was considered wise to exclude groups C and D from the samples identified as positive. Consequently, in 72% of the samples, 6-acetylmorphine could be identified. On the basis of the knowledge that all samples contained at least 0.4 mg l^{-1} morphine and the published evidence [1] that the average amount of free 6-acetylmorphine excreted over a 40.5-h period after heroin administration is 3.5% of the total morphine excretion, very few samples would have been expected to contain less than 5 μg l^{-1} 6-acetylmorphine. In fact, 11 samples (22%) did have concentrations lower than 5 μg l^{-1} which might be an indication that the proportion of heroin metabolites excreted as 6-acetylmorphine is a function of time. Another explanation might be that individuals having a long history of heroin addiction possess a higher deacetylating capacity. Elliot et al. [1] reported that during a 7.5-h constant infusion experiment, 6-acetylmorphine excretion started to decrease before

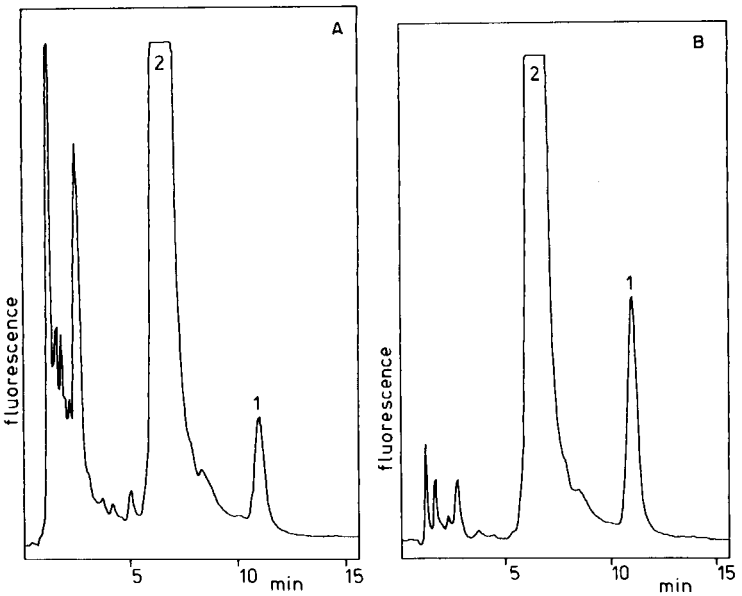


Fig. 3. Chromatograms obtained from (A) a urine sample and (B) a standard solution (h.p.l.c. system II). In (B), the peaks represent: (1) 17 ng of 6-acetylmorphine; (2) 4.2 μg of morphine.

infusion was terminated (between 2.5 and 5.5 h after the beginning of infusion). This might indicate that, even on such a small time scale, alteration of heroin metabolism is possible.

Recently, a new reversed-phase h.p.l.c. system (system II) was developed because the eluent used in the original system caused excessive wear of the piston seal of the high-pressure pump. The new system did not have this problem and showed a comparable absolute detection limit and calibration linearity. However, in chromatograms obtained from blank urine samples, no interfering peaks were present at or near the position of the mixed "dimer" peak. Figure 3 shows chromatograms obtained for a standard and a urine sample. The absence of interference in the chromatograms obtained for blank urine samples is expected to improve the performance of the method considerably. Current investigations are directed to this end.

Mrs. J. P. Weijers-Everhard (Jellinek Laboratory, Amsterdam) is gratefully acknowledged for providing urine samples after having determined their morphine content. The Technical Evaluation Committee of the Morphine Dispensing Experiment is acknowledged for stimulating discussions.

REFERENCES

1 H. W. Elliot, K. D. Parker, J. A. Wright and N. Nomof, Clin. Pharmacol. Ther., 12 (1971) 806.
2 S. Y. Yeh, R. L. McQuinn and C. W. Gorodetzky, J. Pharm. Sci., 66 (1977) 201.
3 A. E. Takemori, Biochem. Pharmacol., 17 (1968) 1627.
4 I. Jane and J. F. Taylor, J. Chromatogr., 109 (1975) 37.
5 P. E. Nelson, S. L. Nolan and K. R. Bedford, J. Chromatogr., 234 (1982) 407.

Analytica Chimica Acta, 170 (1985) 21—27
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

COMBINATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND CHEMILUMINESCENT IMMUNOCHEMICAL DETECTION OF HORMONAL ANABOLICS AND THEIR METABOLITES

E. H. J. M. JANSEN*, R. H. VAN DEN BERG, G. ZOMER, R. BOTH-MIEDEMA, C. ENKELAAR-WILLEMSEN and R. W. STEPHANY

Laboratory for Endocrinology and Laboratory for Clinical Chemistry and Haematology, National Institute for Public Health and Environmental Hygiene, P.O. Box 1, 3720 BA Bilthoven (The Netherlands)

(Received 5th September 1984)

SUMMARY

A specific identification method is presented for a number of hormonal anabolics, especially 19-nortestosterone, methyltestosterone and zeranol and their metabolites. The method is based on a combination of selective fractionation by h.p.l.c. and immunochemical detection with chemiluminogenic steroid/isoluminol conjugates.

In the Netherlands, screening for the presence of legally forbidden anabolic steroids in urine, such as diethylstilbestrol and 19-nortestosterone, in veterinary practice is usually done by radioimmunoassay (r.i.a.) after chromatographic purification of urine extracts. In practice, between screening and final confirmation by gas chromatography/mass spectrometry [1], other identification methods are used to correct as soon as possible any false-positive result obtained in the screening [2]. Besides high-performance liquid chromatography (h.p.l.c.) with diode-array detection, specially developed for application sites [3] and thin-layer chromatography (t.l.c.), a method was introduced based on a combination of characteristic h.p.l.c. retention times and specific radioimmunochemical detection [4]. This paper describes the development of this so-called immunogram method for a number of anabolic steroids and metabolites with various h.p.l.c. systems and chemiluminescent labels for detection.

EXPERIMENTAL

Materials

N-(4-Aminobutyl)-*N*-ethylisoluminol (ABEI) was prepared as described by Schroeder et al. [5]. 17α-Methyltestosterone (MT) and 19-nortestosterone (NT) 3-carboxymethyloxime derivatives and zeranol (Z) 7-carboxymethyl derivative (zearalanon) were coupled to ABEI via the *N*-hydroxysuccinimide

0003-2670/85/$03.30

ester [6] to produce the chemiluminescent labels MT-ABEI, NT-ABEI and Z-ABEI, respectively. The labelled compounds were purified by t.l.c. and characterized by u.v. spectroscopy. The antisera against MT, NT and Z were raised in rabbits by immunization with the corresponding carboxymethyl-oxime conjugate of bovine serum albumin.

H.p.l.c. procedure

The h.p.l.c. equipment consisted of the following components. An automatic injector (WISP; Waters Associates), two solvent delivery systems (model M45 and 6000A, Waters) controlled by a system controller (model 720; Waters). The h.p.l.c. column (150 mm × 4.6 mm, Chrompack) was packed with Hypersil ODS (5 μm; Shandon), Hypersil (5 μm; Shandon) or Lichrosorb Diol (10 μm; Merck) using a column-packing Instrument (Shandon), and was protected by a precolumn (75 mm × 2.1 mm; Chrompack) filled with Bondapak Corasil C18 or Corasil (Waters). The main column was thermostated at 30°C with a home-made metal heat exchanger. Flow conditions were as follows: for Hypersil ODS, methanol/water (60:40 v/v) from 0 to 7 min and pure methanol from 7 to 9 min both at 2.0 ml min^{-1}; for Hypersil and Lichrosorb Diol, isooctane/ethanol (97:3 v/v) from 0 to 7 min and iso-octane/ethanol (60:40 v/v) from 7 to 9 min at 2.0 ml min^{-1}.

Samples (1 ml) of bovine urine were purified after enzymatic hydrolysis by ether extraction as described for diethylstilbestrol [4]. An aliquot (0.4 ml) of urine was applied to the h.p.l.c. column; 65 12-s fractions were collected automatically over 13 min. The fraction-collecting system consisted of a modified fraction collector (model Redirac, LKB) equipped with an electric three-way valve (model PSV-3, Pharmacia). Both fraction collector and valve were operated by the timed events of the integrator (Data Module; Waters) mediated by a home-build interface (model SE 459). The electronic circuit of this interface is available upon request. After each run, the tube between the valve and the fraction collector was washed automatically with 1 ml of eluent to avoid cross-contamination.

Chemiluminescence immunoassay

Chemiluminescence assays for MT, NT and Z were done as follows. The dry h.p.l.c. fractions of a urine extract were dissolved in 0.15 ml of phosphate buffer (0.1 mol l^{-1} phosphate-buffered saline, pH 7.0) and incubated with 0.05 ml of a solution (20 000 counts) of the corresponding ABEI label and 0.5 ml of the corresponding antiserum (final dilutions, v/v, were MT/antiserum, 1:10 000; NT/antiserum, 1:27 000; Z/antiserum, 1:15 000) for 2 h at 37°C and 1 h at 4°C. Separation of bound and free label was accomplished by centrifugation after addition of 0.25 ml of dextran-coated charcoal. After addition of 0.15 ml of 5 mol l^{-1} sodium hydroxide to 0.25 ml of the supernatant, the bound label was heated for 60 min at 80°C prior to chemiluminescent emission measurement. After addition of 0.05 ml of microperoxidase solution (10 μg ml^{-1}, MP 11; Sigma) the chemiluminescence was initiated by

addition of 0.1 ml of 0.3% (v/v) hydrogen peroxide. Light intensity was measured for 6 s with an adapted biocounter M 2010 (Lumac/3M, Schaesberg, The Netherlands). A series of 100 tubes could be counted within 30 min.

RESULTS

Chromatographic fractionation

The fractionation of urine extracts by h.p.l.c. can be accomplished on different bonded phases with a variety of solvents. For routine use, the following three columns with the corresponding mobile phases were employed: Hypersil ODS (5 μm) with methanol:water (6:4, v/v) [7], Hypersil silica (5 μm) or Lichrosorb Diol (10 μm) with iso-octane:ethanol (97:3 v/v). The difference in selectivity is shown in Table 1 where the retention times of some frequently used anabolic compounds are listed.

Immunoassay for the anabolic compounds

19-Nortestosterone (NT). In the Netherlands at present, NT is probably the most frequently used anabolic steroid in the fattening of cattle for slaughter [3, 8]. In some sports, NT also appears to be widely used. For forensic control of illegal veterinary use of anabolic steroids, urine is the most suitable material for investigation. However, the concentration of NT in bovine urine after injection of NT laurate turned out to be relatively low because of its rapid metabolism [9]. Therefore a study was initiated to find a major target metabolite of NT in bovine urine. In Fig. 1, an immunogram is shown of a sample urine (H 153592) from a suspect animal. Two significant immunochemical responses are observed, corresponding to NT itself (4.7 min) and probably one of the possible metabolites of the 5β-reductase route, 5β-estrane-3α,17β-diol (12.2 min). Although the cross-reactivities of NT metabolites towards the antisera are rather low (Table 2), still it is possible to detect high concentrations of these metabolites.

TABLE 1

Retention times of some anabolic compounds on Hypersil ODS, Hypersil Silica and Lichrosorb Diol (for experimental conditions, see text)

Compound	Retention times (min)		
	ODS	Silica	Diol
17β-Trenbolone	4.4	12.0	7.1
19-Nortestosterone	5.3	9.8	5.4
Zeranol	5.9	10.2	14.1
17β-Testosterone	6.8	8.2	4.9
Trans-Diethylstilbestrol	7.1	6.9	14.5
Dienestrol	7.8	8.4	15.7
Hexestrol	8.5	7.2	13.6
17α-Methyltestosterone	8.8	6.1	3.9
Medroxyprogesterone	12.7	6.2	4.8

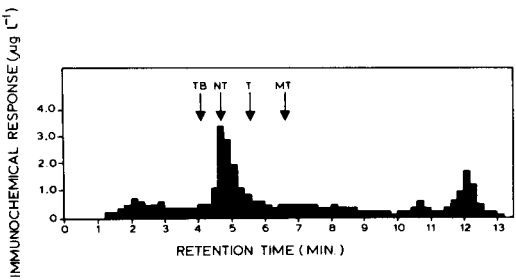


Fig. 1. H.p.l.c./immunogram on Hypersil ODS of an extract of bovine urine (H 153592), using detection via NT chemiluminescence immunoassay. The retention times of standards are indicated by arrows; T = 17β-testosterone, TB = 17β-trenbolone.

TABLE 2

Relative cross-reactivities of two antisera (H 154212 and H 154213) against 19-nortestosterone as determined by chemiluminescence immunoassay and calculated according to Abraham [10]

Compound	Relative cross-reactivity (%)	
	H 154212 (1:27 000, v/v)	H 154213 (1:15 000, v/v)
19-Nortestosterone	100	100
19-Nor-epitestosterone	0.7	0.8
5β-Estrane-3α,17β-diol	2.4	0.8
5β-Estrane-3β,17β-diol	4.5	3.6
19-Norethiocholanolone	0.2	0.1
17α-Testosterone	<0.1	<0.1
17β-Testosterone	34	15
17-Methyltestosterone	4.8	2.1
17α-Estradiol	<0.1	<0.1
17β-Estradiol	0.3	1.5

17α-Methyltestosterone (MT). For the anabolic steroid MT, the development of a r.i.a. for screening purposes is hampered by lack of a commercial radio-label. Therefore a chemiluminescent immunoassay was developed for MT [11] based on our own antisera. The advantage of the selectivity of the various h.p.l.c. systems is shown in Fig. 2 for MT and 17β-testosterone (T) in bovine urine. The cross-reactivity for T was 11% in this system. In the immunograms done with reversed-phase (Fig. 2A) and normal-phase (Fig. 2B) systems, two significant immunochemical responses are shown. On both columns, these responses can be correlated with the presence of T and MT, which gives additional selectivity for the identification of both compounds.

Zeranol (Z). The estrogenic anabolic agent Zeranol is widely used in fattening cattle. To elucidate the metabolism, a model experiment was conducted (at ILOB, Wageningen) with calves implanted with Z and trenbolone acetate.

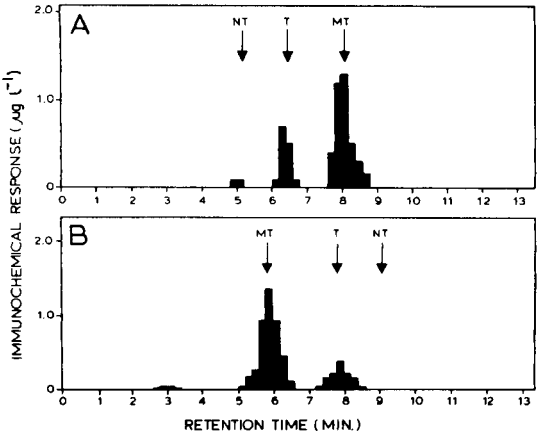


Fig. 2. H.p.l.c./immunograms of an extract of bovine urine (H 154441) enriched with standard MT fractionated on: (A) Hypersil ODS; (B) Hypersil Silica. Detection via MT chemiluminescence immunoassay; retention times of standards are indicated by arrows.

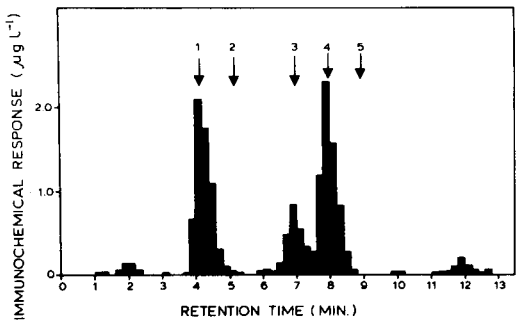


Fig. 3. H.p.l.c./immunogram of an extract of urine (H 140872) of a calf three days after treatment with Zeranol, with detection via Z chemiluminescence immunoassay. The retention times of standards are indicated by arrows: (1) β-Zearalanol (Taleranol); (2) β-Zearalenol; (3) α-Zearalanol (Zeranol); (4) α-Zearalenol, Zearalanone; (5) Zearalenone.

Figure 3 shows the immunogram of an extract of urine taken three days after treatment. From this figure, the metabolism of Z can be observed qualitatively. Immunoresponses are observed at the retention times of Z (7.1 min), the oxidized form zearalanone (8.1 min) and β-zearalanol (4.2 min), the epi-isomer of Z. The cross-reactivities of antisera developed in rabbits are listed in Table 3 and are comparable with those reported for r.i.a. [12, 13]. Further work, including quantification of the metabolites as a function of time, is in progress.

TABLE 3

Relative cross-reactivities (%) of an antiserum (H 154546)[a] against Zeranol and related compounds as determined by chemiluminescence immunoassay and calculated according to Abraham [10]

Compound	Relative cross-reactivity (%)[a]
α-Zearalanol (Zeranol)	100
β-Zearalanol (Taleranol)	60
Zearalanone	134
α-Zearalenol	69
β-Zearalenol	49
Zearalenone	38

[a]Used 1:20 000 v/v (final dilution).

DISCUSSION

From the examples described above, it can be concluded that the combination of h.p.l.c. fractionation and immunochemical detection represents a highly selective and powerful identification method both in forensic investigation for the presence of anabolic steroids in bovine urine and in metabolism studies.

The three h.p.l.c. columns mentioned in Table 1 show different selectivity. In cases of doubt, combined immunograms of a urine extract on various h.p.l.c. systems can increase the certainty of identification of a compound substantially. The selectivity of the immunochemical detection is mainly governed by the antiserum used, although the introduction of chemiluminescence detection may offer additional selectivity by the use of different labels [14].

Although for forensic purposes the antisera must be as selective as possible, group-specific antisera were also developed for metabolism studies. The NT antiserum here described, raised against the 3-carboxymethyloxime conjugate showed higher cross-reactivities towards NT metabolites and other androgens than the NT antisera raised against the NT-BSA conjugate coupled at the 7-position of the steroid moiety. Also the antiserum against Z shows a high cross-reactivity against the main metabolites. This antiserum was raised against a zearalanon-BSA conjugate, coupled at position 7 where the main metabolism is supposed to occur. Details will be reported elsewhere.

Chemiluminescence detection was introduced principally for its high sensitivity and rapid measurement. Two immunograms can be obtained within 6 h by one technician, whereas for r.i.a. 18 h are necessary. At present, immunogram procedures with chemiluminescence detection are under development for stilbenes and steroid estrogens.

This investigation was made as part of projects 368301, 368303 and 378303 on behalf of and for the account of the Dutch Veterinary Chief

Inspectorate of Public Health. The experimental assistance of P.W. Zoontjes, J. van de Siepkamp and H. van Blitterswijk is acknowledged. The authors are indebted to Dr. J. C. Bouffault (Roussel-Uclaf, France), Mr. J. T. Craig (International Minerals and Chemical Corporation, U.S.A.) and Mr. H. A. E. Kuys (Organon, The Netherlands) for reference materials.

REFERENCES

1 H. J. G. M. Derks, J. Freudenthal, L. J. M. Litjens, R. Klaassen, L. G. Gramberg and V. Borrias-van Tongeren, Biomed. Mass Spectrom., 10 (1983) 209.
2 E. H. J. M. Jansen and R. W. Stephany, Vet. Quart., 7 (1985) 35.
3 E. H. J. M. Jansen, H. van Blitterswijk and R. W. Stephany. Vet. Quart., 8 (1984) 60.
4 E. H. J. M. Jansen, R. H. van den Berg, H. van Blitterswijk, R. Both-Miedema and R. W. Stephany, Vet. Quart., 6 (1984) 5.
5 H. R. Schroeder, R. C. Boguslastei, R. J. Carinco and R. T. Buckler, Methods Enzymol., 57 (1978) 424.
6 M. Pazzagli, J. B. Kim, G. Messeri, F. Kohen, G. F. Bolelli, A. Tommasi, R. Salerno, G. Moneti and M. Serio, J. Steroid Biochem., 14 (1981) 1005.
7 E. H. J. M. Jansen, R. Both-Miedema, H. van Blitterswijk and R. W. Stephany, J. Chromatogr., 299 (1984) 450.
8 E. H. J. M. Jansen, G. Zomer, R. H. van den Berg and R. W. Stephany, Vet. Quart, 6 (1984) 101.
9 W. R. Jondorf and M. S. Moss, Xenobiotica, 8 (1978) 197.
10 G. E. Abraham, J. Clin. Endocrinol. Metab., 29 (1969) 866.
11 G. Zomer, R. H. van den Berg and E. H. J. M. Jansen, Anal. Chim. Acta, 170 (1985) 29.
12 S. N. Dixon, J. Vet. Pharm. Therap., 3 (1980) 177.
13 A. P. Carter, S. N. Dixon and M. H. Bew, J. Vet. Pharm. Therap., 7 (1984) 17.
14 G. Zomer, R. H. van den Berg and E. H. J. M. Jansen, Pharm. Weekbl. Sci. Ed., 6 (1984) 257.

Analytica Chimica Acta, 170 (1985) 29—33
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

A SOLID-PHASE CHEMILUMINESCENCE IMMUNOASSAY FOR 17α-METHYLTESTOSTERONE

E. H. J. M. JANSEN*, C. A. LAAN, R. H. VAN DEN BERG and R. W. STEPHANY

Laboratory for Endocrinology, National Institute for Public Health and Environmental Hygiene, P.O. Box 1, 3720 BA Bilthoven (The Netherlands)

G. ZOMER

Laboratory for Clinical Chemistry and Haematology, National Institute for Public Health and Environmental Hygiene, P.O. Box 1, 3720 BA Bilthoven (The Netherlands)

(Received 5th September 1984)

SUMMARY

A solid-phase immunoassay based on chemiluminescence detection is described for the anabolic steroid 17α-methyltestosterone (MT). The reproducibility is increased substantially by introduction of the solid-phase principle and the assay can therefore be used to quantify MT in bovine urine. The detection limit is 10 pg.

Hormonal anabolics are used frequently in fattening cattle to achieve an increased nitrogen conversion resulting in 10—15% extra bodyweight. Diethylstilbestrol (DES) represents a cheap and effective anabolic compound which, however, has been banned in all countries. In the Netherlands, an effective control system for the presence of DES in the urine of cattle was introduced in 1982 [1, 2]. Initial screening is done by radioimmunoassay (r.i.a.), followed by confirmation by gas chromatography/mass spectrometry (g.c./m.s.). Probably on account of the introduction of this control programme, the use of DES and the other "stilbenes", dienestrol and hexestrol, has decreased to almost zero [3]. The results of an investigation of anabolic residues in application sites [4] also indicated a decrease in the use of DES. Other anabolics like 19-nortestosterone (NT), medroxyprogesterone (MP), trenbolone (TB) and 17α-methyltestosterone (MT) in combination with estradiol (E2) or their esters were observed more frequently in such samples.

Immunochemical screening methods have been developed for NT and TB and are currently under development for MP. A radioimmunoassay (r.i.a.) for MT was hampered by lack of the commercial availability of a tritium tracer. To overcome this problem an immunoassay was developed in which a chemiluminescent MT label was used instead of a radioactive label; details will be reported elsewhere. The chemiluminescent immunoassay for MT was done in a similar way to the r.i.a. for DES or NT, in which the separation of bound and free material was accomplished using dextran-coated charcoal.

0003-2670/85/$03.30

The chemiluminescent reaction was done on the bound fraction in the presence of matrix components which resulted frequently in unacceptably high blank values. This paper reports the development of a solid-phase immunoassay which is less sensitive to interferences and which is also applicable to the determination of MT in bovine urine and application sites of cattle.

EXPERIMENTAL

Chemicals and chromatographic equipment

All solvents used were of analytical grade. The steroid standards were checked for purity by melting point, high-performance liquid chromatography (h.p.l.c.) and infrared spectroscopy. The synthesis and purification of the chemiluminescent *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI) conjugate of MT and the MT—3-carboxymethyloxime conjugate of bovine serum albumin were as outlined previously [5].

The equipment used for h.p.l.c. was as described previously [5]. The h.p.l.c. column was packed with Hypersil ODS (5 μm; Shandon) and the precolumn was filled with Bondapak Corasil C18 (Waters). The flow conditions were as follows: methanol/water (6:4 v/v) from 0 to 7 min and pure methanol from 7 to 9 min, both at a flow rate of 2.0 ml min^{-1}. Monitoring was at 254 nm with a fixed wavelength u.v. monitor (model 440, Waters). Signal recording and fraction collecting were achieved as described previously [5].

Methods

Isolation of MT from bovine urine. A 0.5-ml sample of bovine urine was hydrolyzed enzymatically with glucuronidase and sulfatase (Suc d'Helix Pomatia, IBF Reactifs, France) for at least 1 h at 37°C. The free MT was extracted twice with diethyl ether. After addition of 0.225 ml of distilled water, the ether was evaporated under nitrogen, and 0.400 ml of methanol was added. From the methanol/water extract, 0.250 ml was applied to a reversed-phase h.p.l.c. column. The MT fraction was collected automatically for 1 min at the retention time of MT determined previously with a MT standard.

Isolation of MT from an application site. The sample of the application site was investigated by sectioning for the presence of injected preparations like pellets, oils, creams or suspensions. After photography on color slides, the residual preparation was isolated from the sample. Part of this preparation was hydrolyzed enzymatically with esterase followed by extraction of the free anabolic steroids with diethyl ether. After evaporation of the extract, the h.p.l.c. eluent (methanol/water, 6:4 v/v) was added to the dry residue.

Coating of the tubes. The immunoglobulin fraction of an antiserum against MT was purified by affinity chromatography using Protein A-Sepharose (Pharmacia). Polystyrene tubes (Lumacuvettes, Lumac/3M) were coated with 0.200 ml of a 1:2000 (v/v) dilution of the purified antiserum in 0.05 mol l^{-1} sodium hydrogencarbonate for 1 h at room temperature. The

tubes were washed twice with 0.03% Tween in distilled water. Before use, the tubes were washed with assay buffer. Coated tubes can be stored at 4° C for several days without loss of binding capacity.

Assay procedure. The MT fraction of the h.p.l.c. eluate was evaporated and dissolved in 1.50 ml of assay buffer (0.1 mol l^{-1} PBS, pH 7.0) and aliquots (in triplicate) of 0.150 ml were applied to the immunoassay. After addition of 0.05 ml of MT-ABEI solution (20 000 counts) in assay buffer, the tubes were incubated at 4°C for 30 min, and then washed with assay buffer. After addition of 0.20 ml of 2 mol l^{-1} sodium hydroxide the tubes were heated at 80°C for 45 min. The calibration graph was calculated after logit/log transformation and linear regression [6].

Chemiluminescence measurement. Light intensities were measured with an adapted Biocounter M2010 (Lumac/3M, Schaesberg, The Netherlands) using polystyrene Lumacuvettes as reaction tubes. The rapidity of the chemiluminescent emission required no delay time after automatic injection and adjustment of the integration times. Stock solutions of MT and its ABEI conjugate were prepared in ethanol and diluted to the desired concentration in assay buffer before use. After incubation, 0.05 ml of a solution of 10 μg ml^{-1} microperoxidase MP-11 (Sigma) in distilled water was added to the tubes. The working oxidant solution, 0.3% (v/v) hydrogen peroxide in distilled water, was added semi-automatically to the sample in the luminometer. Luminescence was measured over the first 6 s.

RESULTS AND DISCUSSION

Chromatographic purification

Direct application of an ether extract of urine to the solid-phase chemiluminescent immunoassay resulted in high blank values originating from both matrix components and cross-reacting compounds. Therefore a h.p.l.c. purification and separation step was included prior to the assay, in which the cross-reacting compounds were separated from MT. Characteristic retention times are: 17β-testosterone, 6.6 min; 5α-dihydrotestosterone, 11.5 min; NT, 4.9 min; MT 8.0 min [7]. The isocratic h.p.l.c. purification was automated by using an auto-injector and an automatic fraction-collecting system. In this configuration, 46 urine extracts could be purified automatically overnight (15 h).

Specificity of antisera

Cross-reactivities of the MT antiserum towards possible metabolites and related (anabolic) steroids are given in Table 1. Only with 5α-dihydromethyltestosterone (DHMT), 17β-testosterone (T), 5α-dihydrotestosterone (DHT) and NT was appreciable cross-reactivity found. All other related steroids which were tested showed cross-reactivities of less than 0.1%.

TABLE 1

Relative cross-reactivities of some steroids and DES with an antiserum (H 152458) against 17α-methyltestosterone as determined by the solid-phase chemiluminescent immunoassay and calculated according to Abraham [8]

Compound	Cross-reactivity (%)	Compound	Cross-reactivity (%)
17α-Methyltestosterone	100	17α-Trenbolone	<0.01
5α-Dihydromethyltestosterone	36	17β-Trenbolone	<0.01
17α-Testosterone	<0.01	Progesterone	0.08
17β-Testosterone	9	Medroxyprogesterone	<0.01
5α-Dihydrotestosterone	11	17α-Estradiol	<0.01
19-Nortestosterone	3	17β-Estradiol	0.04
		Diethylstilbestrol	<0.01

Assay parameters

The intra-assay variation and the noise of the solid-phase chemiluminescence immunoassay for MT were determined by using blank bovine urine samples. The immunochemical response as determined in 40 different blank urine samples (in triplicate) in two independent assays was 0.20 ± 0.26 $\mu g\ l^{-1}$ (mean ± s.d., n = 80). The relative standard deviations for blank urine samples enriched with MT to 1 and 2 $\mu g\ l^{-1}$ were 16% and 22%, respectively, in two independent assays (n = 12). The relative standard deviation for triplicate determinations was 9.7%, calculated from 91 samples with two independent assays. These values were calculated as described by McDonagh et al. [9]. In these assays, a relative binding of 50% was correlated with a MT content of 2 $\mu g\ l^{-1}$. An absolute lowest detection limit of about 10 pg of MT per tube can be achieved.

Applications

It is concluded from the above results that the solid phase chemiluminescent immunoassay (s.p.l.i.a.) for MT can be used for the detection and quantification of MT in bovine urine. Cross-reacting compounds like T and NT can also be determined after h.p.l.c. fractionation [10]. These anabolic steroids also can be detected in application sites because almost no interfering matrix components are present in this material. For this material, quantification is difficult and of minor importance on account of the large amounts of anabolic compounds present.

The s.p.l.i.a. for MT is here applied mainly to extracts of urine and to application sites, after h.p.l.c. fractionation. In the so-called h.p.l.c./immunogram [5], the retention times of the immunochemical responses can be correlated with those of the standards. Examples of this technique are given in Fig. 1A for an 1-ml sample of bovine urine, enriched with 5 ng of MT. Besides MT the presence of endogenous T can also be observed. In Fig. 1B, an immunogram of an extract from an application site is shown. The presence of large amounts of MT and NT was observed and confirmed by both h.p.l.c. with diode-array detection [4] and g.c./m.s. As a consequence, for forensic

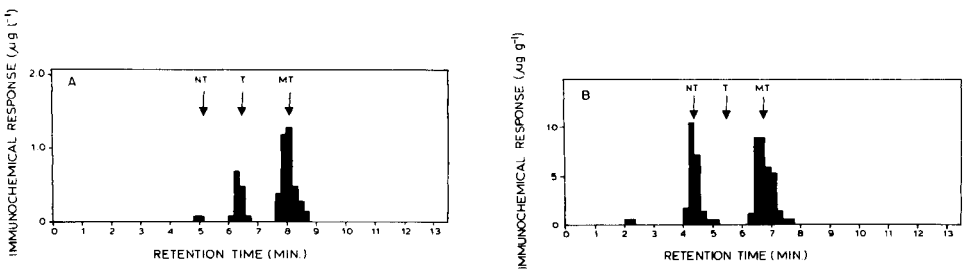


Fig. 1. H.p.l.c./immunograms with detection by solid-phase chemiluminescence immunoassay of MT: (A) in urine from a 2-year old bull, enriched with 5 ng ml^{-1} MT; (B) in an extract from an application site (H 151223). The retention times are indicated by arrows; h.p.l.c. conditions as described for the purification of MT from urine.

purposes, the animal concerned was condemned according to the regulations and rejected for human consumption.

The reported solid-phase chemiluminescence immunoassay for MT turned out to be suitable and sensitive enough for detection and quantification of MT in urine and application sites. Compared to the original chemiluminescence immunoassay inter- and intra-assay variations are improved, but the greatest advantage is the low background measured in urine. Also the time of analysis is decreased, and the assay simplified. The assay can be done within 4 h, including coating the tubes. Two immunograms can be produced within 6 h by one technician. The relative standard deviations, however, obtained at the 1—2 $\mu g\ l^{-1}$ level must still be considered as too high for quantitative purposes in screening for forensic investigation. Therefore an analogous solid-phase assay will be developed using polystyrene spheres in order to increase the reproducibility [11]. Such assays are also under development for other anabolic steroids like NT, DES, TB and Zeranol to replace the need for radioactive labels in r.i.a. procedures.

This investigation was done as part of projects 368301, 368303 and 378303 on behalf of and for the account of the Dutch Veterinary Chief Inspectorate of Public Health. The technical assistance of R. Both-Miedema is acknowledged.

REFERENCES

1 E. H. J. M. Jansen, R. H. van den Berg, H. van Blitterswijk, R. Both-Miedema and R. W. Stephany, Vet. Quart., 6 (1984) 5.
2 H. J. G. M. Derks, J. Freudenthal, L. J. M. Litjens, R. Klaassen, L. G. Gramberg and V. Borias-van Tongeren, Biomed. Mass Spectrom., 10 (1983) 209.
3 E. H. J. M. Jansen and R. W. Stephany, Vet. Quart., 7 (1985) 35.
4 E. H. J. M. Jansen, H. van Blitterswijk and R. W. Stephany, Vet. Quart., 6 (1984) 60.
5 E. H. J. M. Jansen, R. H. van den Berg, G. Zomer, R. Both-Miedema, C. Enkelaar-Willemsen and R. W. Stephany, Anal. Chim. Acta, 170 (1985) 21.
6 D. Rodbard and J. E. Lewald, Acta Endocrinol., Suppl., 147 (1970) 79.

7 E. H. J. M. Jansen, R. Both-Miedema, H. van Blitterswijk and R. W. Stephany, J. Chromatogr., 299 (1984) 450.
8 G. E. Abraham, J. Clin. Endocrinol. Metab., 29 (1969) 866.
9 B. F. McDonagh, P. J. Munson and D. Rodbard, Comp. Prog. Biomed., 7 (1977) 179.
10 E. H. J. M. Jansen, G. Zomer, R. H. van den Berg and R. W. Stephany, Vet. Quart., 6 (1984) 101.
11 A. Gadow, H. Fricke, C. J. Strasburger and W. G. Wood, J. Clin. Chem. Clin. Biochem., 21 (1983) 789.

Analytica Chimica Acta, 170 (1985) 35—44
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

PHARMACEUTICAL APPLICATIONS OF VARIABLE-ANGLE SYNCHRONOUS SCANNING FLUORESCENCE SPECTROSCOPY

BRIAN J. CLARK[a,*], ANTHONY F. FELL[a], KENNETH T. MILNE[b], DONALD M. G. PATTIE[b] and M. HOWARD WILLIAMS[b]

Analytical Research Group, [a]Department of Pharmacy and [b]Department of Computer Science, Heriot-Watt University, 79 Grassmarket, Edinburgh EH1 2HJ (Great Britain)

(Received 7th September 1984)

SUMMARY

The development of variable-angle synchronous scanning (v.a.s.s.) in fluorescence spectrometry is reported, based on a computer-aided spectrofluorimeter. The technique permits a linear path to be scanned at any preselected angle through the emission-excitation matrix defined by (I_{em}, λ_{em}, λ_{ex}), by effectively scanning the emission and the excitation monochromators at different speeds under computer control. When applied to pharmaceutical dosage forms, v.a.s.s. gave good selectivity for chlorpromazine in the presence of its principal degradation product, chlorpromazine sulphoxide, and for oxytetracycline in the presence of the additives vitamin C, thiamine, nicotinamide and riboflavin. Good calibration linearity, precision and recovery were observed for both principal drug components. The angle of the scan trajectory can also be varied continuously through the emission-excitation matrix, to describe any desired path under computer control. This novel technique of non-linear v.a.s.s. can provide an improved method for generating diagnostic profiles of drugs, degradation products and metabolites.

In luminescence studies, high sensitivity and selectivity are generally expected. However, problems of selectivity and ultimately of sensitivity can occur in multicomponent analysis because of the overlap of the broad-band spectra observed. Specificity is a particular problem in the determination of luminescent drugs in their dosage forms, which are often multicomponent preparations. In consequence, a number of procedures has been proposed to increase selectivity in quantitative luminescence spectrometry.

One approach is to exploit a multichannel detector, based either on the vidicon tube [1, 2], which effectively captures the 2-dimensional emission-excitation matrix (EEM), or on the linear photodiode array [3, 4], which operates only in the emission wavelength domain. These detectors are capable of generating a large amount of luminescence data in just a few milliseconds. Moreover, the utility of these detectors can be extended to allow quantitation by incorporating deconvolution algorithms [5]. However, the vidicon is at present relatively expensive, while the linear photodiode array spectrofluorimeter is not yet widely accessible. An alternative to multichannel detection involves the improvement of spectral resolution of

0003-2670/85/$03.30

overlapping peaks, captured by conventional means, by utilising computer-based mathematical techniques such as least-squares deconvolution [6], derivative transformation [7, 8] and matrix-isolation spectroscopy [9].

A further technique in this category of spectral resolution improvement is synchronous luminescence spectroscopy, also described as synchronous excitation spectroscopy. This method was originally proposed by Lloyd [10] and has been recently reviewed by Vo-Dinh [11]. It involves simultaneous scanning of the excitation and emission monochromators, separated by a constant wavelength interval, $\Delta\lambda$. The resulting spectra represent the intensity profile of a 45° section cut through the EEM, and are generally characterised by decreased bandwidths, which can lead to improvements in selectivity. The main areas of application have been in air pollution studies [11, 12] and as an aid to 'fingerprinting' in the oil industry [13, 14]. Relatively few applications have been reported in the field of pharmaceutical and biomedical analysis [15—20].

One drawback to conventional synchronous scanning spectroscopy is that it is limited to producing 45° sections, whose locus is defined by the constant wavelength interval $\Delta\lambda$ employed. This is also true of the constant-energy modification in the frequency domain ($\Delta\nu$), proposed recently by Inman and Winefordner [21]. A novel approach which offers considerable flexibility is variable-angle synchronous scanning (v.a.s.s.), where the wavelength separation between the monochromators is continuously varied [22]. This can be achieved either mechanically, by varying the relative scan speeds of each monochromator [23], or digitally, where the luminescence data are stored for subsequent processing by microcomputer [24]. In the present work, the increased selectivity afforded by the v.a.s.s. technique has been examined in studies on the determination of psychotropic and antibiotic drugs in their respective dosage forms.

EXPERIMENTAL

Instrumentation and software

All fluorescence spectra were recorded on a Perkin-Elmer LS-5 spectrofluorimeter which includes a quantum-corrected reference system for fully-corrected excitation spectra. Slit-widths for both monochromators were set at 5 nm. The spectrofluorimeter was operated in the computer-controlled mode via the RS232C serial interface by either of the following systems. In one, a 48 kbyte Apple II microcomputer with dual disk-drive was used with a Houston DMP4 Hiplot digital plotter (Bausch and Lomb, TX); instrument control, data control and manipulation were achieved by a suite of programs written by the authors in UCSD Pascal. In the other, a Perkin-Elmer Model 3600 Data Station microcomputer was used; instrument control and data collection were achieved by using the commercially available Perkin-Elmer Computerised Luminescence Software (PECLS). 'OBEY' programs using this software were written to perform certain tasks.

Programs were developed here to permit variable-angle synchronous scanning using either of the two systems. Two approaches were used. In the first, the initial wavelengths for the emission and excitation monochromators were sequentially stepped in specified increments over the required range. A variable-angle scan at the desired angle can be obtained by selecting the appropriate value and sign for each of the two increments. In the second approach, which was implemented only for the Apple system, the EEM is first collected and stored on disc for processing at a later stage. The angle is selected by choosing the appropriate increments at the processing stage. With this method, it is possible to obtain a number of sequential segments, each at a different angle. Each pair of adjacent segments can be linked together, thereby defining any desired trajectory through the EEM.

A contour-plotting routine was written in BASIC for the Perkin-Elmer system. Contours in the (λ_{em}, λ_{ex}) plane were produced by joining points of equal fluorescence intensity. The routine was based on a recent algorithm developed to present the 3-dimensional data obtained from photodiode-array u.v. detection in high-performance liquid chromatography [25].

Reagents, solutions and materials

Oxytetracycline, thiamine, riboflavin, nicotinamide and ascorbic acid were obtained from the Sigma Chemical Co. Chlorpromazine hydrochloride and its sulphoxide were kindly supplied by May and Baker (Dagenham, U.K.). Anhydrous aluminium chloride was a pure grade (Koch-Light) and potassium dihydrogenphosphate was AnalaR grade (BDH).

Standard solutions. Standard solutions of oxytetracycline and of the vitamins were prepared each day in 0.05 M potassium dihydrogenphosphate (pH 5.0). Aluminium chloride (0.80 M) was added to these solutions and to the dosage form solutions to give a final concentration of 0.20 M. The solutions were allowed to stand for 15 min before measurement. These solutions were found to be stable for at least 1 h. Chlorpromazine hydrochloride (1—10 μg ml^{-1}) and its sulphoxide (0.1—1.0 μg ml^{-1}) were prepared in 0.05 M potassium dihydrogenphosphate (pH 3.0).

Capsule and tablet dosage forms. The capsules analysed for oxytetracycline (Terramycin-SF; Pfizer, Sandwich, U.K.) contained oxytetracycline (250 mg), ascorbic acid (75 mg), thiamine (2.5 mg), riboflavin (2.5 mg) and nicotinamide (25 mg). The contents of each capsule were extracted into phosphate buffer, pH 5.0, followed by dilution to the working range of 1—10 μg ml^{-1} oxytetracycline. For chlorpromazine tablets (Largactil, 25 mg; May and Baker) 20 tablets were weighed and ground and the equivalent of one tablet was extracted with phosphate buffer, pH 3.0, as previously described [20].

RESULTS AND DISCUSSION

The determination of tetracycline and phenothiazines in their dosage forms is possible by various methods. Among these, fluorescence spectro-

metry would be expected to be suitable, although the method in its conventional implementation suffers from the fact that the major peak of interest overlaps with peaks attributable to degradation products or other formulation constituents.

Tetracyclines may be administered in combination with a vitamin complex, either as an intravenous infusion (extemporaneously prepared) or as one of several commercially available dosage forms. In one of these preparations, oxytetracycline is formulated with vitamin C (ascorbic acid) and with the B-complex vitamins thiamine, nicotinamide and riboflavin. Overlap of the emission spectra is not observed for oxytetracycline in the presence of these vitamins, with the exception of riboflavin for which the overlapping emission spectrum is shown in Fig. 1. The enhanced fluorescence intensity for oxytetracycline observed in this spectrum was achieved by complexation with aluminium chloride; tetracyclines in general are only weakly fluorescent. The complexation of oxytetracycline with aluminium has been previously reported to increase fluorescence intensity tenfold [26].

The extent of this overlap can usefully be examined by interfacing a microcomputer to the spectrofluorimeter to obtain the total fluorimetric information available in the EEM. With suitable computer programs,

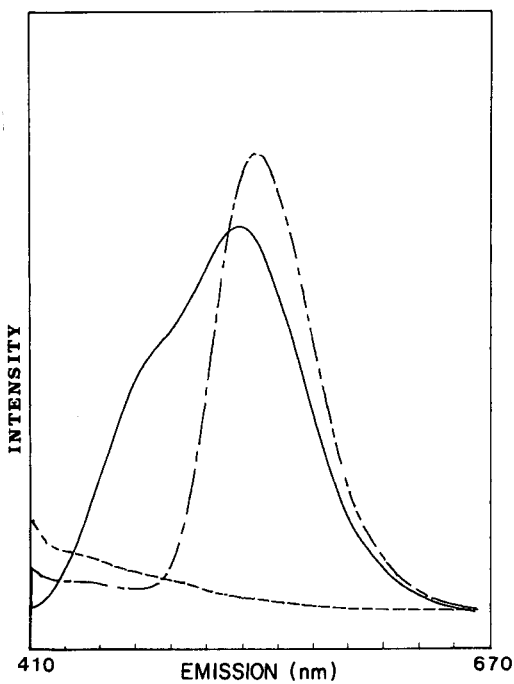


Fig. 1. Emission spectra (λ_{ex} = 378 nm): (—) oxytetracycline (5.01 μg ml^{-1}) and riboflavin (0.189 μg ml^{-1}) in phosphate buffer (pH 5.0)/aluminium chloride (0.2 M); (—·—) riboflavin alone in the buffer; (--) pH 5.0 buffer alone.

3-dimensional spectra can be obtained and presented as the isometric projection, where the emission spectra at stepped increments of excitation wavelength are recorded and plotted (Fig. 2A). A reversed projection of the data can sometimes indicate emission peaks hidden by the foreground (Fig. 2B). Alternatively, the 3-dimensional spectra can be effectively transformed to a plot in the two dimensions of excitation and emission wavelength by linking points of equal intensity to form contours, as shown for riboflavin (Fig. 3A) and for a standard mixture of oxytetracycline and riboflavin (Fig. 3B). This contour presentation has generally been found to be more useful than the isometric projection for indicating the presence of hidden emission peaks. In this case, however, the position of the riboflavin peak is not readily discriminated from the large oxytetracycline emission in the contour plot (Fig. 3B), whereas in the forward projection (Fig. 2A) and more particularly in the reversed projection (Fig. 2B), the riboflavin peak can be more easily seen.

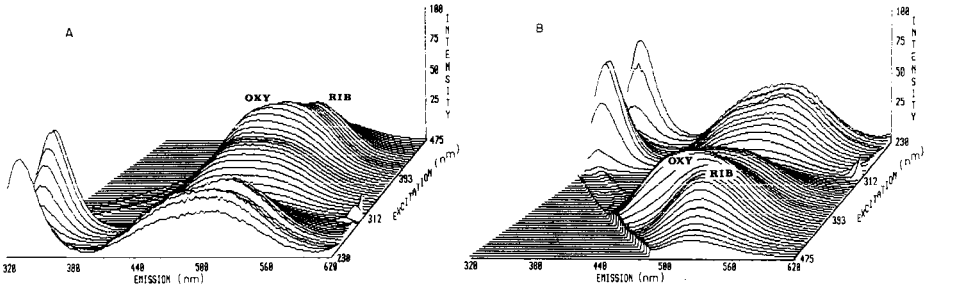


Fig. 2. Isometric plots of the emission-excitation matrix of oxytetracycline (OXY, 10.75 μg ml^{-1}) and riboflavin (RIB, 0.94 μg ml^{-1}) in phosphate buffer (pH 5.0)/ aluminium chloride (0.2 M). (A) Forward projection; (B) reversed projection. Data were obtained using the Perkin-Elmer Model 3600 microcomputer; excitation and emission bandwidths, 5 nm; increments in excitation wavelength, 5 nm for each emission scan; scan speed 240 nm min^{-1}.

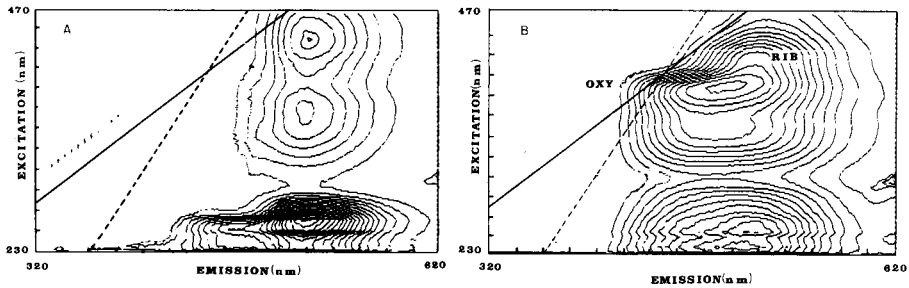


Fig. 3. Contour plots: (A) riboflavin fluorescence; (B) oxytetracycline and riboflavin fluorescence. (—) The conventional synchronous luminescence path slicing the data matrix at 45° ($\Delta\lambda$ = 40 nm); (---) the v.a.s.s. path at 63.4° from (λ_{ex}, λ_{em}) = (230, 340 nm). Experimental conditions as for Fig. 2.

The contour plots are probably of greatest use in indicating the most suitable trajectory to follow in the EEM, in order to obtain conventional synchronous luminescence spectra for the complete resolution of overlapping component peaks. The synchronous scan describes a 45° trajectory through the EEM and, for oxytetracycline and riboflavin, the scan ($\Delta\lambda$ = 40 nm) shown on the contour plot (Fig. 3B) yielded the best attainable resolution of the two drug components, within the constraints of the method. The resolution is shown in Fig. 4. It did not, however, afford sufficient selectivity to resolve completely the overlapping emission peaks of oxytetracycline and riboflavin.

A similar degree of overlap was observed in the spectra of a number of phenothiazines and their spectrally similar sulphoxide oxidation products, as exemplified by the antipsychotic drug chlorpromazine, which is formulated in a number of dosage forms. The spectra are shown in Fig. 5B. The synchronous scan illustrated in Fig. 5A at an optimum separation of $\Delta\lambda$ = 20 nm gave a single sharp peak (λ_{ex} = 294 nm) for the sulphoxide as previously described [20]. At $\Delta\lambda$ = 130 nm (Fig. 5C), which is the most favourable value for the determination of the parent phenothiazine, the major peak shows a small degree of fine structural overlap with the oxidation product (λ_{ex} = 281 nm), which does not, however, interfere in the assay. By employing variable-angle synchronous scanning, complete resolution of the overlapping emission spectra can be achieved, as discussed below.

For the overlapping peaks of oxytetracycline and riboflavin, it was possible

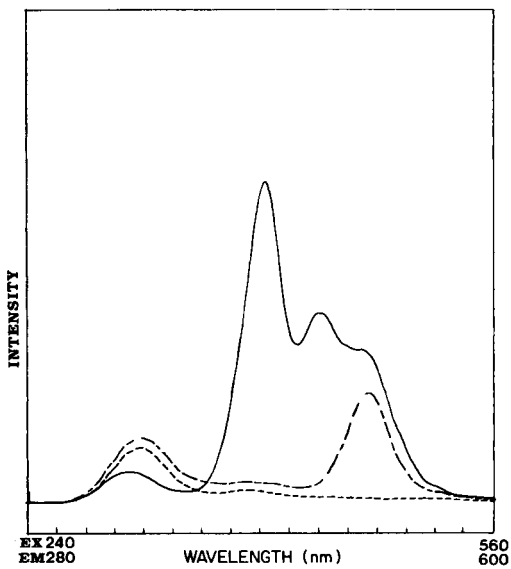


Fig. 4. Synchronous luminescence spectra ($\Delta\lambda$ = 40 nm): (—) oxytetracycline and riboflavin in phosphate buffer (pH 5.0)/aluminium chloride (0.2 M); (— · —) riboflavin alone; (---) pH 5.0 buffer alone. Concentrations and instrumental conditions as for Fig. 2.

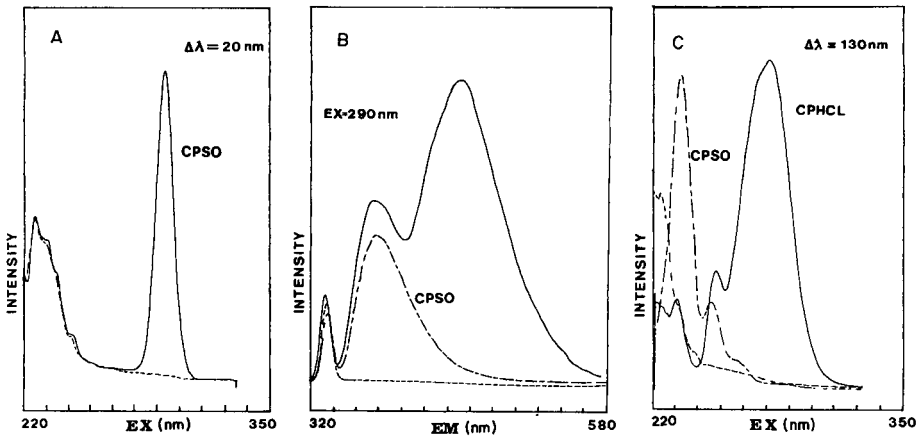


Fig. 5. (A) Synchronous luminescence spectra: (—) chlorpromazine sulphoxide ($\Delta\lambda$ = 20 nm) in pH 3.0 buffer; (---) buffer alone. (B) Emission spectra of chlorpromazine and its sulphoxide impurity, illustrating the extent of spectral overlap (λ_{ex} = 290 nm): (—) chlorpromazine hydrochloride (10.40 μg ml^{-1}) with chlorpromazine sulphoxide (0.258 μg ml^{-1}, CPSO) in phosphate buffer (pH 3.0); (— - —) CPSO alone (0.258 μg ml^{-1}) in phosphate buffer (pH 3.0); (---) buffer alone. (C) Synchronous luminescence spectra in pH 3.0 buffer: (—) chlorpromazine hydrochloride (CPHCl) ($\Delta\lambda$ = 130 nm); (— - —) CPSO; (---) buffer alone.

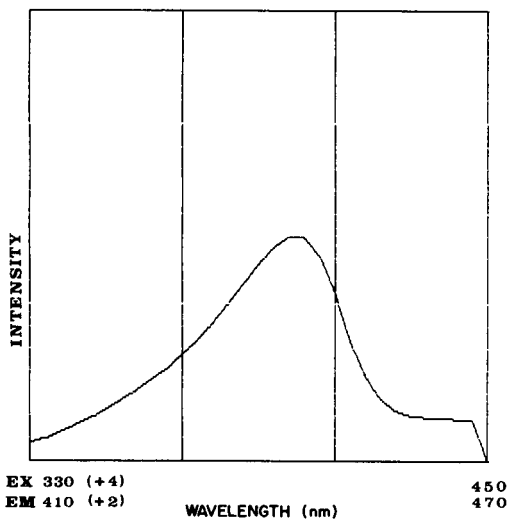


Fig. 6. Variable-angle synchronous scanning spectrum of oxytetracycline at 63.4° from (λ_{ex}, λ_{em}) = (330, 410 nm) through the emission-excitation matrix. Excitation monochromator increment, +4 nm; emission monochromator increment, +2 nm; concentration and instrumental conditions as for Fig. 2.

to obtain spectral resolution by slicing the matrix at 63.4° (Fig. 6). The v.a.s.s. technique allowed the quantitation of oxytetracycline (Table 1), independently of the riboflavin concentration up to 15% (w/w). Attempts to quantify riboflavin in the presence of oxytetracycline were not successful. However, riboflavin could readily be assayed by measurement in the absence of aluminium, when the tetracycline contribution is very slight. The calibration graph was linear for 0—6.1 mg ml^{-1} oxytetracycline in the presence of a constant concentration of riboflavin. The regression line was $y = 12.30\ x + 16.1$ ($n = 5$, $r = 0.991$). The relative standard deviation (r.s.d.) at 4.88 $\mu g\ ml^{-1}$ was 1.96% ($n = 10$).

The chlorpromazine hydrochloride (CPHCl) emission peak was similarly resolved from the interfering sulphoxide by slicing the EEM at a 'reversed' angle of 135° (i.e., λ_{ex} values decreased with increasing λ_{em}). In Fig. 7, both the contour plot and the optimum v.a.s.s. plot for CPHCl are shown. A linear calibration graph was obtained for the parent phenothiazine over the range 0—7.35 $\mu g\ ml^{-1}$, independently of the amount of sulphoxide up to 10% degradation. The regression line was $y = 26.2\ x + 10.24$ ($n = 5$, $r = 0.9894$). The r.s.d. at 5.88 $\mu g\ ml^{-1}$ was 2.34% ($n = 10$). The results shown in Table 1 indicate that the amount of the parent drug in each of the two dosage forms was in reasonable agreement with declared label strength.

The ability to describe any path through the data matrix by non-linear v.a.s.s., without the restriction of scanning in a straight line, raises a number of possibilities. In a complex system, the maximum and minimum emission intensities can be explored by traversing the peaks and valleys. Or, as illustrated in Fig. 8, a curved trajectory can be described through the EEM which permits the light-scattering peaks to be avoided. Improved resolution of overlapping systems which cannot be separated by linear scanning may be possible by this technique. The use of non-linear v.a.s.s. to generate diagnostic profiles of drugs, their degradation products and metabolites offers a new technique for qualitative characterisation in pharmaceutical analysis.

Conclusions

The flexibility conferred by v.a.s.s. has been shown to be successful in resolving peak overlap experienced in two differing problem areas, that of

TABLE 1

Results for the assay of parent drug in the respective dosage forms by v.a.s.s.[a]

Drug	Found (mg)	Recovery (%)	R.s.d. (%)
Oxytetracycline capsules (250 mg)[b]	236.6	94.6	1.96
Chlorpromazine hydrochloride tablets (25 mg)[b]	24.1	96.4	2.34

[a]Conditions and experimental parameters as in Figs. 6 and 7. [b]Nominal content.

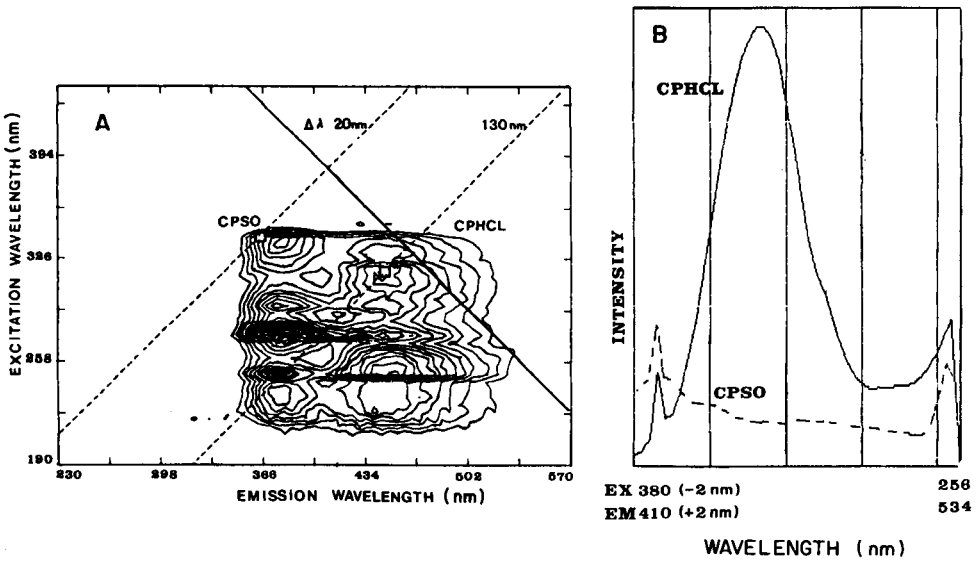


Fig. 7. (A) Contour plot of the fluorescence of chlorpromazine hydrochloride (CPHCl) and its sulphoxide impurity (CPSO): (---) conventional synchronous luminescence paths at 45° through the data matrix for $\Delta\lambda$ = 20 nm and 130 nm; (—) v.a.s.s. path at a 'reversed' angle of 135°. (B) V.a.s.s. spectra at a 'reversed' angle of 135° from (380, 410 nm) (excitation monochromator increment, −2 nm, emission monochromator increment, +2 nm).

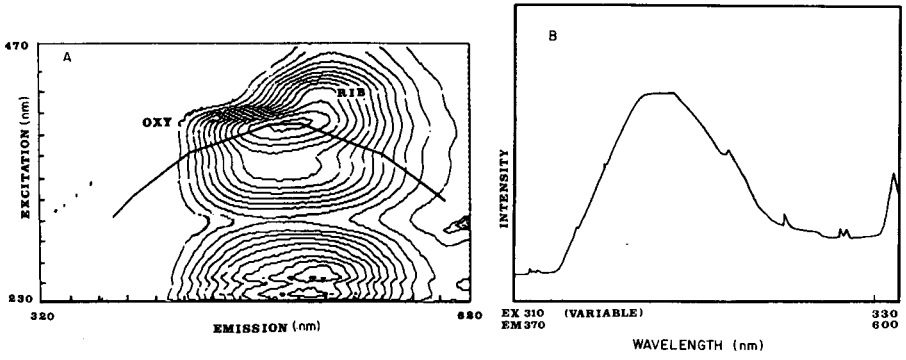


Fig. 8. (A) Contour plot of oxytetracycline and riboflavin demonstrating a curved trajectory cut through the data matrix (cf. Fig. 3B). (B) V.a.s.s. spectrum obtained from the curved trajectory scan. The contour plot was generated by linking 6 consecutive segments in sequence. The excitation and emission monochromator increments varied progressively for each segment, starting at (λ_{ex}, λ_{em}) = (310, 370 nm). Experimental conditions as in Fig. 2, except that the Apple-II microcomputer was used.

a drug and its interfering degradation product and of a drug and its co-formulated components. The technique shows promise as a new approach in the analysis of materials other than pharmaceuticals and could also be applied to further areas of luminescence spectroscopy, such as phosphorescence.

The possibility of describing paths cut at any angle and direction through the data matrix, reported here for the first time, merits further investigation as a novel technique for qualitative and quantitative analysis.

We thank Mr. Barry A. Rowe and Dr. Steven G. Winfield of Perkin-Elmer, Beaconsfield, U.K., for valuable and stimulating discussions. Thanks are also extended to Dr. Hugh P. Scott and Mr. Sandy Yates (Heriot-Watt University) for their valuable assistance in the design of some of the programs employed. The helpful encouragement of Professor James N. Miller (Loughborough University of Technology) in the early stages of this work is gratefully acknowledged.

REFERENCES

1 R. E. Santini, M. J. Milano and H. L. Pardue, Anal. Chem., 45 (1973) 915A.
2 I. M. Warner, J. B. Callis, E. R. Davidson, R. Gouterman and G. D. Christian, Anal. Lett., 8 (1975) 665.
3 M. A. Ryan, R. J. Miller and J. D. Ingle, Anal. Chem., 50 (1978) 1772.
4 Y. Talmi, Appl. Spectrosc., 36 (1982) 1.
5 C. N. Ho, G. D. Christian and E. R. Davidson, Anal. Chem., 53 (1981) 92.
6 W. Lindberg, J. A. Persson and S. Wold, Anal. Chem., 55 (1983) 643.
7 J. N. Miller, Proc. Anal. Div. Chem. Soc., 16 (1979) 203.
8 G. L. Green and T. C. O'Haver, Anal. Chem., 46 (1974) 2191.
9 T. C. O'Haver and W. M. Parks, Anal. Chem., 46 (1974) 1886.
10 J. B. F. Lloyd, Nature (London), Phys. Sci., 231 (1971) 64.
11 T. Vo-Dinh, Modern Fluorescence Spectroscopy, Vol. 4, Plenum Press, New York, 1981, p. 167.
12 T. Vo-Dinh, R. B. Gammage and P. R. Martínez, Anal. Chem., 53 (1981) 253.
13 P. John and I. Soutar, Anal. Chem., 48 (1976) 520.
14 I. Soutar, Anal. Proc., 20 (1983) 19.
15 J. C. André, Ph. Baudot and M. Niclause, Clin. Chim. Acta, 76 (1977) 55.
16 J. N. Miller and A. F. Fell, J. Pharm. Pharmacol., 32 (1980) 70P.
17 J. N. Miller, T. A. Ahmad and A. F. Fell, Anal. Proc., 19 (1982) 37.
18 Ph. Baudot and J. C. André, Anal. Lett., 15 (1982) 471.
19 L. J. C. Love and R. Weinberger, Spectrochim. Acta, Part B, 38 (1983) 1421.
20 B. J. Clark and A. F. Fell, J. Pharm. Pharmacol., 35 (1983) 22P.
21 E. L. Inman, Jr. and J. D. Winefordner, Anal. Chem., 54 (1982) 2018.
22 D. L. Eastwood, in E. L. Wehry (Ed.), Modern Fluorescence Spectroscopy, Vol. 4, Plenum Press, New York, 1981, p. 251.
23 J. N. Miller, Analyst (London), 109 (1984) 191.
24 B. J. Clark, A. F. Fell, I. E. Aitchison, D. M. G. Pattie, M. H. Williams and J. N. Miller, Spectrochim. Acta, Part B, 38 (1983) 61.
25 B. J. Clark, A. F. Fell, H. P. Scott and D. Westerlund, J. Chromatogr., 286 (1984) 261.
26 D. Hall, J. Pharm. Pharmacol., 28 (1976) 420.

Analytica Chimica Acta, 170 (1985) 45—59
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

FLUORESCENCE DECAY OF α-CHYMOTRYPSIN STUDIED BY THE PICOSECOND-RESOLVED SINGLE PHOTON-COUNTING TECHNIQUE

G. DESIE, N. BOENS*, M. VAN DEN ZEGEL and F. C. DE SCHRYVER*

Department of Chemistry, Katholieke Universiteit Leuven, Celestijnenlaan 200F, B-3030 Heverlee (Belgium)

(Received 6th September 1984)

SUMMARY

The fluorescence decay of the multi-tryptophan-containing enzyme α-chymotrypsin in Tris buffer (pH 7.8) at room temperature was studied using a frequency-doubled, synchronously-pumped picosecond rhodamine-6G laser excitation source with time-correlated single photon-counting detection. The fluorescence decay parameters were computed with a non-linear least-squares iterative reconvolution program. The goodness-of-fit was tested with well-known graphical methods such as residuals plots and the autocorrelation function. Numerical tests (reduced chi-square, ordinary runs test and the Durbin—Watson statistic) were included to improve the reliability of the residuals analysis. Normal distribution of the weighted residuals was checked with the normal probability plot, and with computation of the mean and standard deviation of the weighted residuals. α-Chymotrypsin exhibited triple-exponential fluorescence decay kinetics with decay times of 615 ± 76 ps, 1.7 ± 0.2 ns, and 4.3 ± 0.3 ns. The fractional fluorescence contributions depended on the emission wavelength. The fluorescence spectra of the components contributing to the total fluorescence were calculated from the steady-state fluorescence spectrum and fluorescence decays at different emission wavelengths, and from convoluted time-resolved emission spectra and a fluorescence decay measurement.

The fluorescence emission of the tryptophan residue(s) of many proteins has frequently been used as an intrinsic spectroscopic probe [1—7]. The fluorescence behavior of the indole chromophore depends on several parameters such as solvent polarity and the proximity of quenching groups [8—12]. Its fluorescence maximum is shifted to longer wavelengths in polar isotropic media [13—16]. This property has often been used as an indication for solvent accessibility to the chromophore. The tryptophan residues of several proteins have been classified according to their fluorescence maxima [17, 18].

Since time-resolved techniques have become available, the fluorescence kinetics of many proteins have been investigated. Indole and many indole-containing compounds were found to decay mono-exponentially [12]. The fluorescence decay of tryptophan in neutral aqueous solution [19] and the fluorescence of some proteins containing one tryptophan [20] could not be described adequately by a mono-exponential decay function. The photophysics of tryptophan in aqueous solutions has been studied extensively [12, 19, 21—25]. The most plausible interpretation for the non-exponential

0003-2670/85/$03.30

fluorescence decay of tryptophan was given by Szabo and Rayner [23] and Petrich et al. [12]. At room temperature, zwitterionic tryptophan consists of different ground-state rotamers. The C_α substituents (NH_3^+ and COO^-) have different quenching affinities for indole. Because the ammonium group is only present at the end amino acids of proteins, tryptophan has now been abandoned as a model compound for the tryptophanyl residues in proteins. *N*-Acetyl-L-tryptophanamide (NATA) which has two amide bonds is now considered to be a better model compound [26, 27]. Its fluorescence decay is mono-exponential in aqueous solutions [12, 28].

The multi-exponential fluorescence decay of tryptophanyl residues in many proteins has sub-nanosecond components which could not be resolved accurately by conventional time-correlated single photon-counting techniques [28—33]. Recently, the advent of pulsed picosecond laser systems as excitation sources [34] and the use of excitation pulse-shape mimic compounds [35, 36] has pushed the time resolution of the single photon-counting technique into the picosecond region, improving considerably the accuracy of measurements of the short-lived fluorescence decay of protein samples [37—39].

This report describes the fluorescence decay of the multi-tryptophan-containing enzyme, α-chymotrypsin (α-CT) in tris(hydroxymethyl)aminomethane (Tris) buffer pH 7.8 at room temperature. The fluorescence spectrum of α-CT was resolved into contributions of individual tryptophans or classes of tryptophans.

EXPERIMENTAL

Materials

α-Chymotrypsin (EC 3.4.21.1) type I-S (from bovine pancreas, 3× crystallized, lyophilized, essentially salt-free; Sigma) and *N*-acetyl-L-tryptophanamide (NATA; Sigma) were used as received. Thin-layer chromatography of NATA with different solvent mixtures showed a single spot. Benzo[b]-indeno[1:2-e]pyran (BIP) was synthesized by Boyd's procedure [40], and purified by column chromatography over silica gel with dichloromethane as eluent. Freshly prepared enzyme solutions in Tris buffer (pH 7.8) were used in the measurements. Deionized/distilled water was used to prepare buffer solutions.

Instrumentation

The pH was measured with a Radiometer PHM82 meter. Absorption spectra were recorded on a Perkin-Elmer Lambda-5 u.v.-visible spectrophotometer. Fluorescence spectra were obtained with a Spex Fluorolog 1902 which had double monochromators in both excitation and emission, or with a Spex Fluorolog 212/Datamate. Both apparatus were interfaced to a Digital Equipment Corporation PDP-11/23 computer for data analysis. For fluorescence measurements, solutions were not degassed; degassing had no

measurable effect on the fluorescence decay times and quantum yields of the enzyme solutions. The samples were contained in quartz fluorescence cuvettes with a 1-cm optical path length. The fluorescence decay curves of the samples were obtained by using the time-correlated single photon-counting technique [34, 41]. A diagram of the picosecond time-resolved fluorimeter used for the fluorescence decay measurements reported here is shown in Fig. 1. Sample excitation in the range 290—315 nm was achieved with a frequency-doubled, cavity-dumped, mode-locked, synchronously-pumped dye laser system as follows. An extended-cavity 5-W argon-ion laser (Spectra-Physics Model 16509) was mode-locked by using an ultra-stable mode-locking system (Spectra-Physics Model 342A) giving pulses of about 150 ps f.w.h.m. (full width at half maximum), at a repetition rate of 82 MHz, and with an average power of 300 mW. The synchronization photodiode was removed from the cavity dumper and set to monitor the laser beam; this modification improved the long-term stability of the pulse shape and intensity. The argon-ion laser pulses were used to excite rhodamine 6G in ethylene glycol (Spectra-Physics) in a jet-stream dye laser (Spectra-Physics Model 375B) with a cavity length equal to that of the ion laser. An acousto-optic cavity dumper (Spectra-Physics Model 344S) and cavity dumper driver (Spectra-Physics Model 454) were used to output pulses at a rate of 400 or 800 kHz. Wavelength tuning was done with a tuning wedge (Spectra-Physics Model 375) incorporated in the dye laser cavity. After careful alignment, average powers of 30 mW could be obtained at a pulse repetition rate of 800 kHz. The pulse width was ca.

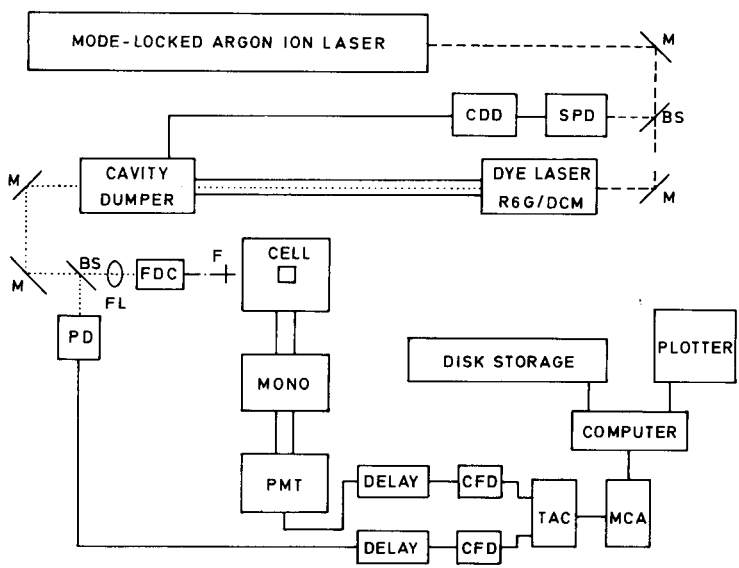


Fig. 1. Picosecond fluorescence spectrometer: BS, beam splitter; CDD, cavity dumper driver; CFD, constant fraction discriminator; F, Corning 7-54 filter; FDC, frequency-doubling crystal; FL, focussing lens; M, mirror; MCA, multichannel analyzer; MONO, monochromator; PD, photodiode; PMT, XP2020Q photomultiplier tube; SPD, synchronization photodiode; TAC, time-to-amplitude converter.

10 ps, measured with a Spectra-Physics autocorrelator. Frequency doubling was achieved with an angle-tuned Inrad model 563-1117 KDP "B" crystal. The dye laser light was focused on the crystal through a lens. The residual undoubled light was removed with a Corning 7-54 filter. The laser system was mounted on a mechanically isolated optical table (Newport Research Corporation) in a room with temperature (22 ± 1°C) and humidity (ca. 60%) control.

Fluorescence was monitored at right angles to the excitation path by a fast photomultiplier tube (Philips XP2020Q) mounted in an Applied Photophysics voltage divider. Because the frequency-doubled light is horizontally polarized, no polarizer was inserted in the emission path. Magic angle detection (through a Karl Lambrecht MUG TS10, angle 54°44′, polarizer) gave the same results but at a reduced count rate. In the emission path a Jobin-Yvon DH-20-Vis double monochromator was used. The f.w.h.m. of the scattered excitation pulse shape measured by the time-correlated fluorimeter was ca. 250 ps. The convoluted mono-exponential fluorescence decay of benzo[b]indeno[1:2-e]pyran or xanthione in iso-octane [36] was used to mimic the excitation pulse shape. With this technique, the sample fluorescence and the instrument response function are monitored at the same wavelength, ruling out any wavelength-dependent characteristics of the photomultiplier tube (PMT) for fluorescence detection.

Conventional time-correlated single photon-counting detection methods were used to obtain the sample fluorescence decay and the excitation pulse shape. The start signal for the time to the pulse-height analyzer (time-to-amplitude converter; TAC; Canberra Model 2043) was taken from a fast photodiode (Spectra-Physics Model 403B) monitoring a fraction of the undoubled dye-laser beam. Quad constant fraction discriminators (Ortec Model 934) were used to eliminate background pulses and to provide the correct voltage signals for the TAC. The TAC was operated in the normal configuration, the voltage ramp being initiated by the photodiode signal and terminated by a signal from the XP2020Q photomultiplier. Pulse pile-up distortion was avoided by working at an average count of 0.02—0.005 photons per excitation pulse. The output pulse of the TAC was fed into a biased amplifier (Canberra Model 1467) to sample a section of the TAC range and expand it to cover the full voltage range of the multichannel analyzer (MCA; Canberra Model 8100) working in the pulse-height analysis mode. The Canberra Model 1467 allows a linear expansion of the MCA time-scale down to about 1 ps/channel. Data were collected in 256 channels of the MCA.

α-Chymotrypsin solutions were excited with 296-nm radiation in order to minimize the contribution of tyrosine residues to the total fluorescence. The absorbance of the samples at 296 nm was 0.06—0.07. Tryptophan emission could be monitored from 300 to 450 nm. Data collection was stopped when the total number of counts of emitted photons reached at least 10^4 in the MCA peak channel. Data accumulated in the multichannel analyzer were transferred to a Digital Equipment Corporation PDP-11/23 computer for processing.

Residuals analysis

The experimental fluorescence decay curve, $I(t)$, is a convolution of the true decay, $i(t)$, with the measured instrument response function, $E(t)$, i.e.,

$$I(t) = \int_0^t E(t-u)\, i(u)\, du \tag{1}$$

When $I(t)$ and $E(t)$ are known, $i(t)$ can be determined by a non-linear least-squares iterative reconvolution method [42, 43], based on the algorithm of Marquardt [44]. The experimental fluorescence decays were analyzed by using trial decay functions containing one, two or three exponentially decaying terms

$$i(t) = \sum_{i=1}^{n} \alpha_i \exp(-t/\tau_i) \quad (n = 1, 2, 3) \tag{2}$$

where α_i are pre-exponential factors and τ_i are decay times.

The differences between the experimental (observed) decay data and the values calculated (predicted) after a decay law had been fitted to the data [36] were carefully inspected. The most widely used technique for examining error terms in time-correlated single photon-counting experiments involves the plotting of the weighted residuals, R_i, (see below) against time (or channel number). The patterns of such a plot are helpful in choosing the correct functional form of the decay law, in determining the need for additional terms in the decay function, and in detecting outliers. There should be no discernible trend in the plot of the residuals, R_i, versus channel number, i, only a random scatter of points about the line $R_i = 0$. In detecting heteroscedasticity [45] (error terms with unequal variances), it is helpful to plot the (squared) residuals versus the calculated values $\hat{Y}_i$. A graph of R_i vs. $\hat{Y}_i$ should reflect a random scatter of points about a line with zero slope. Systematic patterns would indicate an inadequate model for the decay law. Examining the plot of the autocorrelation function can also indicate if the model chosen for the decay function is adequate [46].

Although residual plots can easily be analyzed and interpreted, their interpretation is somewhat subjective and sometimes biased. Therefore several numerical statistical tests were used to make the residuals analysis more reliable. The first numerical test is the calculation of the reduced chi-square statistic, χ^2_ν, given by $\chi^2_\nu = (n-p)^{-1} \Sigma_{i=1}^{n} R_i^2$, where the residual, R_i, is defined as $R_i = (Y_i - \hat{Y}_i)/\sigma_i$. Here, Y_i is the fluorescence signal detected in channel number i with standard deviation σ_i, $\hat{Y}_i$ is the value calculated from the decay function, n is the number of data points and p is the number of adjustable fitting parameters. The quantity $\nu = n - p$ is the number of degrees of freedom. $\hat{Y}_i$ is calculated from $E(t)$ and $i(t)$, according to Eqn. 1. Because the experimental values of the fluorescence decay curves are drawn from Poisson distributions, σ_i can be approximated by $Y_i^{1/2}$ or, more accurately, by $\hat{Y}_i^{1/2}$.

It is convenient to convert the chi-square statistic to a standard normal

deviate, Z_{χ^2} given by $Z_{\chi^2} = (\nu/2)^{1/2}(\chi_\nu - 1)$ [47]. The needed probability can be determined from a table of standard normal probabilities, e.g., $|Z_{\chi^2}| < 1.645$ for testing at the 90% confidence level, to $|Z_{\chi^2}| < 3.891$ at the 99.99% level. Although the chi-square test is a powerful test for goodness-of-fit, it does not take any account of the signs of the residuals, of their distribution and of their correlation. Therefore additional tests are necessary to examine those properties of the residuals.

One method of evaluating the hypothesis that the time sequence of the errors associated with the experimental decay data are randomly distributed is to examine the arrangement of signs (+ or —) of the corresponding residuals [45, 47, 48]. In the "ordinary runs test" the number of sequences of residuals with the same sign (ordinary runs), r, is counted and compared with the number expected, μ, for a set of random numbers. When the number of residuals with plus, n_1, and minus, n_2, signs both exceed 20, the normal approximation can be made and the hypothesis of randomness can be tested from a table of standard normal probabilities

$$Z = (r - \mu + 0.5)/\sigma$$

where $\mu = [2n_1n_2/(n_1 + n_2)] + 1$ and $\sigma^2 = 2n_1n_2(2n_1n_2 - n_1 - n_2)/[(n_1 + n_2)^2(n_1 + n_2 - 1)]$. Here, σ is the standard deviation of the runs distribution.

To test for serial correlation between residuals, the Durbin—Watson test statistic d [49]

$$d = \sum_{i=1}^{n} (R_i - R_{i-1})^2 / \sum_{i=1}^{n} R_i^2$$

was calculated. For a mono-exponential decay with $n \geqslant 100$, the residuals are regarded as uncorrelated at the 0.05 (one-tail) significance level if $d > 1.69$; if $d < 1.65$ the residuals are considered to be correlated; when d lies between these values no conclusions can be drawn. For a double-exponential decay with $n \geqslant 100$, the corresponding critical lower and upper limits are 1.73 and 1.63 respectively.

Checking that the residuals are normally distributed is important especially if confidence intervals are to be calculated. The most popular graphical approach to evaluating the normality of a distribution is the normal probability plot [45, 50], in which the n ordered residuals R_i are plotted against the inverse cumulative normal distribution function $F^{-1}(i/(n + 1))$. If the distribution is normal, the points should lie roughly on a straight line. If a normal distribution with the same mean and standard deviation as the sample data is plotted on the same figure, it can be used as a reference for evaluating the assumption of normality. The other tests for assessing the normality of the error terms include the determination of the percentage of the residuals within the [2, —2] interval, and calculation of the mean and standard deviation of the residuals. These values should be reasonably close to the predicted values of 95.44%, 0.0 and 1.0, respectively.

Time-resolved emission spectra and decay-associated spectra

Time-resolved emission spectra are experimental emission spectra obtained during discrete time intervals after excitation. Decay-associated spectra are derived spectra linked to decay functions and represent the spectral distributions of the individual emitting species which contribute to the total fluorescence. They can be generated from time-resolved single photon-counting data by two procedures.

The first method requires the measurement of a steady-state emission spectrum and decay curves at different emission wavelengths [30, 31, 51, 52]. Each decay curve is resolved into its n decay times, τ_i and n pre-exponential factors, α_i. The fractional contribution, $Q_i(\lambda)$, of each decay component to the total fluorescence is calculated from the decay times and the pre-exponential factors from

$$Q_i(\lambda) = \alpha_i\tau_i / \sum_{i=1}^{n} \alpha_i\tau_i \tag{3}$$

The decay-associated spectrum $F_i(\lambda)$ of the ith component can then be calculated from

$$F_i(\lambda) = F_{ss}(\lambda)Q_i(\lambda) \tag{4}$$

where $F_{ss}(\lambda)$ is the steady-state emission intensity at wavelength λ. This procedure requires much experimental and computation time to produce spectra with high resolution, but it has the advantage of providing time-resolved emission spectra free of convolution artifacts.

The second method requires the measurement of at least n time-resolved emission spectra and one decay curve to evaluate the decay times of the n components [52, 53]. The fluorescence decay of a n-component mixture excited by a δ-pulse is given by Eqn. 2. When the excitation pulse has a finite width, the observed fluorescence decay is given by

$$I(\lambda, t) = \sum_{i=1}^{n} \alpha_i(\lambda) \int_0^t E(u) \exp[-(t-u)/\tau_i]\, du \tag{5}$$

The fluorescence intensity observed in a time-resolved emission spectrum is given by

$$I(\lambda, a \rightarrow b) = \sum_{i=1}^{n} \alpha_i(\lambda) \int_a^b \int_0^t E(u) \exp[-(t-u)/\tau_i]\, du\, dt \tag{6}$$

where a and b define the time-window used to record the spectrum. Similar equations can be written for each selected time-window. Equation 6 can be written in matrix notation

$$[F_j(\lambda)] = [C_{ji}]\,[A_i(\lambda)] \tag{7}$$

where $F_j(\lambda)$ is the time-resolved spectrum measured with time-window j, $A_i(\lambda)$

represents the relative intensity of the decay-associated spectrum of the *i*th component and C_{ji} is the double integral of Eqn. 6 associated with the *i*th component and time-window *j*. The values for $A_i(\lambda)$ can be obtained by a standard matrix inversion program [54]. This method can easily be programmed for a digital computer and the time required to obtain decay-associated spectra with high resolution is short.

RESULTS

The fluorescence decays of α-chymotrypsin (α-CT) and its tryptophanyl-model compounds (1,2-dimethylindole and NATA) were studied at room temperature. The emission was monitored at 370 nm with 296-nm excitation. The fluorescence decays of 1,2-dimethylindole and NATA could be described adequately by a mono-exponential decay function. Their respective lifetimes were found to be 5.78 ± 0.05 ns and 2.90 ± 0.04 ns. A fluorescence decay fitting result of NATA in Tris buffer is shown in Fig. 2. The corresponding normal probability plot is given in Fig. 3. The fluorescence decay of α-CT could not be described adequately by a single- or double-exponential decay

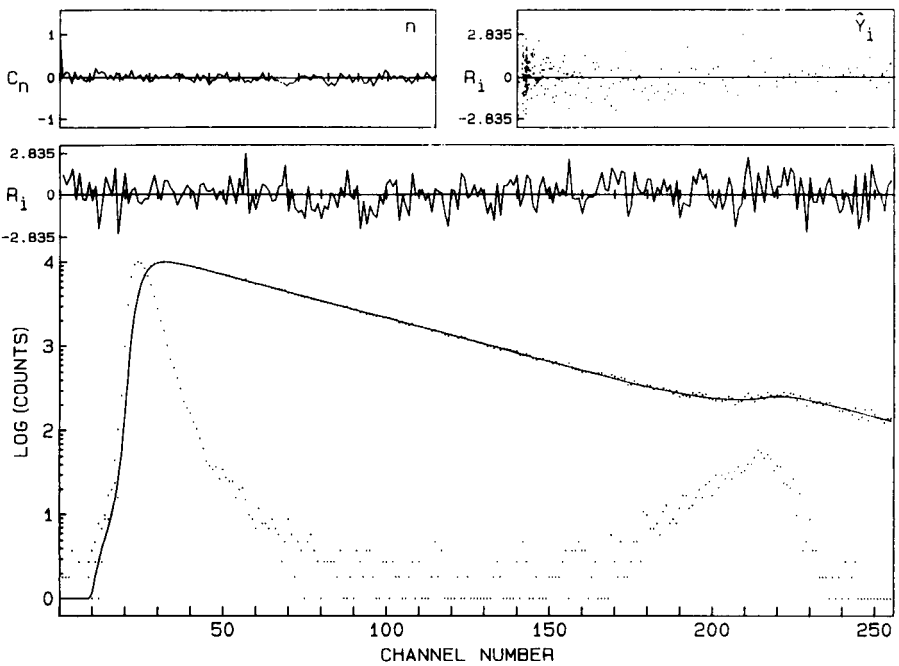


Fig. 2. Experimental fluorescence decay curve (point plot) of NATA in Tris buffer (pH 7.8). The decay function calculated between channels 1 and 255 is shown as a solid line. The instrument response function (point plot) is the convoluted decay of BIP in iso-octane. λ_{ex} = 296 nm; λ_{em} = 370 nm; channel width 71 ps; χ^2_ν = 1.01; Z_{χ^2} = 0.10; ordinary runs Z = —0.72; Durbin—Watson d = 1.92. Also shown are the autocorrelation function C_n and residual plots vs. channel number and vs. the calculated decay function values $\hat{Y}_i$.

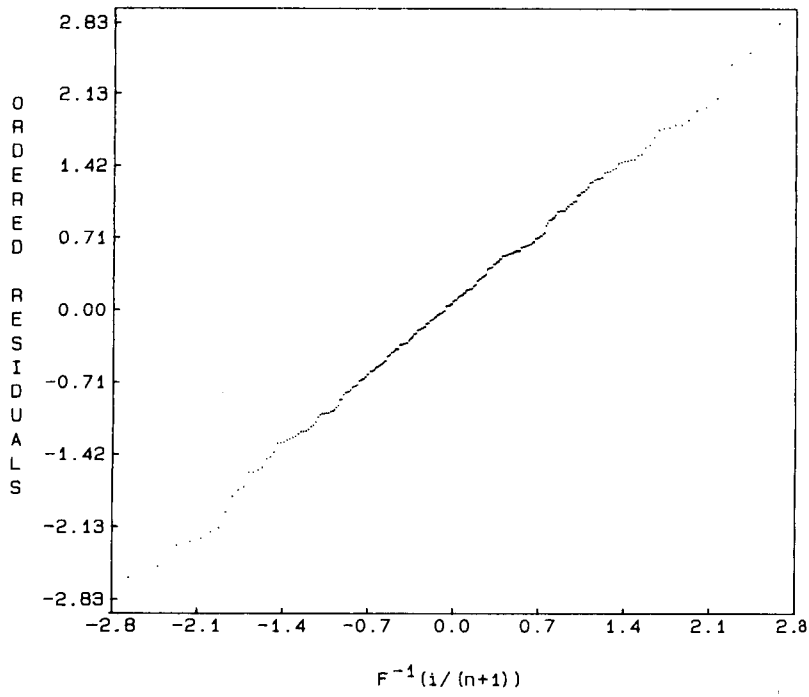


Fig. 3. Normal probability plot for the decay given in Fig. 2; 95.46% of the weighted residuals are in the [—2, 2] interval. Their mean and standard deviation are 0.06 and 1.00, respectively.

function. Statistically acceptable fits were obtained only when the experimental data were fitted to a three-exponential decay law (Fig. 4, Table 1).

The fluorescence decay of NATA and α-CT was analyzed at different emission wavelengths. For NATA, the fluorescence decays were mono-exponential and the lifetimes (2.90 ± 0.04 ns) were independent of the emission wavelength between 330 and 440 nm. The fluorescence decay data of α-CT as a function of emission wavelength could only be described by a sum of three exponential terms. The three kinetic components had decay times of 615 ± 76 ps, 1.7 ± 0.2 ns, and 4.3 ± 0.3 ns. The values of the decay times were independent of the emission wavelength. The contributions (Eqn. 3) of the decay components to the total fluorescence varied with emission wavelength. The contributions of each decay component to the total fluorescence are shown in Table 2. From the decay times and the pre-exponential factors, two different average lifetimes were calculated, namely $\langle\tau\rangle = \Sigma\alpha_i\tau_i^2/\Sigma\alpha_i\tau_i$ and $\tau_m = \Sigma\alpha_i\tau_i/\Sigma\alpha_i$. Here, $\langle\tau\rangle$ approximates the average lifetime measured by the phase-shift method [55] while τ_m approximates the integral of the impulse response obtained from pulse data [46]. Both $\langle\tau\rangle$ and τ_m increase with increasing emission wavelength (Fig. 5).

The decay-associated spectra calculated from the fractional fluorescence

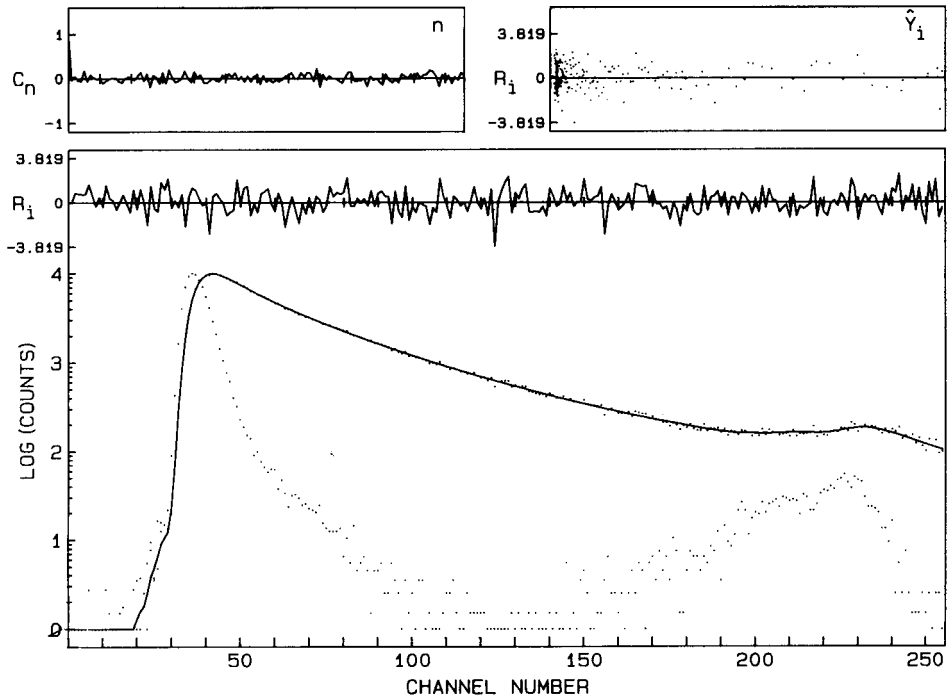


Fig. 4. Experimental fluorescence decay curve (point plot) of α-CT in Tris buffer (pH 7.8). The decay function calculated as a three-exponential is shown as a solid line (α_1 = 0.40, τ_1 = 562 ps, α_2 = 0.49, τ_2 = 1.66 ns, α_3 = 0.11, τ_3 = 4.27 ns). The instrument response function (point plot) is the convoluted decay of BIP in iso-octane. λ_{ex} = 296 nm; λ_{em} = 370 nm; channel width 71 ps; χ^2_ν = 1.03; Z_{χ^2} = 0.35; ordinary runs Z = 0.02; Durbin—Watson d = 2.11. Also shown are the autocorrelation function C_n and residual plots vs. channel number and vs. the calculated decay function values $\hat{Y}_i$.

TABLE 1

Statistical analysis of the non-linear least-squares iterative reconvolution of the decay data of NATA and α-CT (λ_{em} = 370 nm)
Treatment of NATA as a single exponential and α-CT as a single, double and triple exponential

	NATA	α-CT 1-exp	α-CT 2-exp	α-CT 3-exp
χ^2	1.01	34.15	1.63	1.03
Z_{χ^2}	0.10	372.15	7.07	0.35
O.R.[a]	—0.72	—12.61	—2.97	0.02
D.W.[b]	1.92	0.08	1.33	2.11
C.S.[c]	—0.82	—384.76	—10.04	—0.33
%[d]	95.46	18.82	90.59	94.51
μ[e]	0.06	2.03	0.13	0.06
σ[f]	1.00	5.45	1.26	1.00

[a]Ordinary runs statistic Z. [b]Durbin—Watson statistic d. [c]Combined statistic C.S. = $Z - |Z_{\chi^2}|$ [47]. [d]% of weighted residuals in [—2, 2]. [e]Mean of weighted residuals. [f]Standard deviation of weighted residuals.

TABLE 2

Fractional fluorescence contributions $Q_i(\lambda)$ (%) of α-CT in Tris buffer pH 7.8 as a function of emission wavelength[a]

λ_{em} (nm)	Q_1	Q_2	Q_3	λ_{em} (nm)	Q_1	Q_2	Q_3
305	40	47	13	370	19	55	26
310	37	48	15	380	19	52	29
315	23	63	14	390	19	50	31
320	24	59	17	400	18	49	33
330	22	59	19	410	18	47	35
340	22	58	20	420	16	45	39
350	21	57	22	430	15	44	41
360	20	55	25	440	15	41	44

[a]The standard errors of parameters Q_1, Q_2, Q_3 were ±1.

contributions $Q_i(\lambda)$ and the steady-state fluorescence emission spectrum of α-CT in Tris buffer pH 7.8 are plotted in Fig. 6. Three different time-resolved spectra of α-CT in this buffer were recorded at different time intervals after excitation; the spectra, normalized at 380 nm, are given in Fig. 7. The decay-associated spectra calculated from the time-resolved data by the procedure described above are shown in Fig. 8. Figures 6 and 8 show that the maxima of the decay-associated spectra shift to longer wavelengths with increasing decay time of the contributing components.

DISCUSSION

The fluorescence decay of 1,2-dimethylindole and NATA can be fitted to a mono-exponential decay law. Non-exponential fluorescence decay is

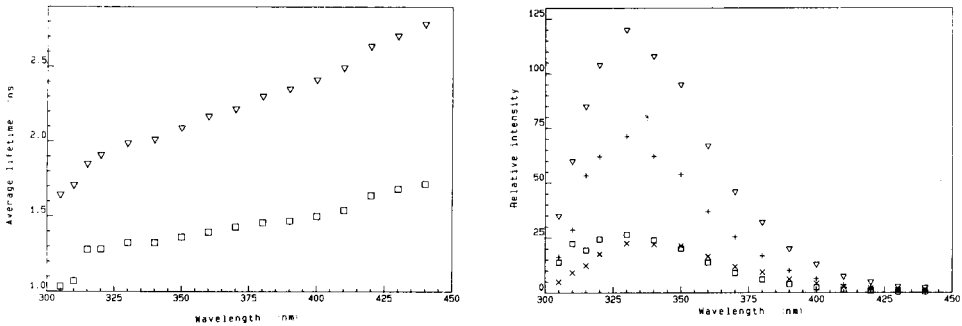


Fig. 5. Wavelength dependence of the average fluorescence lifetimes $\langle\tau\rangle$ and τ_m of α-chymotrypsin in Tris buffer pH 7.8 at λ_{ex} = 296 nm; (▽) $\langle\tau\rangle$; (□) τ_m.

Fig. 6. Decay-associated spectra of α-CT in Tris buffer pH 7.8 calculated from the steady-state fluorescence spectrum and the decays at different emission wavelengths: (□) the 615-ps component; (+) the 1.7-ns component; (×) the 4.3-ns component; (▽) steady-state emission spectrum.

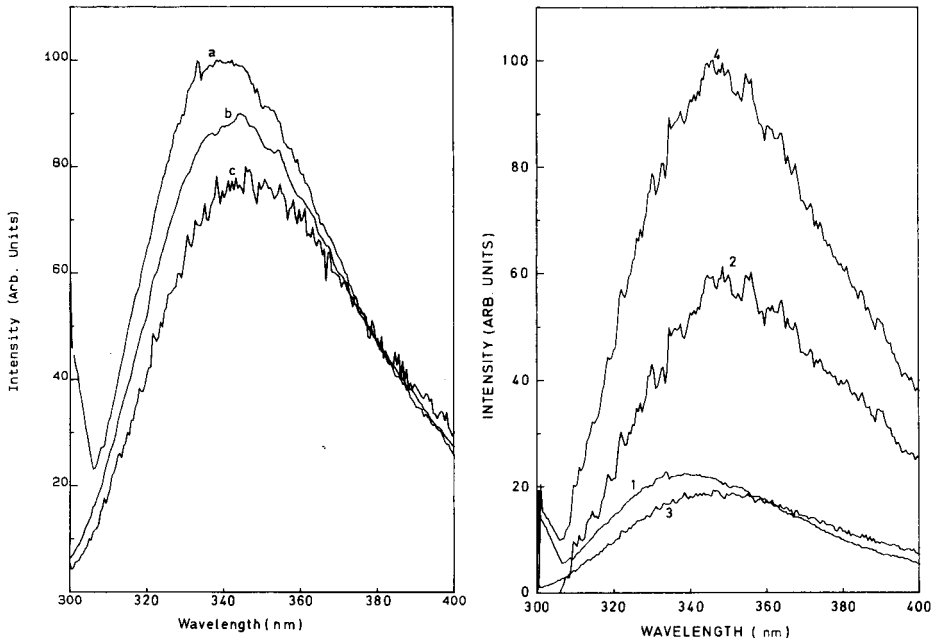


Fig. 7. Convoluted time-resolved emission spectra of α-CT in Tris buffer pH 7.8 at different time intervals after excitation at 296 nm (onset of the excitation pulse is taken as $t = 0$). Time intervals: (a) 0—1.4 ns; (b) 2.3—3.3 ns; (c) 6.9—9.0 ns. The spectra are normalized at 380 nm.

Fig. 8. Decay-associated spectra of α-CT in Tris buffer pH 7.8 generated from the time-resolved data and a fluorescence decay measurement: (1) the 615-ps component; (2) the 1.7-ns component; (3) the 4.3-ns component; (4) sum of 1, 2, 3.

possible if the indole chromophore is substituted with groups which have energy minima for different ground-state rotamers [12, 56, 57]. The two substituents on the C_α atom of NATA contain an amide bond. Both groups have nearly the same electrophilicity and their mean distances from the indole fluorophore are approximately equal. Therefore the different rotamers have nearly identical fluorescence decay times, and the decay can be fitted to a single-exponential function [12, 30]. The residuals analysis (Table 1) of the fluorescence decay fitting of NATA in Tris buffer pH 7.8 corroborates these results. The fluorescence lifetime (2.90 ± 0.04 ns) found for NATA is in excellent agreement with the data reported in the literature (Table 3).

The fluorescence decay kinetics of α-CT in Tris pH 7.8 could only be described by a triple-exponential function (Fig. 4 and Table 1). The statistical evaluations used make it possible to distinguish between single, double- and triple-exponential decays (Table 1). The weighted residuals obtained for the best fit based on single- or double-exponential decay kinetics clearly were not randomly distributed. The values calculated for the ordinary runs test

TABLE 3

Lifetimes of NATA

Excitation system	τ	χ^2	R.m.s.[a]	T (°C)	Ref.
Gated N_2-filled flash lamp	2.99	1.25		20	30
Gated N_2-filled flash lamp	3.79	1.27		10	31
O_2-filled free running lamp	2.93		0.0016		28
Dye laser[b]	2.8		0.0060	20	32
Dye laser[b]	2.95	<1.2		20	12
Dye laser[b]	3.97	<1.2		5	12
Dye laser[b]	3.00[c]			20	23
Dye laser[b]	2.90	1.01		22	This work

[a]Root mean square of the deviations between the calculated and experimental curves: r.m.s. = $\{(1/n)\Sigma_{i=1}^{n}[Y_i - \hat{Y}_i]^2\}^{1/2}$. The values of Y_i are normalized to 1.00 in the peak channel. [b]Rhodamine 6G. [c]Inspection of the residuals plots was used as the goodness-of-fit criterion.

confirm this (Table 1). Furthermore, the residuals were serially correlated as is shown by the Durbin—Watson test statistic. The autocorrelation function also indicates that the fit to a single- or double-exponential decay was unsatisfactory. The χ^2 statistic indicates a lack of fit when the data were fitted to a single- or double-exponential decay law. Only when the data were fitted to a triple-exponential decay was an excellent fit obtained. From Fig. 4 and Table 1, it is clear that in this case the weighted residuals were randomly distributed (Z = 0.02); no heteroscedasticity could be detected; the error terms were not correlated (autocorrelation function and Durbin—Watson d = 2.11). The χ^2 statistic (1.03), Z_{χ^2} (0.35), and the value (—0.33) for the combined statistic are indicative for a good fit [47]. The normal probability plot (not shown) indicated that the error terms are normally distributed. The values for the percentage (94.51) of the weighted residuals in [—2, 2], their mean (0.06) and standard deviation (1.00) are further evidence for normal distribution.

The decay times associated with the three kinetic components are 615 ± 76 ps, 1.7 ± 0.2 ns, and 4.3 ± 0.3 ns. The fractional contributions of the two short decay components (615 ps and 1.7 ns) to the total fluorescence (Eqn. 3, Table 2) decrease with increasing emission wavelength, while the contribution of the 4.3-ns decay component increases with increasing wavelength. Consequently, the average lifetimes $\langle\tau\rangle$ and τ_m increase with increasing wavelength (Fig. 5). The derived decay-associated spectra (Figs. 6 and 8) reveal the presence of at least three different classes of tryptophanyl residues. An explanation for the existence of these different classes is not given here. Further research on single-tryptophan proteins and tryptophan model compounds is necessary to elucidate the origin of the complex fluorescence behavior of α-chymotrypsin.

This research was supported in part by grants from the University Research Fund and the FKKO. G. D. and M. V. d. Z. are predoctoral fellows of the IWONL. N. B. is a research associate of the National Fund for Scientific Research (Belgium).

REFERENCES

1 I. Weinryb and R. F. Steiner, Biochemistry, 9 (1970) 135.
2 S. S. Lehrer, Biochemistry, 10 (1971) 3254.
3 F. A. Cotton, C. J. Bier, V. W. Day, E. E. Hazen, Jr. and S. Larsen, Cold Spring Harbor Symp. Quant. Biol., 36 (1972) 243.
4 C. B. Anfinsen, A. N. Schechter and H. Taniuchi, Cold Spring Harbor Symp. Quant. Biol., 36 (1972) 249.
5 S. Singh and D. M. Lee, Int. J. Biol. Macromol., 5 (1983) 243.
6 T. Hiratsuka, J. Biochem., 93 (1983) 875.
7 M. Chauvet, M. Bourdeaux, C. Briand, M. Dell'Amico, M. Sarrazin, C. Vincent and J. P. Revillard, Int. J. Peptide Protein Res., 21 (1983) 513.
8 L. J. Andrews and L. S. Forster, Photochem. Photobiol., 19 (1974) 353.
9 C. Conti and L. S. Forster, Biochem. Biophys. Res. Comm., 57 (1974) 1287.
10 R. Klein, I. Tatischeff, M. Bazin and R. Santus, J. Phys. Chem., 85 (1981) 670.
11 M. C. Chang, J. W. Petrich, D. B. McDonald and G. R. Fleming, J. Am. Chem. Soc., 105 (1983) 3819.
12 J. W. Petrich, M. C. Chang, D. B. McDonald and G. R. Fleming, J. Am. Chem. Soc., 105 (1983) 3824.
13 F. W. J. Teale, Biochem. J., 76 (1960) 381.
14 B. L. Van Duuren, J. Org. Chem., 26 (1964) 2954.
15 I. Weinryb and R. F. Steiner, in R. F. Steiner and I. Weinryb (Eds.), Excited States of Proteins and Nucleic Acids, Plenum Press, New York, 1971.
16 J. W. Longworth, in R. F. Steiner and I. Weinryb (Eds.), Excited States of Proteins and Nucleic Acids, Plenum Press, New York, 1971.
17 E. A. Burstein, Equilibrium dynamics of the native protein structure, (in Russian), U.S.S.R. Acad. Sci., Pushchino, 1977, p. 60.
18 E. A. Permyakov and V. L. Shnyrov, Biophys. Chem., 18 (1983) 145.
19 D. M. Rayner and A. G. Szabo, Can. J. Chem., 56 (1978) 743.
20 A. Grinvald and I. Z. Steinberg, Biochim. Biophys. Acta, 427 (1976) 663.
21 B. Alpert, D. M. Jameson, R. Lopez-Delgado and R. Schooley, Photochem. Photobiol., 30 (1979) 479.
22 R. J. Robbins, G. R. Fleming, G. S. Beddard, G. W. Robinson, P. J. Thistlethwaite and G. J. Woolfe, J. Am. Chem. Soc., 102 (1980) 6271.
23 A. G. Szabo and D. M. Rayner, J. Am. Chem. Soc., 102 (1980) 554.
24 E. Gudgin, R. Lopez-Delgado and W. R. Ware, Can. J. Chem., 59 (1981) 1037.
25 E. Gudgin, R. Lopez-Delgado and W. R. Ware, J. Phys. Chem., 87 (1983) 1559.
26 R. Lumry and M. Hershberger, Photochem. Photobiol., 27 (1978) 819.
27 D. Creed, Photochem. Photobiol., 39 (1984) 537.
28 A. Grinvald and I. Z. Steinberg, Biochemistry, 13 (1974) 5170.
29 J.-P. Privat, P. Wahl, J.-C. Auchet and R. H. Pain, Biophys. Chem., 11 (1980) 239.
30 J. B. A. Ross, K. W. Rousslang and L. Brand, Biochemistry, 20 (1981) 4361.
31 J. B. A. Ross, C. J. Schmidt and L. Brand, Biochemistry, 20 (1981) 4369.
32 T. C. Werner and L. S. Forster, Photochem. Photobiol., 29 (1979) 905.
33 P. M. Torgerson, Biochemistry, 23 (1984) 3002.
34 K. G. Spears, L. E. Cramer and L. D. Hoffland, Rev. Sci. Instrum., 49 (1978) 255.
35 D. R. James, D. R. M. Demmer, R. E. Verrall and R. P. Steer, Rev. Sci. Instrum., 54 (1983) 1121.

36 N. Boens, M. Van den Zegel and F. C. De Schryver, Chem. Phys. Lett., 111 (1984) 340.
37 T. Torikata, L. S. Forster, C. C. O'Neal, Jr. and J. A. Rupley, Biochemistry, 18 (1979) 385.
38 T. A. Berkhout, A. J. W. G. Visser and K. W. A. Wirtz, Biochemistry, 23 (1984) 1505.
39 A. G. Szabo, D. Krajcarski, M. Zuker and B. Alpert, Chem. Phys. Lett., 108 (1984) 145.
40 G. V. Boyd, Chem. Ind. (London), (1957) 1244.
41 B. T. Turko, J. A. Nairn and K. Sauer, Rev. Sci. Instrum., 54 (1983) 118.
42 A. E. McKinnon, A. G. Szabo and D. R. Miller, J. Phys. Chem., 81 (1977) 1564.
43 D. V. O'Connor, W. R. Ware and J. C. Andre, J. Phys. Chem., 83 (1979) 1333.
44 D. W. Marquardt, J. Soc. Ind. Appl. Math., 11 (1963) 431.
45 R. F. Gunst and R. L. Mason, Regression Analysis and its Application, A Data-Oriented Approach, M. Dekker, New York, 1980.
46 A. Grinvald and I. Z. Steinberg, Anal. Biochem., 59 (1974) 583.
47 R. Catterall and D. A. Duddell, in R. B. Cundall and R. E. Dale (Eds.), Time-Resolved Fluorescence Spectroscopy in Biochemistry and Biology, Plenum Press, New York, 1983, p. 173.
48 J. Dickinson Gibbons, Nonparametric Methods for Quantitative Analysis, Holt, Rinehart and Winston, New York, 1976.
49 J. Durbin and G. C. Watson, Biometrika, 37 (1950) 409; 38 (1951) 159; 58 (1971) 1.
50 D. M. Chilko, SAS Tech. Rep. A-106, 1978.
51 B. Donzel, P. Gauduchon and P. Wahl, J. Am. Chem. Soc., 96 (1974) 801.
52 S. R. Meech, D. V. O'Connor, A. J. Roberts and D. Phillips, Photochem. Photobiol., 33 (1981) 159.
53 J. R. Knutson, D. G. Walbridge and L. Brand, Biochemistry, 21 (1982) 4671.
54 P. B. Bevington, Data Reduction and Error Analysis for the Physical Sciences, McGraw-Hill, New York, 1969, p. 302.
55 M. Inokuti and F. Hirayama, J. Chem. Phys., 43 (1965) 1978.
56 B. Dezube, C. M. Dobson and C. E. Teague, J. Chem. Soc., Perkin Trans. 2, (1981) 730.
57 J. R. Cavanaugh, J. Am. Chem. Soc., 92 (1970) 1488.

Analytica Chimica Acta, 170 (1985) 61—71
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

THE APPLICABILITY OF FLUORESCENCE LINE-NARROWING SPECTROSCOPY IN COMBINATION WITH THIN-LAYER CHROMATOGRAPHY

J. W. HOFSTRAAT, H. J. M. JANSEN, G. PH. HOORNWEG, C. GOOIJER and N. H. VELTHORST*

Department of General and Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam (The Netherlands)

(Received 6th September 1984)

SUMMARY

Laser excitation at low temperatures of compounds on thin-layer chromatographic plates yields, also after elution, highly resolved spectra. The fluorescence line-narrowed spectra permit identification of spectroscopically alike compounds, as is demonstrated for pyrene and halogenated pyrenes. Detection limits are in the low nanogram region even with relatively simple instrumentation. It is shown that quantitative application is possible if an internal standard is used.

Highly resolved fluorescence spectra of molecules in various kinds of matrices at low temperatures can be obtained by using a laser as excitation source. Generally, the luminescence spectra of organic molecules in liquid and solid solutions are severely broadened by site inhomogeneity. Of course, resolution of the vibrational structure in the spectra strongly improves the selectivity of fluorimetric analysis. Applications of fluorescence line-narrowing spectroscopy in analytical chemistry have been reported by Bykovskaya et al. [1], Brown et al. [2, 3] and Chiang et al. [4]. The technique appears to be very selective, especially when variable excitation wavelengths and time resolution are used. A large number of unsubstituted as well as substituted polycyclic aromatic hydrocarbons (PAHs) in glassy matrices have been successfully investigated. Recently, the fluorescence line-narrowed spectra of pyrene on a silica gel thin-layer chromatographic (t.l.c.) plate has been reported [5].

The physical background of fluorescence line narrowing is reasonably well understood [6]. The narrow spectral bandwidth of the exciting light provides an optical selection of the molecules that will fluoresce. Thus a tremendous decrease of the inhomogeneous broadening can be achieved. The resulting spectral bands consist of a narrow line, the zero-phonon line, accompanied by a broad band, the phonon wing, on its long wavelength side. The phonon wings are due to interactions of the solute molecule with matrix phonons.

There are two important prerequisites for obtaining fluorescence line-narrowed spectra. First, the temperature of the solution must be 50 K or less,

0003-2670/85/$03.30

since temperature and the extent of electron—phonon coupling determine the relative intensity of the narrow zero-phonon lines (I_{ZPL}) and the broad phonon wings (I_{PW}), which is generally expressed as the Debye—Waller factor, $\alpha = I_{ZPL}/(I_{ZPL} + I_{PW})$. The weaker the electron—phonon coupling and the lower the temperature, the greater becomes I_{ZPL} and the closer the Debye—Waller factor approximates 1. For some compounds, even at very low (1.2—4.2 K) temperatures, no quasi-line spectra are observed. In this case, the inhomogeneous broadening may be due to strong intra- or inter-molecular (e.g., charge transfer [4]) interactions. As a second requirement, the laser excitation wavelength should be sufficiently close to the 0—0 transition region. Excitation to the vibrational combination band area of the first excited electronic state, generally starting at about 1500 cm^{-1} above the origin, or to higher electronically excited states yields broad-banded spectra.

When excitation is performed in the inhomogeneously broadened purely 0—0 transition, a simple, one-sited fluorescence spectrum emerges, with the laser wavelength operating as origin; i.e., the whole fluorescence line-narrowed spectrum shifts with the excitation wavelength. Only when time-resolved detection techniques are used can the complete spectrum be recorded; otherwise, the short wavelength part is obscured by scattered laser light. This problem can be avoided by accomplishing excitation to the lower energy vibronic bands of the first excited state, but then a more complex spectrum is recorded. The position, number and (relative) intensities of the sites vary with the laser excitation wavelength. This dependence can be used to probe the vibronic structure of the first excited state [7].

In the present paper, the combination of highly resolved fluorescence spectroscopy and thin-layer chromatography is examined in more detail. Especially, attention is paid to the influence of solvents on the spectral shapes and to quantitative aspects of the technique. Pyrene and halogen-substituted pyrenes are used as model compounds. Pyrene has been studied by the fluorescence line-narrowing technique before [2, 3, 5]; its emission and absorption spectra have been fully interpreted [8]. Solvent effects on the vibrational structures of its fluorescence and absorption spectra have also been studied extensively [9—11]. Fluorescence line-narrowed spectra for the substituted pyrenes do not seem to have been reported. The chloro-substituted pyrenes are relevant, because they have been identified in incineration products [12] and in chlorine-treated drinking water [13].

EXPERIMENTAL

Instrumentation

The experimental set-up for the fluorescence line-narrowing experiments has been described [5, 14]. A Schott GG 375 cut-off filter was used to remove stray light, which was particularly abundant in the measurements on the t.l.c. plates.

The broad-band fluorescence spectrum of pyrene was obtained with a 450-W xenon lamp, a grating monochromator (λ_{ex} = 320 nm, bandpass

10 nm), and a 1-m monochromator as described recently [5]. For the absorption measurements, a Cary 14 spectrophotometer was used.

The fluorescence line-narrowing experiments were done at approximately 10 K. The samples were cooled in a Cryodyne model 21 closed-cycle refrigerator (CTI Cryogenics) equipped with a home-built gilded copper radiation shield. The cooling of the sample took 50 min. The broad-banded fluorescence spectrum was recorded at about 20 K [5]. The absorption spectra were obtained at 77 K. The samples were contained in 1-mm quartz cuvettes and placed in a TRL-3 liquid nitrogen cooling system (Cryoson). In 10 min the samples reached their final temperature.

Chemicals

Pyrene (Goldmarke; EGA-Chemie) was used as received. 1-Fluoropyrene and 1-chloropyrene were provided by Prof. Dr. J. Cornelisse of the Rijksuniversiteit, Leyden, the Netherlands. The substituted pyrenes were synthesized by the method of Vollman et al. [15] and recrystallized from ethanol. All solvents, n-hexane (Aldrich; >99%), n-nonane and n-dodecane (Baker grade; >99%), 3-methylpentane (Janssen Chimica; >99%), cyclohexane (Merck Uvasol; >99.7%), absolute ethanol (Baker analyzed; >99.5%) and glycerol (Merck analyzed), were used as received; they showed no fluorescence in the wavelength region of interest. Silica-gel t.l.c. plates (Merck) with and without fluorescence indicator (DC-Alufolien Kieselgel 60-F_{254} and 60-H, respectively) were used. The presence of a fluorescence indicator did not interfere with the experiments.

Procedure

Spots (300 nl) of solutions containing the pyrene (6—3000 μg ml^{-1}) were applied on the t.l.c. plate with a specially constructed apparatus as described by de Vries and Brinkman [16]. As recommended by Mosnaim et al. [17] a cyclohexane/n-hexane (1:1, v/v) mixture was used for elution. After development over a distance of about 5 cm, R_F values of 0.38 for pyrene, 0.47 for 1-chloropyrene and 0.63 for dichloropyrene(s) were established. The plates were air-dried before measurements.

For the fluorescence line-narrowing experiments in glassy matrices, 5.0×10^{-4} M samples of pyrene and 1-fluoropyrene were used. In the absorption experiments, 5.00×10^{-3} M solutions were used.

RESULTS AND DISCUSSION

The fluorescence line-narrowed spectra; temperature and solvent effects

Excitation of pyrene on a silica-gel t.l.c. plate with broad-band light from a conventional xenon lamp source yields the fluorescence spectrum shown in Fig. 1. The badly resolved spectrum has five discernible bands with maxima at 371, 378, 382, 388 and 392 nm, and bandwidths at half maximum of 3 nm (about 200 cm^{-1}).

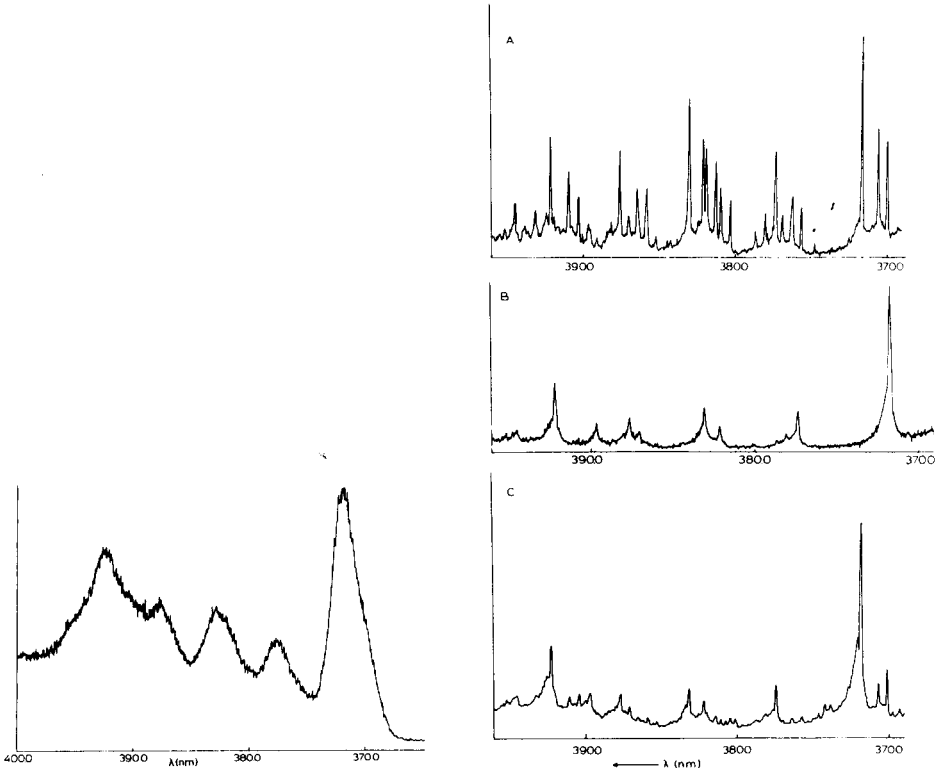


Fig. 1. Fluorescence spectrum of 7 μg of pyrene on a t.l.c. plate with broad-band excitation (T = 20 K, $\lambda_{ex} \approx$ 320 nm).

Fig. 2. Fluorescence line-narrowed spectra of pyrene (T = 10 K, λ_{ex} = 363.8 nm): (A) in 3-methylpentane (5.0×10^{-4} M); (B) in ethanol (5.0×10^{-4} M); (C) on a t.l.c. plate (50 ng; pyrene was applied in n-dodecane).

Excitation with the 363.8-nm line of the argon-ion laser induces great narrowing of the inhomogeneously broadened spectral lines. Figure 2 shows spectra of pyrene in 3-methylpentane, in ethanol and on a t.l.c. plate, recorded at 10 K. The zero-phonon lines have a bandwidth at half maximum of about 0.05—0.1 nm (3—6 cm^{-1}), partially determined by the resolution of the monochromator. Two interesting features emerge. First, the spectrum in Fig. 2C shows less pronounced phonon wings than the one reported previously [5], which was recorded at 20 K. The electron-phonon coupling appears to be stronger for pyrene on the t.l.c. plate than in the glassy solutions. This explains why the temperature reduction hardly influences the latter spectra. The second feature is the matrix dependence of the spectra: the intensities are strongly influenced, but the line positions are hardly affected. Whereas for 3-methylpentane three clearly discernible sites are found at 370.04, 370.64 and 371.73 nm, for ethanol only one site, at

371.85 nm, is observed. The spectrum of pyrene on the t.l.c. plate consists of one strong site at 371.90 nm and two weak sites at 370.70 and 370.13 nm. As can be deduced from the data given above, there is only a small shift in the position of the 0—0 transition. Because the bands in the absorption spectra (Fig. 3) are relatively broad, the slight shift cannot be fully responsible for the strongly varying site intensities.

The site structure gives information about the vibrational modes of the molecule in the first excited state [7]. Because excitation is done at 363.80 nm, the sites at 371.73, 370.64 and 370.04 nm in 3-methylpentane lie at 587, 508 and 464 cm^{-1} from the excitation wavenumber. These data can be correlated with the 583-cm^{-1} a_g mode, and the 496- and 456-cm^{-1} b_{3g} modes reported by Bree and Vilkos [8] for the S_1-state of pyrene in a biphenyl single crystal matrix at 10 K. The latter, non-totally symmetric modes are not observed in the site structure of the fluorescence spectrum recorded in ethanol. This is in line with other investigations which have shown that the relative intensities of the weaker, non-totally symmetric transitions of pyrene in nonpolar solvents are strongly enhanced with respect to the 0—0 transition [9—11]. This observation, based on experiments in liquids, can be supported by absorption measurements at 77 K; the spectrum in 3-methylpentane (Fig. 3A) shows a more pronounced non-totally symmetric contribution to the absorption spectrum in the 365-nm region than that in the more polar ethanol (Fig. 3B). In the 3-methylpentane spectrum a shoulder at 365.4 nm, at approximately 460 cm^{-1} from the 0—0 transition, is clearly visible next to the totally symmetric 363.8 nm band at 590 cm^{-1}. The spectrum recorded in ethanol only shows a band at 364.0 nm. The band at

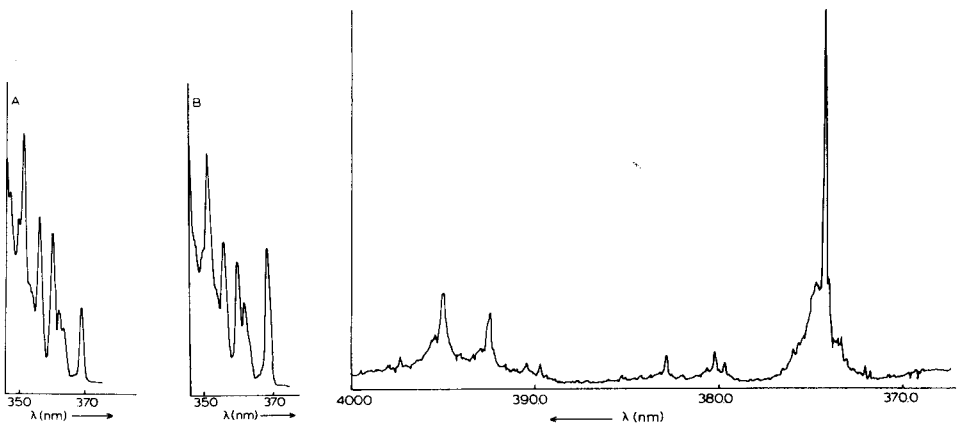


Fig. 3. Absorption spectra of pyrene (5.00×10^{-3} M; T = 77 K): (A) in 3-methylpentane; (B) in ethanol.

Fig. 4. Fluorescence line-narrowed spectrum of 5.0×10^{-4} M 1-fluoropyrene (T = 10 K, λ_{ex} = 263.8 nm) in glycerol/water/ethanol (2:1:1, v/v/v).

about 365.0 nm (500 cm^{-1} from the 0—0 transition) cannot be discerned in either spectra, because it is obscured by the two other transitions. Thus, the matrix dependency of the site structure of the fluorescence line-narrowed spectra can be clarified via simple absorption measurements of the corresponding samples. The t.l.c. plate, with its less pronounced sites at 370.13 and 370.70 nm, seems to occupy an intermediate position between 3-methylpentane and ethanol. The intensity distribution in the fluorescence spectra is also influenced by the solvent. In 3-methylpentane, the vibronic bands are much more intense, in comparison with the 0—0 transition, than in the two other matrices. This is in agreement with previous observations in liquid solutions [9—11].

The fluorescence line-narrowed spectrum of 1-fluoropyrene in glycerol/water/ethanol (2:1:1, v/v/v) is depicted in Fig. 4. An assignment of the most intense vibronic bands is given in Table 1. The potential of fluorescence line-narrowing as a selective analytical technique becomes obvious if the spectra given in Figs. 2 and 4 are compared. The 1-fluoropyrene spectrum has its 0—0 transition at 374.12 nm, clearly distinct from the purely electronic transition of pyrene at 371.9 nm. In addition, the vibronic band at 382.83 nm (608 cm^{-1} from the origin) in the spectrum of 1-fluoropyrene does not have a pendant in the unsubstituted one. The 0—0 transition occurs mainly in one site. In more apolar solvents (see Fig. 5) a two site structure is observed. The solvent dependence of the 1-fluoropyrene site structure thus seems to be similar to that of pyrene.

Quantitative aspects

Brown et al. [2] reported the direct quantification of fluorescence line-narrowing spectra in glasses. Unfortunately, in the present experimental set-up, reproducible positioning of the sample was difficult, so that an internal standard was utilized to eliminate instrumental and adjustment effects. Attention was focussed on quantifying 1-fluoropyrene with pyrene as the reference compound.

TABLE 1

Major bands in the narrow-line fluorescence spectrum of 1-fluoropyrene in glycerol/water/ethanol at 10 K

λ (nm)	$\bar{\nu}$ (cm^{-1})	$\Delta\bar{\nu}$ (cm^{-1})	Intensity	Assignment
374.12	26729	0	vs	0—0
379.65	26340	389	w	0—389
380.20	26302	427	w	0—427
382.83	26121	608	w	0—608
389.68	25662	1067	w	0—1067
390.41	25614	1115	w	0—1115
392.42	25483	1246	m	0—1246
394.44	25352	1377	m	0—1377
397.25	25173	1556	w	0—1556

The heights of the 374.32-nm peak of 1-fluoropyrene and the 371.90-nm peak of pyrene (Fig. 5) were used for calibration. The internal standard was present in concentrations varying between 0.5 and 5.0 times the concentration of 1-fluoropyrene. This did not affect the results, indicating that energy transfer is negligible at the concentration levels used. As can be seen from Fig. 5 for 1-fluoropyrene at a t.l.c. plate, in addition to the site at 374.32 nm another site at 372.15 nm is observed, close to the principal peak of pyrene. Therefore a correction had to be made for the residual emission of 1-fluoropyrene at 371.90 nm. The intensities of the two sites in the 1-fluoropyrene spectrum depend on the solvent used to apply the sample on the t.l.c. plate. In n-dodecane, the 374.32-nm peak is the more intense, whereas in n-nonane the 372.15-nm peak is the stronger. In the latter solvent, both sites show enhanced intensities with respect to the pyrene main peak. The dependence of the spectral intensities on the solvent used to dissolve the compounds, disappears if the t.l.c. plate is dried overnight. This procedure, though, is detrimental to the detection limits. The solvent dependence of the intensities of the spectral bands probably also explains the increase in sensitivity when the plate is sprayed with n-heptane, the sample having been applied in diethyl ether [5]. In the present experiment, n-alkanes were used for application and spraying with n-heptane did not improve the sensitivity.

Calibration data were collected in a number of independent experiments, using several n-alkane solvents to apply the samples on the t.l.c. plates. The repeatibility for measurements on five, separately applied, identical sample

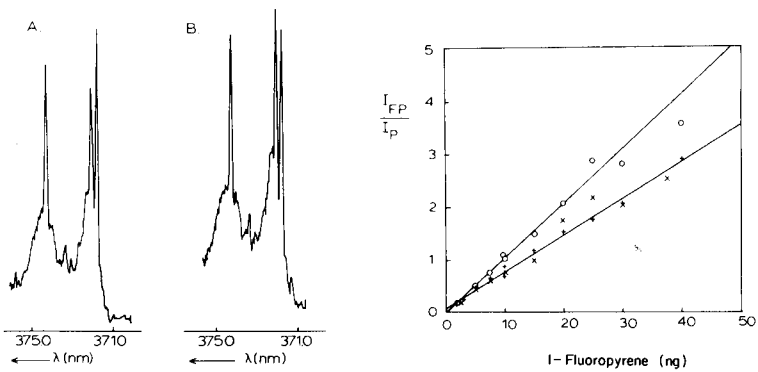


Fig. 5. Fluorescence line-narrowed spectra of the 0—0 transition area of mixtures of 25 ng of 1-fluoropyrene and 40 ng of pyrene on a t.l.c. plate: (A) applied in n-dodecane; (B) applied in n-nonane.

Fig. 6. Calibration plots for 1-fluoropyrene in the low concentration region. The 374.32-nm peak of 1-fluoropyrene is weighted with the 371.90-nm peak of pyrene, the internal standard. (○) Samples were applied in n-nonane; slope = 0.094, intercept = 0.12, correlation coefficient = 0.99 (n = 11). (+, ×) Samples were applied in n-dodecane in two separate experiments; slope = 0.069, intercept = 0.010, correlation coefficient = 0.99 (n = 20).

spots was better than 8%. Figure 6 shows two calibration plots for samples applied in n-nonane and n-dodecane. Both plots show good linearity in the low concentration region. The higher slope observed for n-nonane is due to the relative enhancement of the 1-fluoropyrene emission intensity in this solvent compared to that of pyrene. If the same solvent is used in different experiments, similar plots are found, as demonstrated for n-dodecane.

An advantage of the t.l.c. plate as a matrix compared to glassy solutions lies in the fact that many samples (up to 25 in the present experimental set-up) can be studied in one batch. In this way, equal experimental conditions are ensured and time-consuming sample changes and coolings are avoided.

Detection limits

With the experimental set-up used, 1 ng of pyrene and 1-fluoropyrene could be detected. Various improvements could be made to decrease the detection limit further. A continuously scannable dye laser would provide excitation at more favorable wavelengths; from Fig. 3 it can be deduced that excitation of pyrene in 3-methylpentane at 361.5 nm, for instance, is more effective than at 363.8 nm. The use of time-resolved techniques and/or a double monochromator would decrease the interfering effects from laser stray light, which produces a relatively high background, especially when t.l.c. plates are used as matrices. More sophisticated detection techniques (photon counting, optical multichannel analyzer) would lower the detection limits still further. Finally, measurements at lower temperatures (e.g., 4.2 K) would yield higher zero-phonon lines and thus increase the sensitivity.

Application: impure 1-chloropyrene

The 1-chloropyrene sample appeared to contain some impurities. The fluorescence line-narrowed spectrum of this compound obtained by excitation with the 363.80-nm line of the argon ion laser is shown in Fig. 7A. Apart from the 1-chloropyrene fluorescence, with its most prominent bands at 374.10 and 375.15 nm (compare, e.g., the highly resolved Shpol'skii spectra reported for this compound by Colmsjö et al. [18]), bands from pyrene start at about 372 nm, and a third compound has a strong emission at about 380 nm. The last emission could be attributed to dichloropyrene(s), with the help of gas chromatography/mass spectrometry (g.c./m.s.). The structure of the dichloropyrene(s) could not be established, because no spectroscopic data on these compounds seem to be available in the literature. However, further electrophilic substitution reactions of 1-substituted pyrenes are known to yield a mixture of 1,6- and 1,8-disubstituted products [19]. It is noted that the relative intensities of the emission lines do not give direct information on the concentration of the compounds in the samples, because they are strongly influenced by the absorptivities at the laser excitation wavelength and the fluorescence quantum yields. The g.c./m.s. analysis showed that the 1-chloropyrene sample contained 3 mol-% pyrene and 15 mol-% dichloropyrene. In the fluorescence spectrum, the pyrene and

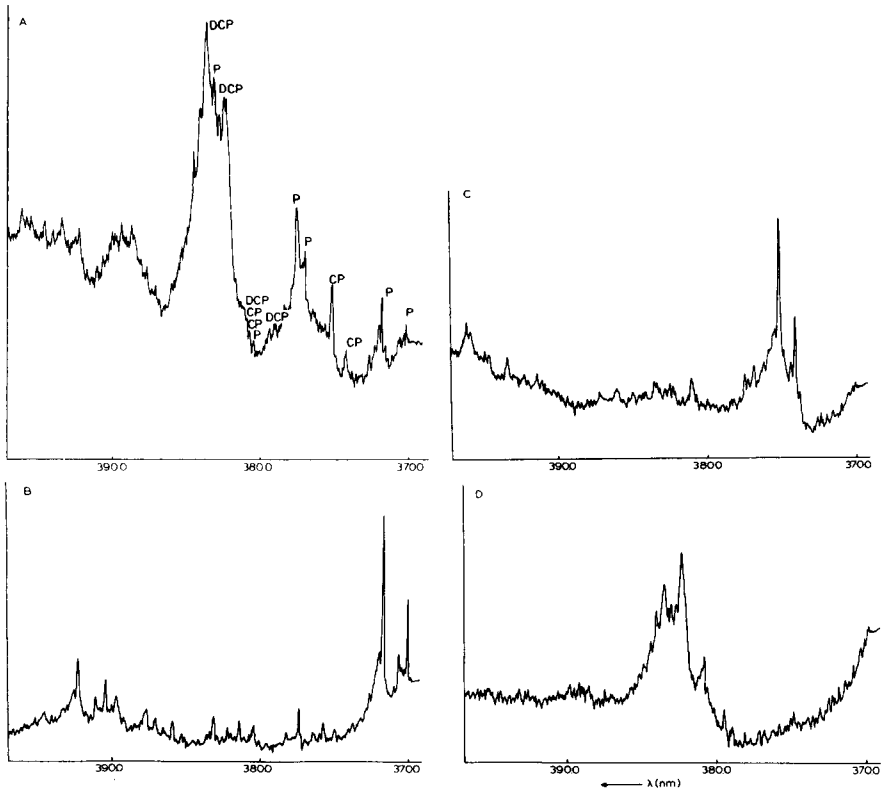


Fig. 7. Fluorescence line-narrowed spectra of impure 1-chloropyrene on a t.l.c. plate (T = 10 K, λ_{ex} = 363.8 nm). (A) 100 ng of the sample applied in n-dodecane without elution (P = pyrene, CP = 1-chloropyrene, DCP = dichloropyrene); (B) spectrum of pyrene after elution; (C) spectrum of 1-chloropyrene after elution; (D) spectrum of dichloropyrene after elution. For B—D, elution was done with cyclohexane/n-hexane (1:1, v/v); a 1-μg sample of impure 1-chloropyrene dissolved in n-dodecane was applied on the t.l.c. plate.

dichloropyrene bands appear with relatively strong intensity, indicating that these compounds are more efficiently excited at 363.8 nm than 1-chloropyrene. With a dye laser, producing a continuously variable wavelength output, all three compounds can be excited at their optimal wavelengths so that the selectivity of the fluorescence line-narrowing method can be increased.

After elution of the 1-chloropyrene sample on a silica-gel t.l.c. plate with cyclohexane/n-hexane (1:1, v/v), three separate spots appeared, which contained pyrene (at R_F = 0.38), 1-chloropyrene (at R_F = 0.47) and dichloropyrene (R_F = 0.63). The spectra of the separated compounds are shown in Fig. 7B—D. It can be seen that especially pyrene and 1-chloropyrene give well resolved fluorescence line-narrowed spectra. Dichloropyrene, which has its 0—0 transition at about 1500 cm^{-1} from the laser exciting light, gives a less resolved spectrum. For all three compounds, however, spectra with bands at typical wavelengths are recorded, so that in principle they can be identified unambiguously.

Conclusion

The application of fluorescence line-narrowing methods to compounds on t.l.c. plates in principle yields highly resolved spectra, both with and without elution. For the compounds investigated, the spectra show a slightly stronger electron-phonon coupling than those recorded in glasses, emphasizing the importance of measuring at very low temperatures. The positions of the spectral lines in the pyrene spectrum are hardly affected by the nature of the solvent, so that they can be used for identification purposes. In contrast, the site structure and the relative intensities of the spectral lines are dependent on the solvent. Such effects have been observed for other aromatic molecules as well [20, 21].

The combination of fluorescence line-narrowing spectroscopy and t.l.c. plates has several advantages. Many samples can be examined in one batch, thus ensuring the same experimental conditions and saving time. Furthermore, the method gives some freedom in treating samples. The compounds can be dissolved in various solvents and can be applied in volumes of 0.05–250 μl, when automatic spotting devices are employed [22]. Only a small sample is needed for a single measurement. When a complex sample is studied, the (partial) separation achieved by t.l.c. can be followed by selective identification with the highly resolved fluorescence technique. A disadvantage is the strong scattering of the laser excitation light by the t.l.c. matrix, which can only be reduced by utilizing expensive apparatus.

Quantitative applications of the fluorescence line-narrowing method in combination with thin-layer chromatography appear feasible in the present simple experimental set-up if an internal standard is employed. With this set-up, detection limits lie in the low nanogram regime, but can be decreased further by the use of more sophisticated instrumentation.

U. A. Th. Brinkman is gratefully acknowledged for stimulating interest in this work.

REFERENCES

1 L. A. Bykovskaya, R. I. Personov and Yu. V. Romanovskii, Anal. Chim. Acta, 125 (1981) 1.
2 J. C. Brown, M. C. Edelson and G. J. Small, Anal. Chem., 50 (1978) 1394.
3 J. C. Brown, J. A. Duncanson, Jr. and G. J. Small, Anal. Chem., 52 (1980) 1711.
4 I. Chiang, J. M. Hayes and G. J. Small, Anal. Chem., 54 (1982) 318.
5 J. W. Hofstraat, M. Engelsma, W. P. Cofino, G. Ph. Hoornweg, C. Gooijer and N. H. Velthorst, Anal. Chim. Acta, 159 (1984) 359.
6 R. I. Personov, in V. M. Agranovich and R. M. Hochstrasser (Eds.), Spectroscopy and Excitation Dynamics of Condensed Molecular Systems, North-Holland, Amsterdam, 1983.
7 R. I. Personov and E. I. Al'shits, Chem. Phys. Letters, 33 (1975) 85.
8 A. Bree and V. V. B. Vilkos, Spectrochim. Acta, Part A, 27 (1971) 2333.
9 A. Nakajima, Bull. Chem. Soc. Jpn., 44 (1971) 3272.
10 K. Hara and W. R. Ware, Chem. Phys., 51 (1980) 61.

11 D. C. Dong and M. A. Winnik, Photochem. Photobiol., 35 (1982) 17.
12 C. Rappe, S. Marklund, P.-A. Berqvist and M. Hansson, Chem. Scr., 20 (1982) 56.
13 A. R. Oyler, R. J. Liukkonen, M. K. Lukasewycz, D. A. Cox, D. A. Peake and R. M. Carlson, Env. Health Perspect., 46 (1982) 73.
14 W. P. Cofino, J. W. Hofstraat, G. Ph. Hoornweg, C. Gooijer, C. MacLean and N. H. Velthorst, Chem. Phys. Lett., 89 (1982) 17.
15 H. Vollman, H. Becker, M. Corell and H. Streeck, Justus Liebigs Ann. Chem., 531 (1937) 1.
16 G. de Vries and U. A. Th. Brinkman, Mikrochim. Acta (Wien), 11 (1984) 47.
17 A. D. Mosnaim, M. Wolf, L. Saavedra, A. Rosenkranz, S. Diaz and D. C. Nonhebel, J. Chrom., 80 (1973) 259.
18 A. Colmsjö, Y. Zebühr and C. Ostman, Chem. Scr., 20 (1982) 123.
19 C. Tintel, Thesis, Rijksuniversiteit, Leyden, The Netherlands, 1983.
20 G. Durocher and C. Sandorfy, J. Mol. Spectrosc., 20 (1966) 410.
21 M. Koyanagi, J. Mol. Spectrosc., 25 (1968) 273.
22 J. C. Touchstone, R. E. Levitt and G. J. Hansen, in J. C. Touchstone and D. Rogers (Eds.), Thin Layer Chromatography: Quantitative Environmental and Clinical Applications, Wiley, New York, 1980.

Analytica Chimica Acta, 170 (1985) 73—80
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CONTINUOUS KINETIC ASSAY OF ARYLSULFATASES WITH NEW CHROMOGENIC AND FLUOROGENIC SUBSTRATES

ERNST KOLLER and OTTO S. WOLFBEIS*

Institut für Organische Chemie, Karl-Franzens-Universität, A-8010 Graz (Austria)

(Received 26th September 1984)

SUMMARY

Arylsulfatases are determined, even in weakly acidic solution, by a direct and continuous kinetic method using new coumarin-derived sulfates as substrates. After enzymatic hydrolysis, the substrate dissociates to form intensely colored and strongly fluorescent phenolates, with absorption maxima ranging from 383 to 497 nm, and fluorescence emission maxima between 470 and 577 nm. Rates of enzymatic hydrolysis, optimum pH values and detection limits of arylsulfatase from *Aerobacter aerogenes* and *Patella vulgata* are determined.

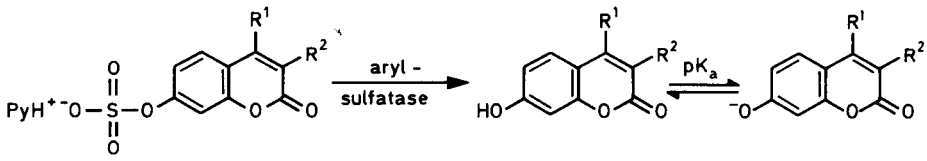
Arylsulfatase determinations with synthetic substrates are used in the diagnosis of metachromatic leukodystrophy and Maroteaux—Lamy syndrome [1—4]. The most frequently used spectrophotometric assays for arylsulfatase make use of *p*-nitrophenyl sulfate [5] or nitrocatechol sulfate (2-hydroxy-5-nitrophenyl sulfate) [6]. These substrates are cleaved enzymatically to give, after addition of base, the colored phenolates. However, the methods have disadvantages in that they do not allow a continuous assay in weakly acidic solutions (which is the optimum pH range for several arylsulfatases), and in not being very sensitive. Fluorimetric assays offer an interesting alternative [7]. Many fluorogenic arylsulfatase substrates derived from highly fluorescent aromatic hydroxy compounds have therefore been prepared [8]. Among these, the sulfate of 7-hydroxy-4-methylcoumarin (4-methyl-umbelliferone, 4-MU) [9] is now commercially available and has found the most widespread application. It shows little fluorescence, but is enzymatically cleaved to give 4-MU. The latter, having a pK_a of 7.8 [10], can dissociate at physiological pH values to give the highly fluorescent 4-MU anion. In acidic solutions, however, almost no dissociation takes place, which prevents a continuous assay of arylsulfatase by following the formation of the anion (λ_{ex}^{max} = 360 nm, λ_{em}^{max} = 450 nm). Excitation is therefore at 338 nm [8], where uncharged 4-MU absorbs strongly. Unfortunately, most biological matter displays strong NADH fluorescence under excitation at 330—360 nm, thus giving rise to strong background signals.

In continuation of a search for enzyme substrates that fluoresce at long wavelengths [11, 12], it was found that 7-hydroxycoumarins bearing

0003-2670/85/$03.30

electron-withdrawing substituents in position 3 of the coumarin ring show both a spectral shift to longer wavelengths and a decrease in the pK_a value. It has also been noticed [13] that introduction of a cyano group into position 4 of 7-hydroxycoumarins causes considerable shifts to longer wavelengths in the absorption and fluorescence maxima. As can be seen from Table 1, the absorption and fluorescence maxima of the phenolate form of compound **2d** are at considerably longer wavelengths than those of the phenolates of compounds 2a—c. In addition, the pK_a value is lowered to 6.0.

To take advantage of these properties, the sulfates of several 7-hydroxycoumarins absorbing and fluorescing at long wavelengths were prepared. The structures are given below. Results are presented which were obtained with these new substrates for spectrophotometric and fluorimetric determinations of arylsulfatases.



1a - d **2a - d**

1,2	R^1	R^2
a	H	phenyl
b	H	2-benzoxazolyl
c	H	2-benzothiazolyl
d	CN	2-(5-chlorobenzoxazolyl)

EXPERIMENTAL

Chemicals and apparatus

Enzymes. All enzymes were purchased from the Sigma Chemical Company. Arylsulfatase (EC.3.1.6.1) from *Aerobacter aerogenes*, type VI, in 50% glycerol solution containing 0.01 M Tris, pH 7.5, showed an activity of 3.2 U mg^{-1} of protein. One unit will hydrolyze 1.0 μmol of *p*-nitrophenyl sulfate per minute at pH 7.1 and 37°C. Arylsulfatase (EC.3.1.6.1) from limpets (*Patella vulgata*), type V, contained low β-glucuronidase activity and showed an activity of 9 U mg^{-1} of solid. One unit will hydrolyze 1.0 μmol of nitrocatechol sulfate per hour at pH 5.0 and 37°C.

Substrates. Compounds **1a—d** were synthesized as described elsewhere [14]; 50-mg samples ($35 each) are available on request. Solutions of substrates (0.1033 mM in 0.1 M acetate buffer for arylsulfatase from *Patella vulgata* and 0.1 mM in 0.1 M Tris buffer for arylsulfatase from *Aerobacter aerogenes*) were freshly prepared each day by dissolving the substrate in buffer. A dilution series was made with buffer in the substrate concentration range 0.1—0.01 mM for the determination of the K_m and v_{max} values.

TABLE 1

Absorption and emission maxima of coumarins 2a—d in aqueous solution of pH 9 containing 10% methanol

Coumarin	Maxima (nm)		Stokes' shift	pK_a (at 23° C)
	Absorption	Emission[a]		
2a	383	472	89	7.80
2b	427	471	44	6.84
2c	440	490	50	7.02
2d	497	577	80	6.00

[a]With excitation at the absorption maximum.

Acetate buffer. Acetate buffer (0.1 M) was prepared by mixing the appropriate amount of freshly distilled acetic acid with triply distilled water. Sufficient 1 M sodium hydroxide was added to adjust the pH to the desired value, and the volume was brought to the required value with triply distilled water.

Tris buffer. Tris(hydroxymethyl)aminomethane buffer (0.1 M) was prepared by dissolving the appropriate amount of Sigma 7—9 buffer (Sigma Chemical Co.) in triply distilled water. Hydrochloric acid (1.0 M) was added to adjust the pH to the desired value, and the solution was diluted to the required volume with triply distilled water.

Apparatus. A Perkin-Elmer Lambda-5 spectrophotometer and an Aminco SPF-500 spectrofluorimeter, fitted with a 250-W xenon lamp were used. The digital output of the latter was processed by using a Hewlett-Packard 9815-A desk calculator and a 7725-A plotter. The monochromators of the instruments were set at the wavelengths given in Table 1.

Procedure

All measurements were made at 25° C. A 3-ml portion of substrate solution was placed in a rectangular quartz cell. The absorbance and fluorescence intensity of these solutions were very low. At zero time, 0.1 ml of the enzyme solution to be assayed was added. For arylsulfatase from *Aerobacter aerogenes*, the commercial enzyme solution was added in amounts between 0.3 and 5.0 μl, whereas arylsulfatase from *Patella vulgata* had first to be dissolved in water. The increase of absorbance (ΔA) or fluorescence (ΔF) caused by hydrolysis of the substrate was then automatically recorded vs. time, usually for 1—10 min. From the slope of this plot, the activity of the unknown enzyme was calculated in the usual way. The K_m and v_{max} values were obtained from Lineweaver—Burk plots.

RESULTS AND DISCUSSION

The new substrates were investigated with respect to optimum pH, rates of enzymatic and non-enzymatic hydrolysis, and detection limits for two arylsulfatases.

Assay principle

Substrates **1a—d** are hydrolyzed enzymatically to form the corresponding 7-hydroxycoumarins, which, owing to their relatively low pK_a values (6—7, except for compound **2a**) under assay conditions, partially dissociate to form their anions (see above). With the exception of compound **2a**, these anions show strong absorption ($\epsilon > 30\,000$ l mol^{-1} cm^{-1}) and intense fluorescence. Table 1 compiles the absorption and emission data of the anions of compounds **2a—d** and thus the wavelengths appropriate for enzyme determinations.

In addition to the enzymatic cleavage, the substrates also suffer some non-enzymatic hydrolysis, the rate of which is dependent on pH. Figure 1 shows the pH dependence of the non-enzymatic hydrolysis of compound **1b**. Because cleavage becomes significant at pH values above 8, assays should be done at lower pH values to obtain sufficient precision. Slow non-enzymatic hydrolysis is also found in weakly acidic solutions, an effect that has to be taken into account when enzyme activities are determined.

Arylsulfatase from Aerobacter aerogenes

The rate of enzymatic hydrolysis of substrates **1a—c** is maximal at pH > 8 (see, for instance, Fig. 1). For the determination of its kinetic properties, pH

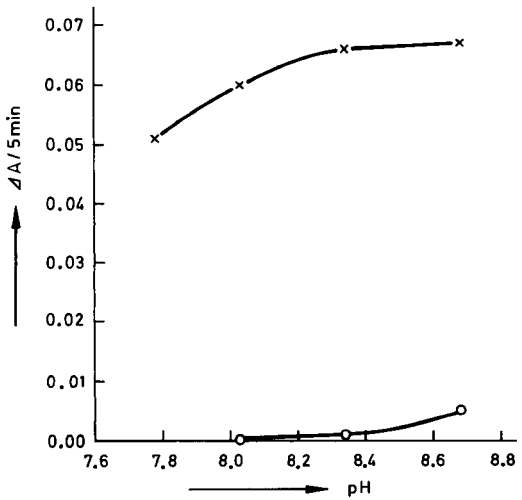


Fig. 1. Spectrophotometrically determined pH dependences for substrate **1b** (0.1 mM) by arylsulfatase from *Aerobacter aerogenes* (0.04 U ml^{-1}) in 0.1 M Tris buffer at 25°C: (○) non-enzymatic hydrolysis; (×) enzymatic hydrolysis.

values between 7.2 and 8.0 were chosen as a compromise between enzymatic and non-enzymatic hydrolysis (Table 2). Substrate **1d** was not investigated because of its instability in alkaline solutions.

It is evident from Table 2 that the rate of enzymatic hydrolysis is strongly affected by the substituent in position 3 of the coumarin ring. Substrate **1c** undergoes rather slow enzymatic cleavage. Substrate **1b**, possessing a benzoxazolyl substituent, exhibits relatively rapid enzymatic hydrolysis. A Lineweaver—Burk plot gives a K_m value of 0.10 mM and a v_{max} value of 0.237 μmol min^{-1} mg^{-1} for the enzymatic hydrolysis of **1b** by arylsulfatase from *Aerobacter aerogenes* at pH 8.0. Larger values of K_m and v_{max} were found for the enzymatic cleavage of substrate **1a**; a K_m value of 0.07 mM and a v_{max} value of 2.161 μmol min^{-1} mg^{-1} were found at pH 7.2.

A fairly high sensitivity can be achieved in spectrofluorimetric assays of arylsulfatase from *Aerobacter aerogenes* by using substrates **1a** and **1b** (Table 3). They are also useful in spectrophotometric assays, but give higher detection limits. The respective values are given in Table 3.

Substrate **1a** is more sensitive than **1b**. With **1a** as substrate (0.1 mM), a spectrophotometric assay of arylsulfatase from *Aerobacter aerogenes* can be made in the range 2.6×10^{-3}—1.7×10^{-2} U ml^{-1} with an accuracy of ±2.5%.

Compound **1b** was tested in a spectrofluorimetric assay. A linear relation between enzyme activity and rate of enzymatic hydrolysis was found for

TABLE 2

Initial rates of enzymatic hydrolysis of substrates **1a—c** by arylsulfatase from *Aerobacter aerogenes* at constant substrate concentration (0.1 mM in 0.1 M Tris buffer) and 25° C (at the wavelengths given in Table 1)

Substrate	pH	Arylsulfatase (U ml^{-1})	Initial rate		
			ΔA/5 min	ΔF min^{-1} [a]	nmol min^{-1} [b]
1a	7.2	0.0085	0.135	0.75	9.16
1b	8.0	0.0426	0.331	1.55	4.65
1c	8.0	0.0426	0.032	0.17	0.44

[a]Total spectrofluorimeter range = 10.0. [b]Spectrophotometric measurement.

TABLE 3

Detection limits for arylsulfatase from *Aerobacter aerogenes* by using 0.1 mM substrates in 1-min spectrophotometric and spectrofluorimetric assays (at the pH values given in Table 2)

Substrate	Detection limit (U ml^{-1})	
	Spectrophotometric	Spectrofluorimetric
1a	0.0026	0.0008
1b	0.004	0.002
1c	0.04	0.02

4×10^{-3}—4×10^{-2} U ml^{-1}, with an average error of ±4.9%. Higher activities were not investigated. An increase in sensitivity may be achieved by use of a longer reaction time.

Arylsulfatase from Patella vulgata

This enzyme was chosen because it exhibits an activity maximum at pH 5. It was noticed that the initial enzymatic hydrolysis of substrates **1a—d** is relatively slow, but reaches a constant rate only a few minutes after the enzyme and substrate solutions have been mixed. The experimental data obtained after a 2-min incubation time were used for the evaluation of rates of enzymatic hydrolysis (Table 4).

The optimum pH values for the direct and continuous assay, defined as the pH at which the rates of change in absorbance or fluorescence are maximal, were found to be 6.6 for substrates **1b** and **1c** and 5.9 for substrate **1d** in 0.1 M acetate buffer. The pH value at which the most sensitive continuous enzyme determination can be made (5.9—6.6) is different from the pH value of maximal enzyme activity (pH 5). This can be explained by the proximity of the optimal pH value of enzyme activity and the pK_a value of the phenol formed through hydrolysis. The anion absorbance or fluorescence produced by enzymatic hydrolysis is a function of both the enzyme activity and the amount of anion present at the pH used. This situation has recently been discussed in more detail in a related paper on the continuous spectrophotometric and spectrofluorimetric determination of acid phosphatases [12].

Except for compound **1a**, the new substrates are useful for determining arylsulfatase from *Patella vulgata* by a direct and continuous method. A pK_a value of 7.8 for 7-hydroxy-3-phenylcoumarin, which is the hydrolysis product of substrate **1a**, prevents a continuous assay in weakly acidic solutions. All substrates **1a—d** can, of course, be applied in non-continuous assays.

With respect to the rate of enzymatic hydrolysis, compound **1c** is the most suitable substrate. A Lineweaver—Burk plot gives a K_m value of 0.10 mM and a v_{max} value of 0.033 μmol min^{-1} mg^{-1} for the enzymatic cleavage of **1c**

TABLE 4

Rates of enzymatic hydrolysis of substrates **1b—d** by arylsulfatase from *Patella vulgata* (0.134 U ml^{-1}) at constant substrate concentration (0.1 mM in 0.1 M acetate buffer) and 25° C (at the wavelengths given in Table 1)

Substrate	pH	Rate		
		ΔA/10 min	ΔF min^{-1} [a]	nmol min^{-1} [b]
1b	6.6	0.023	0.10	0.18
1c	6.6	0.061	0.86	0.70
1d	5.9	0.029	0.71	0.26

[a,b] As in Table 2.

TABLE 5

Detection limits of arylsulfatase from *Patella vulgata* by using substrates **1b—d** (0.1 mM) (pH values as given in Table 4)

Substrate	Detection limits (U ml^{-1})	
	Spectrophotometric[a]	Spectrofluorimetric[b]
1b	0.03	0.10
1c	0.01	0.03
1d	0.02	0.03

[a]Reaction time, 10 min. [b]Reaction time, 1 min.

by arylsulfatase from *Patella vulgata* at pH 6.6 in 0.1 M acetate buffer. It is remarkable that this enzyme cleaves substrate **1c** most rapidly, whereas arylsulfatase from *Aerobacter aerogenes* favors substrate **1b** over **1c**.

Compound **1c** is the most sensitive substrate for a spectrophotometric assay (Table 5). Because of the higher sensitivity of spectrofluorimetry, the respective detection limits for arylsulfatase from *Patella vulgata* are lower (Table 5). Enzyme activities as low as 0.03 U ml^{-1} can be determined within 1 min. An increase in sensitivity may be achieved by prolonging the reaction time, or by increasing the substrate concentration, because the substrate concentrations (0.1 mM) are similar to the respective K_m values.

With **1c** or **1d** as a substrate (0.1 mM), a spectrofluorimetric assay of arylsulfatase from *Patella vulgata* can be done in the range 0.029—0.23 U ml^{-1} with an accuracy of ±2.1%. Substrate **1d** (K_m = 0.2 mM; v_{max} = 0.017 μmol min^{-1} mg^{-1}) has advantages over **1c** in that its hydrolysis product has longer-wavelength absorption and fluorescence maxima.

Unlike other fluorogenic substrates such as the sulfates of 7-hydroxy-4-methylcoumarin [8, 9] and β-naphthol [8], substrates **1b—d** do not require u.v. excitation. Substrate **1d** especially allows measurements far into the visible region, where no strong background fluorescence from biological materials is to be expected. In addition, the large Stokes' shift (80 nm) can prevent interferences from Raman scatter and stray light, in particular when measurements are made in the re-emission mode (front face technique).

Financial support from the "Fonds zur Förderung der wiss. Forschung" (Project P5014) is gratefully acknowledged.

REFERENCES

1 E. H. Kolodny and R. A. Mumford, Adv. Exp. Med. Biol., 68 (1976) 239.
2 H. Christomanou and K. Sandhoff, Clin. Chim. Acta, 79 (1977) 527.
3 G. Dubois, J. C. Turpin and N. Baumann, Adv. Exp. Med. Biol., 68 (1976) 233.
4 J. Potter, G. D. Timmons, L. Rinehart and E. J. Witmer, Clin. Chim. Acta, 39 (1972) 518.

5 Y. A. Leon, R. D. Bulbrook and E. D. Comer, Biochem. J., 75 (1960) 612.
6 A. B. Roy, Biochem. J., 62 (1956) 41.
7 G. G. Guilbault, Enzymatic Methods of Analysis, Pergamon Press, Oxford, 1970.
8 G. G. Guilbault and J. Hieserman, Anal. Chem., 41 (1969) 2006.
9 W. R. Sherman and E. F. Stanfield, Biochem. J., 102 (1967) 905.
10 G. J. Yakatan, R. J. Juneau and S. G. Schulman, Anal. Chem., 44 (1972) 1044.
11 O. S. Wolfbeis and E. Koller, Anal. Biochem., 129 (1983) 365.
12 E. Koller and O. S. Wolfbeis, Anal. Biochem., 143 (1984) 146.
13 O. S. Wolfbeis, E. Koller and P. Hochmuth, Bull. Chem. Soc. Jpn., in press.
14 E. Koller and O. S. Wolfbeis, Monatsh. Chem., 116 (1984) 65.

Analytica Chimica Acta, 170 (1985) 81—87
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

FURTHER STUDIES ON THE REACTION OF AMINES AND PROTEINS WITH 4-FLUORO-7-NITROBENZO-2-OXA-1,3-DIAZOLE

HIROSHI MIYANO, TOSHIMASA TOYO'OKA and KAZUHIRO IMAI*

Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113 (Japan)

(Received 6th September 1984)

SUMMARY

The reaction of glycine with NBD-F (4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole) is investigated to establish conditions that provide a high formation rate of NBD-glycine and a low hydrolysis rate of the reagent. The reaction rate increases with higher temperature, larger contents of organic solvent and a sodium borate buffer. The use of borate buffer decreases the rate of hydrolysis of the reagent. For low-molecular-weight amines, conditions for a suitable liquid chromatographic postcolumn reactor include a high content of acetonitrile and 0.1 M sodium borate (pH 8.0—8.5). For proteins, separated by molecular-exclusion chromatography, water is needed for sensitive reactions. Suitable postcolumn reactor conditions include borate buffer (pH 7.9) containing 0.1 M potassium chloride, a 0.02% (w/v) NBD-F solution in acetonitrile with reaction at 50°C for about 45 s. The detection limits for human serum albumin, β-lactoglobulin and myoglobin are 6.6 pmol, 8.4 pmol and 11 pmol, respectively.

4-Fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) was introduced [1] as a fluorogenic reagent to replace 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). It reacts under rather mild conditions (at pH 8.0 and 60°C for 1 min) with both primary and secondary amines to give fluorescent adducts (λ_{ex} = 470 nm, λ_{em} = 530 nm). Its derivatives with amino and imino acids [2, 3], catecholamines and polyamines [4] have been separated and quantified sensitively by high-performance liquid chromatography (h.p.l.c.). However, the hydrolyzed product (NBD-OH; 4-hydroxy-7-nitrobenzo-2-oxa-1,3-diazole) of the reagent can cause problems in attempts to enhance the sensitivity because it fluoresces weakly (λ_{ex} = 470 nm, λ_{em} = 555 nm); the fluorescence is less than a fifth of that of NBD-glycine, and is decreased further at about pH 1. This was used successfully for postcolumn reaction and detection of amino and imino acids in h.p.l.c. [5].

In this paper, the various conditions which affect the yields of NBD-amine and NBD-OH are examined and suitable conditions are given for obtaining a high ratio of NBD-amine to NBD-OH. The applicability of NBD-F to the postcolumn reaction of some proteins separated on h.p.l.c. is also reported.

0003-2670/85/$03.30

EXPERIMENTAL

Chemicals

Glycine (Ajinomoto Co., Tokyo) was used as received. Samples of proteins, i.e., human serum albumin (HSA, Cohn Fraction V; Wako Pure Chemical Co., Tokyo), β-lactoglobulin (Bovine; ICN Pharmaceuticals) and myoglobin (from Equine Skeletal Muscle; Sigma Chemical Co.), were kindly donated by Shimazu Seisakusho Co. (Kyoto). The NBD-F and NBD-glycine (m.p. 185—187°C) were synthesized by the methods of Nunno et al. [6] and Ghosh [7], respectively. 4-Methoxy-7-nitrobenzo-2-oxa-1,3-diazole (NBD-OCH_3) and NBD-OH (m.p. 199—200°C) were synthesized as described by Dal Monte et al. [8]. The NBD-Cl was from Merck. The buffer pH 4.01 solution (0.05 M potassium phthalate buffer) was from Nakarai Chemicals Co. (Kyoto). All other chemicals were of reagent grade. Water was deionized and distilled.

Procedures

Reaction of glycine with NBD-F. Glycine in the buffer solution in a 10-ml brown glass test tube was treated with a freshly prepared solution of NBD-F, NBD-Cl or NBD-OCH_3 in the selected organic solvent. The final concentration of glycine was 11.5 μM and that of the reagent was 1.15 mM. At certain time intervals, an aliquot was treated with 1 M hydrochloric acid to stop the reaction, and then subjected to h.p.l.c.

Separation of NBD derivatives. The l.c. system consisted of a Milton-Roy minipump MS1-33R, an auto-sampling unit (KST-24) and a high-pressure auto injector (KMH1000; Kyowa Seimitsu Co., Tokyo) with a column of TSK LS-160 (polyethyleneglycol dimethacrylate gel, 5 μm, 150 × 8 mm i.d., Toyo Soda Manufacturing Co., Tokyo). The detector was a Schoeffel FS-970 spectrofluorimeter equipped with 5-μl flow cell and a cut-off filter at 490 nm (excitation at 470 nm) and a C-RIA Chromatopac (Shimazu Seisakusho Co.) which measured each peak area. The column temperature was ambient. The eluent was a mixture of methanol and 0.05 M phthalate buffer solution of pH 4.01 (6:4, v/v); the flow rate was 0.67 ml min^{-1}. The respective elution times for NBD-glycine and NBD-OH were 11.5 min and 14 min.

Postcolumn reaction of proteins with NBD-F. Two Model 6000A pumps (one for the eluent and the other for NBD-F solution) equipped with a U6K universal injector (Waters Assoc.) were used. The proteins dissolved in water were separated at ambient temperature on a column of Shim-Pak Diol-150 (500 × 7.9 mm i.d., 5 μm) kindly donated from Shimazu Seisakusho; backpressures were maintained at about 1000 psi. The eluent was 50 mM sodium borate buffer (pH 7.9) containing 0.1 M KCl. A solution of NBD-F in acetonitrile (recommended concentration is 0.02% (w/v)) was used for the postcolumn reaction. The eluent and the reagent solution were mixed with a three-way T-joint (type K3T-16S; Kyowa Seimitsu). All flow lines after the column were made with 0.25 mm i.d. PTFE tubing. Reaction time was set at about 45 s. A Hitachi 650-10S spectrofluorimeter equipped with a 18-μl

flow cell was used for detection with an excitation wavelength at 470 nm ± 10 nm and emission at 530 nm ± 10 nm. In a separate experiment, the column effluent was monitored for proteins at 210 nm (spectrophotometric detector SPD-2A, range 0.04 absorbance full scale; Shimazu Seisakusho).

RESULTS AND DISCUSSION

Reaction rate constants of glycine with NBD-F

Glycine was selected as the model amine because authentic NBD-glycine was available in a pure form. The reaction rate constants of glycine with NBD-F were measured under various conditions of temperature, pH, buffer components and organic solvents. Under the present conditions, i.e., with a 100-fold excess of NBD-F for glycine, the reaction followed pseudo-first-order kinetics. It was found that high temperature, high pH of the buffer and high contents of organic solvent gave large k_{obs} values (Table 1). The borate buffer gave a larger k_{obs} value than the other buffers (Table 1). The different solvents for NBD-F gave very similar rate constants (0.11—0.17 min^{-1}) except for methanol (Table 1).

For a comparison of the reactivities of glycine with the fluorogenic reagents having the benzofurazan ring structure (NBD-F, NBD-Cl and NBD-OCH_3) the conditions selected were: sample in 0.1 M borate buffer (pH 8.0), reagent in ethanol (8:2, v/v), and 40°C. As shown in Table 2, the reaction of NBD-F was about 500 and 60 times faster than those of NBD-Cl and NBD-OCH_3, respectively. Those results are compatible with previous results which showed that NBD-F is more reactive than NBD-Cl [1] and that NBD-OCH_3 is more reactive than NBD-Cl [9]. Among those reagents, NBD-OCH_3 itself had a weak fluorescence (λ_{ex} = 400 nm, λ_{em} = 470 nm) which was about 1/100 times weaker than that of NBD-glycine; this reagent would therefore be unsuitable for highly sensitive detection of amines.

The reaction rate constants of NBD-OH were also measured; this was possible because pure authentic NBD-OH was also available. The reaction rate of glycine was about twice as fast in the borate buffer pH 8.0 as in the phosphate buffer at the same pH and the hydrolysis of NBD-F was much slower in the borate buffer than in the phosphate (Table 1). In this respect, borate is the best buffer among those tested. The hydrolysis rates of NBD-F were of the same order in the different organic solvents tested (Table 1) except for methanol in which the rate constant was difficult to measure. As the temperature and the pH of the reaction mixture were increased, the hydrolysis rate of NBD-F increased (Table 1). The percentage content of ethanol had little effect on the hydrolysis (Table 1).

In previous papers [2, 3], the recommended conditions were 50% ethanol/0.1 M sodium borate (pH 8.0) at 60°C for 1 min to obtain complete reaction of amines with NBD-F; at that time the hydrolysis of NBD-F was not considered. The present results suggest that ethanol may be replaced by acetonitrile, especially in the case of the postcolumn reaction, because in acetonitrile

TABLE 1

Effect of different experimental variables on the reaction rate constants for glycine with NBD-F and hydrolysis of NBD-F

Variable	Range	k_{obs} (min^{-1})[a]		k_1/k_2
		Glycine (k_1)	Hydrolysis (k_2)	
Temp. (°C)[a]	40	9.7×10^{-3}	1.7×10^{-2}	0.57
	50	3.0×10^{-2}	7.1×10^{-2}	0.42
	60	8.1×10^{-2}	1.6×10^{-1}	0.51
pH[b]	8.0	1.8×10^{-2}	1.7×10^{-3}	11
	8.5	5.1×10^{-2}	4.5×10^{-3}	11
	9.0	1.1×10^{-1}	1.1×10^{-2}	10
	9.5	1.9×10^{-1}	2.0×10^{-2}	9.5
	10.0	2.1×10^{-1}	3.4×10^{-2}	6.2
Ethanol content (%)[c]	10	5.9×10^{-2}	1.1×10^{-2}	5.4
	20	1.7×10^{-1}	1.0×10^{-2}	17
	40	4.1×10^{-1}	1.3×10^{-2}	32
Buffer type[d]	Phosphate (Na)	8.4×10^{-2}	4.5×10^{-2}	1.9
	Borate (Na)	1.7×10^{-1}	1.0×10^{-2}	17
	Collidine (HCl)	1.4×10^{-3}	1.5×10^{-3}	0.93
Solvent type[e]	Ethanol	1.7×10^{-1}	1.0×10^{-2}	17
	Isopropanol	1.7×10^{-1}	1.1×10^{-2}	15
	Acetone	1.7×10^{-1}	1.1×10^{-2}	15
	Acetonitrile	1.3×10^{-1}	7.1×10^{-3}	18
	n-Propanol	1.1×10^{-1}	1.1×10^{-2}	6.9
	Methanol	5.6×10^{-2}	—[f]	

[a]The rate constants were obtained from the reaction of glycine (11.5 μM) in 0.067 M phosphate buffer (pH 7.0) with NBD-F (1.15 mM) in ethanol (8:2 v/v). [b]As in (a), except for 0.1 M sodium borate buffer, ethanol (9:1 v/v) solvent, and reaction temperature (30°C). [c]As in (a), except for 0.1 M sodium borate (pH 8.0) and variable ethanol concentration; temperature 40°C. [d]As in (a), except for buffer type (0.1 M, pH 8.0) and temperature (40°C). [e]As in (c) except for organic solvent (8:2 v/v). [f]Not measured.

TABLE 2

Reaction rate constants for glycine with NBD-F, NBD-Cl and NBD-OCH_3

Reagent	k_{obs} (min^{-1})[a]
NBD-F	1.7×10^{-1}
NBD-OCH_3	2.7×10^{-3}
NBD-Cl	3.6×10^{-4}

[a]The rate constants were obtained from the reaction of glycine (11.5 μM) in 0.1 M sodium borate (pH 8.0) with reagents (1.15 mM) in ethanol (8:2 v/v) at 40° C.

the ratio of the rate constants for glycine and for hydrolysis was almost the same as that in ethanol, and there was less formation of bubbles when the column eluate and the organic solvent containing NBD-F were mixed. Experience indicated that larger contents of acetonitrile, borate buffer at pH 8.0—8.5 and shorter reaction times were appropriate for the postcolumn reaction.

Postcolumn reaction of proteins with NBD-F without hydrochloric acid

The recommended conditions for the postcolumn reaction were applied for the determination of proteins without hydrochloric acid (in contrast to the determination of amino acids) after their separation by molecular exclusion chromatography [5].

For preliminary investigations of the reactivities of proteins with NBD-F, a flow-injection system was adopted without the separation column. The pH of the carrier solution was adjusted to pH 7.9, which was the highest pH to be used for the column. The results showed, unexpectedly, that the reactivities of HSA (m.w. 69 000) and myoglobin (m.w. 17 000) with NBD-F decreased when larger contents of acetonitrile were present (Fig. 1a). Therefore, 50% acetonitrile seemed to be suitable for the reaction. Thus, the flow rates of both the carrier stream and the NBD-F solution in acetonitrile were selected as 1.0 ml min^{-1}. It is noteworthy that the best concentrations of organic solvent are different for the reaction of NBD-F with low-molecular-weight substances such as glycine and with HSA and other large molecules; the appropriate content of water is necessary for the reaction of proteins. After further investigations on the reaction temperature and the concentration of NBD-F in acetonitrile, the most suitable reaction conditions were settled as

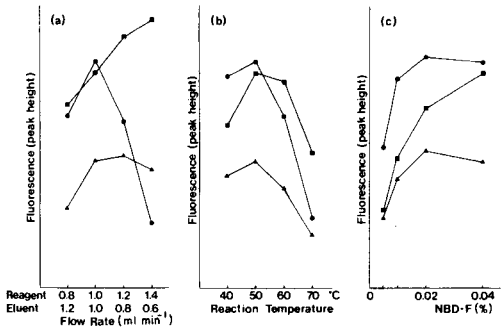


Fig. 1. Effects of (a) the content of acetonitrile, (b) reaction temperature and (c) concentration of NBD-F, on the reaction of proteins with NBD-F in a flow-injection system. Compounds tested: (•) HSA; (▪) β-lactoglobulin; (▲) myoglobin. (a) The flow rates of the carrier stream (50 mM sodium borate, pH 7.9, containing 0.1 M KCl) and the reagent solution (NBD-F in acetonitrile) were changed; the NBD-F concentration was kept at 1.1 mM in the reaction mixtures; 250 pmol of each protein in 10 μl of water was injected and allowed to react at 50°C for about 45 s. (b) The flow rates of both the carrier stream and the reagent solution (0.04% NBD-F, 2.2 mM, in acetonitrile) were 1.0 ml min^{-1}; other conditions as for (a) except for temperature. (c) Reaction temperature was 50°C; other conditions as for (b) except the NBD-F content.

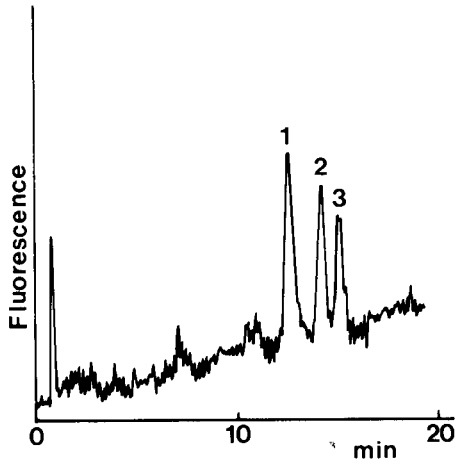


Fig. 2. A chromatogram obtained from the postcolumn reaction of proteins with NBD-F. Peaks: (1) HSA; (2) β-lactoglobulin; (3) myoglobin. The flow rates of both the eluent (50 mM sodium borate buffer pH 7.9 containing 0.1 M KCl) and the reagent (0.02% NBD-F in acetonitrile) were 1.0 ml min^{-1}. Protein solution (5 μl containing 60 pmol of each) was injected onto the recommended column. Reactor at 50°C for about 45 s.

0.02% NBD-F in acetonitrile at 50°C for about 45 s (Fig. 1b and c). Thus the detection limits for HSA, β-lactoglobulin (m.w. 36 000) and myoglobin which had been separated by molecular exclusion chromatography and quantified in the postcolumn reactor were 6.6 pmol, 8.4 pmol and 11 pmol (S/N = 2), respectively (Fig. 2). Although the detection limits are larger than those obtained by a u.v. detector at 210 nm (about 0.17 pmol, 0.33 pmol and 1.9 pmol (S/N = 2) for HSA, β-lactoglobulin and myoglobin, respectively), the fluorescence detection could have advantages in selectivity. With regard to the sensitivity for the present l.c. method, higher sensitivity could probably be obtained by using more elaborate pumps for the delivery of the eluent and the reagent solution to reduce the variation of the baseline.

In conclusion, the present studies indicate that suitable reaction conditions for both low-molecular-weight amines and proteins with NBD-F are available and are applicable to postcolumn reactions in h.p.l.c.

The authors thank Prof. T. Nakajima, University of Tokyo, for his valuable suggestions and discussions.

REFERENCES

1 K. Imai and Y. Watanabe, Anal. Chim. Acta, 130 (1981) 377.
2 Y. Watanabe and K. Imai, Anal. Biochem., 116 (1981) 471.
3 Y. Watanabe and K. Imai, J. Chromatogr., 239 (1982) 723.
4 T. Toyo'oka, Y. Watanabe and K. Imai, Anal. Chim. Acta, 149 (1983) 305.

5 Y. Watanabe and K. Imai, Anal. Chem., 55 (1983) 1786.
6 L. D. Nunno, S. Florio and P. E. Todesco, J. Chem. Soc., Sect. C, (1970) 1433.
7 P. B. Ghosh, J. Chem. Soc., Sect. B, (1968) 334.
8 D. Dal Monte, E. Sandri and P. Mazzaracchio, Boll. Sci. Fac. Chim. Ind. Bologna, 26 (1968) 165.
9 L. Johnson, S. Lagerkvist, P. Lindroth, M. Ahnoff and K. Marinsson, Anal. Chem., 54 (1982) 939.

Analytica Chimica Acta, 170 (1985) 89—94
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

QUINOLIZINIUM SALTS AS FLUORESCENT PROBES FOR *N*-NUCLEOPHILES

M.A. MARTÍN, B. DEL CASTILLO*, J. EZQUERRA and J. ALVAREZ-BUILLA

Cátedras de Técnicas Instrumentales y Química Orgánica, Facultad de Farmacia, Universidad Complutense, 28040 Madrid (Spain)

(Received 6th September 1984)

SUMMARY

Quinolizinium salts are effective fluorescent reagents for the detection of amines. A series of carbamylquinolizinium salts was synthesized to take advantage of the influence of the carbamyl group on the fluorescence of the heterocyclic aromatic system. The native fluorescence of carbamylquinolizinium derivatives is described. The polycyclic compounds exhibit intense fluorescence which can be measured for 10^{-10}—10^{-11} M solutions. With the diphenyl derivatives, it is not possible to establish correlations between the Hammett parameters from different substituents and the fluorescence emission. The fluorescence of reaction products with piperidine is described; the emission intensity observed is highest for the polycyclic quinolizinium derivatives, which can be considered as the most appropriate for the detection of amines.

Many 5-membered heterocyclic compounds do not show native fluorescence, but do fluoresce when they are attached to a benzene ring. Analytical methods have been developed in which a fluorophore is produced after several reactions. For example, Nakamura and Tamura [1] described a determination of carbonyl compounds based on reaction with *N*-methylnicotinamide chloride. Other methods have been based on the enhanced emission obtained when molecular rigidity is increased, e.g., condensation of amino acids with formaldehyde.

A fluorescent probe is a compound that will show fluorescence when it is bound to nonfluorescent compounds, providing an emission different from that of the reaction medium as well as high quantum efficiency. Such probes are very useful in qualitative and quantitative analysis; they can be used to characterize compounds after chromatographic separation and to detect compounds with no or little native fluorescence. The fluorogenic reaction can be versatile, rapid and not necessarily drastic; the products and fluorophores must be stable in the reaction medium.

Many different fluorescent probes are now available; the selected probe will depend on the characteristics of the functional group. For example, *o*-phthalaldehyde [2] and fluorescamine [3] are often used in analysis for amines. Quinolizinium salts can be considered as fluorescent probes for amines and nucleophiles in general. In previous work, the utility of these

0003-2670/85/$03.30

compounds for detecting and quantifying amines in pharmaceutical analysis was demonstrated [4]. Necessary conditions for optimizing the reaction between 1,2-dicarbonyl compounds and 2-picolinium salts were established; this reaction produces the quinolizinium ring [5].

In the present paper, the fluorescent properties of carbamylquinolizinium salts and 2-vinyl derivatives of quinolizinium salts are studied.

EXPERIMENTAL

Apparatus, reagents and chemicals

Absorption spectra were measured with a Beckman DU-7 u.v.-visible spectrophotometer equipped with thermostated cells and a videocopier. Fluorescence spectra were obtained with a Perkin-Elmer Hitachi 204 spectrofluorimeter (xenon lamp XBO, 150 W) equipped with a synchronized recorder. A thermostated Selecta bath was used to control the temperature of the samples.

The solvents used were of spectrofluorimetric grade; acetone was obtained from Merck, and methanol and acetonitrile from Carlo Erba. All carbamylquinolizinium salts and vinyl derivatives of quinolizinium salts were synthesized in the Organic and Pharmaceutical Chemistry Laboratory.

Procedures

The stock solution of the quinolizinium salt was 1.3×10^{-4} M in acetone/methanol (1:1, v/v). From this stock solution, aliquots were taken to prepare different solutions in order to establish the relationship between emission intensity and fluorophore concentration. The reaction between quinolizinium salts and nucleophiles has already been described [6].

RESULTS AND DISCUSSION

Native fluorescence of the derivatives

These salts show notable native fluorescence produced by the basic fluorophore, the quinolizinium ring; structural variations at different positions of the fluorophore change the emission of the salts in solution. Previous studies [4] demonstrated that a carboxylate group at position 4 of the quinolizinium ring greatly enhanced the fluorescent emission, because of the presence of a new chromophore attached directly to the quinolizinium ring. The carbamylquinolizinium salts were more water-soluble than the quinolizinium salts themselves, and also provided enhanced fluorescence compared with the unsubstituted quinolizinium salts.

When homologous series of quinolizinium and carbamylquinolizinium salts were compared, greater emission intensity was observed for the latter salts. 7-Ethoxycarbonylmethylacenaphtho(1,2-b)quinolizinium bromide was the only quinolizinium derivative giving a better limit of detection (10^{-11} M) than the carbamylquinolizinium derivatives. The most likely explanation is that the carboxylate group increased the fluorescent emission.

The u.v.-visible absorption spectra for all these compounds were recorded. A band was observed between 330 nm and 350 nm for all the diphenyl derivatives of the quinolizinium and carbamylquinolizinium salts. A weak interaction between the carbamyl group and the quinolizinium ring in the ground state of the molecule was indicated by the minor shifts of this absorption band. However, interactions between both chromophore groups were produced when the molecules were in excited state; thus, each of these compounds showed characteristic peaks, but different from their homologues without the carbamyl group.

The fluorescence peak was shifted to higher wavelengths in carbamylquinolizinium salts compared to quinolizinium salts, although the excitation wavelengths for both series of salts were quite close together. The excitation peak shifts were very small (ca. 5 nm). These results suggest that the interactions between the heterocyclic system and different substituents in the ground state and in the excited state are very different.

In the polycyclic compounds (9 and 10, Table 1) with flat aromatic substituents the influence of the carbamyl group was cancelled. These compounds showed different absorption spectra (a broad band with peaks at 330 nm, 360 nm, 400 nm and 410 nm) and different emission spectra. However, the influence of the carbamyl group was confirmed by the shift of the emission peak to higher wavelengths.

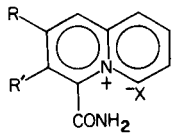
The different influence of the same substituent attached to different positions (*m*- or *p*-) on the phenyl substituents was demonstrated in the 2,3-diphenyl derivatives. Isomeric compounds (6 and 8, Table 1) exhibited different characteristic excitation and emission wavelengths. These results prove that the existence of different electronic effects, related to the position of substitution, change the shape of the fluorescence spectra [7]. In some compounds, the fluorescent emission was almost entirely quenched by electron-attracting substituents such as nitro groups. For the dinitro derivative (3, Table 1), the emission was not completely quenched because of the benzene ring between the fluorophore and the quenching group; the emission was decreased only slightly. For the diphenyl derivatives, it was not possible to establish a correlation between the Hammett parameters for different substituents and the intensity and wavelengths of excitation and emission (Table 2). The emission peak was shifted to higher wavelengths for the dinitro derivative, which also had the largest Hammett parameter.

The polycyclic compounds appeared to be very suitable as fluorescent probes. These compounds were detected at very low concentrations because the molecular rigidity improved the emission process [8, 9].

A series of vinyl derivatives of quinolizinium salts was also studied; the ethylene bond was placed between the quinolizinium system and the R′ substituent (see Table 3), which was a heterocycle (compounds 12, 13 and 14) or a benzene ring (compound 11). The absorption spectra of all these compounds showed a bathochromic shift of the maximum wavelengths compared to the carbamylquinolizinium and the simple quinolizinium

TABLE 1

Native fluorescence parameters and linear ranges found for ten carbamylquinolizinium salts



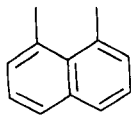
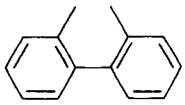
Compound No.	R	R′	X	Solvent	λ_{ex} (nm)	λ_{em} (nm)	Linear range (M)
1	CH_3	CH_3	Cl^-	Acetone/MeOH	400	495	1.2×10^{-4}—1.3×10^{-6}
				Water	400	485	1.2×10^{-4}—1.3×10^{-5}
2	C_6H_5	C_6H_5	Cl^-	Acetone/MeOH	355	390	5.5×10^{-4}—1.3×10^{-6}
				Water	345	390	1.3×10^{-4}—1.3×10^{-5}
3	$3\text{-}NO_2C_6N_4$	$3\text{-}NO_2C_6H_4$	BF_4^-	Acetone/MeOH	415	445	1.2×10^{-4}—1.3×10^{-7}
					395	445	— —
4	$3\text{-}CH_3OC_6H_4$	$3\text{-}CH_3OC_6H_4$	BF_4^-	Acetone	350	510	2.1×10^{-5}—2.1×10^{-3}
5	$3\text{-}ClC_6H_4$	$3\text{-}ClC_6H_4$	Cl^-	Acetone/MeOH	345	365	1.2×10^{-4}—1.3×10^{-5}
				Acetone	345	365	2.3×10^{-5}—4.6×10^{-6}
6	$3\text{-}CH_3C_6H_4$	$3\text{-}CH_3C_6H_4$	BF_4^-	Acetone	355	450	2.0×10^{-5}—2.2×10^{-7}
7	$4\text{-}ClC_6H_4$	$4\text{-}CH_3C_6H_4$	Cl^-	Acetone	350	390	
					330	390	2.0×10^{-5}—2.3×10^{-8}
8	$4\text{-}CH_3C_6H_4$	$4\text{-}ClC_6H_4$	Cl^-	Acetone	355	420	2.5×10^{-5}—7.7×10^{-7}
9			Cl^-	Acetone/MeOH	370	455	
				Acetone	415	460	2.5×10^{-5}—2.8×10^{-10}
10			Cl^-	MeOH	430	510	1.3×10^{-4}—1.3×10^{-8}
			Cl^-	Acetone	420	510	1.2×10^{-4}—1.3×10^{-11}

TABLE 2

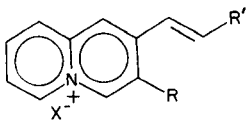
Relation between the Hammett parameter (σ) and fluorescence intensity for 2,3-diphenyl derivatives of carbamylquinolizinium salts

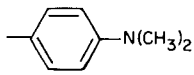
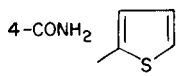
Compound no.	σ	λ_{ex} (nm)	λ_{em} (nm)	RFI[a]	Compound no.	σ	λ_{ex} (nm)	λ_{em} (nm)	RFI[a]
2	0	355	390	47	6	—0.07	355	450	22
3	0.71	415	445	39	7	0.23	350	390	25
4	0.12	350	510	21	8	—0.17	355	420	28
5	0.37	345	365	13					

[a]Relative fluorescence intensity.

TABLE 3

Native fluorescence parameters and linear ranges for four vinyl derivatives of quinolizinium salts



Compound No.	R	R'	X	Solvent	λ_{ex} (nm)	λ_{em} (nm)	Linear range (M)
11	CH_3		Br^-	Acetone	475	590	1.3×10^{-5}—1.3×10^{-10}
				Methanol	475	590	1.3×10^{-5}—2.7×10^{-10}
12	CH_3		Cl^-	Acetone	410	490	1.3×10^{-4}—1.3×10^{-6}
				Methanol	415	490	1.3×10^{-4}—3.0×10^{-6}
13	CH_3		Cl^-	Acetone/MeOH	390	480	1.3×10^{-4}—1.3×10^{-6}
14	CH_3		Cl^-	Methanol	405	590	1.3×10^{-4}—1.3×10^{-7}

salts. The characteristic band of the quinolizinium system was observed between 330 nm and 350 nm. The fluorescence emission was also shifted to higher wavelengths (590 nm) for the vinyl series. The Stokes loss was greater than for the diphenyl derivatives of the quinolizinium and carbamylquinolizinium salts. Stokes loss and full width at reciprocal "e" (FWRE) parameters were related to the degree of planarity of the compounds [10]. Electron-donating substituents at position 2- of the quinolizinium ring increased the fluorescence intensity (compound 11); 10^{-7}—10^{-10} M solutions were easily quantified. Deactivating substituents attached directly to the quinolizinium system decreased the fluorescence intensity.

The special fluorescent characteristics of 2(*p*-dimethylamino)styryl-3-methylquinolizinium bromide were studied in detail. A mathematical study of the relationship between fluorescence intensity and quinolizinium salt concentration showed that linear response was obtained between 10^{-5} M and 10^{-10} M.

Reaction between quinolizinium salts and N-nucleophiles

The quinolizinium ring with its quaternary nitrogen provides a system with a high electron deficiency. These compounds are excellent substrates for studying nucleophilic aromatic substitution reactions. Many of them react with N-, C- and S-nucleophiles. When the nucleophilic reagent was an

amine, the quinolizinium ring was opened, as in the process described by Moerler and Kröhnke [11]. The nucleophilic attack was produced at position 6 of the quinolizinium ring when positions 2 and 3 were substituted.

To study the reactions between quinolizinium salts and nucleophiles, the most representative compounds of the series were chosen. Piperidine was selected as the reference amine because it is one of the most nucleophilic amines and because it is a secondary, aliphatic and cyclic amine; consequently, the nitrogen pair of electrons is free for nucleophilic aromatic substitution.

2,3-Diphenylquinolizinium bromide was selected from the quinolizinium salts without a carbamyl group because it is very reactive and versatile and it is easily synthesized. Its reaction products showed a very long Stokes loss (100 nm) depending on the nucleophilic reagent used. The polycyclic compounds and 2,3-diphenyl-4-carbamylquinolizinium chloride were outstanding in comparison to the other carbamylquinolizinium salts tested. Amine concentrations around 10^{-12} M could be detected with 7-carbamylacenaphtho(1,2-b)quinolizinium chloride as fluorescent probe. The reaction with compound 11 (Table 3) was also studied. This reaction with nucleophiles was not easily produced because the presence of the (*p*-dimethylamine)styryl substituent in position 2 of the quinolizinium ring decreased the electron deficiency of the heterocyclic system. The positive charge on the heterocyclic nitrogen can be transferred to the exocyclic nitrogen, and so the nucleophilic attack depends on the polarity of the medium.

The use of these salts was examined for amines with pharmacological activity, such as adrenaline, noradrenaline, phenylephrine, ephedrine, benzocaine and bromhexine, as will be described elsewhere.

REFERENCES

1 H. Nakamura and Z. Tamura, Anal. Chem., 50 (1978) 2047.
2 P. Lindroth and K. Mopper, Anal. Chem., 51 (1979) 1667.
3 H. Nakamura and Z. Tamura, Anal. Chem., 52 (1980) 2087.
4 C. Serrano, A. García de Marina, B. Del Castillo and J. Alvarez-Builla, An. R. Acad. Farm., 48 (1982) 75.
5 G. Gonzalez-Trigo, M. E. Fombella, J. Alvarez-Builla, B. Del Castillo, A. García de Marina and J. A. Avila-Santiago, An. Quim., 77C (1981) 225.
6 B. Del Castillo, A. García de Marina, M. E. Fombella and J. Alvarez-Builla, An. Quim., 77C (1981) 357.
7 A. Bowd and J. H. Turnbull, J. Chem. Soc. Perkin Trans. 2, (1977) 121.
8 I. B. Berlman, J. Phys. Chem., 66 (1977) 4231.
9 T. Takemura, P. K. Das, G. Hug and R. S. Becker, J. Am. Chem. Soc., 98 (1976) 7099.
10 I. B. Berlman, Handbook of Fluorescence Spectra of Aromatic Molecules, Academic Press, New York, 1971, p. 51.
11 D. Moerler and F. Kröhnke, Justus Liebigs Ann. Chem., 744 (1971) 65.

Analytica Chimica Acta, 170 (1985) 95—100
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

THE INFLUENCE OF SOLVENT POLARITY AND VISCOSITY ON FLUORESCENCE OF QUINOLIZINIUM SALTS

M. A. MARTÍN, M. BALLESTEROS and B. DEL CASTILLO*

Cátedra de Técnicas Instrumentales, Facultad de Farmacia, Universidad Complutense, 28040 Madrid (Spain)

(Received 6th September 1984)

SUMMARY

The effects of dielectric constant, viscosity and polarity of the solvents used on the relative fluorescent emission of 2,3-diphenylquinolizinium bromide and 2(*p*-dimethylamino)styryl-3-methylquinolizinium bromide are described. These salts were entirely dissolved in ten different solvents. The 2(*p*-dimethylamino)styryl derivative produced different ionic species, and was a fluorescent acid-base indicator. The fluorescence of these quinolizinium salts is shown to increase with greater viscosity of the medium.

Fluorimetric detection is often used in high-performance liquid chromatography (h.p.l.c.). Many studies have been made concerning interactions between solvents and sample solutes. Interaction between the solute and mobile-phase components can affect the sensitivity of the method. For example, the emission of catecholamines [1], indole [2], pyrene [3, 4] and other compounds can be quenched by water, with an important decrease in the limit of detection for these compounds. Quinolizinium salts could be useful in derivatization reactions for h.p.l.c., so that it is necessary to know the characteristics of solvent—solute interaction.

Viscosity is an important parameter directly related to the solvent polarity and with a significant influence on emission processes. Normally, when viscosity is considered in fluorimetric studies [5, 6], it is related to temperature variation. However, viscosity in itself can be an important factor in increasing fluorescence intensity.

In this paper, the effects of solvent and viscosity are studied simultaneously. Different variables describing solvent polarity are used, such as dielectric constant, Kosower's Z values [7] and dipole moment [8]. A mathematical treatment of variables which can define the influence of viscosity on emission is also reported.

EXPERIMENTAL

Apparatus, reagents and chemicals

Absorption spectra were measured with a Beckman DU-7 u.v.-visible spectrophotometer equipped with thermostated cells and a videocopier.

0003-2670/85/$03.30

Fluorescence spectra were obtained with a Perkin-Elmer Hitachi 204 spectrofluorimeter (xenon lamp XBO, 150 W) equipped with a synchronized recorder. A Spandomatic SS-2 pH meter was used. Samples were thermostated in a Selecta bath.

The solvents used were acetic acid, butanol and propanol (spectroscopic grade) and ethanol and acetone (spectrofluorimetric grade; all from Merck); and formamide and dimethylsulfoxide (spectroscopic grade) and methanol and acetonitrile (spectrofluorimetric grade; all from Carlo Erba).

2,3-Diphenylquinolizinium bromide and 2(*p*-dimethylamine)styryl-3-methylquinolizinium bromide were synthesized in this Institute.

Procedures

The pK_a value of 2(*p*-dimethylamine)styryl-3-methylquinolizinium bromide was determined spectrophotometrically. Measurements at specific pH values involved the use of McIlvaine buffers (citric acid/disodium phosphate) covering the range pH 2.2—8.8.

For studies of the effect of polarity of solvents, 10^{-4} M solutions of quinolizinium salts were prepared. The concentration of quinolizinium salts for spectrophotometric measurements was 2.5×10^{-5} M. For spectrofluorimetric determinations, the concentrations were about 10^{-5} M to avoid the inner filter effect.

The effect of viscosity was studied for each solvent. The quinolizinium salt concentration was always 10^{-5} M and the concentration of glycerol was varied between 10% and 90% in water. The solutions were prepared as follows: to 9 ml of glycerol solution, obtained by mixing glycerol with water, 1 ml of 10^{-4} M quinolizinium salt was added, giving a final concentration of 10^{-5} M. Water was not used to change the viscosity when the solvents were acetic acid and butanol; viscosity was then changed by using the same solvents.

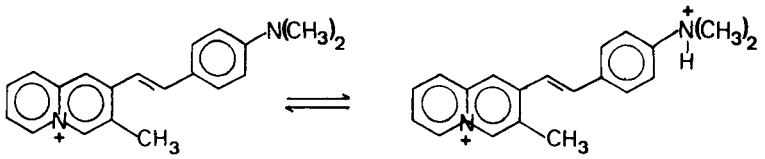
All solutions were prepared at 20°C.

RESULTS AND DISCUSSION

2,3-Diphenylquinolizinium bromide and 2(*p*-dimethylamino)styryl-3-methylquinolizinium bromide were considered as effective fluorescent probes for nucleophiles [9]. 2,3-Diphenylquinolizinium bromide is reactive with diverse nucleophiles, giving products with remarkable fluorescence [10]. The styryl derivative is not only a good fluorescent probe but has the following characteristics: it is an acid-base indicator; the u.v.-visible absorption spectra show remarkable solvatochromic effects [11]; it is very stable in methanolic solution, the fluorescence emission being constant for a long time; and the useful linear range is 10^{-5}—10^{-10} M. These two compounds were therefore studied in detail.

The pK_a value for 2(*p*-dimethylamino)styryl-3-methylquinolizinium bromide was found to be 4.11. This compound can be protonated when

the medium is acidic (structure II). The acid form absorbed at λ_{max} = 362 nm, and was not fluorescent. In alkaline media, the unprotonated structure (I) shows maximal absorption at 436 nm, and maximal fluorescence at 590 nm (λ_{ex} = 470 nm).



I II

When the solvent was changed, a significant solvatochromic effect was observed for this salt. Notable shifts in the absorption maximum (433 nm to 488 nm) were caused by the different orientation of solvent molecules surrounding the solute. According to Kamlet et al. [12, 13], the electronic spectral transitions, $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$, are affected by the characteristic polarity and polarizability of the solvent. The shifts are probably due to transfer between solvent donor and solute acceptor, hydrogen bonds being established between solvent and solute.

In Table 1 the shifts in the wavelength of maximum absorption are compared with the dielectric constants and Kosower's Z values. When the dielectric constant increased, a hypsochromic shift of the absorption maximum was produced. The smallest value (433 nm) was obtained with water. There was a linear relationship between the dielectric constant or Z value and the maximum absorption wavelength for different hydroxyl-containing solvents (including water). Formamide, which has a higher dielectric constant than water, should show a greater hypsochromic shift but the absorption maximum in formamide was close to that obtained in solvents with dielectric constants of 30—40. Solvatochromic effects were not significant in solutions of 2,3-diphenylquinolizinium bromide.

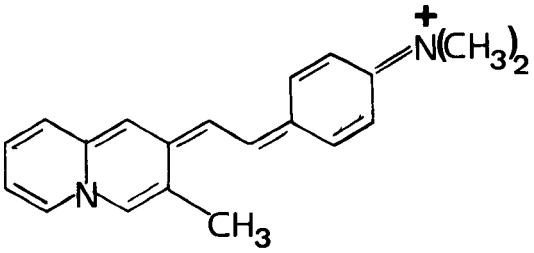
TABLE 1

Solvent effect on absorption spectra of 2.5 × 10^{-5} M 2(*p*-dimethylamino)styryl-3-methylquinolizinium bromide

Solvent	ϵ^a	Z^b	λ_{max} (nm)	A^c	Solvent	ϵ^a	Z^b	λ_{max} (nm)	A^c
Acetic acid	6.1	79.2	481.5	0.309	Methanol	33.6	83.6	475.0	0.926
1-Butanol	17.8	77.7	488.5	0.894	Acetonitrile	38.3	71.3	470.5	1.038
1-Propanol	20.1	78.3	486.5	0.780	DMSO	45.0	71.1	476.0	0.869
Acetone	20.7	65.7	473.5	0.949	Water	80.3	94.6	433.0	0.603
Ethanol	24.3	79.6	483.5	0.934	Formamide	109.0	83.3	474.0	0.820

[a] Dielectric constant. [b] Kosower value. [c] Absorbance.

Changes in the luminescent characteristics of these compounds by solvent effects were also studied. The excitation and emission peaks were shifted insignificantly (only 5 nm). However, the fluorescent emission intensity was enhanced greatly for 2(*p*-dimethylamino)styryl-3-methylquinolizinium bromide. For 2,3-diphenylquinolizinium bromide, the enhancement was weaker. The most intense emission for both compounds was obtained in solvents with low dielectric constants. As the dielectric constant of the solvents and *Z*-values increased, the fluorescence emission decreased. The smallest fluorescence was observed in water, possibly because of hydrogen bonding between solvent molecules and the exocyclic nitrogen. In the excited state, a 2(*p*-dimethylamino)styryl-3-methylquinolizinium bromide complex could be stabilized by hydrogen bonding decreasing the emission (structure I). The other possible form (III) with a positive charge on the exocyclic nitrogen predominated in the solvents of low dielectric constant. Hydrogen bonding between the solvent (in this case, alcohols) and the heterocyclic nitrogen would be difficult, because the ring nitrogen is inaccessible sterically. The existence of the resonance hybrid was confirmed by



III

the shifts of the spectral absorption peak and by the distinct reactivity shown in nucleophilic substitution reactions in different solvents. The reactivity was greater when the solvent polarity increased. When the solvent was formamide, the fluorescence intensity was greater than expected on the basis of the dielectric constant. This result was in line with the increased viscosity of the medium.

The existence of a resonance hybrid seemed improbable in the case of 2,3-diphenylquinolizinium bromide. Although there was some variation in the fluorescence with solvent polarity, in agreement with that of the styryl compound, the variation in emission intensity was much less.

The plots of relative fluorescence intensity versus dielectric constant (Fig. 1), *Z* values (Fig. 2), and dipole moments (Fig. 3) showed similar behaviour for both dissolved salts, except for four solvents. Thus, in acetic acid, the fluorescence intensity was less than the "expected" value, probably because of the relative instability of these compounds in acidic medium. Deviations were also found with the acetone, acetonitrile and DMSO (solvents 4, 7 and 8); these deviations were greatest in the plot against dipole

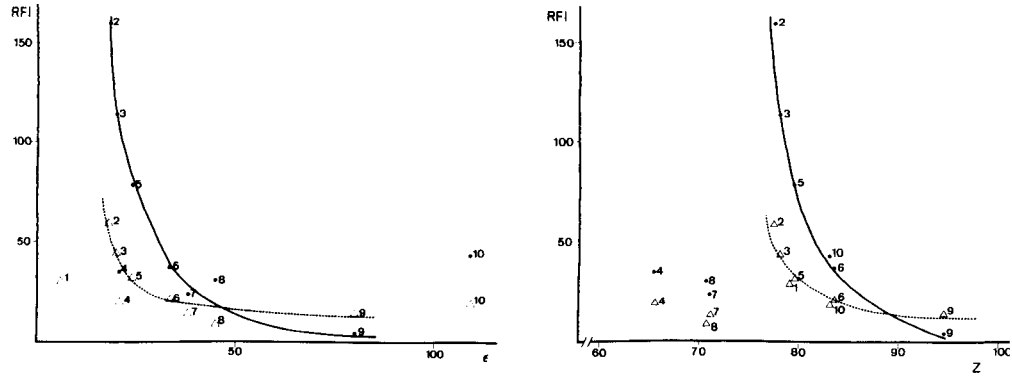


Fig. 1. Relative fluorescence intensity (RFI) vs. dielectric constant (ϵ): (•) 2(*p*-dimethylamino)styryl-3-methylquinolizinium bromide; (△) 2,3-diphenylquinolizinium bromide. Solvent: (1) acetic acid; (2) butanol; (3) propanol; (4) acetone; (5) ethanol; (6) methanol; (7) acetonitrile; (8) DMSO; (9) water; (10) formamide.

Fig. 2. Relative fluorescence intensity (RFI) vs. Kosower's (Z) values. Symbols and solvents as in Fig. 1.

moment (Fig. 3), probably because these solvents, which have essentially similar structure, have large dipole moments.

When the temperature was constant, the fluorescence intensity increased as the viscosity of the solvent increased (Fig. 4). The effect of viscosity on the fluorescence intensity for each solvent was tested further: aqueous solutions with different concentrations of glycerol were prepared and a constant concentration of the quinolizinium salt dissolved in solvents 1—10 was added to these solutions. The fluorescence intensity vs. viscosity relationships were exponential curves. A mathematical treatment of the experi-

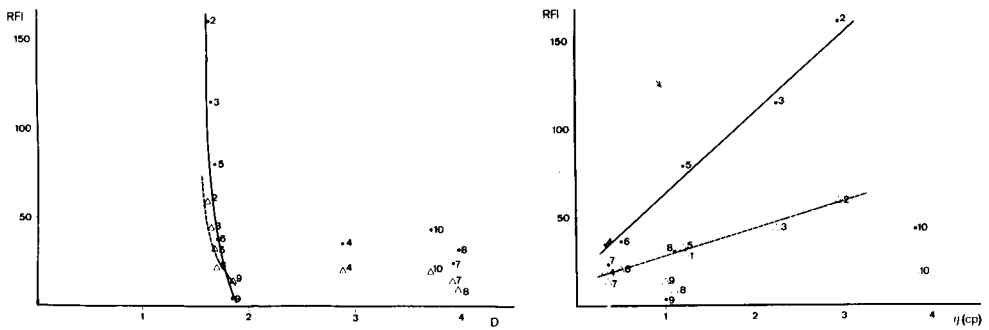


Fig. 3. Relative fluorescence intensity (RFI) vs. dipole moment (D). Symbols and solvents as in Fig. 1.

Fig. 4. Relative fluorescence intensity (RFI) vs. viscosity (η). Symbols and solvents as in Fig. 1.

TABLE 2

Equations obtained from viscosity study (compound 1, 2(*p*-dimethylamino)styryl-3-methylquinolizinium bromide; compound 2, 2,3-diphenylquinolizinium bromide)

Solvent	Compound 1		Compound 2	
	Equation	r^2	Equation	r^2
Acetic acid	—	—	$y = 9.33 \exp(0.03x)$	0.97
1-Butanol	$y = 249.69 \exp(0.01x)$	0.98	$y = 22.08 \exp(0.02x)$	0.94
1-Propanol	$y = 10.03 \exp(0.03x)$	0.98	$y = 2.95 \exp(0.04x)$	0.99
Acetone	$y = 11.81 \exp(0.03x)$	0.99	$y = 3.63 \exp(0.03x)$	1.00
Ethanol	$y = 10.93 \exp(0.04x)$	0.99	$y = 3.21 \exp(0.04x)$	0.99
Methanol	$y = 10.02 \exp(0.02x)$	1.00	$y = 2.99 \exp(0.04x)$	0.99
Acetonitrile	$y = 9.90 \exp(0.03x)$	0.98	$y = 3.63 \exp(0.03x)$	0.99
DMSO	$y = 8.11 \exp(0.04x)$	0.99	$y = 3.05 \exp(0.04x)$	0.99
Water	$y = 2.39 \exp(0.04x)$	1.00	$y = 3.01 \exp(0.03x)$	0.98
Formamide	$y = 8.06 \exp(0.03x)$	0.96	$y = 1.73 \exp(0.04x)$	0.96

[a] y = relative fluorescence intensity; x = % (w/w) glycerol; r^2 = correlation.

mental values obtained was applied. The exponential equations are listed in Table 2; the correlation coefficients obtained were very satisfactory. Thus viscous media can be used to increase the fluorescence emission intensity, when the relationship between the viscosity increase and fluorescence increase is known.

In this work, studies of polarized fluorescence demonstrated the polarization of these compounds in solution, with values $1 > p > 0$. The p value increased with viscosity, as would be expected.

The authors are grateful to the Fondo de Investigaciones Sanitarias de la Seguridad Social for a research fellowship.

REFERENCES

1 P. M. Froenlich and M. Yeats, Anal. Chim. Acta, 87 (1976) 185.
2 T. R. Hopkins and R. Lumry, Photochem. Photobiol., 15 (1972) 555.
3 A. Nakajima, Bull. Chem. Soc. Jpn., 44 (1971) 3272.
4 K. Kalyanasunderam and J. K. Thomas, J. Am. Chem. Soc., 99 (1977) 2039.
5 L. A. King, J. N. Miller and D. Thorburn Burns, Anal. Chim. Acta, 68 (1974) 205.
6 S. A. Carlson and D. M. Hercules, J. Am. Chem. Soc., 93 (1971) 5611.
7 E. M. Kosower, J. Am. Chem. Soc., 80 (1958) 3253.
8 R. C. Weast, Handbook of Chemistry and Physics, 57th edn., CRC Press, Boca Raton, E-56 and E-65.
9 M. A. Martín, B. Del Castillo, J. Ezquerra and J. Alvarez-Builla, Anal. Chim. Acta, 170 (1985) 89.
10 B. Del Castillo, A. Garcia de Marina, M. E. Fombella and J. Alvarez-Builla, An. Quim., 77C (1981) 357.
11 S. Arai, M. Yamazaki, K. Nagakura, M. Ishikawa and M. Hide, J. Chem. Soc. Chem. Commun., (1983) 1037.
12 M. Kamlet, M. Abbound, M. Abraham and R. W. Toft, J. Org. Chem., 48 (1983) 2877.
13 M. Kamlet, T. Hall, J. Boykin and R. W. Toft, J. Org. Chem., 44 (1979) 2599.

Analytica Chimica Acta, 170 (1985) 101—107
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

ENHANCED LUMINESCENCE DETERMINATION OF HORSERADISH PEROXIDASE CONJUGATES
Application of Benzothiazole Derivatives as Enhancers in Luminescence Assays on Microtitre Plates

G. H. G. THORPE*, S. B. MOSELEY, L. J. KRICKA, R. A. STOTT and T. P. WHITEHEAD

Department of Clinical Chemistry, Wolfson Research Laboratories, Queen Elizabeth Medical Centre, Birmingham B15 2TH (Great Britain)

(Received 23rd September 1984)

SUMMARY

The benzothiazole derivatives, 2-cyano-6-hydroxybenzothiazole, 6-hydroxybenzothiazole and dehydroluciferin, enhance light emission from the horseradish peroxidase-catalysed oxidation of cyclic diacylhydrazides such as luminol. The relatively intense and prolonged light emission from reactions enhanced by benzothiazole derivatives is easily detected and is utilised in a rapid assay for specific antibody against cytomegalovirus done on black polystyrene microtitre plates. Rapid measurements are possible when a prototype manually-operated microtitre plate reader is used. Light emission from individual wells was quantified by an end-window photomultiplier tube positioned either just above the microtitre plate surface, or some distance away, the light being collected through a fibre optic light guide. The assay was also done on transparent poly(vinyl chloride) microtitre plates with simultaneous measurement of light emission from several wells; this was achieved with simple instrumentation and a 20 000-ASA Polaroid instant photographic film.

Chemiluminescent quantitation of horseradish peroxidase (HRP) conjugates by using cyclic diacylhydrazides and an oxidant at pH 7—10 is improved by the addition of firefly luciferin [1—3]. Benzothiazole derivatives such as 6-hydroxybenzothiazole and dehydroluciferin also enhance light emission from such reactions and 500—1000-fold increases in light emission from HRP conjugate in solution have been demonstrated in this laboratory. Enhanced reactions produce relatively intense, prolonged light emission, which simplifies monitoring and initiation of luminescent reactions on solid supports such as microtitre plates. This allows simple and rapid quantitation of HRP conjugate in immunoassays.

Instruments (plate readers) for monitoring luminescent immunoassays done on conventional transparent microtitre plates have been described [4, 5], but none is available commercially. These were designed for luminescent reactions with short-lived light emission and thus contained integral injectors for the initiation of individual reactions in front of single or multiple

0003-2670/85/$03.30

photodetectors. In transparent microtitre plates, light transmission from partly spent reactions in neighbouring wells can occur and this "carryover" is minimised by reading every second well at 10-s intervals [4]. Monitoring enhanced luminescent reactions in black polystyrene microtitre plates circumvents these problems and allows the use of simpler instrumentation. Light emission from enhanced reactions is also sufficiently intense to allow luminescent immunoassays done on transparent microtitre plates to be monitored by using instant photographic film [6]. As light emission is constant for several minutes, reactions can be initiated before exposure to the film.

This paper describes the application of benzothiazole derivative-enhanced luminescence to an enzyme immunoassay for specific antibody to cytomegalovirus (CMV) monitored by using microtitre plate readers or instant photographic film.

EXPERIMENTAL

Reagents

Luminol (Sigma Chemical Co.) was purified by recrystallisation from hot sodium hydroxide solution and decolourisation with charcoal. Dehydroluciferin was kindly supplied by M. DeLuca (Chemistry Department, University of California), and 6-hydroxybenzothiazole and 2-cyano-6-hydroxybenzothiazole by N. Baggett (Chemistry Department, University of Birmingham). Synthetic firefly luciferin was from Sigma Chemical Co. Cytomegalovirus complement fixation antigen (Flow Laboratories) and goat anti-human immunoglobulin (IgG) horseradish peroxidase conjugate (Miles Laboratories) were used. Serum specimens (A—D) with a range of concentrations of antibody against CMV (previously determined by a spectrophotometric enzyme immunoassay with an alkaline phosphatase conjugate [7]) were kindly supplied by the Regional Blood Transfusion Service, Birmingham. Poly(vinyl chloride) M24 (PVC) and black polystyrene MicroFLUOR "B" plates were obtained from Dynatech Laboratories.

Apparatus

Photomultiplier-based luminescence microtitre plate readers. An end-window photomultiplier tube (9924A, Thorn EMI Electron Tubes) was fitted with a small circular aperture and positioned close to the upper surface of a black microtitre plate contained in a light-tight box. The glowing plate could be manually positioned rapidly and precisely so that individual wells were aligned with the aperture. The photomultiplier tube was operated in the photocurrent mode and results were displayed on a digital voltmeter. Alternatively, a fibre optic light guide (Fibrox A181/50902, Rank Precision Industries) was manually positioned above a microtitre plate well and the image of the output end was focussed on the photomultiplier via a lens contained in a light-tight housing. As this prototype instrument had no

mechanical means for positioning the fibre optic within a light-tight chamber, measurements were made in a dark room.

Camera luminometer. The camera luminometer consisted of a light-tight box mounted on top of a Polaroid film pack [8]. A glowing transparent microtitre plate (contained in a metal mask to isolate light emission from individual wells) was positioned on top of Polaroid 612 (ASA 20 000) instant photographic film by removal of a simple sliding shutter. After exposure (90 s), the shutter was re-inserted and the film developed (35 s).

Cytomegalovirus antibody assay procedures

Photomultiplier-based microtitre plate reader measurements. Dispense 200 μl of a 1:200 dilution of CMV antigen in 0.05 M carbonate—hydrogencarbonate buffer (pH 9.6) into each well of a black polystyrene microtitre plate. Cover the microtitre plate and store at 0—4° C overnight. Empty the contents from the coated plate and wash three times with phosphate-buffered saline (pH 7.3; Oxoid) containing 0.1% Tween 20 (PBST). Dilute serum samples or reference material 1:20 in PBST and add 200 μl to individual wells from an 8-place multiple pipette. Cover the plate and place on a microtitre plate vibrator (model 804; Luckham) for 30 min at room temperature. Discard the contents of the plate and repeat the washing procedure. Pipette 200 μl of anti-human IgG/HRP conjugate (diluted 1:25 000 with PBST) into each well and leave for 30 min at room temperature. Empty the contents from each well and wash the plate three times with PBST. Immediately after the final wash procedure, initiate the luminescent reactions in each well by adding 200 μl of a luminol/hydrogen peroxide/enhancer mixture. This mixture was prepared by addition of 200 μl of 6-hydroxybenzothiazole, 2-cyano-6-hydroxybenzothiazole, dehydroluciferin or firefly luciferin (0.5 mg ml^{-1} in dimethyl sulphoxide) to 20 ml of a luminol (60 μM)/hydrogen peroxide (2 mM) solution in 0.1 M Tris buffer, pH 8.5 or 8.0. Finally, record the light intensity from individual wells at various times after initiation.

Photographic measurements. Repeat the assay as described above but use transparent PVC U-bottomed microtitre plates and extend the incubations with diluted sample or HRP conjugate to 90 min or 60 min, respectively. Initiate the luminescent reactions at pH 8.5, using the 6-hydroxybenzothiazole-enhanced system, and introduce the glowing microtitre plate, supported in the metal shield, into the camera luminometer. Expose the plate to the photographic film for 90 s, remove the film and develop for 35 s.

RESULTS AND DISCUSSION

Light emission from the chemiluminescent oxidation of luminol, catalysed by horseradish peroxidase conjugates in the immunometric assay for CMV-specific antibody, is enhanced by the addition of 6-hydroxybenzothiazole, 2-cyano-6-hydroxybenzothiazole, or dehydroluciferin (Table 1). The enhanced reactions improve discrimination between antibody concentrations,

TABLE 1

Light emission from bound HRP conjugate in an enhanced luminescent enzyme-linked immunosorbent assay done on black microtitre plates for CMV-antibody in four serum samples (A—D)[a]

Enhancer	Light intensity (mV)							
	A		B		C		D	
	1[b]	5[b]	1[b]	5[b]	1[b]	5[b]	1[b]	5[b]
None[c]	42	44	48	49	84	77	126	116
2-Cyano-6-hydroxybenzothiazole[c]	42	36	45	38	123	122	284	282
6-Hydroxybenzothiazole[c]	40	44	48	45	1230	1070	2815	2286
Dehydroluciferin[c]	910	675	1185	807	3840	2636	5415	4002
6-Hydroxybenzothiazole[d]	40	39	43	46	519	452	974	824
Dehydroluciferin[d]	368	245	400	280	1444	920	2185	1415

[a]Serum CMV antibody levels were determined by enzyme immunoassay with an alkaline phosphatase conjugate. Absorbance changes for specimens A, B, C, and D were 0.04, 0.07, 0.6 and 0.9, respectively. [b]Time (min) after initiation. [c]At pH 8.5. [d]At pH 8.0.

TABLE 2

Discrimination between sera (A—D)[a] with differing CMV antibody concentrations

Enhancer	Ratio of light emission from sera A—D to that of A[b]			
	A	B	C	D
None	1.0	1.1	2.0	3.0
Firefly luciferin	1.0	1.2	3.7	7.5
6-Hydroxybenzothiazole	1.0	1.2	30.7	70.4

[a]Absorbance changes as in Table 1. [b]The intensity from bound HRP conjugate was recorded 60 s after initiation. Individual assays at pH 8.5.

6-hydroxybenzothiazole producing greater differentiation than firefly luciferin (Table 2). Light emission is prolonged and relatively constant. Even at high conjugate concentrations, where, under the conditions used, decay of light emission is more pronounced, emission continues for at least 15 min (Fig. 1). The magnitude of the enhancement depends on the reaction conditions and the enhancer employed; dehydroluciferin and 6-hydroxybenzothiazole produced the largest increases in intensity.

Light emission cannot be transmitted through the body of opaque black microtitre plates. Such plates therefore form a convenient solid support for luminescence-monitored immunoassays, and light emission from individual wells can easily be measured by using a simple photomultiplier-based instrument. Multiple reactions can be initiated manually outside the luminometer as the prolonged light emission eliminates the need to initiate individual reactions in front of the photodetector. Light measurement and positioning

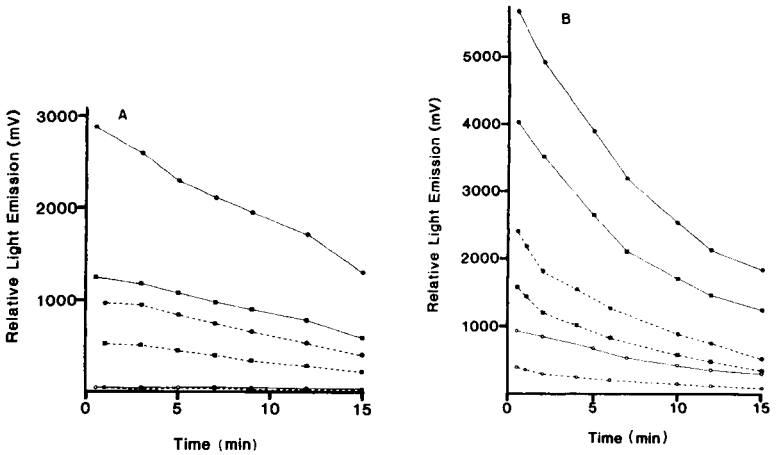


Fig. 1. Kinetics of light emission in the enhanced luminescent enzyme-linked immunosorbent assay for CMV-specific IgG: (A) 6-Hydroxybenzothiazole-enhanced; (B) dehydroluciferin-enhanced. Serum specimens (cf. Table 1): (○) A; (▪) C; (●) D. Solid lines: pH 8.5, broken lines: pH 8.0.

of individual wells under the detector could be completed in ca. 1 s, and therefore very rapid sequential measurements from a series of wells were possible. Carryover of light from neighbouring wells was not significant. For wells from which relative light emissions in the assay corresponded to 252, 274, 2384 and 3264 mV, the mean signals from the eight immediately surrounding wells were 47, 49, 49 and 50 mV, respectively. Rapid sequential measurements from a series of wells were possible, allowing a group of measurements to be made with a single detector several minutes after reactions had been initiated. Good differentiation between standards was possible even when incubation times with sample and conjugate were decreased to 15 min (Table 3). Rapid measurements, or initiation of small numbers of reactions at a time, minimised the effects of any changes in the rate of light emission.

The assay showed acceptable within-batch precision. In the dehydroluciferin-enhanced assay, the mean light emission after 60 s for standards A, B, C and D (n = 8) was 917, 992, 3422 and 4719 mV, with coefficients of variation of 12.7, 12.7, 5.7 and 5.4%, respectively. The use of a fibre optic light guide enabled light to be measured with the photomultiplier tube positioned remote from the glowing microtitre plate. Discrimination between standards was possible, although with the simple apparatus used measurements were less efficient than those achieved by positioning the photomultiplier tube directly above individual wells (Table 3).

The use of transparent microtitre plates and an exposure time of 90 s, allowed the relatively intense and prolonged enhanced light emission from bound HRP conjugate to be detected with high-speed instant photographic film. Multiple samples were assayed simultaneously using simple equipment requiring no external power source. Reactions were initiated prior to exposure

TABLE 3

Intensities measured in the 6-hydroxybenzothiazole-enhanced luminescence assay for CMV-specific IgG [15 min incubation of sample and conjugate, emission at pH 8.5 recorded by photomultiplier tube (PMT) after 60 s; sera A—D as in Table 1]

Measurement	Intensity recorded (mV)			
	A	B	C	D
PMT directly above microtitre plate	19	20	572	1404
Light transferred by fibre optic light guide to PMT	9	9	120	220

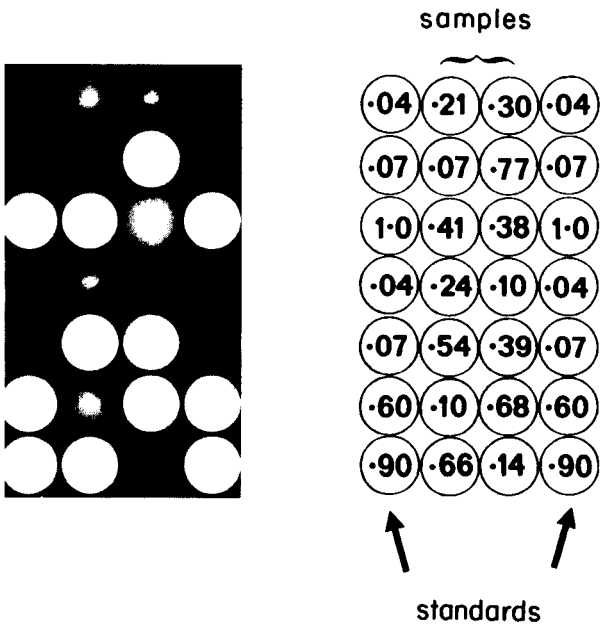


Fig. 2. Photograph on instant film of the 6-hydroxybenzothiazole-enhanced emission for assay of CMV-specific IgG. Absorbance changes for standards and specimens in the spectrophotometric alkaline phosphatase immunoassay are indicated in the corresponding circles.

to the film and a permanent record of the semi-quantitative results was obtained (Fig. 2). Full exposure of the film was observed for samples containing relatively high concentrations of CMV-specific antibody whereas only partial exposure occurred with low or undetectable levels, thus enabling immediate identification of these specimens.

Enhanced luminescent techniques based on benzothiazole derivatives allow rapid quantitation of HRP conjugates. The ability to run such assays, with simple instrumentation, in conjunction with solid supports such as

beads, tubes [3] and microtitre plates should promote the rapid and widespread application of luminescence-monitored immunoassays.

The financial support of the Department of Health and Social Security is gratefully acknowledged.

REFERENCES

1 T. J. N. Carter, C. J. Groucutt, R. A. W. Stott, G. H. G. Thorpe and T. P. Whitehead, Eur. Patent Pub., 87959, 1982.
2 T. P. Whitehead, G. H. G. Thorpe, T. J. N. Carter, C. J. Groucutt and L. J. Kricka, Nature (London), 305 (1983) 158.
3 G. H. G. Thorpe, R. Haggart, L. J. Kricka and T. P. Whitehead, Biochem. Biophys. Res. Commun., 119 (1984) 481.
4 H. R. Schroeder, C. M. Hines, D. D. Osborn, R. P. Moore, R. L. Hurtle, F. F. Wogoman, R. W. Rogers and P. O. Vogelhut, Clin. Chem., 27 (1981) 1378.
5 J. E. F. Holley, Eur. Patent Pub., 0025350, 1981.
6 G. H. G. Thorpe, T. P. Whitehead, R. Penn and L. J. Kricka, Clin. Chem., 30 (1984) 806.
7 J. C. Booth, G. Hannington, T. A. G. Aziz and H. Stern, J. Clin. Pathol., 32 (1979) 122.
8 T. P. Whitehead, G. H. G. Thorpe, L. J. Kricka, J. E. C. Gibbons and R. A. Bunce, Eur. Patent Pub., 110610, 1983.

Analytica Chimica Acta, 170 (1985) 109—116
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

SENSITIVE ASSAY FOR NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE AND ITS REDUCED FORM BASED ON THE BIOLUMINESCENCE METHOD

JOSÉ RAMACLE*, ANDRÉE HOUBION and MARTINE RAES

Unité de Biochimie Cellulaire, Facultés Universitaires Notre-Dame de la Paix, B-5000 Namur (Belgium)

(Received 28th June 1984)

SUMMARY

An assay of reduced nicotinamide adenine dinucleotide phosphate (NADPH) by bioluminescence was investigated and applied for $NADP^+$. The $NADP^+$ is first reduced by glucose-6-phosphate dehydrogenase and then assayed in a mixture containing a NADPH/flavin mononucleotide oxidoreductase which in turn activates luciferase. Many interferences were observed and the method was modified accordingly. $NADP^+$ and NADPH can be assayed separately or simultaneously within the range 1—100 pmol, which is sufficiently sensitive to be applied to biological materials. Many details and precautions must be taken into consideration.

Nicotinamide adenine dinucleotide phosphate (NADP) is a natural coenzyme present in all animal and plant organisms. The ratio of the reduced form (NADPH) to the oxidized form ($NADP^+$) is usually high [1]. Each of these forms has a particular role in enzyme regulation [2], during differentiation [3, 4] and similarity processes [5—7] as well as being target functions in the cytotoxic action of ionizing radiations [8, 9] and alkylating agents [10, 11]. The earliest methods for the assay of NADPH were based on its absorbance at 340 nm; the detection limit is about 10 nmol but many other biological materials interfere. The fluorescence determination is more sensitive and much used but some macromolecules also fluoresce and quenching is normally very high in biological preparations. Lowry and coworkers [1, 12] developed an elegant method based on $NADP^+$ in a cycling system and then assayed for the products. The method is sensitive but it is also very delicate and all the enzymes tested for the cycling process have shortcomings [12] such as sensitivity to anions, destruction of NADPH, lack of selectivity for NADPH and binding of $NADP^+$. Another laborious sensitive method involves conversion of the coenzyme to 5′-adenosine monophosphate (AMP) followed by radio-immunoassay [13].

Bioluminescence with luciferase as a photon emitter was first developed for the determination of adenosine triphosphate [14]. The system has been adapted for NADH and NADPH [15—18]. The assay for NADPH is very

0003-2670/85/$03.30

sensitive and rapid; however, as will be shown below, adaptation of the method for $NADP^+$ shows up the sensitivity of the enzyme mixture to its environment and makes it necessary to examine carefully the parameters influencing light emission by the luciferase enzyme. In a recent paper, Wienhausen and DeLuca [19] adapted the bioluminescence method with insolubilized enzymes but did not discuss the interferences that are described in this paper.

EXPERIMENTAL

Standard NADPH (sodium salt), flavin mononucleotide (FMN), luciferase and NADPH:FMN oxidoreductase were obtained from Lumac (Schaesberg, The Netherlands); $NADP^+$ (sodium salt; 98—100% pure) and glucose-6-phosphate (98—100% pure) were from Sigma Chemical Co. Glucose-6-phosphate dehydrogenase (grade 1 from yeast) was from Boehringer (Mannheim). Other chemicals were from Merck.

The assay of NADPH was usually done as recommended by Lumac: to 0.1 ml of the reconstituted mixture containing FMN, aldehyde, luciferase and the NADPH:FMN oxidoreductase in 0.1 mol l^{-1} phosphate buffer (pH 6.9) was added 0.1 ml of the NADPH solution in 0.01 mol l^{-1} sodium hydroxide containing 0.5 mmol l^{-1} cysteine. The emissions during periods of 1 s were recorded until maximum emission was obtained, usually after 0.2—1 min. The use of fluorescent lighting had to be avoided. After 8 min, the emission was usually back near the control value and 20 μl of standard NADPH solution (40 pmol) was added to obtain internal standardization. Given the stimulation of the luciferase during the 8 min of the assay, each series of experiments was started and ended by a standard assay with internal standardization in order to correct for this stimulation. An M2010 biocounter (Lumac Systems, Basel, Switzerland) was used.

The NADPH standards were reconstituted in 0.01 mol l^{-1} sodium hydroxide containing 0.5 mmol l^{-1} cysteine and kept at —70° C. The $NADP^+$ standards were freshly prepared in distilled water.

When NADPH was assayed selectively, the alkaline cysteine-containing solution was heated at 60° C for 10 min to destroy any residual $NADP^+$. For selective assay of $NADP^+$, NADPH was first destroyed by lowering the pH to 1.4 by the addition of hydrochloric acid to give a final concentration of 0.04 mol l^{-1}. When the alkaline cysteine-containing solution was used, enough hydrochloric acid was added to neutralize the solution and then reach the required pH. After 5 min, the pH was increased by the addition of Tris-HCl buffer (pH 7.4) to give final concentrations of 0.03 mol l^{-1} and 0.04 mol l^{-1} sodium hydroxide. Then glucose-6-phosphate to give a final concentration of 1 mmol l^{-1} and glucose-6-P dehydrogenase (0.1 mg ml^{-1}) were added. After 5 min at 20° C, the pH was increased to 12 by the addition of 0.067 mol l^{-1} sodium hydroxide and the solution was heated at 70° C for 10 min. The pH was decreased to 7.4 by the addition of 0.067 mol l^{-1} hydrochloric acid before the bioluminescence assay was conducted.

For both $NADP^+$ and NADPH assays, the solution was adjusted to pH 7.4 with 0.03 mol l^{-1} Tris-HCl buffer before glucose-6-phosphate and glucose-6-phosphate dehydrogenase were added; the experiment was then conducted as for the $NADP^+$ assay.

RESULTS

The stabilities of $NADP^+$ and NADPH are affected by many factors such as pH and neutral salts [20]. The stability of both forms at very low concentrations was tested at alkaline, neutral and acid pH under the conditions used here (Fig. 1). NADPH was stable at alkaline and neutral pH but immediately destroyed in acid. $NADP^+$ was stable in acid but was affected gradually at alkaline pH. $NADP^+$ was completely destroyed by heating the solution at 60°C for 10 min.

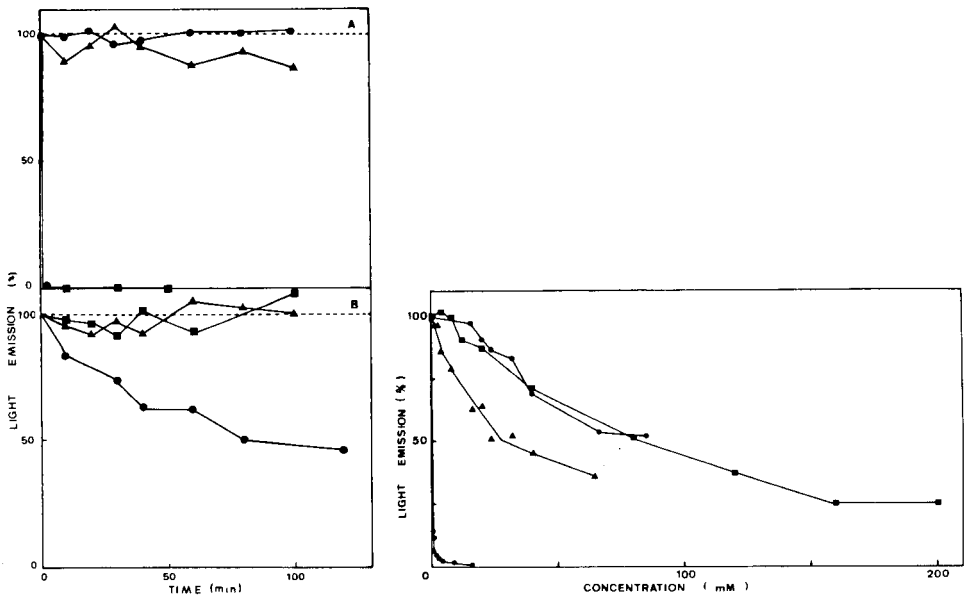


Fig. 1. Variation of stability of NADPH (A) and $NADP^+$ (B) with pH. The nucleotides (0.2 μmol l^{-1}) were incubated at 20°C in 0.5 mmol l^{-1} cysteine also containing: (•) 0.01 mol l^{-1} NaOH; (▪) 0.04 mol l^{-1} HCl; or (▲) 0.03 mol l^{-1} Tris-HCl buffer pH 7.4. After different times, the solutions were neutralized and the samples assayed as described under Experimental. The results are expressed as percentage compared to the zero-time incubation.

Fig. 2. Effect of anions on the NADPH assays. NADPH (0.2 μmol l^{-1}) was assayed (see Experimental) in the presence of various concentrations of sodium salts: (•) Tris-HCl; (▪) NaCl; (▲) Na_2SO_4; (★) Na ascorbate. Results are expressed as % of the maximum light emission obtained in the absence of these anions.

Effects on the luciferase system

During this work, the susceptibility of the photon emission system to anions was noted. A systematic study of this effect is presented in Fig. 2. It can be seen that sulfate and especially ascorbate are very detrimental to the enzymes; however, all the anions affect the enzyme activity to some extent. Cysteine is very peculiar; it scarcely influences the NADPH assay at first but it does affect the internal standardization (Fig. 3). The inactivation of the enzyme is delayed at high cysteine concentrations (Fig. 4).

The NADPH assay is based on the emission of photons by luciferase. The maximum of this emission depends not only on the NADPH concentration but also on the activity of the luciferase. In the assay, the emission increases rapidly to give a peak of emission after a few seconds and then decreases because of the depletion of NADPH (Fig. 5). The presence of glucose-6-phosphate and glucose-6-phosphate dehydrogenase in the mixture also greatly affects the evolution of the system because NADPH is continuously regenerated (Fig. 5). Even the small residual activity present after heating for 10 min at 65°C was enough to modify the assay. Only heating at 70°C for 10 min completely abolished the effect. In Fig.5, a 10-fold excess of glucose-6-

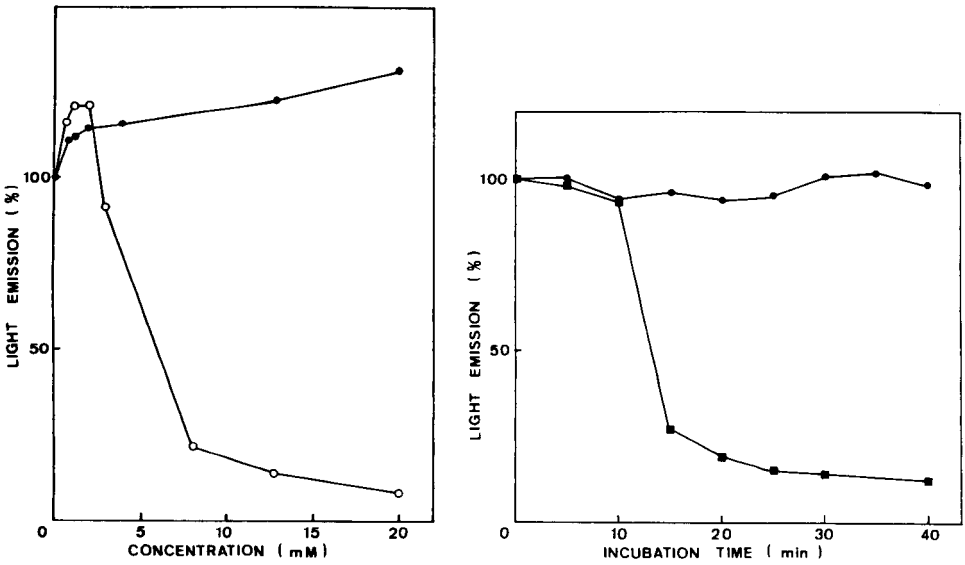


Fig. 3. Effect of cysteine on the NADPH assays: (•) NADPH (0.2 μmol l^{-1}) was assayed (see Experimental) in the presence of various concentrations of cysteine; (○) counting of the internal standards 15 min after the first assay. Results are expressed as % of the maximum emission obtained with 0.5 mmol l^{-1} cysteine.

Fig. 4. Evolution of the activity of the luciferase system incubated in the presence of cysteine: (•) 0.1 mmol l^{-1}; (▪) 20 mmol l^{-1} cysteine. The incubated mixture was then used for NADPH determinations. The results are expressed as % of the maximum light emission obtained at zero-time incubation.

phosphate and glucose-6-phosphate dehydrogenase was used and the quenching of the $NADP^+$ assay was about 50%. When corrections were made by internal standardization, the recovery for NADPH was 100%, but 183% when the solution was not heated, 140% when the solution was heated at 65°C and 104% when it was heated at 70°C. Complete deactivation of glucose-6-phosphate dehydrogenase is thus essential for obtaining correct results.

As mentioned above, the emission maximum depends on the luciferase activity. The same assay mixture increases its activity throughout the few hours of a normal assay. In order to decrease this variation, the enzyme mixture was prepared and kept for 16 h at 4°C before use. Corrections for variations in the enzyme activity were obtained by internal standardization. However, NADPH assays usually gave excessive recoveries. Variation of the luciferase activity upon stimulation with NADPH was suspected. Figure 6 shows that after the first stimulation, luciferase gives a higher emission (ca. 105%) but on successive stimulations the activity falls gradually. This type of activation was usually observed in practice; however, when old and inefficient mixtures were used, the stimulation could reach much more than 105%.

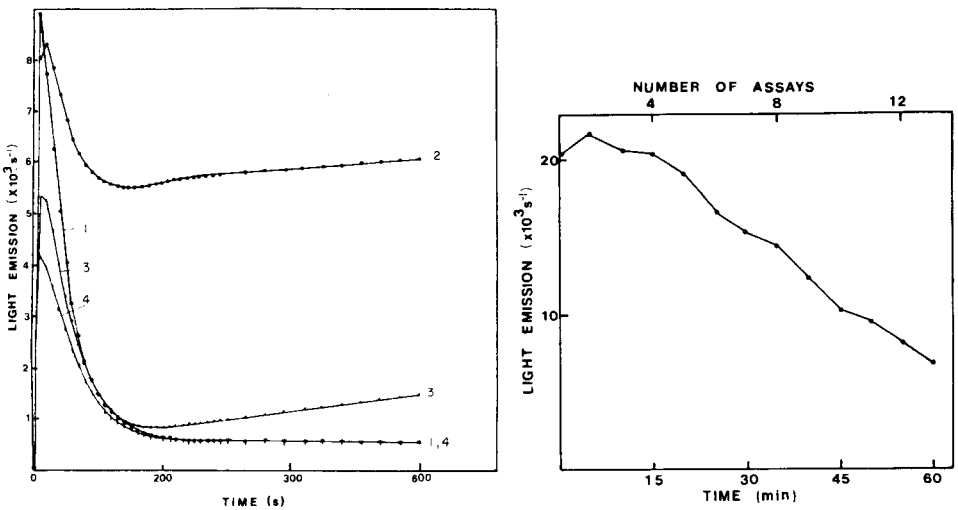


Fig. 5. Effect of NADPH regeneration on the activity of the luciferase system. Curve 1: NADPH (0.2 μmol l^{-1}) was assayed directly as described under Experimental, the emission being followed with time. Curves 2—4: $NADP^+$ (0.24 μmol l^{-1}) was incubated with glucose-6-phosphate and glucose-6-phosphate dehydrogenase as described and then assayed in the luciferase system: (2) without heating; (3) after heating for 10 min at 65°C; (4) after heating for 10 min at 70°C.

Fig. 6. Variation in the activity of luciferase on repeated stimulations. The NADPH was assayed directly as described above but every 5 min, 10 μl of NADPH solution (20 pmol) was added to the same enzyme mixture and the maximum emission recorded. The values are corrected for the dilution effect.

Assay for NADP⁺ and NADPH

Calibration graphs for $NADP^+$ and NADPH alone or simultaneously are presented in Fig. 7. The maximum emission is proportional to the amount of NADPH in the 1—100 pmol range and $NADP^+$ in the 1—50 pmol range. The slopes of the plots differ because of the difference in quenching and the variation in the luciferase activity from one experiment to the other. The variations in the luciferase activity throughout the few hours of one assay were minimal here. When corrected by internal standardization, recoveries of NADPH and $NADP^+$ ranged between 90 and 110%. Recoveries for $NADP^+$ and NADPH when simultaneously assayed, were 80—90%.

To test the simultaneous assay further, a solution of NADPH (250 pmol ml^{-1}) was prepared in 0.01 mol l^{-1} sodium hydroxide containing 0.5 mmol l^{-1} cysteine and a solution of $NADP^+$ (250 pmol ml^{-1}) in water. Equal volumes of the two solutions were mixed and immediately divided into three parts which were used, as described under Experimental, to assay NADPH after inactivation of $NADP^+$, then $NADP^+$ after inactivation of NADPH in acidic medium, and then for the sum of $NADP^+$ and NADPH, each assay being done in duplicate. The results found were 121.9 ± 14.5 pmol ml^{-1} for NADPH, 135.4 ± 9.0 pmol ml^{-1} for $NADP^+$, and 263.7 ± 26.0 pmol ml^{-1} for both coenzymes.

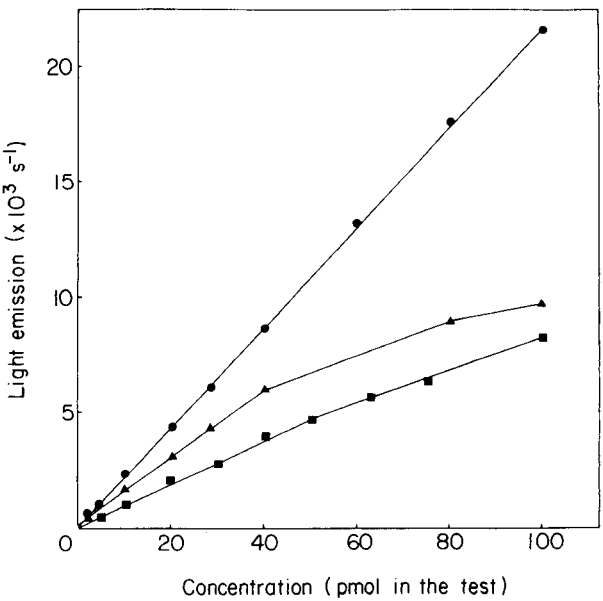


Fig. 7. Calibration graphs: (■) $NADP^+$; (●) NADPH; (▲) $NADP^+$ plus NADPH. Standard solutions of $NADP^+$, NADPH and a mixture of both were freshly prepared in 0.01 mol l^{-1} NaOH containing 0.5 mmol l^{-1} cysteine and diluted appropriately. Maximum emission during 1 s was recorded.

DISCUSSION

The assay of $NADP^+$ and NADPH whether simultaneously or separately is not easy, and much attention has been given to the problem [1, 12, 13, 15—17]. The bioluminescence method was applied here for $NADP^+$ assays. The procedures are based on the principle that $NADP^+$ is stable in acidic medium in which NADPH is not, and NADPH is stable at alkaline pH at which $NADP^+$ is not. This principle was applied by Lowry and coworkers [1, 20] to assay specifically for both coenzymes. Their methodology was modified here: sulfate was replaced by chloride and ascorbic acid was omitted because of its strong inhibitory effect on luciferase (Fig. 2). Cysteine, which also deactivates the enzyme but at higher concentrations (Figs. 3 and 4) was retained because of its protective effect against the oxidation of NADPH by heme-containing molecules [20]. The effects of temperature and pH on the luciferase activity had already been investigated [15].

The method proposed gives the individual amounts of NADPH and $NADP^+$ and the sum of both, with limited quenching and good sensitivity. The method is easy and relatively fast, especially if automatic photometers are used. In this work, variations of 10—20% in the results were often observed. The application of the method to biological materials is immediate because correction of quenching is made by internal standardization. The removal of ascorbic acid is, however, a great limitation because this reducing agent was introduced into the test in order to prevent the oxidation of reduced nucleotides by hemoglobin [1]. Another shortcoming of the method is the variation of the luciferase activity from day to day and also throughout one day of work.

Martine Raes was supported by the Fonds National de la Recherche Scientifique, Belgium.

REFERENCES

1 H. B. Burch, M. E. Bradley and O. H. Lowry, J. Biol. Chem., 242 (1967) 4546.
2 A. Omachi, C. B. Scott and H. Hegarty, Biochim. Biophys. Acta, 184 (1969) 139.
3 A. I. Caplan, M. J. Rosenberg, Proc. Natl. Acad. Sci. U.S.A., 72 (1975) 1852.
4 C. G. Blomquist, K. E. Larson and L. Taddeini, Exp. Cell Res., 199 (1976) 447.
5 R. K. Morton, Nature (London), 181 (1958) 540.
6 M. H. Briggs, Nature (London), 187 (1960) 249.
7 J. B. Clark, A. L. Greenbaum and P. McLean, Biochem. J., 98 (1966) 546.
8 H. Maas, G. H. Rathgen, H. A. Huenkel and O. Schubert, Z. Naturforsch., 13b (1958) 735.
9 H. Hilz, M. V. Gossler, M. Oldekop and M. Scholz, Biochem. Biophys. Res. Commun., 6 (1962) 379.
10 I. M. Roitt, Biochem. J., 63 (1956) 300.
11 J. Rüter, H. Vachek, M. Oldekop, I. Wüppen and H. Hilz, Biochem. Z., 344 (1966) 153.
12 M. M.-Y. Chi, C. V. Lowry and O. H. Lowry, Anal. Biochem., 89 (1978) 119.
13 R. Bredehorst, H. Lengyel and H. Hilz, Eur. J. Biochem., 99 (1979) 401.

14 P. E. Stanley, S. G. Williams, Anal. Biochem., 19 (1969) 381.
15 P. E. Stanley, Methods Enzymol., 57 (1978) 215.
16 J. Lavi, R. Raunio, T. Stahlberg and T. Lovgren, J. Clin. Chem. Clin. Biochem., 19 (1981) 749.
17 S. E. Brolin, C. Berne and U. Isacsson, Anal. Biochem., 50 (1972) 50.
18 L. A. Idahl, Uppsala J. Med. Sci., 86 (1981) 131.
19 G. Wienhausen and M. Deluca, Anal. Biochem., 127 (1982) 380.
20 O. H. Lowry, J. V. Passonneau and M. K. Rock, J. Biol. Chem., 236 (1961) 2756.

Analytica Chimica Acta, 170 (1985) 117—123
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

SERUM ESTRADIOL MEASUREMENT BY SOLID-PHASE CHEMILUMINESCENCE IMMUNOASSAY AND DIRECT RADIOIMMUNOASSAY

JOZEF DE BOEVER*, FORTUNA KOHEN[a], MARC DHONT and DIRK VANDEKERCKHOVE

Department of Obstetrics and Gynaecology, Academic Hospital, De Pintelaan 185, B-9000 Gent (Belgium)

GEORGES VAN MAELE

Department of Medical Information, Academic Hospital, De Pintelaan 185, B-9000 Gent (Belgium)

(Received September 5th 1984)

SUMMARY

Estradiol-17β is determined in serum extracts by solid-phase chemiluminescence immunoassay. The results are compared with those obtained from unextracted serum in routine conditions with a commercial radioimmunoassay (r.i.a.) kit. For the chemiluminescence procedure, a purified monoclonal antibody to estradiol-6-carboxymethyloxime/bovine serum albumin and the homologous chemiluminescent marker conjugate estradiol-6-carboxymethyloxime aminobutylethylisoluminol are used. Bound and free ligand are separated by washing and simple centrifugation. Results obtained by the chemiluminescence assay (y) and by r.i.a. (x) on 170 serum specimens from women during ovulation induction showed good correlation ($y = 1.01x - 16$ with $r = 0.95$). The methods are similar in selectivity, detection limit (ca. 10 ng l^{-1}) and precision (interassay relative standard deviation, 8—13%).

Successful monitoring of ovulation induction requires the sequential analysis of one or several hormones in peripheral blood or in urine [1—3]. Daily measurement of estrogens (e.g., estradiol) in serum or plasma has become the method of choice in many laboratories [4—6]. This demands rapid and reliable assays, which have indeed been developed; for some years, commercial reagent kits for the direct measurement of estradiol by radioimmunoassay (r.i.a.) have been available.

Radioimmunoassay, however, is associated with health hazards, the short half-life and radiolysis of labelled reagents and radioactive waste disposal. Recent studies from several laboratories have indicated that chemiluminescent markers can be a feasible alternative to radiolabelling. Such markers have been used in developing reliable solid-phase chemiluminescence immuno-

---

[a]Permanent address: Department of Hormone Research, The Weizmann Institute of Science, Rehovot 76100, Israel.

0003-2670/85/$03.30

assays for steroids in serum or plasma [7—9]. This paper compares the results of estradiol measurement by a solid-phase chemiluminescence immunoassay after extraction from serum, with those obtained routinely with direct r.i.a. without extraction.

EXPERIMENTAL

Sample collection and extraction

Blood samples were obtained by venipuncture from patients during treatment for ovulation inducation with menopausal gonadotropins and choriogonadotropin. Sera were stored frozen at −20° C until assayed. A 1-ml portion of serum was extracted with 7.5 ml of diethyl ether. The mean efficiency of extraction, as found by measuring the recovery of tritiated estradiol added to the serum samples, was 96.0% (s.d. = 2.6%). The ether phase was evaporated under nitrogen and the dry residue was dissolved in 0.5 ml of assay buffer. Duplicate 0.1-ml aliquots were analysed by chemiluminescence immunoassay.

Procedures

Chemiluminescence immunoassay. Serum levels of estradiol were assayed by the solid-phase chemiluminescence assay technique described by De Boever et al. [9]. The assay uses a monoclonal antibody to estradiol-6-carboxymethyloxime/bovine serum albumin and the chemiluminescent marker conjugate, estradiol-6-carboxymethyloxime aminobutylethyl-isoluminol (E_2-ABEI). Portions (0.1 ml) of solid-phase antibody, reconstituted serum extract or standard and steroid-marker conjugate (i.e., 100 pg of E_2-ABEI) are added to lumacuvettes (12 × 50 mm polystyrene cuvettes; Lumac Systems, Basel, Switzerland). After incubation for 1 h or overnight at 4°C, bound and free ligand are separated by adding 0.9 ml of wash solution, centrifugation (10 min, 2000*g*, room temperature), and decantation of the supernate. To the precipitate, 0.2 ml of 2 mol l^{-1} sodium hydroxide is added. The mixture is incubated for 30 min at 60° C and then cooled to room temperature. The amount of bound ligand marker is measured by adding 0.1 ml of diluted (10 μg/ml) microperoxidase and 0.1 ml of $x\%$ = 0.2% hydrogen peroxide solution and integrating the light emitted for 10 s with a luminometer (Biocounter M-2000; Lumac).

Direct radioimmunoassay. The commercial EIR ^{125}I-estradiol direct r.i.a. kit (code ER-155, EIR, Radio-isotopen Service, 5303 Würenlingen, Switzerland) was used. The assay has been described in detail [10]. Briefly, duplicate 50-μl amounts of standards or unknown serum samples are added to glass tubes and incubated with 100 μl of ^{125}I-labelled estradiol and 100 μl of primary antiserum for 2 h at 37° C. Incubation with 0.5 ml of solid-phase second antibody for 30 min at 37° C is followed by centrifugation (2000*g*, 10 min, 4° C), and decantation to separate the bound and free ligand. The radioactivity in the pellet was measured with a model 1270 Rackgamma-II counter (LKB Wallac, Bromma, Sweden).

Data processing. Non-specific blank values were subtracted from the counts obtained in the bound fraction. Calibration graphs were constructed by plotting B/B_0 values vs. log dose of the standards (B = net signal at a particular estradiol concentration, B_0 = net signal in the absence of estradiol). The extraction efficiency data were used to calculate estradiol concentrations in serum as determined by the chemiluminescence immunoassay. Best-fit linear regression lines were calculated as described by Davies [11]. The method described by Jeffcoate [12] was used to construct precision profiles; data from more than 100 duplicate measurements in consecutive assays were used to calculate the linear least-squares fit of the mean counts from the bound material (response) and the standard deviation (s.d.) of the duplicates. Response replicates whose s.d. was 7% or more were rejected. The mean dose/response curve and the response/s.d. relationship (i.e., the error-envelope) was constructed. With this curve, the error in dose over the entire dose range was calculated and precision profiles were constructed by plotting the error in dose (corrected for the use of duplicates) vs. the dose.

RESULTS

Sensitivity and selectivity

The least amount of estradiol that could be distinguished from zero (mean − 2s.d.) was calculated from five chemiluminescence immunoassay and sixteen r.i.a. consecutive calibration curves prepared in duplicate (Fig. 1). The values were 12.5 ng l^{-1} for chemiluminescence assay and 7.4 ng l^{-1} for direct r.i.a. Similar values were obtained from the precision profiles.

For both methods, cross-reactivity data are given for 50% inhibition of binding of labelled ligand. For r.i.a., the cross-reactivities as indicated by the kit manufacturer were (estradiol = 100%): estrone and estriol, 2%; ethinylestradiol, progesterone, testosterone, and rostanediol, <0.1%; estradiol-3-glucuronide, 0.04%; estradiol-17-glucuronide, 0.07%. The cross-reactivities found in the solid-phase chemiluminescence immunoassay were (estradiol = 100%): estrone, 0.7%; estriol, 45%; ethinylestradiol, 0.5%; progesterone, testosterone, 5α-androstane-3α,17β diol, estrone-3-sulphate and estradiol-17β-glucuronide, <0.01%.

Precision and accuracy

Figure 2 shows within-batch precision profiles for both assays. At relative standard deviations of ⩽10%, the working range is 12—625 ng l^{-1} for the chemiluminescence procedure and 15—1000 ng l^{-1} for the r.i.a. Within-batch precisions for the chemiluminescence assay estimated from 9 replicate samples analysed in duplicate were 7.2%, 9.4% and 8.4% at 150, 700 and 1800 ng l^{-1}, respectively, within a single assay. The within-batch precision of the r.i.a. calculated from the variation of duplicate means in 30 consecutive assays was 8.9% (51 pairs) and 11.9% (76 pairs) over the ranges 23—145 and 151—850 ng l^{-1}. Between-batch precision, calculated from replicate determinations in consecutive assays were, for the chemiluminescence immuno-

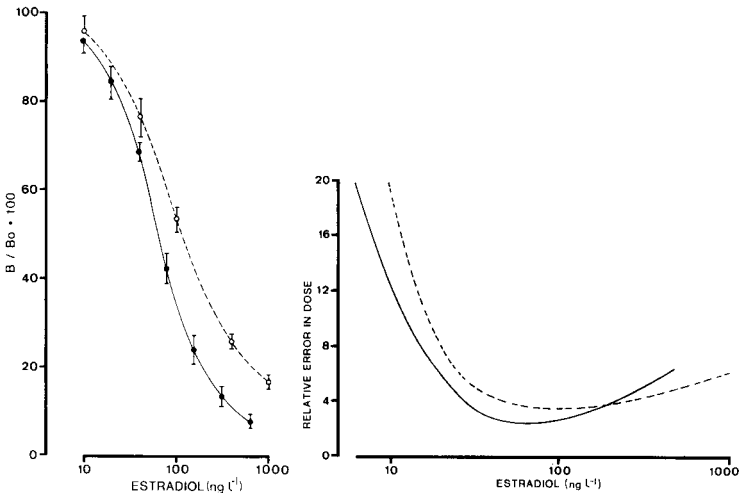


Fig. 1. Dose response curves for estradiol: (•) chemiluminescence immunoassay; (○) direct r.i.a. Error bars indicate ±s.d. for 5 and 16 consecutive determinations, respectively.

Fig. 2. Precision profiles: (——) solid-phase chemiluminescence with extraction; (— — —) direct r.i.a. Relative error is expressed as relative standard deviation (%).

assay, 13.4% and 8.2% at 56 and 150 ng l^{-1}, respectively, (9 replicates) and, for r.i.a., 11.5%, 11.4% and 8.6% at 25, 121 and 540 ng l^{-1}, respectively (⩾9 replicates).

To establish the accuracy of the chemiluminescence method, the recovery of estradiol (25—500 ng l^{-1}), added to 1 ml of serum from men, was measured.

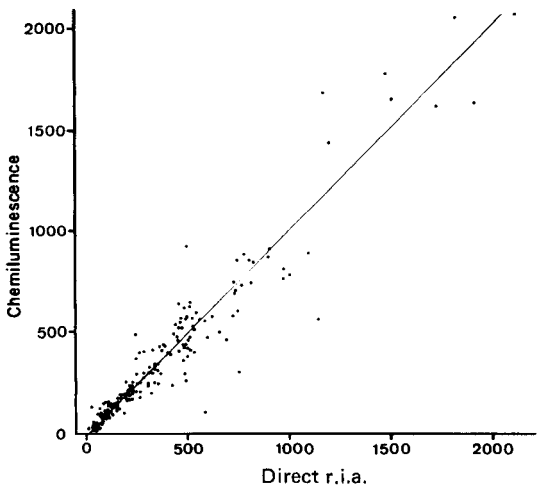


Fig. 3. Comparison of the estradiol concentrations (ng l^{-1}) in serum measured by the chemiluminescence immunoassay (y) and direct r.i.a. (x). The regression equation is $y = 1.01x - 16$, for $n = 170$ with $r = 0.95$.

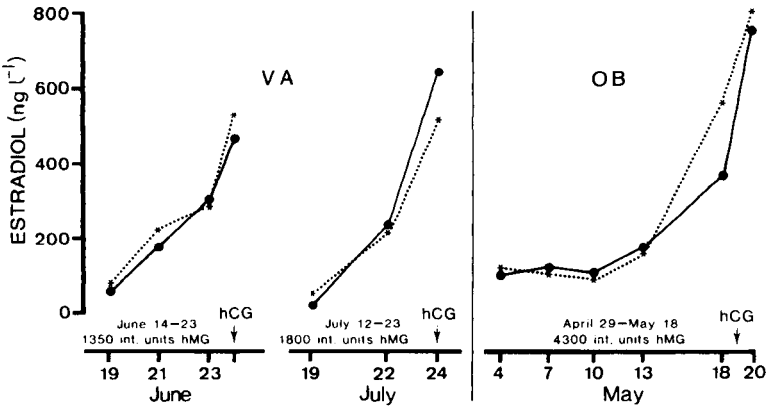


Fig. 4. Estradiol concentrations in serum from women (VA, OB) undergoing ovulation induction therapy: (——) chemiluminescence immunoassay; (· · ·) direct r.i.a. The patients received various doses of human menopausal gonadotropins (hMG) and a single dose (5000 int. units) of human choriogonadotropin (hCG) at the times indicated.

The mean recovery was 97.9%. For direct r.i.a., recoveries stated by the kit manufacturer and by Mertens et al. [10] average 109% and 101.9%, respectively.

Sera containing high concentrations of estradiol were assayed at various dilutions (1+1 to 1+7) prepared in assay buffer (chemiluminescence procedure) or in steroid-free serum (r.i.a.). The correlations between the expected (x) and found (y) estradiol concentrations (ng l^{-1}) were, for chemiluminescence immunoassay (n = 15 sera), $y = 1.03x + 24$ (r = 0.99), and for r.i.a. (n = 21 sera), $y = 0.91x + 14$ (r = 0.94).

Serum estradiol values obtained by the solid-phase chemiluminescence immunoassay correlated well with those from direct r.i.a. (Fig. 3). When both methods were used, increasing estradiol concentrations in the serum of women were observed during ovulation induction therapy with human menopausal gonadotropins and human choriogonadotropin (Fig. 4).

DISCUSSION

Both the solid-phase chemiluminescence immunoassay and the direct r.i.a. provide satisfactory accuracy and precision. Significant bias between results is not observed, as evidenced by the correlation between the estradiol concentrations determined by both methods. Both procedures have similar sensitivities and detection limits, and their lower working ranges with relative standard deviations $\leqslant$10% show no differences (15 ng l^{-1} (r.i.a.), 12 ng l^{-1} (chemiluminescence)). Both calibration curves can thus be used over the complete range. At 10 ng l^{-1}, the concentration of the lowest standard used,

the relative errors in dose derived from the precision profiles were 16% (r.i.a.) and 12.5% (chemiluminescence).

The direct r.i.a. has the advantage of a shorter total assay time, requiring about 6 h vs. 8 h for the chemiluminescence assay (with extraction of sera) for 15—20 patients. The difference is partly due to the extraction procedure and evaporation of the organic phase prior to the chemiluminescence assay, and partly to the washing step. The time required for the latter up to the heating with sodium hydroxide is considerable, being 2 h for samples from 20 patients. However, the measurement of luminescence is very fast (15—20 s per sample). Readings of duplicate standards and unknowns are thus completed within 20 min.

Chemiluminescence immunoassays that do not require separation of bound and free hormone (homogeneous assays) have been developed for progesterone and estriol in plasma [13]. These assays, however, are affected by interference from luminescent compounds present in plasma extracts. Another approach is to shorten the time required for the enhancement of chemiluminescence. The use of luminol or isoluminol as a chemiluminescent label requires the addition of sodium hydroxide to all samples and heating for 30 min. The use of acridinium ester derivatives [14, 15] that do not require a catalyst would obviate the need of heating with sodium hydroxide. It is uncertain, however, if these esters could be used as chemiluminescent labels for steroid hormones. A third possibility would be to work out a direct chemiluminescence immunoassay for estradiol in serum or plasma. Recently, such direct methods for steroid hormones in serum have been described for cortisol (detection limit 5.4 $\mu g\ l^{-1}$) [16], estriol (0.2 $\mu g\ l^{-1}$) [17], and progesterone (0.17 $\mu g\ l^{-1}$) [18].

For the determination of estradiol in unextracted serum, the sensitivity of the present method (ca. 11 $ng\ l^{-1}$) is adequate but the amount of serum required per assay tube would by far exceed the 10—20 μl that have been used for direct r.i.a. for estriol, progesterone or cortisol [16—18].

Despite the longer working time required, the solid-phase chemiluminescence assay with extraction of sera can be done within acceptable time limits, i.e., one working day. The assay is simple and stable and the results show that it can be used for clinical application.

We thank Dr. P. Stanley and Mr. J. Vossen of Lumac 3M, Schaesberg, the Netherlands for the loan of a Biocounter M-2000 luminometer. This work was supported by grants to F. K. from the Bosch Foundation and the Binational Science Foundation (Jerusalem).

REFERENCES

1 C. H. Wu, Obstet. Gynecol., 49 (1977) 308.
2 WHO, Am. J. Obstet. Gynecol., 138 (1980) 383.
3 C. M. Branch, P. O. Collins and W. P. Collins, J. Steroid Biochem., 16 (1982) 345.
4 M. M. Shaaban and A. Klopper, J. Obstet. Gynaecol. Br. Commonw., 80 (1973) 783.

5 D. R. Tredway, U. Goebelsmann, I. H. Thorneycroft and D. R. Mishell, Jr., Am. J. Obstet. Gynecol., 120 (1974) 1035.
6 J. S. G. Biggs, J. Hennessey and I. Jones, Obstet. Gynecol., 51 (1978) 10.
7 F. Kohen, M. Pazzagli, M. Serio, J. De Boever and D. Vandekerckhove, in W. P. Collins (Ed.), Alternative Immunoassays, Wiley, London, 1985, p. 103.
8 F. Kohen, J. B. Kim, H. R. Lindner and W. P. Collins, Steroids, 38 (1981) 73.
9 J. De Boever, F. Kohen and D. Vandekerckhove, Clin. Chem., 29 (1983) 2068.
10 R. Mertens, R. J. Liedtke and J. D. Batjer, Clin. Chem., 29 (1983) 1961.
11 R. G. Davies, Computer Programming in Quantitative Biology, Academic Press, London, 1981, p. 208.
12 S. L. Jeffcoate, Efficiency and Effectiveness in the Endocrine Laboratory, Academic Press, London, 1981, p. 72.
13 F. Kohen, J. B. Kim and H. R. Lindner, in M. A. Deluca and W. D. McElroy (Eds.), Bioluminescence and Chemiluminescence, Academic Press, New York, 1981, p. 357.
14 F. McCapra, Acc. Chem. Res., 9 (1976) 201.
15 I. Weeks, I. Beheshti, F. McCapra, A. K. Campbell and J. S. Woodhead, Clin. Chem., 29 (1983) 1474.
16 L. Lindström, L. Meurling and T. Lövgren, J. Steroid Biochem., 16 (1982) 577.
17 W. Klingler, O. Haupt, G. v. Postel and R. Knuppen, Steroids, 42 (1983) 123.
18 J. De Boever, F. Kohen, D. Vandekerckhove and G. Van Maele, Clin. Chem., 30 (1984) 1637.

Analytica Chimica Acta, 170 (1985) 125—131
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

APPLICATION OF CHEMILUMINESCENCE IMMUNOASSAYS FOR STEROID HORMONES IN CLINICAL ENDOCRINOLOGICAL INVESTIGATIONS IN WOMEN

JOZEF DE BOEVER*, FORTUNA KOHEN[a], RUDY SERREYN and DIRK VANDEKERCKHOVE

Department of Obstetrics and Gynaecology, Academic Hospital, De Pintelaan 185, B-9000 Gent (Belgium)

GEORGES VAN MAELE

Department of Medical Information, Academic Hospital, De Pintelaan 185, B-9000 Gent (Belgium)

(Received 5th September 1984)

SUMMARY

Immunoassays based on chemiluminescence for the measurement of serum and plasma steroids (estradiol, estriol, progesterone, testosterone, and cortisol), urinary steroid conjugates (estrone-3-glucuronide, estriol-16α-glucuronide and pregnanediol-3α-glucuronide) and peptide hormones (choriogonadotropin and luteinizing hormone) are surveyed briefly. These immunoassays are simple, robust and valid alternatives to radioimmunoassay. Homogeneous procedures and recent solid-phase assays based on purified specific antibodies, covalently coupled to polymer beads are discussed. Some new results are presented for solid-phase chemiluminescence immunoassays: estradiol is quantified in extracts of serum by using a monoclonal antibody to estradiol with estradiol-6-carboxymethyloxime-aminobutylethyl isoluminol as the marker ligand, and progesterone is quantified in unextracted serum by using a polyclonal antibody to progesterone, progesterone-11-hemi-succinyl-aminobutylethyl isoluminol as the marker ligand, and danazol (17α-pregna-2,4-dien-20-yno[2,3-d]-isoxazol-17-ol) to displace progesterone from serum binding-proteins. Their clinical utility is demonstrated.

In the last two decades radioimmunoassay (r.i.a.) techniques have offered a means for simple and reliable determinations of many hormones and their metabolites. Such techniques, therefore, have become invaluable tools for endocrinological investigations. The use of radioactive labels, however, is associated with health hazards and stability problems. Therefore, for many years, alternatives to r.i.a. based on the use of enzymes, fluorescent, and bio- and chemi-luminescent compounds have been studied.

In the field of steroid hormones, the chemiluminescent label isoluminol [1] has been used successfully for the development of sensitive and reliable

[a]Permanent address: Department of Hormone Research, The Weizmann Institute of Science, Rehovot 76100, Israel.

0003-2670/85/$03.30

immunoassay systems [2] for the measurement of serum and plasma levels of cortisol [3—5], progesterone [6—9], estradiol [10, 11] and estriol [12] in the peripheral circulation and of the glucuronides of estrone [13, 14], estriol [15] and pregnanediol [13] in urine. Some of these chemiluminescence immunoassays have been used to assess ovarian function [9, 13, 14, 16] and fetal well-being [12] and to monitor the treatment of functional infertility through ovulation induction with gonadotropins [9, 11].

This article reviews briefly the chemiluminescence immunoassays that have been developed for steroid hormones in the peripheral circulation and for their conjugates in urine. New data are presented for two solid-phase chemiluminescence immunoassays, one for estradiol after extraction from plasma [11] and a direct assay for progesterone (i.e. without extraction) in serum [9].

Experimental methods

The chemiluminescent label used in all assays has been isoluminol, substituted in its aromatic amino group by alkyl chains, $(CH_2)_n$ (n = 2—6). This substitution augments the light output when trace metals are determined [17]. Carboxy derivatives of steroids or their glucuronides can be covalently attached to these alkyl chains, yielding chemiluminescent marker conjugates.

Monoclonal antibodies have been generated by using the hybridoma technique of Köhler and Milstein [18]. Polyclonal antibodies have been raised in rabbits. Both types of antibodies have been used in the different chemiluminescence immunoassays without significant differences arising in the quality of the assays [11, 19].

Several types of chemiluminescence immunoassay have been studied; in some, the bound and free ligand fractions must be separated (heterogeneous or separation assays), whereas in others, there is no separation (homogeneous). Heterogeneous assays can further be divided into liquid-phase methods in which separation of the bound and free fractions is obtained by, e.g., dextran-coated charcoal, and solid-phase methods in which the antibodies are either coated on the tube wall or covalently coupled to polymer beads.

Finally, most of the chemiluminescence methods have been applied to extracts of serum, plasma or urine. Only three direct assays (i.e., for unextracted serum or plasma) have been described [5, 9, 12].

RESULTS AND DISCUSSION

Tables 1 and 2 summarize the chemiluminescence immunoassays that have been developed in different laboratories for the measurement of steroid hormones in serum and plasma (Table 1) and of steroid conjugates in urine (Table 2). The sensitivity of determination of steroids by chemiluminescence immunoassay has improved since the first assays, e.g., for progesterone, from 25 to 2 pg/tube. The homogeneous assay for progesterone [6] is based on the higher total light emission of the luminescent marker conjugate in the

TABLE 1

Measurement of plasma steroids by chemiluminescence immunoassay

Steroid	Method	Sensitivity (pg/tube)	Precision[a]		Ref.
			Intra-assay	Inter-assay	
Progesterone	Homogeneous	25	—	—	6
	Charcoal	5	6.8	15.2	7
	Antibody-coated tube	10	7.0	10.1	8
	Direct assay, covalently coupled antibodies	2	8.9	10.3	9
Cortisol	Homogeneous	20	7.5	8.6	3
	Charcoal	10	7.2	14.0	4
	Direct assay, antibody-coated tube	54	10.0	12.5	5
Testosterone	Charcoal	2	8.8[b] 10.5[c]	19.9[b] 16.1[c]	20
	Covalently coupled antibodies	5	—	—	13
Estradiol	Antibody-coated tube	2	9.4	7.9	10
	Covalently coupled antibodies	5	8.1	10.0	11
Estriol	Coupled antibodies	4	9.5	10.5	12

[a]Expressed as relative standard deviation (%). [b]Male. [c]Female.

antibody-bound state compared to the free state. This method, however, is susceptible to interference from luminescent compounds in biological samples. Consequently, assays requiring separation of bound and free ligand have been developed. Except for the direct assay for cortisol [5], the sensitivities of the heterogeneous assays are better than those of the homogeneous assays. The precisions, both intra- and inter-assay, of all the methods described in Tables 1 and 2 are very similar.

Homogeneous assays were developed before the liquid and solid-phase heterogeneous types. The latter assays which use antibodies coated on the tube wall have proved to be robust and reliable but still have some disadvantages in the limited amount of antibody that can be bound to the tube wall, the slower reaction, the potential loss of immunoreactivity of adsorbed antibodies, and the considerable batch-to-batch variation in the plastic tubes that is reflected by variation in the behaviour of adsorbed antibodies [13].

TABLE 2

Measurement of urinary steroid metabolites by chemiluminescence immunoassay methods

Metabolite	Method	Sensitivity (pg/tube)	Precision[a]		Ref.
			Intra-assay	Inter-assay	
Estriol-16α-glucuronide	Homogeneous	10	7.0	9.5	15
	Antibody-coated tube	200	7.4	11.1	21
Pregnanediol-3α-glucuronide	Antibody-coated tube	100	6.8	9.0	22
	Antibody-coated tube	30	7.0	8.0	16
Estrone-3-glucuronide	Antibody-coated tube	2	9.1	12.0	14
	Covalently coupled antibodies	5	4.2	6.2	13

[a] Expressed as relative standard deviation (%).

As a result, the solid phase has been changed and in the latest chemiluminescence immunoassays reported the antibodies are covalently coupled to small polymer beads, 5—10 μm in diameter. Separation of bound and free ligand is achieved by washing followed by simple centrifugation. This solid-phase system has yielded sensitive assays for estradiol [11], testosterone [13], progesterone [9] and estrone-3-glucuronide [14].

Some of the assays listed in Tables 1 and 2 have been applied for clinical endocrinological investigation in women. These studies include: (a) the variations of concentrations of urinary pregnanediol-3α-glucuronide [13, 16] and estrone-3-glucuronide [14] and of estradiol [10] and progesterone [9] during the normal menstrual cycle; (b) the increases of estradiol and progesterone concentration in the peripheral circulation during ovulation induction therapy with gonadotropins [9, 11, 19]; (c) normal concentrations of estriol in the serum of pregnant women [12].

To illustrate the application of chemiluminescence immunoassays in clinical endocrinological investigations in women, new data are presented here that were obtained with two solid-phase chemiluminescence immunoassays based on antibodies covalently coupled to immunobeads, one for estradiol in extracts of serum [19] and one for progesterone in unextracted serum [9]. Figure 1 illustrates the changes in the concentrations of estradiol and progesterone in serum of two women treated with human menopausal gonadotropins and human choriogonadotropin. These concentrations (y) correlated well with those obtained with r.i.a. (x). The linear regression equation for estradiol ($n = 19$) was $y = 0.98x + 7.23$ ($r = 0.98$) and for progesterone ($n = 10$) was $y = 1.01x - 2.4$ ($r = 0.99$). Normal values of progesterone

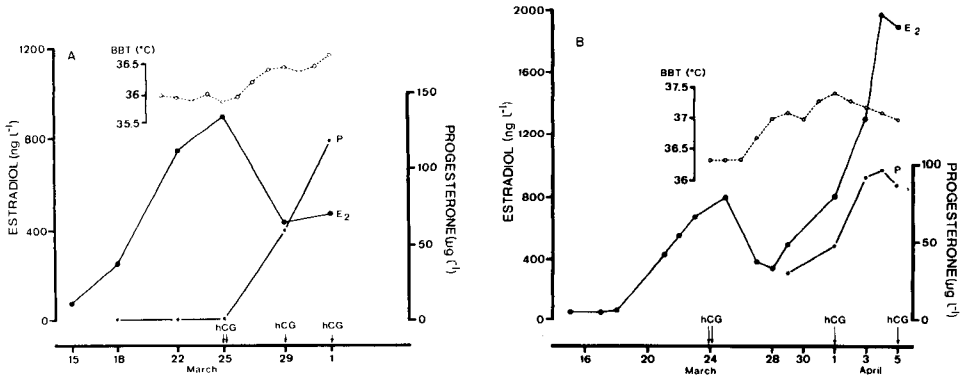


Fig. 1. Concentrations of estradiol and progesterone in serum, determined by chemiluminescence immunoassay, during ovulation induction: E2, estradiol; P, progesterone; BBT, basal body temperature. For A, 4000 international units of human menopausal gonadotropins (hMG) was administered during March 4—24; for B, 3150 units of hMG was administered during March 8—23. Arrows denote one dose of human choriogonadotropin (hCG; 5000 international units).

in serum during the menstrual cycle, determined by direct solid-phase chemiluminescence assay are listed in Table 3. As with the results for ovulation induction, a good correlation between the concentrations determined by r.i.a. (x) and chemiluminescence immunoassay (y) was obtained, i.e., $y = 0.93x + 0.37$ ($r = 0.95, n = 94$).

The data presented here and in the references listed in Tables 1 and 2, indicate that chemiluminescence immunoassays can be done with similar sensitivity, accuracy and precision as is achieved in r.i.a. Results obtained by both methods correlate well. Chemiluminescence immunoassay can therefore provide an analytically valid and convenient non-isotopic alternative to r.i.a. for routine determinations of steroids in blood and their metabolites in urine.

Some technical problems concerning the selectivity of the monoclonal and polyclonal antibodies, the type of technique used for phase separation, and the correct choice of the conjugate (a problem that is related to the length of the alkyl chain), cannot be overlooked during the development of new chemiluminescence immunoassays. However, once suitable conditions have been established, the assays prove to be robust and reliable. The present authors have used antibodies to estradiol, covalently coupled to polymer beads and the corresponding conjugate [11] for over two years without loss of activity or light yield. Other conjugates show the same stability, but the progesterone-isoluminol conjugates suffer from a similar loss of product as is noticed with progesterone itself on serial dilution. This can be avoided by the injection of small amounts of ethanolic solutions of the conjugates into the assay buffer.

The problems of bridge recognition that are observed with ^{125}I-progesterone to cause poor precision and sensitivity in r.i.a. [23, 24] are not encountered

TABLE 3

Serum progesterone concentrations measured during the menstrual cycle

Day of menstrual cycle	No. of samples	Progesterone (nmol l^{-1})		
		Arithmetic mean (and S.D.)	Geometric mean	Range
1—11	41	0.7(0.5)	0.6	0.3—2.5
12—15	7	4.6(3.8)	3.6	2.0—10.8
16—25	46	33.9(8.7)	32.8	19.4—59.8

in the direct chemiluminescence assay for progesterone [9]. The sensitivity of this direct assay (0.54 nmol l^{-1} [9]) and that for estriol (0.7 nmol l^{-1} [12]) are adequate for the determination of the two steroids in 10—20 μl of serum. These small volumes help to minimize possible interference from other substances in the serum in the measurement of light. The washing step after incubation further decreases interference and is responsible for the optimal separation of the bound and free fractions. This is illustrated by the low assay blanks, that are at most 0.8% [12] and 0.5% [9] of the total counts.

As discussed previously [19], a limitation of the present chemiluminescence immunoassays that use isoluminol as the chemiluminescent marker is the time required for initiation of light emission. The use of alternative chemiluminescent probes, that need less time to produce light, could overcome this problem. Further decrease in the assay time can be attained if extraction of the samples is not necessary and phase separation can be omitted.

We thank Dr. P. Stanley and Mr. J. Vossen of Lumac 3M, Schaesberg, The Netherlands, for the loan of a Biocounter M-2000 luminometer. This work was supported by grants to F. K. from the Bosch Foundation and the Binational Science Foundation (Jerusalem).

REFERENCES

1 H. R. Schroeder, P. O. Vogelhut, R. J. Carrico, R. C. Boguslaski and R. T. Buckler, Anal. Chem., 48 (1976) 1933.
2 F. Kohen, M. Pazzagli, M. Serio, J. De Boever and D. Vandekerckhove, in W. P. Collins (Ed.), Alternative Immunoassays, Wiley, London, 1985, p. 103.
3 F. Kohen, M. Pazzagli, J. B. Kim and H. R. Lindner, Steroids, 36 (1980) 421.
4 M. Pazzagli, J. B. Kim, G. Messeri, F. Kohen, G. F. Bolelli, A. Tommasi, R. Salerno and M. Serio, J. Steroid Biochem., 14 (1981) 1981.
5 L. Lindström, L. Meurling and T. Lövgren, J. Steroid Biochem., 16 (1982) 577.
6 F. Kohen, M. Pazzagli, J. B. Kim, H. R. Lindner and R. C. Boguslaski, FEBS Lett., 104 (1979) 201.
7 M. Pazzagli, J. B. Kim, G. Messeri, G. Martinazzo, F. Kohen, F. Franceschetti, A. Tommasi, R. Salerno and M. Serio, Clin. Chim. Acta, 115 (1981) 287.
8 F. Kohen, J. B. Kim, H. R. Lindner and W. P. Collins, Steroids, 38 (1981) 73.

9 J. De Boever, F. Kohen, D. Vandekerckhove and G. Van Maele, Clin. Chem., 30 (1984) 1637.
10 J. B. Kim, G. J. Barnard, W. P. Collins, F. Kohen, H. R. Lindner and Z. Eshhar, Clin. Chem., 28 (1982) 1120.
11 J. De Boever, F. Kohen and D. Vandekerckhove, Clin. Chem., 29 (1983) 2068.
12 W. Klingler, O. Haupt, G. v. Postel and R. Knuppen, Steroids, 42 (1983) 123.
13 F. Kohen, H. R. Lindner and S. Gilad, J. Steroid Biochem., 19 (1983) 413.
14 D. A. Weerasekera, J. B. Kim, G. J. Barnard, W. P. Collins, F. Kohen and H. R. Lindner, Acta Endocrinol. (KBH), 101 (1982) 254.
15 F. Kohen, J. B. Kim, G. Barnard and H. R. Lindner, Steroids, 36 (1980) 405.
16 Z. Eshhar, J. B. Kim, G. Barnard, W. P. Collins, S. Gilad, H. R. Lindner and F. Kohen, Steroids, 38 (1981) 89.
17 H. R. Schroeder and F. M. Yeager, Anal. Chem., 50 (1978) 1114.
18 G. Köhler and C. Milstein, Eur. J. Immunol., 6 (1976) 511.
19 J. De Boever, F. Kohen, M. Dhont, G. Van Maele and D. Vandekerckhove, Anal. Chim. Acta, 170 (1985) 117.
20 M. Pazzagli, M. Serio, P. Munsun and D. Rodbard, in IAEA (Ed.), Radioimmunoassay and Related Procedures in Medicine, IAEA, Vienna, 1982, p. 747.
21 G. Barnard, W. P. Collins, F. Kohen and H. R. Lindner, J. Steroid Biochem., 14 (1981) 945.
22 G. Barnard, W. P. Collins, F. Kohen and H. R. Lindner, in M. A. Deluca and W. D. McElroy (Eds.), Bioluminescence and Chemiluminescence, Academic Press, New York, 1981, p. 311.
23 J. E. T. Corrie, W. A. Ratcliffe and J. S. Macpherson, Steroids, 38 (1981) 709.
24 J. E. T. Corrie, W. M. Hunter and J. S. Macpherson, Clin. Chem., 27 (1981) 594.

Analytica Chimica Acta, 170 (1985) 133—137
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

Short Communication

FLUORESCENCE IMMUNOASSAY FOR ANGIOTENSIN I

A. REYES and M. MORELL
Department of Biochemistry, Faculty of Medicine, University of Malaga (Spain)
J. J. LASERNA*
Department of Analytical Chemistry, Faculty of Sciences, University of Malaga (Spain)
(Received 5th September 1984)

Summary. A heterogeneous fluorescence immunoassay for angiotensin I is described. Fluorescein isothiocyanate (FITC) is used as fluorescent label; it is covalently linked to angiotensin I by reacting in pH 9.2 carbonate buffer at 18°C for 24 h. A Sephadex G-10 gel filtration column permits separation of conjugate from excess of FITC. The assay is applied to 4—40 ng ml^{-1} angiotensin I, with a relative standard deviation of 5%.

The advantages and disadvantages of fluorescence immunoassay compared with radioimmunoassay (r.i.a.) have been widely discussed [1—6]. However, they form a rather specialized area because of the difficulties presented by both methods. These arise because the principal assay reagent, the antibody, is a naturally occurring protein and also because the procedures involve micro and trace techniques. In both methods, moreover, much of the decision making and the trial and error approach usually employed in the optimization phases of the determinations demand a high degree of skill and experience.

It is often possible to take advantage of well established r.i.a. procedures to develop a fluorescence immunoassay for a given analyte, thus giving access to a considerable amount of information which may save much time and effort. However, in general, the specific model of experimental conditions used in an r.i.a., which serves to define the performance of the assay, is no longer valid for the corresponding fluorescence assay because of the different nature of the method of determining the end-point in fluorescence immunoassays, which requires several optimization stages in which there is a need to decrease the non-specific signal.

This report describes one type of fluorescence immunoassay for angiotensin I. The configuration of the assay, similar to r.i.a. [7—9], is heterogeneous, and measures the free antigen fraction. The background fluorescence from the reagents limits the sensitivity of the assay, but the precision is similar to that of r.i.a.

0003-2670/85/$03.30

Experimental

Chemicals. All chemicals used were analytical-reagent grade. Water was distilled and demineralized. Phosphate buffer (pH 7.1) was prepared by mixing appropriate amounts of 0.1 M sodium monohydrogenphosphate and 0.1 M potassium dihydrogenphosphate. Carbonate buffer (pH 9.2) was prepared by mixing appropriate volumes of 0.2 M sodium carbonate and 0.2 M sodium hydrogencarbonate. Ileu^5-angiotensin I (Sigma Chemical Co.) was used as supplied. Standard solutions were prepared daily by dissolving the peptide in phosphate buffer. Further dilutions were made with buffer. Fluorescein isothiocyanate was obtained from Merck. Radioimmunoassay was done with a commercial kit (Serono Diagnostic). The antiserum in this kit was used in the fluorescence immunoassay.

Apparatus. The Perkin-Elmer 650 spectrofluorimeter used was equipped with a 150-W xenon lamp, excitation and emission grating monochromators, and 1-cm quartz cells.

Synthesis of angiotensin-fluorescein conjugate. FITC (7.5 mg) was dissolved in 2 ml of carbonate buffer. This solution was slowly added with constant stirring to a solution prepared by dissolving 2.5 mg of angiotensin I in 2 ml of carbonate buffer. The reaction mixture was stirred for 24 h at room temperature, before 0.5 ml of the reaction mixture was applied to a Sephadex G-10 (Pharmacia) gel filtration column (1 × 30 cm). The eluent was 0.1 M phosphate buffer (pH 7.1), and the flow rate was 0.2 ml min^{-1}. The eluate was fractionated in 0.2-ml fractions and stored at −20°C until use. The development of the labelling reaction was monitored by thin-layer chromatography on 0.25-mm thick silica gel plates. The eluent was n-butanol/acetic acid/water (60/20/20 by volume). The development tank was presaturated with the eluent for 1 h.

Fluorescence immunoassay procedure. The whole procedure was done at low temperature (2—4°C). Angiotensin I (100 μl) was placed in plastic test tubes and 100 μl of labelled angiotensin I and 100 μl of antiserum were added. The samples were mixed rapidly and stored for 150 min in a refrigerator. After incubation, 100 μl of angiotensin-free carrier serum and 1.0 ml of polyethylene glycol 6000/water (20/80 v/v) were added. After mixing (Vortex) and centrifugation at 1500g for 20 min at 4°C, the fluorescence of 1.0 ml of the supernatant fluid was measured at 525 nm, with excitation at 496 nm.

Results and discussion

Fluorescein isothiocyanate (FITC) was selected as the fluorescent label for angiotensin I because of the ready availability of the reagent and the ease of the coupling reaction, in which there are no extreme reaction conditions that might damage the immunoreactivity of the peptide.

Three experimental variables were checked to optimize the labelling reaction: pH, reaction time and temperature. The development of the reaction was followed by thin-layer chromatography, using ninhydrin to visualize

angiotensin I spots and ultraviolet radiation to detect both FITC and labeled angiotensin fluorescence. In the chromatographic system used, the unreacted angiotensin I and FITC were found to have R_F values of 0.30 and 0.95, respectively, whereas the conjugate had an R_F value of 0.45. Strict adherence to the chromatographic conditions mentioned under Experimental gave reproducible R_F values. As the labeled angiotensin I does not respond to ninhydrin, FITC must be masking the *N*-terminal primary amine of the molecule.

Aliquots of the reaction mixture were taken at zero time and at intervals of 2, 4, 8, 16, 24, 36 and 48 h. At 4°C, conjugation appeared only after 8 h of reaction, and increased slowly for at least 48 h. At room temperature (18—20°C), the first conjugation occurred 2 h after labelling, and there was no substantial increase from 24 h onwards. The extent of labelling was constant in the pH range 9.0—9.5, decreasing at pH <8.5. Although the rate of the labelling reaction seemed to be unaffected by the relative concentrations of the reactants, three conjugations, done at angiotensin/FITC mole ratios of 1:1, 1:2 and 1:10, revealed a progressive decrease in the final spot corresponding to angiotensin. This study suggests that the labelling of angiotensin I with FITC at a mole ratio of 1:10, at pH 9.2 (carbonate buffer), for 24 h at room temperature is practicable.

Isolation and properties of the conjugate. The Sephadex G-10 gel filtration column permits the separation of the conjugate from the excess of FITC on the basis of molecular size. Aliquots (0.5 ml) of the 24-h reaction mixture were applied to the column which resolved two peaks (Fig. 1). The first contained labelled angiotensin I and the second was unreacted FITC. Aliquots from the respective peak fractions were tested for their antigenicity by r.i.a., using anti-angiotensin I antiserum. All the antigenicity resided in the first peak, revealing no loss of immunoreactivity of the labelled angiotensin I. The concentration of angiotensin-fluorescein in each fraction was determined by its absorbance at 492 nm in 0.1 M phosphate buffer (pH 7.1), assuming the molar absorptivity of the conjugate to be the same as for fluorescein isothiocyanate (calculated to be 3.55×10^4 l mol^{-1} cm^{-1} at pH 7.1). Taking into

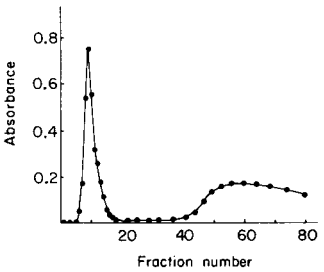


Fig. 1. Fractionation of angiotensin I-fluorescein on Sephadex G-10 (measured at 492 nm).

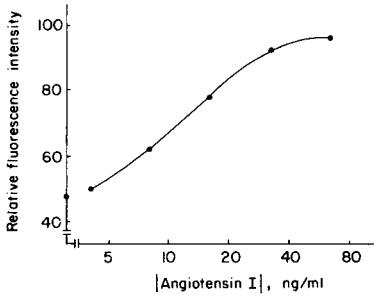


Fig. 2. Calibration graph for the fluorescence immunoassay of angiotensin I.

account a 1:1 mole ratio in the conjugate, the concentrations determined spectrophotometrically agreed closely with those given by r.i.a. Consequently, for routine determinations of conjugate, spectrophotometric measurements were used.

The fluorescence excitation and emission maxima of the tracer shifted 3.5 and 5 nm, respectively, to longer wavelengths relative to those of FITC. These shifts may be accounted for by the covalent linkage of FITC to the peptide. The intensity was linearly proportional to the concentration of conjugate in phosphate buffer from 10^{-10} to 10^{-7} mol l^{-1}. The detection limit, defined as that tracer concentration in the measured solution which gives a signal-to-noise ratio of 2:1, was found to be 0.4 ng ml^{-1}. Tracer solutions stored at −20°C showed no change in properties over at least six months.

Calibration graph. The calibration graph obtained is shown in Fig. 2. Intra-assay precision, determined for a series of 11 replicates at the 36 ng ml^{-1} angiotensin I level, was 5% (relative standard deviation). By comparison with r.i.a., the present assay is approximately one order of magnitude less sensitive, because of the reagent-produced fluorescence. As discussed above, the detection limit for labelled angiotensin I at pH 7.1 is 0.4 ng/ml^{-1}, but this value rises to 1.1 ng ml^{-1} when the tracer is processed by the entire fluorescence immunoassay protocol. It should be noted that the r.i.a. detection limit (typically 0.2—0.5 ng ml^{-1}) represents the concentration of angiotensin I contained in the solution introduced into the incubation mixture, while the above-mentioned detection limits refer to the concentration of tracer in the measured solution. Consequently, the minimum concentration of tracer in the solution introduced to the incubation mixture must be 15.5 ng ml^{-1}, according to the established protocol. As a result, in order to achieve antibody competition, the range of concentrations for fluorescence immunoassay must be similar to the tracer concentration. In fact, by using a conjugate concentration of 30 ng ml^{-1}, maximum sensitivity is obtained in the range 4—40 ng ml^{-1}, and 4 ng ml^{-1} is the minimum amount clearly distinguishable from the assay blank.

Improvements in the detection limit could be achieved by changing the solvent used for the separation of the antibody-bound and free fractions of antigen. For example, although the fluorescence of labelled angiotensin I in ethanol is 30% less than in polyethylene glycol as a result of the decreased solvent viscosity, the overall signal-to-noise ratio is almost doubled in ethanol. However, at present, the possibility of using ethanol reproducibly to separate the bound and free fractions of angiotensin I has not been investigated fully.

REFERENCES

1 D. S. Smith, M. H. H. Al-Hakiem and J. Landon, Ann. Clin. Biochem., 18 (1981) 253.
2 C. M. O'Donnell, J. H. McBride, A. Broughton and S. C. Suffin, Clin. Chem., 25 (1979) 1077.
3 D. S. Smith, M. Hassan and R. D. Nargessi, in E. L. Wehry (Ed.), Modern Fluorescence Spectroscopy, Vol. 3, Plenum, New York, 1981, p. 143.

4 E. Soini and I. Hemmilä, Clin. Chem., 25 (1979) 353.
5 C. M. O'Donnell and S. C. Suffin, Anal. Chem., 51 (1979) 33A.
6 G. C. Visor and S. G. Schulman, J. Pharm. Sci., 70 (1981) 469.
7 G. C. Zucchelli, R. Malvano, U. Rosa and A. Salvetti, J. Nucl. Biol. Med., 17 (1973) 187.
8 E. Haber, T. Koerner, L. B. Page, B. Kliman and A. Purnode, J. Clin. Endocrinol., 23 (1969) 1349.
9 D. K. Krause, W. Hummerich and K. Poulsen, (Eds.), Radioimmunoassay Renin-Angiotensin, Thieme, Stuttgart, 1978.

Analytica Chimica Acta, 170 (1985) 139—142
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

Short Communication

A FLUOROIMMUNOASSAY FOR SCREENING OF HEPATITIS B SURFACE ANTIGEN

GUIDO DE SCHRIJVER, ROGER JOZEF WIEME and LUC PITTOORS

Laboratory of Internal Medicine, Academic Hospital, De Pintelaan 185, B-9000 Gent (Belgium)

(Received 5th September 1984)

Summary. A fast, sensitive sandwich fluoroimmunoassay for hepatitis B surface antigen (HB_s-ag) is described. Rabbit antibodies to HB_s-ag are covalently bound to the centre of a glass-fiber filter disk. The second antibody is a swine anti-human HB_s-ag conjugated with β-D-galactosidase from *E. coli*. The substrate is 4-methylumbelliferyl-β-D-galactopyranoside. The technique is applied with the Stratus system which does the entire test in 8 min. The detection limit is 1 ng ml^{-1}, and the results correlate well with a radioimmunoassay technique.

Solid-phase techniques are popular in radioimmunoassay, enzyme immunoassay and recently also in fluoroimmunoassay. Different materials can be used to immobilize molecules. Polystyrene beads or microtiter plates are most frequently used [1—4]. Recently, the U.S. company Dade produced an instrument which uses a glass-fiber filter fixed in a plastic support as the solid phase. The immunoassay procedure used is based on the sequential saturation method [5]. The antibody is precipitated in the central area of the filter by conventional double-antibody techniques. After application of the sample, enzyme-labelled antigen is applied, to react with the remaining specific antibody sites. After a short incubation, substrate solution applied to the center of the reaction area washes out any unbound label to the periphery of the filter. The enzyme reaction at the center is quantified by front-surface fluorescence. The microprocessor-controlled automated instrument processes the filter paper disk through the above sequence and calculates the final concentration. The total reaction time is 8 min.

This communication deals with application of this technique to the detection of hepatitis B surface antigen (HB_s-ag), with two major modifications. First, a covalent-linking procedure is used to immobilize the antibody in the center of the glass fiber with a bifunctional bis-diazotized reagent. This gives better immobilization of the antibody and better exposure of the binding sites. Secondly, the sandwich technique is preferred to the commercially available procedures.

0003-2670/85/$03.30

Experimental

Materials. *m*-Maleimidobenzoyl *N*-hydroxysuccinimide ester (MBS), β-D-galactosidase (EC.3.2.1.23) and 4-methylumbelliferyl-β-D-galactopyranoside (MUG) were from the Sigma Chemical Company. A rabbit antibody to human hepatitis B surface antigen (Behringwerke, Marburg) and swine anti-HB_s antigen (Nordic Immunologic Laboratories) were used. Sepharose 6B, G25 PD10 columns (Pharmacia Fine Chemicals, Uppsala), glass microfiber disks (Whatman GF/F, 4.7 cm), dioxan and 2-mercaptoethanol (Merck) and Fast Blue B salt (Janssen Chimica) were other requirements.

Conjugation procedure. The conjugation procedure used was a modification of the method described by Schechter et al. [6]. To a solution of 1.5 mg of swine anti-HB_s in 950 μl of 0.05 M sodium phosphate buffer, pH 7.3, containing 0.05 M NaCl, 0.001 M $MgCl_2$ and 0.1% (w/v) sodium azide (buffer A), three 5-μl portions of 127 μM MBS in dioxan were added. The mixture was incubated for 60 min at 30°C, and five 5-μl aliquots of the MBS solution were added at 10-min intervals. After a total incubation time of 2 h, the mixture was added to a Sephadex G25 PD10 column and eluted with buffer to an end volume of 2 ml. A 2-ml portion of a solution containing 1.5 mg of β-D-galactosidase in buffer A was added to the activated IgG and incubated for 1 h at 30°C, and 25 μl of a 1% (w/v) 2-mercaptoethanol solution in the buffer was added; the mixture was incubated again for 2 h at room temperature. This stopped the reaction and prevented formation of polymers.

The conjugate was fractionated on a column (0.9 × 60 cm) filled with Sepharose 6B and eluted with buffer A at a flow rate of 8 ml h^{-1}. The eluent was monitored at 280 nm with a LKB u.v. monitor; 1.5-ml fractions were collected. The enzyme activities of the collected fractions were assayed with 7.5×10^{-5} M 4-methylumbelliferyl-β-D-galactopyranoside in 0.01 M phosphate buffer, pH 7.3, containing 0.1 M NaCl, 0.001 M magnesium chloride, 0.1% sodium azide and 0.1% (w/v) bovine serum albumin (buffer B). A 5-μl portion of each fraction was measured in 3 ml of substrate solution on a fluorimeter (model MPS; Vitatron). The fraction with the highest enzyme activity and which agreed with the expected molecular mass of the conjugate (±650 000 dalton) was used for the immunoassay. In this procedure, fraction numbers 13—15 were useful.

Immobilization procedure. Coupling of proteins to inorganic support materials with a bifunctional bis-diazotized reagent was described by Messing and Stinson in 1974 [7]. This technique was adapted for the glass fiber disks in the present work. A 100-μl aliquot of 0.036 M sodium hydroxide was added to the centre of a glass fiber filter disk, and eluted three times with 50 μl of distilled water. Then 100 μl of 0.01% Fast Blue B salt solution in distilled water containing 0.05% Tween-20 was applied. After 30 min, 100 μl of a (1 + 9) solution of the rabbit anti-HB_s (precipitated with ammonium sulfate) in 0.05 M phosphate buffer, pH 8, containing 0.05 M NaCl, 0.001 M magnesium chloride and 0.1% sodium azide, was applied. After a reaction time of 30 min, 100 μl of 0.05 M phosphate buffer, pH 8, containing 2% bovine

serum albumin (BSA) was applied to neutralize unreacted Fast Blue B salt. Next, 50 μl of a 0.1% (w/v) BSA solution in 0.005 M phosphate buffer, pH 7.3, was applied three times and the disks were left to dry. The disks could be stored in a dry atmosphere at room temperature for about 30 days.

Analytical procedures. The disks were cut to the appropriate dimensions and fixed in empty plastic supports. They were loaded on the instrument as prescribed. The second antibody solution was a (1 + 49) dilution of the conjugate in buffer A. The substrate wash solution was 7.5×10^{-4} M MUG in buffer A. All serum samples were diluted (1 + 4) and inactivated for 35 min at 56°C before processing further. The digoxin program on the instrument was used. The cut-off values were established as recommended for the Ausria II techniques [8].

Results and discussion

Fifty seven serum samples were analyzed for hepatitis B surface antigen. Twenty were negative, 17 were low positives (Ausria $\leqslant$ 350 cpm) and 20 were high positives (Ausria $\geqslant$ 500 cpm) of the 37 positives, 35 were also positive by fluoroimmunoassay; of the 20 negatives, 18 were negative by fluoroimmunoassay. A dilution curve for a positive control serum with known antigen content is given in Fig. 1, which also shows a comparison with radioimmunoassay values.

Two remarks can be made. First, the sensitivity of the fluorescence immunoassay seems to be the same as that of the radioimmunoassay. Both have a detection limit of 1 ng ml^{-1}. Secondly, the fluorescence immunoassay curve shows a high dose hook effect above 15 ng ml^{-1}. The same effect was seen with the positive samples which gave negative results in fluorescence immunoassay. Once diluted, they gave a positive value. An explanation for this effect may be steric hindrance. If a large amount of conjugate is bound to the filter disk, the substrate is inaccessible to the enzyme, so there is no fluorescence. The same effect was described by Gibbons et al. [9] for a β-galactosidase conjugate in a system for the detection of anti-immunoglobulin.

To avoid the problem of a negative result being obtained for a sample with a high concentration of HB_s antigen, the test should be done once at the

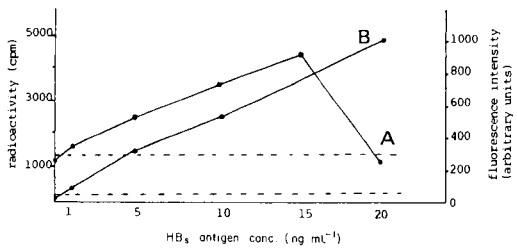


Fig. 1. Dilution curve for a positive control sample with known concentration of hepatitis B surface antigen (20 ng ml^{-1}). Dilutions were made in negative serum. Measurements by: (A) fluoroimmunoassay; (B) radioimmunoassay.

standard dilution (1 + 4) and once at greater dilution (1 + 49) instead of running duplicates at the same dilution.

Conclusions

A modification of the conjugation procedure of Schechter et al. [6] has been used to conjugate β-galactosidase to anti-HB_s. The procedure gives a highly reproducible conjugate composition and conjugate quality, and a large amount of monomolecular conjugates. This is extremely important in the sandwich technique on glass fiber filters where good elution of unbound material during the wash procedure is essential to achieving a low background. The covalent coupling method of an antibody to the glass fiber is preferable. There is better adherence of the complex during the wash cycle. This procedure allowed a sandwich fluoroimmunoassay for HB_s antigen in the nanogram range to be completed within 8 min.

REFERENCES

1 L. R. Overby, J. P. Miller, I. D. Smith, R. H. Decker and C. M. Ling, Vox Sang., 24 (1973) 102.
2 O. P. Lehtonen and M. K. Viljanen, J. Immunol. Methods, 34 (1980) 61.
3 A. R. Neurath and N. Strick, J. Virol. Methods, 3 (1981) 155.
4 I. Ionescu-Matiu, Y. Sanchez, H. A. Fields and G. R. Dreesman, J. Virol. Methods, 6 (1983) 41.
5 J. L. Giegel, M. R. Brotherton, P. Cronin, M. D'Aquino, S. Evans, Z. H. Heller, W. S. Knight, K. Krishnan and M. Sheiman, Clin. Chem., 28/9 (1982) 1894.
6 Y. Schechter, J. Schlessinger, S. Jacobs, K. J. Chang and P. Cuatrecasas, Proc. Natl. Acad. Sci. U.S.A., 75 (1978) 2135.
7 R. A. Messing and H. R. Stinson, Mol. Cell. Biochem., 4/3 (1974) 217.
8 C. M. Ling, R. H. Decker, R. S. Foemmel and C. R. Overby, J. Lab. Clin. Med., 86 (1975) 690.
9 I. Gibbons, C. Skold, G. L. Rowley and E. F. Ulman, Anal. Biochem., 102 (1980) 167.

Analytica Chimica Acta, 170 (1985) 143—148
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

Short Communication

DOUBLE-ANTIBODY HOMOGENEOUS FLUORESCENCE IMMUNOASSAY OF PHENYTOIN

JEFFREY S. O'NEAL and STEPHEN G. SCHULMAN*

College of Pharmacy, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32610 (U.S.A.)

PERRY O. TEAGUE

College of Medicine, University of Florida, Gainesville, FL 32610 (U.S.A.)

(Received 5th September 1984)

Summary. A homogeneous fluorescence immunoassay suitable for quantifying 5—25 mg l^{-1} phenytoin (5,5-diphenyl-2,4-imidazolidenedione) in serum is described. The fluorophor-labeled phenytoin and unlabeled phenytoin (analyte) compete for a limited number of anti-phenytoin antibody binding sites in the presence of anti-fluorophor antibodies. The label employed is a sulfonamido derivative of 2-naphthol-8-sulfonic acid (2-8) which, at neutral pH, undergoes excited-state proton transfer. Binding of anti-2-8 antibodies to the drug-fluorophor conjugate results in quenching of the conjugate base emission at 480 nm. The relative standard deviations are about 5%.

Immunoassay methods have become increasingly important in the field of biomedical analysis. Their ability to quantify very selectively a large number of analytes has proved invaluable. Traditional immunoanalytical systems have employed radioactive labels. Although these labels provide high sensitivity, they suffer from many disadvantages including short shelf life, health hazards, etc. Alternatives include fluorescent labels which, in addition to solving most of the problems presented by radiolabels, offer greater flexibility in the development of the immunoassay. Environmental effects imposed on specific label populations can lead to signal modification obviating the necessity for a separation step. The homogeneous methods reduce operator handling of the sample, thus reducing potential sample loss during physical transfer steps. Traditional fluorescent labels include fluorescein and rhodamine. Alternative labels which have greater emission red shifts from the excitation wavelengths are proposed here. This class of potential derivatizing reagents also possesses aromatic hydroxyl functional groups. In the lowest excited singlet electronic state of the label, the hydroxyl group becomes much more acidic so that at physiological pH electronic absorption by the undissociated acid is followed by emission from the excited conjugate base. Large energy stabilization of the base form in the excited electronic state results in a considerable shift to longer wavelengths of the conjugate base

0003-2670/85/$03.30

emission relative to that of the acid form. This shift to longer wavelengths is particularly desirable when the analytical signal originates from a biological matrix. Not only is interference from scattered incident radiation decreased but emissions from fluorescent molecules of biological origin tend to emit at shorter wavelengths.

The proposed homogeneous fluorescence immunoassay for phenytoin (5,5-diphenylhydantoin; 5,5-diphenyl-2,4-imidazolidenedione) is based on the partitioning of a 2-naphthol-8-sulfonamido derivative of phenytoin (2-8-P) between anti-fluorophor and anti-phenytoin antisera. Displacement of 2-8-P from anti-phenytoin binding sites by unlabeled phenytoin results in increased availability of the fluorophor for anti-fluorophor binding. Previous approaches have used fluorescein as a label for assays of albumin [1] and human IgG [2]. Fluorescein is known to be quenched on binding to anti-fluorescein antibodies [3—5], and advantage was taken of this in the development of immunoassay systems. Binding of anti-2-naphthol-8-sulfonamido-poly-(L-lysine) to 2-8-P results in a substantial inhibition of excited-state proton transfer. Prior binding of the conjugate to anti-phenytoin antisera sterically excludes the binding of a second antiserum directed against the fluorophor, allowing the development of the double-antibody homogeneous immunoassay.

Phenytoin is a widely prescribed anticonvulsant drug which is characterized by a narrow therapeutic index. Serum concentrations above 21 mg l^{-1} are toxic whereas those below 9 mg l^{-1} are generally ineffective. It is therefore necessary to monitor serum phenytoin concentrations to maximize clinical efficacy [6, 7]. Techniques which have been used to quantify phenytoin include gas chromatography [8], high-performance liquid chromatography [9], radioimmunoassay [10] and homogeneous enzyme immunoassay [11]. Recent fluorescence immunoassays for phenytoin include a reactant-labeled assay [12], a magnetizable solid-phase procedure [13], a fluorescence polarization immunoassay [14], and a double-antibody technique [15].

Experimental

Chemicals. 5,5-Diphenylhydantoin (99% purity; Aldrich Chemical Co.) was used. 3-[(2″-Hydroxynaphthyl-8″-sulfonyl)-2′-amino]ethyl-5,5-diphenylhydantoin was synthesized as previously described [16]. Poly(L-lysine) hydrobromide, having an average polymerization number of 100, was from Sigma Chemical Co. Rabbit anti-phenytoin-3-ω-valeryl-bovine serum albumin (anti-P) was from Miles-Yeda Company (Rehovot, Israel). Antiserum stock solutions were prepared by diluting the antisera (1 + 9) with a phosphate buffer (pH 7.5, ionic strength 1.1 M) containing 0.1% (w/v) sodium azide.

Fluorescence was measured with a Perkin-Elmer MPF-2A fluorimeter. All measurements and incubations were done at 30° C.

Synthesis of 2-8—poly(L-lysine). A 2-8—poly(L-lysine) immunogenic conjugate was synthesized as follows. Poly(L-lysine) hydrobromide (31 mg) was dissolved in 10 ml of deionized water and the pH was increased by adding 2 ml of 0.5 M borate buffer (pH 10). A solution of 7.4 mg of 2-acetoxy-8-naphthalenesulfonyl chloride [16] in 10 ml of acetone was added dropwise

to the stirred mixture, and stirring was continued for 30 min to complete hydrolysis of the acetate protecting group. The mixture was dialyzed against five 1-l volumes of phosphate buffer (pH 7.5, I = 0.05 M). Spectrophotometric estimation of the extent of conjugation indicated an approximate 10:1 mole ratio of fluorophor to macromolecule. The dialyzed product was immediately frozen and stored at −20°C.

Preparation of anti-2-8 antisera. Two groups of two New Zealand white rabbits were used. One group received 5-mg doses of immunogen and the other received 0.5 mg. The first inoculation, via multiple subcutaneous injections, consisted of the immunogen dissolved in an emulsion containing 0.5 ml of complete Freund's adjuvant and 0.5 ml of phosphate buffer pH 5.7 adjusted to an ionic strength of 0.10 with NaCl. After 3 weeks the rabbits were re-inoculated similarly except that incomplete Freund's adjuvant was substituted. A third inoculation was done after 10 days using only phosphate-buffered saline as a vehicle, and the animals were bled after an additional week. The IgG proteins were purified by a standard ammonium sulfate precipitation followed by chromatography on DEAE cellulose in 0.07 M sodium phosphate pH 6.3 [17]. Pooled fractions were immediately lyophylized and stored at −20°C.

Phenytoin assay. Relative abilities of the different antiserum fractions to quench the long-wavelength fluorescence of 2-8-P were evaluated by titrating 5×10^{-7} M 2-8-P with antiserum stock solutions and monitoring the fluorescence at the 480-nm emission maximum with excitation at 337 nm. The spectral bandpass for all measurements was 10 nm. A typical plot obtained is depicted in Fig. 1. A rabbit which had received 5 mg of immunogen produced the most desirable antisera and this was used for all future experiments.

The anti-P concentration was optimized, all other reagent concentrations being fixed. Into a cuvette were placed 2 ml of phosphate buffer (pH 7.5, I = 0.10 M) containing 5×10^{-7} M 2-8-P and 50 µl of normal human serum. Varying amounts of anti-P were added and the mixture was incubated for 10 min, after which 1 µl of anti-2-8 was added. Measurements were made after a further 10 min. A blank was subtracted to correct for variable amounts of background serum fluorescence; the blank consisted of the same mixture except that the buffer did not contain the labeled drug. A plot illustrating the effect of varying the anti-P concentration on the fluorescence signal is depicted in Fig. 2.

A similar procedure, with a fixed addition of 3 µl of anti-P stock solution and normal human serum spiked with phenytoin was used to produce a calibration graph. A typical plot is shown in Fig. 3.

Results and discussion

Six samples of normal human serum spiked with phenytoin (5—25 mg l^{-1}) were assayed 10 times on one day and 5 times on different days. Precision data, listed in Table 1, are good, demonstrating the effectiveness of the method. If more complete quenching of the long-wavelength emission had

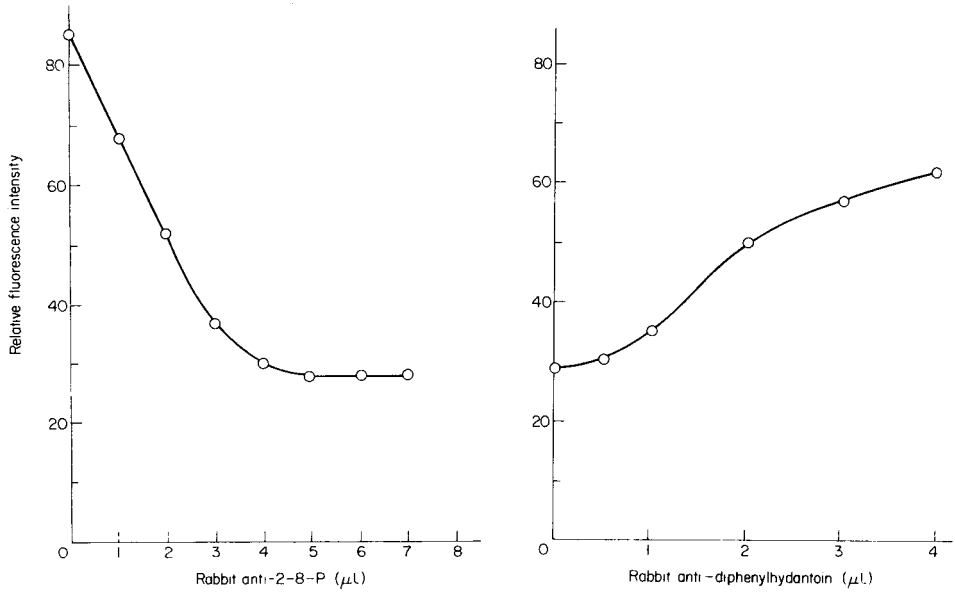


Fig. 1. Effect of rabbit anti-2-8-P on the 480-nm emission of 5×10^{-7} M 2-8-P in phosphate buffer (pH 7.5, I = 0.10 M).

Fig. 2. Effect of anti-phenytoin on the fluorescence intensity at 480 nm of 5×10^{-7} M 2-8-P.

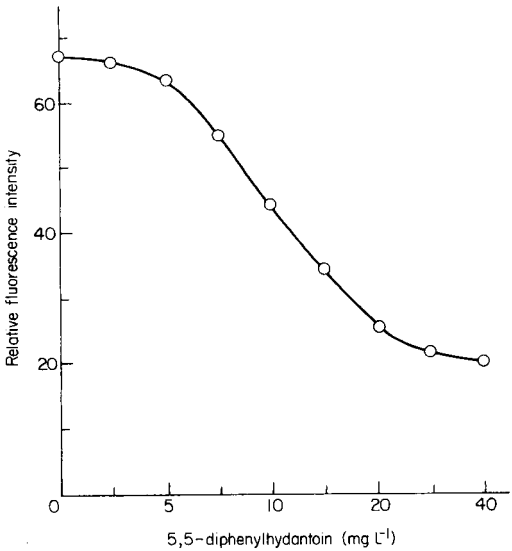


Fig. 3. Calibration graph for phenytoin.

TABLE 1

Precision data[a] for the assay of phenytoin added to normal serum

Phenytoin added (mg l^{-1})[b]	5	10	15	20	25
R.s.d. (%) within day	3.7	5.2	4.7	5.6	5.3
R.s.d. (%) between day	5.2	9.3	4.3	7.7	7.2

[a]Percent relative standard deviation (%) are given. [b]Final concentration.

been obtained, better sensitivity could have been gained. The mechanism by which the excited-state proton transfer of the label is inhibited upon binding to the 2-8-P antibody is uncertain. Probably, some of the label is bound by that fraction of the antibody population which specifically recognizes the region around the hydroxyl group of the label. That the label fluoresces, even when entirely sequestered by the antibody, must be due to the heterogeneity of the antibody population. A monoclonal antibody with a higher affinity for the hydroxy-containing portion of the label might completely inhibit proton transfer and so improve the assay.

It has been suggested that homogeneous fluorescence immunoassays of this type would be impossible because of the bridging effects of small molecules [18]. From the above work, this is clearly not the case for all labeled haptens. Subsequent to 2-8-P binding to antiphenytoin, the hydroxyl portion of the fluorophor must be oriented away from the bulk solution, making it unavailable for reaction with anti-2-8 antibodies and so allowing the immunoassay.

Support for this work was provided by a fellowship granted to Mr. O'Neal by the United States Pharmacopeial Convention.

REFERENCES

1 R. D. Nargessi, J. Landon and D. S. Smith, Clin. Chim. Acta, 89 (1978) 461.
2 R. F. Zuk, G. L. Rowley and E. F. Ullman, Clin. Chem., 25 (1979) 1554.
3 A. J. Portman, S. A. Levison and W. B. Dandliker, Biochem. Biophys. Res. Commun., 43 (1971) 207.
4 D. E. Lopatin and E. W. Voss, Biochemistry, 10 (1971) 208.
5 R. M. Watt and E. W. Voss, Immunochemistry, 14 (1977) 533.
6 H. Kutt and F. McDowell, J. Am. Med. Assoc., 203 (1968) 167.
7 H. Kutt and J. K. Penry, Arch. Neurol. (Chicago), 31 (1974) 283.
8 A. Richens, in H. Schneider, D. Janz, C. Gardiner-Thorpe, H. Meinardi and A. L. Sherwin (Eds.), Clinical Pharmacology of Anti-Epileptic Drugs, Springer, Berlin, 1975, p. 293.
9 P. M. Kabra, B. E. Stafford and L. J. Marton, Clin. Chem., 23 (1977) 1284.
10 J. W. Paxton, F. J. Rowell and J. G. Ratcliffe, J. Immunol. Methods, 10 (1976) 317.
11 H. E. Booker and B. A. Darcey, Clin. Chem., 21 (1975) 1766.
12 R. C. Wong, J. F. Burd, R. J. Carrico, R. T. Buckler, J. Thoma and R. C. Boguslaski, Clin. Chem., 25 (1979) 686.

13 R. S. Kamel, J. Landon and D. S. Smith, Clin. Chem., 26 (1980) 1281.
14 A. R. McGregor, J. O. Crookall-Greening, J. Landon and D. S. Smith, Clin. Chim. Acta, 83 (1978) 161.
15 M. J. Kurtz, M. Billings, T. Koh, G. Olander, T. Tyner, B. Weaver and L. Stone, Clin. Chem., 29 (1983) 1015.
16 J. S. O'Neal and S. G. Schulman, Anal. Chem., 56 (1984) 2888.
17 A. Johnstone and R. Thorpe, Immunochemistry in Practice, Blackwell, Oxford, 1982, p. 45.
18 R. D. Nargessi and J. Landon, in J. J. Langone and H. V. Vunakis (Eds.), Methods of Enzymology, Vol. 74, Academic Press, New York, 1981, p. 60.

Analytica Chimica Acta, 170 (1985) 149—152
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

Short Communication

RAPID EXTRACTION AND SAMPLE CLEAN-UP FOR THE FLUORESCENCE DENSITOMETRIC DETERMINATION OF AFLATOXIN M1 IN MILK AND MILK POWDER

J. BIJL* and C. VAN PETEGHEM

Laboratory of Food Analysis, Pharmaceutical Institute, State University of Ghent, Harelbekestraat 72, B-9000 Ghent (Belgium)

(Received 7th September 1984)

Summary. The diluted sample is passed through a SepPak C18 cartridge and the toxin is eluted with acetonitrile/water (3:7, v/v). The extract is cleaned up on a SepPak silica cartridge. The antidiagonal spot application technique is used for two-dimensional thin-layer chromatography. Spots are quantified by fluorescence densitometry. Recoveries of aflatoxin M1 added in the range 0.03—0.1 ng g^{-1} of milk are 86—97%. The detection limit is about 0.005 ng g^{-1} for milk and 0.05 ng g^{-1} for milk powder.

Aflatoxins, produced by certain fungi of the genera *Aspergillus* and *Penicillium*, occur naturally in a wide variety of foods. The most often encountered of the group, and usually in the highest concentrations, aflatoxin B1, is a recognized carcinogen. When B1 is ingested by cows, some (1—3%) of it is metabolized to M1 which may be excreted in the milk. Because M1 is also a potent carcinogen, the detection and determination of M1 in milk and milk products is of increasing interest. Published methods [1—3] are laborious, lack sensitivity, or cause problems by emulsion formation. In this communication, a more convenient sensitive method is described. The aflatoxin is extracted as described by Winterlin et al. [4], with a SepPak C18 column. For quick clean-up, a SepPak silica column is proposed. Finally, two-dimensional thin-layer chromatography (t.l.c.) based on the antidiagonal application technique [5, 6] is combined with fluorodensitometry. The resolving power is excellent.

Experimental

Apparatus and reagents. A Zeiss MQ-3 fluorodensitometer was used with a Minigrator integrator (Spectra-Physics). For extraction and clean-up, SepPak C18 and SepPak silica cartridges (Waters Associates) were applied. The t.l.c. plates were silica gel 60 (without fluorescence indicator; 10 × 10 cm; Merck).

Chemicals were of analytical grade. Acetonitrile was redistilled from glass apparatus. Water was twice-distilled. Diethyl ether was dried and made peroxide-free. The aflatoxin M1 standard was purchased from I.A.R.C. and checked spectrophotometrically.

0003-2670/85/$03.30

Extraction. Dilute 20 ml of milk or reconstituted milk with 30 ml of water. Prewash a SepPak C18 cartridge with 5 ml of acetonitrile and 5 ml of water successively, and pass the sample through at 4—5 ml min^{-1}. Rinse with 5 ml of water and 20 ml of acetonitrile/water (1:9, v/v) and discard the eluate. Elute with 4 ml of acetonitrile/water (3:7, v/v) into a centrifuge tube. Extract the eluate twice with 3 ml of dichloromethane on a Vortex mixer and centrifuge for 3 min at 4000 rpm. Combine the organic phases and evaporate the dichloromethane at 30° C under a gentle stream of nitrogen. Remove traces of water by adding 200 µl of toluene/ethanol (1:1) and evaporate. Dissolve the residue in 1 ml of dichloromethane.

Cleanup. Prewash the SepPak silica cartridge with 5 ml of dichloromethane. Apply the residue and rinse the tube twice with 1 ml of dichloromethane. Elute and discard the eluate. Wash the SepPak with 5 ml of diethyl ether and discard. Elute aflatoxin M1 with 8 ml of chloroform/acetone (4:1) into a test tube. Evaporate at 30° C under a gentle stream of nitrogen and dissolve the residue in 100 µl of benzene/acetonitrile (9:1).

Thin-layer chromatography. Apply standard solutions and an aliquot of the extract as in the antidiagonal technique (Fig. 1). Develop in the first direction with diethyl ether/methanol/water (95:4:1). Leave to dry for 10 min in a ventilated room under subdued light. Develop in the second direction with chloroform/acetone (70:30). Let dry, observe under 366-nm radiation and mark the aflatoxin M1 spots with a pencil.

Fluorescence densitometry. To quantify the aflatoxin M1, use an excitation wavelength of 365 nm (mercury line) with a 430-nm cut-off filter for

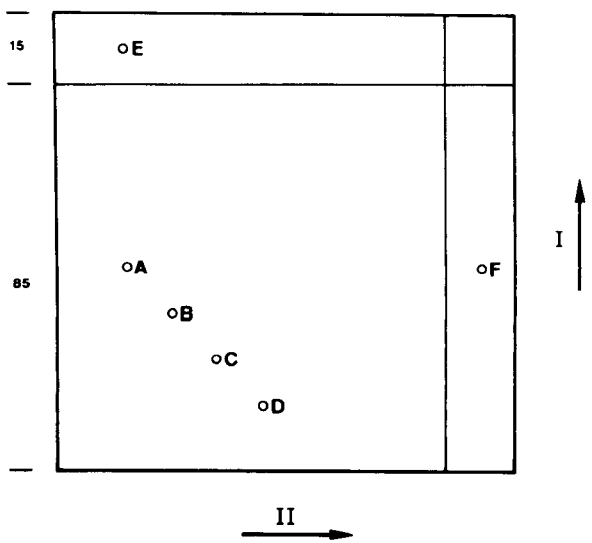


Fig. 1. Application of standards and extract. (A) Extract (20—50 µl); (B—F) standards (20 µl of different concentrations).

TABLE 1

Recovery and repeatability of the determination

Sample no.	Aflatoxin M1 (ng g^{-1})		Recovery (%)
	Added	Found[a]	
1	0.030	0.028 ± 0.002	97 ± 7
2	0.100	0.089 ± 0.002	89 ± 2

[a]Mean and standard deviation for 4 determinations.

the emission. Calculate the area automatically by means of the integrator. Calculate the concentration of the extract from the regression line of the standard concentrations versus areas.

Confirmation of identity. The identity of aflatoxin M1 was confirmed by derivative formation and rechromatography. To prepare the acetyl derivative [1], dry aliquots of the standard solution and of the extract solution under nitrogen. Add 50 µl of acetic acid anhydride and 50 µl of pyridine. Warm for 15 min at 50°C, dry under nitrogen and dissolve in 50 µl of benzene/acetonitrile (9:1). Apply in the classical way for two-dimensional t.l.c. and develop in the same solvent systems as above. To prepare the hemiacetal derivative (aflatoxin M2a) [3], proceed as for the acetyl derivative but add 100 µl of trifluoroacetic acid and 10 µl of water as reagents.

Results and discussion

The use of SepPak C18 cartridges for extraction allows very quick and easy isolation of aflatoxin M1, avoiding the often encountered emulsification. Solvent consumption is low and a fairly clean extract is obtained. In order to remove any remaining interferences, a fast clean-up on SepPak silica cartridges conveniently replaces the laborious methods on silica-gel columns.

The advantage of antidiagonal spot application over the classical procedure for two-dimensional t.l.c. is that both the standards and the extract are developed in the two directions; thus all spots become similarly diffuse. As the standard and extract spots are situated close to each other, even visual comparison can provide acceptable precision.

Overall recovery and repeatability for samples spiked at 0.03 ng g^{-1} and 0.1 ng g^{-1} of milk are summarized in Table 1. The detection limit is about 0.005 ng g^{-1} of milk and 0.05 ng g^{-1} of milk powder.

This work was supported by the N.F.W.O. Krediet aan Navorser to C.V.P. and by F.G.W.O. grant 3.0033.83.

REFERENCES

1 Official Methods of Analysis, 13th Edn., AOAC, Arlington, VA, 1980, sections 26.090—26.095.

2 H. Cohen, M. Lapointe and J. M. Fremy, J. Assoc. Off. Anal. Chem., 67 (1984) 49.
3 R. M. Beebe and D. M. Takahashi, J. Agric. Food Chem., 28 (1980) 481.
4 W. Winterlin, G. Hall and D. Hsieh, Anal. Chem., 51 (1979) 1873.
5 P. Beljaars, C. Verhulsdonk, W. Paulsch and D. Liem, J. Assoc. Off. Anal. Chem., 56 (1973) 1444.
6 I. Hais, J. Chromatogr., 48 (1970) 200.

Analytica Chimica Acta, 170 (1985) 153—158
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

Short Communication

THE IDENTIFICATION OF FLUNITRAZEPAM AND ITS METABOLITES IN URINE SAMPLES

H. H. VAN ROOIJ*, A. FAKIERA, R. VERRIJK and W. SOUDIJN

University of Amsterdam, Division of Pharmaceutical Chemistry, Plantage Muidergracht 24, 1018 TV Amsterdam (The Netherlands)

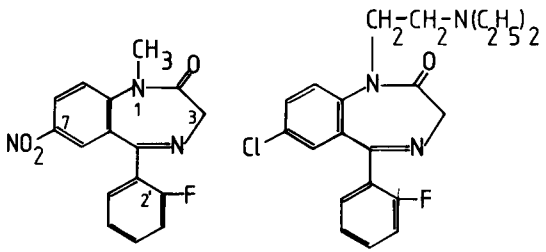
J. P. WEIJERS-EVERHARD

Laboratorium Jellinek Centrum, Sarphatistraat 75, 1018 EX Amsterdam (The Netherlands)

(Received 26th September 1984)

Summary. A thin-layer chromatographic method with fluorescence detection is described for the identification of flunitrazepam and its metabolites in urine. The method is based on the hydrolysis and subsequent formation of acridine derivatives by cyclization in dimethylformamide and is selective for benzodiazepines with a 2-fluorophenyl group. The method is applicable to urine samples even after an oral dose of only 2 mg of flunitrazepam. Flunitrazepam and flurazepam consumption can be distinguished.

Flunitrazepam [I; Rohypnol; 5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H,1,4-benzodiazepin-2-one] is used as a hypnotic (dose 1—2 mg) and also in pre-medication for anaesthesia. Abuse of benzodiazepines by drug



I II

addicts has been described [1, 2]; among the different benzodiazepines, flunitrazepam is popular among the addicts in Amsterdam.

A chemical method of establishing the extent of abuse by analysing urine samples from these patients is often hindered by the presence of other drugs and their metabolites. Moreover, flunitrazepam is rapidly transformed and subsequently excreted in conjugate products [3]. Among the analytical methods available for flunitrazepam and/or its metabolites in biological samples are gas-liquid chromatography with electrochemical (g.c./e.c.) [4], (radio)-immunological methods [5] and high-performance liquid

0003-2670/85/$03.30

chromatography (h.p.l.c.) with u.v. detection [6, 7]. However, g.c./e.c. is frequently designed for pharmacokinetic studies, and commercially available immunological methods do not discriminate between benzodiazepines. The h.p.l.c. methods are probably not selective or sensitive enough for the compounds under investigation in urine [5]. However, benzodiazepines with a fluorine atom substituted at the 2-position in the benzene ring are easily converted to highly fluorescent acridine derivatives through the benzophenones obtained after hydrolysis, as has been described for flurazepam (II) [8].

The present communication describes the optimization and application of this method for the identification of flunitrazepam and its metabolites in urine samples from drug addicts after thin-layer chromatography (t.l.c.).

Experimental

Apparatus. A thin-layer scanner (Zeiss PMQ-II; Zeiss, Oberkochen, F.R.G.) equipped with a mercury light source. Mass spectra were recorded by the Organic Chemistry Department, using electron impact or field desorption as the ionization technique.

Drugs and chemicals. All chemicals were of analytical grade (Merck). Flurazepam, flunitrazepam and the metabolites of flunitrazepam were kindly donated by Hoffman LaRoche (Basle, Switzerland).

Procedures. Urine samples were stored at −18° C. In the experiments to establish the nature of the metabolites, the samples were first either heated at 60° C with glucuronidase/arylsulfatase (Helix Pomatia; Merck) for 1 h and subsequently extracted with two 5-ml portions of ethyl acetate at pH 10 or just extracted without applying the enzymatic treatment. The residue obtained after the organic extract had been evaporated with a gentle stream of nitrogen at 50° C was further treated as described below.

The residues obtained as described above, or 5-ml portions of thawed urine samples, were heated with 5 ml of 6 M hydrochloric acid for 30 min at 100° C; 5 min before the end of the reaction a tin pellet was added. The reaction was stopped by cooling in ice and mixture was carefully adjusted to pH 10 with 10 M sodium hydroxide (bromothymol blue indicator solution). The mixture was extracted twice with 5-ml portions of ethyl acetate. The combined organic extracts were then evaporated on a water bath at 50° C with a gentle stream of nitrogen. The residue was dissolved in 1 ml of a saturated solution of sodium nitrite in dimethylformamide and transferred to a reaction vial, which was then firmly closed with an aluminium cap. The vials were placed in an oil bath at 160° C for 60 min.

For t.l.c., 1-μl aliquots of the reaction mixture were spotted. In the case of unknown samples, the reaction mixture was evaporated to dryness at 50° C in a vacuum oven in order to remove traces of dimethylformamide. The residue obtained was dissolved in methanol and subjected to t.l.c.

Silica-gel plates without a fluorescence indicator (Merck) were used. Chromatography tanks were saturated with solvent by using filter paper

lined against the wall. Solvent A consisted of chloroform and acetone (85:15) and solvent B contained ethyl acetate/ethanol/ammonia (100:10:3). The plates were inspected under u.v. radiation (254 nm and 365 nm) or scanned with the thin-layer scanner at an excitation wavelength of 365 nm and an emission wavelength of 445 nm.

Results and discussion

The development of a sensitive qualitative urine test for flunitrazepam taken by patients and/or drug addicts is made difficult by the many possible metabolic transformations of flunitrazepam [3]. Reduction of the nitro group, followed by acetylation, hydroxylation at the 3-position followed by conjugation with, for example, glucuronic acid, and demethylation at the nitrogen atom may occur in all possible combinations (see Fig. 1). Accordingly, many different metabolites may be present in urine in low concentrations; further, the dosage of flunitrazepam is usually low (<10 mg). However, by making use of the chemical properties of that part of the molecule common to all metabolites, the sensitivity requirements can be made less stringent.

The method optimized here for flunitrazepam and its metabolites is based on a method developed for the identification of flurazepam (II) in biological samples [8]. The benzodiazepines are hydrolysed to benzophenones (see Fig. 1) by boiling with 6 M hydrochloric acid; these benzophenones are extracted and converted to highly fluorescent acridine derivatives [9] by utilizing the active fluorine atom in the benzene ring [10].

The hydrolysis of flunitrazepam and its metabolites appeared to be complete within 30 min. As the hydrolysis is done at low pH, the reduction of the aromatic nitro group by tin was investigated. Reduction of the nitro group increases the concentration of the amino-containing metabolites of flunitrazepam and therefore the overall sensitivity. This reduction seemed to

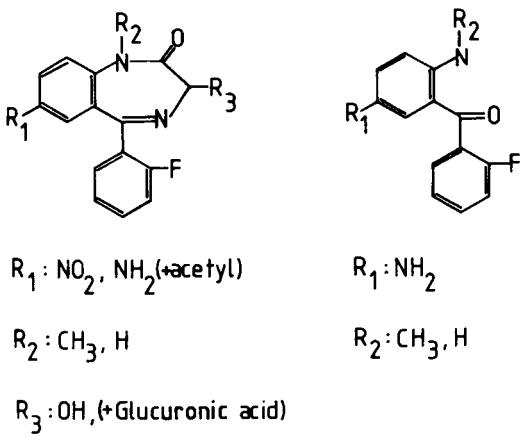
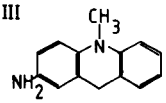


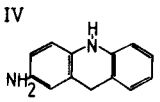
Fig. 1. Left: Structural formulae of metabolites of flunitrazepam (I). Right: Structural formulae of benzophenones derived from the metabolites of (I) by hydrolysis.

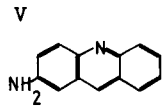
take place within 5 min. A longer reduction time also produced other compounds, which were not identified.

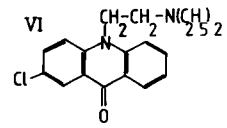
Thin-layer chromatography showed that the benzophenones derived from the flunitrazepam derivatives had fluorescent properties in situ after several hours, probably because of oxidation of the aromatic amine part. However, this fluorescence was not reproducible and had a low sensitivity. Only $\geqslant$80 ng applied on the t.l.c. plate could be detected visually; this was not improved by spraying oxidizing reagents.

Acridine derivatives are formed from 2-fluoro-substituted benzophenones by refluxing in dimethylformamide with a base as catalyst [10]. Anhydrous potassium carbonate was used as the base for flurazepam, but sodium nitrite was preferred here in order to prevent oxidation of the formed aminoacridines in the following extraction procedure. The formation of acridine derivatives from flunitrazepam and its metabolites was found to be reproducible and reasonably efficient only if the reaction temperature was at least 160° C, in contrast to the temperature needed for conversion of flurazepam (100°).

III 

IV 

V 

VI 

The structure of the acridine derivatives formed was established by t.l.c. and by mass spectrometry based on field-desorption or electron-impact ionization. From these measurements, it was concluded that flunitrazepam, having an *N*-methyl group, was converted to aminomethylacridan (III), while desmethylflunitrazepam was converted to aminoacridan (IV) and/or aminoacridine (V). Compounds IV and V could not be separated by t.l.c. and the ionization technique used for mass spectrometry interfered with an exact mass determination. Flurazepam, however, was completely converted to the corresponding chloracridanone derivative (VI) at 160° or 100° C, regardless of the base catalyst used.

After the condensation, the derivatives were extracted and the extracts were evaporated to dryness, as it was necessary to remove traces of dimethylformamide, which interfered with the t.l.c. procedure as well as with the fluorescence detection, especially when low amounts of the acridine derivatives were concerned. The t.l.c. procedure was examined with the two solvent systems previously described for flurazepam derivatives [8]; these made it possible to discriminate between flurazepam and flunitrazepam intake by patients.

The limit of detection of the fluorescence was 5—10 ng of compound IV with visual detection; 0.5 ng could be detected with a signal/(peak-to-peak) noise ratio of 3 when the thin-layer scanner was used. The fluorescence discrimination could be enhanced by spraying with 50% sulfuric acid in ethanol (Table 1).

The sensitivity of the method was demonstrated by the analysis of a urine sample containing metabolites of flunitrazepam after an oral dose of 2 mg

TABLE 1

T.l.c. and fluorescence data for the acridine derivatives

Compound	R_F value				Fluorescence with λ_{ex} = 365 nm	
	System A		System B			$+H_2SO_4$
	Found	Lit. [8]	Found	Lit. [8]		
III	56	—	62	—	Blue/445 nm	Blue-green/473 nm
IV/V	38	—	62	—	Blue/443 nm	Blue-green/460 nm
VI	12	15	32	45	Blue/445 nm	Blue-green/478 nm

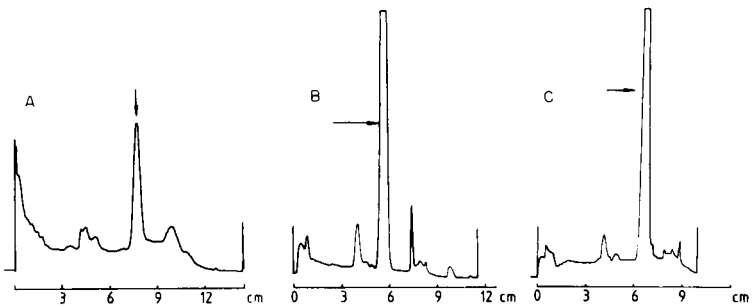


Fig. 2. T.l.c. scans of urine extracts: (A) urine extract from volunteer (dose, 2 mg of flunitrazepam); (B) urine extract of patient 44 (solvent A); (C) same extract with solvent B.

had been taken by a volunteer. In the first urine portion (300 ml) collected in the morning, compound IV or V could be detected. A t.l.c. scan of this sample is shown in Fig. 2. Obviously demethylation of flunitrazepam had taken place in this volunteer. However, in the urine of a patient who had taken six tablets (12 mg), only compound III could be detected (Fig. 2). The identity of this compound was confirmed by field-desorption mass spectrometry after the compound had been eluted with methanol from the t.l.c. plate.

The nature of the metabolites was further characterized by enzymatic hydrolysis and extraction prior to the actual procedure. After an oral dose of flunitrazepam, 10% of the metabolites present in urine are in a lipid-soluble form, 10% are conjugated to glucuronic acid and 80% are probably present as the hydrophilic acetamino derivative. In addition, no flunitrazepam or a nitro-containing metabolite could be detected in urine samples, which eliminates the need for a reduction step.

The method developed can selectively detect benzodiazepines with a 2-fluorophenyl group in urine samples and can also discriminate between flunitrazepam and flurazepam intake. The method also provides qualitative information on the metabolic products of flunitrazepam excreted in urine, and has been applied successfully to urine samples of volunteers and drug addicts.

REFERENCES

1 M. L. Stitzer, R. R. Griffiths, A. T. McLellan, J. Grabovski and J. W. Hawthorne, Drug Alcohol Dependence, 8 (1981) 189.
2 R. R. Griffiths, D. R. McLeod, G. E. Bigelow, I. A. Liebson, J. D. Roache and P. Nowowieski, J. Pharmacol. Exp. Ther., 229 (1984) 501.
3 J. P. Cano, J. Guintrand, C. Aubert and A. Viala, Arzneim.-Forsch., 27 (1977) 338.
4 R. Jochemsen and D. D. Breimer, J. Chromatogr., 227 (1982) 199.
5 R. H. Drost, T. A. Plomp and R. A. Maes, J. Toxicol. Clin. Toxicol., 19 (1982) 303.
6 T. B. Vree, B. Lenselink, E. van der Kleijn and G. M. M. Nijhuis, J. Chromatogr., 143 (1977) 530.
7 K. Harzer and R. Barchet, J. Chromatogr., 132 (1977) 83.
8 J. A. F. deSilva and N. Strojny, J. Pharm. Sci., 60 (1971) 1303.
9 G. Weber and F. W. Teale, Trans. Faraday Soc., 53 (1957) 646.
10 R. I. Fryer, J. Earley and L. H. Sternbach, J. Chem. Soc., (1963) 4979.

(Continued from outside back cover)

0003-2670/85/$03.30

Printed in The Netherlands

CONTENTS

(Abstracted, Indexed in: Anal. Abstr.; Biol. Abstr.; Chem. Abstr.; Curr. Contents Phys. Chem. Earth Sci.; Life Sci.; Index Med.; Mass Spectrom. Bull.; Sci. Citation Index; Excerpta Med.)

(Continued on inside back cov