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Application of synchronous luminescence to the separate determination of cochromatographing metabolites of the carcinogen, 7-methylbenz[*c*]acridine

J. H. GILL and G. M. HOLDER*

Department of Pharmacy, University of Sydney, New South Wales 2006, Australia

Abstract: A synchronous fluorescence assay for the cochromatographing metabolites of 7-methylbenz[c]acridine, 7-hydroxymethylbenz[c]acridine and *trans*-7-methylbenz[c]-acridine-10,11-dihydrodiol is described and applied to the analysis of these metabolites formed by rat liver microsomes.

Keywords: *Synchronous fluorescence; 7-methylbenz[c]acridine.*

Introduction

The technique of synchronous luminescence, which was first described by Lloyd [1] and has also been called synchronous fluorescence or synchronous excitation spectrofluorimetry, has been applied to multicomponent analytical problems [2,3], to the analysis of oil [4] and polycyclic aromatic hydrocarbons [2, 3], and to forensic problems [5]. It offers high sensitivity, and by selection of appropriate $\Delta\lambda$ ($\lambda_{em}-\lambda_{exc}$) values, it offers selectivity. This selectivity is shown with mixtures of polycyclic aromatic hydrocarbons which exhibit broad complex emission spectra, while synchronous luminescence spectra of the same mixtures show sharp peaks corresponding to the individual components [2, 3].

The carcinogen, 7-methylbenz[c]acridine (7MBAC), has been the subject of extensive metabolic studies from this laboratory directed towards the identification of its proximate and ultimate carcinogenic species. Quantitative studies involved the use of gradient reverse-phase HPLC to separate metabolites which were then assayed radiochemically [6]. Amongst the principal metabolites formed in liver and lung preparations were 7-hydroxymethylbenz[c]acridine (7HOMBAC), *trans*-8,9-dihydro-8,9-dihydroxy-7-methylbenz[c]acridine and its 5,6-isomer [7]. Later, studies by gas chromatography–mass spectrometry (GC–MS) indicated that the 7HOMBAC chroma-

*To whom all correspondence should be addressed.

togram peak was not homogeneous, and that it also contained *trans*-10,11-dihydro-10,11-dihydroxy-7-methylbenz[c]acridine (7MBAC-10,11-DHD) [8]. Insofar as the proximate carcinogen may be a minor metabolite, it was important to quantitate the extent of formation of both 7HOMBAC and 7MBAC-10,11-DHD which were previously measured together [8, 9]. This is especially relevant since both compounds have some activity in mutagenicity tests [13]. This communication reports the development of a synchronous luminescence assay for each compound without their separation, and results obtained with rat liver microsomal metabolism of 7MBAC.

Experimental

Chemicals

7MBAC [10], 7HOMBAC [11] and 7MBAC-10,11-DHD [12] were synthetically available. [^{3}H]-7MBAC was prepared by catalytic reduction of 7-bromomethylbenz[c]-acridine with tritium gas by Amersham Ltd, Bucks, U.K. Biochemicals were obtained from Sigma Chemical Co., St Louis, MO, U.S.A., and Boehringer-Mannheim, Sydney, Australia. HPLC grade methanol was used throughout the study.

Spectroscopy and analysis

Fluorescence spectra were recorded in the corrected mode with a Perkin–Elmer MPF-44B fluorescence spectrophotometer fitted with a DCSU accessory in methanol or 56% v/v methanol in water, which was the solvent in which the analytes were eluted from the HPLC column. Emission and excitation spectra of pure compounds were recorded with slit widths of 2 nm. For synchronous spectra the excitation and emission monochromator slit widths were 2 and 10 nm respectively. The 7MBAC metabolites were separated by HPLC using a methanol–water gradient, and an RP-8 reverse-phase column (Brownlee Laboratories, Santa Clara, CA, U.S.A.) [6]. The metabolite fraction which chromatographed with retention time identical with those of 7HOMBAC and 7MBAC-10,11-DHD was adjusted to 5.0 ml with 56% v/v methanol–water, and a portion was removed for radioactivity determination [6]. Synchronous fluorescence spectra were recorded from 300 nm (emission) with $\Delta\lambda$ = 55 nm, and the intensities of peaks at 445 and 368 nm were used to determine the concentrations of 7HOMBAC and 7MBAC-10,11-DHD respectively. Standard curves were prepared for each compound, both in the absence and presence of the other analyte, and were corrected by use of a solvent blank.

Animals and metabolism

Groups of six male Wistar rats were pretreated intraperitoneally with the enzyme-inducing agent 7MBAC (20 mg/kg) in corn oil for 2 days, or were untreated. After the rats were sacrificed, livers of pretreated and untreated animals were removed, and microsomes were prepared [8]. Microsomal metabolism of [^{3}H]-7MBAC (40 μM) was conducted in 1.0 ml volumes such that about 20% conversion to metabolites occurred [8]. The total ethyl acetate-soluble radioactivity (metabolites and unchanged 7MBAC) was applied to the HPLC column and metabolite distributions were determined radiochemically by liquid scintillation counting of a small aliquot of each chromatographic fraction [6]. This allowed the proportion of metabolites cochromatographing with 7HOMBAC and 7MBAC-10,11-DHD to be calculated. The proportion of each metabolite in these chromatographic fractions was measured by synchronous fluorescence after adjusting the volume to 5.0 ml as described above.

Results and Discussion

The 7MBAC metabolite peak eluting from the reverse-phase column with a retention time identical to that of the synthetic 7HOMBAC and 7MBAC-10,11-DHD was shown by GCMS to contain 7HOMBAC and a minor amount of the 10,11-dihydrodiol. Separate determination of the two compounds within the HPLC fraction was achieved without further chromatography by exploiting the technique of synchronous fluorescence.

The most suitable $\Delta\lambda$ values for the assay were investigated by reference to the fluorescence excitation and emission spectra (Fig. 1) of the pure analytes. Figure 2 presents synchronous luminescence spectra for both 7HOMBAC and 7MBAC-10,11-DHD and shows the relative intensity of the signal for a fixed concentration of each analyte as $\Delta\lambda$ was varied. A $\Delta\lambda$ that maximized sensitivity for the minor component, 7MBAC-10,11-DHD, and that gave peaks for each component which were unaffected by the presence of the other was desired.

The choice of $\Delta\lambda$ = 89 nm which matched the excitation and emission λ_{max} of 7MBAC-10,11-DHD of 279 and 368 nm, respectively, and would have been most sensitive for this compound, was not suitable because under these conditions the detectability of the 7HOMBAC was compromised. A $\Delta\lambda$ of 67 nm which matched the emission λ_{max} of 7MBAC-10,11-DHD with the smaller excitation maximum at 301 nm would have been suitable, but a $\Delta\lambda$ of 55 nm was chosen because this presented the best compromise between 7MBAC-10,11-DHD and 7HOMBAC detection in the mixtures to be analysed. Under these conditions the quantification could be carried out using values of 368 and 445 nm for 7MBAC-10,11-DHD and 7HOMBAC measurement respectively.

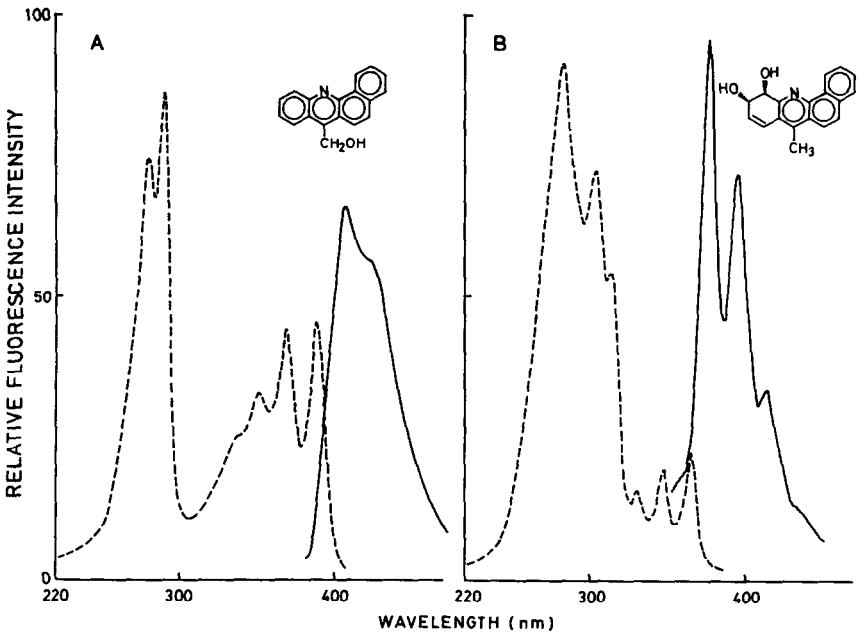

Figure 1
Excitation (– – –) and emission (———) spectra of 7HOMBAC (A) and 7MBAC-10,11-DHD (B).

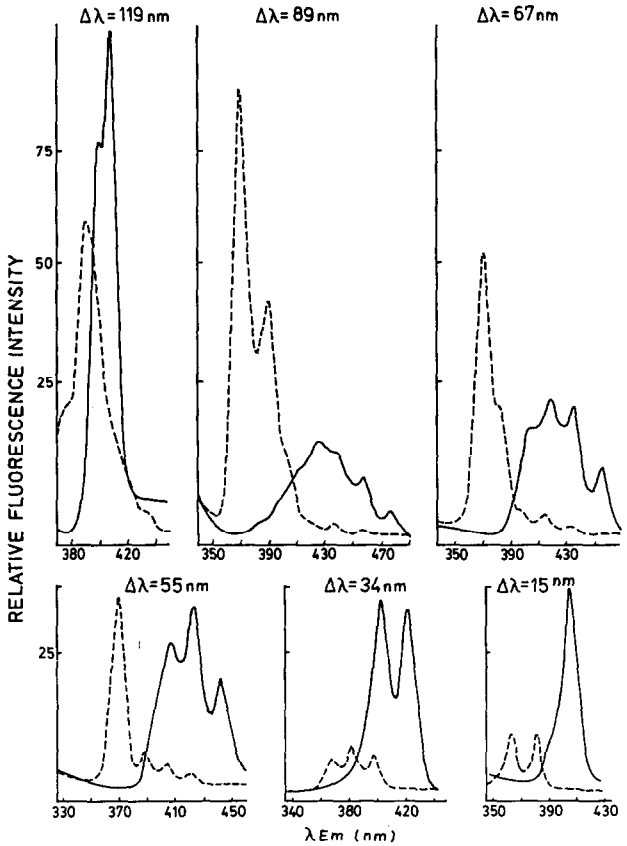

Figure 2
Synchronous luminescence spectra of 8.3×10^{-8}M 7HOMBAC (———) and 3.3×10^{-8}M 7MBAC-10,11-DHD (– – –) recorded at various Δλ values. For all scans the spectrofluorimeter slit widths, range and sensitivity controls were unaltered.

There was limited overlap of the synchronous luminescence signals of the two components at $\Delta\lambda = 55$ nm, but no interference at the two wavelengths chosen for the analysis. The standard curve was linear for $0.2–20 \times 10^{-7}$M 7HOMBAC ($r^2 = 0.9997$, slope $= 2.72 \times 10^{7}M^{-1}$, y intercept $= -0.112$), and the presence of a range of 7MBAC-10,11-DHD concentrations ($1.13–11.3 \times 10^{-7}$M) did not alter the relative fluorescence intensity of 1.71×10^{-8}M 7HOMBAC. For 7MBAC-10,11-DHD the standard curve was linear from 0.4 to 60×10^{-8}M ($r^2 = 0.9993$, slope $= 1.08 \times 10^{8}M^{-1}$, y intercept $=$ 0.281), and the presence of 7HOMBAC ($1.71–42.8 \times 10^{-8}$M) did not alter the relative fluorescence intensity of 5.63×10^{-8}M 7MBAC-10,11-DHD. Linearity of the fluorescent response with concentration was also obtained in the presence of other analyte.

When repeated analyses were performed for 7MBAC metabolites from untreated rats, 7MBAC-10,11-DHD was found to constitute about 13% of the metabolites in the chromatographic fraction which eluted with the same retention time as that of 7HOMBAC and 7MBAC-10,11-DHD (Table 1). When microsomes which had been prepared from rats induced with 7MBAC were used, 7MBAC-10,11-DHD constituted

Table 1
7HOMBAC and 7MBAC-10,11-DHD metabolites of ^{3}H-7MBAC formed by liver microsomes prepared from untreated and 7MBAC-induced rats

	Untreated	Induced
7HOMBAC concentration (range × 10^7M)	1.17 – 2.98†	1.15 – 1.84
Percent 7HOMBAC*	22.5 ± 1.9†	16.3 ± 2.8
7MBAC-10,11-DHD concentration (range × 10^8M)	1.57 – 4.63	2.34 – 3.49
Percent 7MBAC-10,11-DHD*	3.5 ± 1.0	3.1 ± 0.5

Total metabolites were separated by HPLC [6], and those fractions which contained 7HOMBAC and 7MBAC-10,11-DHD were combined and assayed by synchronous luminescence as described in the Methods.

*Expressed as a proportion of total metabolites formed during the incubations.

†The range or mean ± S.E. for three batches of liver microsomes is shown.

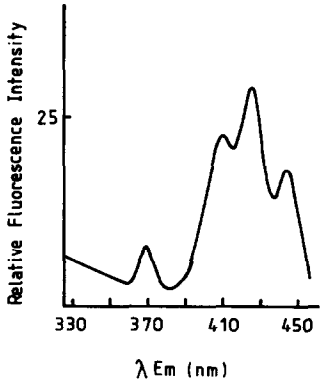

Figure 3
The synchronous luminescence spectrum ($\Delta\lambda$ = 55 nm) of a mixed 7HOMBAC and 7MBAC-10,11-DHD fraction obtained by HPLC of ^{3}H-7MBAC metabolites from liver microsomes of untreated rats. Concentrations determined in this experiment were 1.81×10^{-7}M for 7HOMBAC and 1.57×10^{-8}M for 7MBAC-10,11-DHD.

about 16%. Figure 3 shows the synchronous luminescence of the mixed metabolites obtained from the HPLC separation of metabolites formed by liver microsomes of untreated rats. Results presented in Table 1 show the range of analyte concentrations encountered in the metabolic work and the fraction of each analyte expressed as a percentage of the total metabolites formed in the incubations. The range of concentrations found for induced and untreated rat liver microsomes is wide because varying amounts of radioactivity were applied to the HPLC column.

The assay was very simple to perform and is highly sensitive, allowing concentrations of 7MBAC-10,11-DHD down to 0.4×10^{-8}M to be detected. This is equivalent to 20 pmol (or 5.5 ng) of 7MBAC-10,11-DHD recovered from the HPLC column. The assay for 7HOMBAC was not optimized since it was known that this was the major metabolite. A $\Delta\lambda$ of 119 nm matching the major excitation and emission bands of this compound would be most sensitive, but this would not be selective in the presence of 7MBAC-10,11-DHD (Fig. 2). A $\Delta\lambda$ of 34 or 15 nm would both improve the sensitivity to

7HOMBAC approximately two-fold over levels detectable at λ_{em} 445 nm using $\Delta\lambda$ = 55 nm if quantitation were carried out at Δ_{em} 420 or 402 nm respectively. However, a $\Delta\lambda$ of 15 nm allows greater selectivity and greater sensitivity to 7MBAC-10,11-DHD.

The use of synchronous luminescence allows very sensitive detection of polycyclic azaaromatic compounds and their metabolites, and should have application for other carcinogens such as polycyclic aromatic hydrocarbons, naphthylamines and aflatoxins.

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Note added in proof: Since the acceptance of this manuscript an application of synchronous luminescence to the direct estimation of benzo[a]pyrenediolepoxide–DNA adducts in modified DNA to the level of 1 adduct per 1.4×10^7 nucleotides has appeared (K. Vahakangas, A. Haugen and C. C. Harris, *Carcinogenesis* **6**, 1109–1116 (1985)).