Journal of Pharmaceutical & Biomedical Analysis
Vol. 6, Nos 6–8, pp. 843–852, 1988
Printed in Great Britain

0731–7085/88 $3.00 + 0.00

Digital techniques for luminescence detection in liquid chromatography with an intensified linear photodiode array*

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Abstract: The use of a rapid-scanning fluorescence detector in liquid chromatography is examined in the context of its potential contribution in pharmaceutical and biomedical analysis. The data generated by an intensified linear photodiode array detector is presented as an isometric plot of (I_{em},λ_{em},t) at a defined excitation wavelength. Techniques examined for peak homogeneity assessment include: emission spectral normalisation at points through the chromatographic peak profile, second order differentiation (d^2I/dt^2) and the fluorescence emission ratio chromatogram, generated by calculating the ratio of emission intensities at two defined emission wavelengths, at all points in the time domain elution profile. These techniques are illustrated with reference to some polynuclear aromatic hydrocarbons and to the β-blocker drug atenolol and its related impurities. Future developments of this new detector technology in LC are also considered.

Keywords: *HPLC; multichannel luminescence detectors; intensified linear photodiode array; chromatographic peak purity assessment; atenolol; polynuclear aromatic hydrocarbons.*

Introduction

Since the first integral commercial UV-visible multichannel spectrophotometer was introduced in 1979, multichannel technology has made a considerable contribution in pharmaceutical analysis and development laboratories [1]. Spectrophotometric instrumentation almost exclusively based on the linear photodiode array (LDA) has continued to expand and exploit the advantages of rapid spectral scanning capability, particularly when associated with time dependent studies [2, 3].

However, it is in the domain of liquid chromatography (LC) that LDA technology has produced its major impact [4]. This is particularly the situation in pharmaceutical analysis where complex multicomponent mixtures are often found for drugs and their degradation products and synthetic route precursors, or in the field of quality control, where the additional dimension of wavelength presents the analyst with spectral

*Presented at the "International Symposium on Pharmaceutical Biomedical Analysis", September 1987, Barcelona, Spain.

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information that can be useful for solute identification, especially when stored in a matrix of response, wavelength and time data, with the support of a microcomputer [5].

Absorptiometric detectors have dominated over this period of rapid development, although a number of elegant applications of multichannel luminescence detectors have been reported, in particular by Warner's group, based on the vidicon [6, 7] and on the LDA [8]. However, these applications were carried out essentially on research equipment of modular design. Only recently has the first complete commercial unit for rapid scanning luminescence detection emerged in the market place. This instrumentation, comprising an intensified linear photodiode array spectrofluorimetric detector, opens up several new possibilities for enhanced detection sensitivity and selectivity for fluorogenic solutes. The capability of rapidly scanning the emission spectrum during elution allows the generation of a matrix of (I_{em},λ_{em},t) data, albeit at a fixed excitation wavelength, λ_{ex}. This large data set can then be usefully exploited through the use of digital algorithms similar to those well characterised in absorptiometric detection [9]. Initially the total data matrix can be presented as the contour plot or as an isometric projection generated at any suitable viewing angle. Suitable elution profiles can be extracted at any wavelength (λ_{em}) from the matrix together with emission spectra at any elution time (t). The combination of emission spectra and retention data provides a powerful data set, not only for direct solute identification, but also for assessment of chromatographic peak purity. This latter aspect is of particular interest to the pharmaceutical analyst and can be approached using a number of digital techniques.

In its simplest form, interrogation of the chromatographic peak can be achieved by capturing and normalising spectral data throughout the elution profile. An indication of peak purity can then be presented, provided that sufficient differences exist in the emission spectra of any coeluting components. This proviso also applies to the method of fluorescence emission ratioing, where the fluorescence intensity at defined wavelength pairs is ratioed as a function of time at a fixed λ_{ex}. A further technique for peak homogeneity validation is second order differentiation of the peak profile as a function of time (d^2I/dt^2), which has the added advantage that overlapping peaks can be resolved, and also quantitative information can be obtained [10]. The utility of these digital techniques in pharmaceutical analysis is reported by examining the impurity profile of the β-adrenoceptor blocking drug atenolol, in terms of its possible synthetic route precursors and degradation products and of some polynuclear aromatic hydrocarbons (PNAH) of toxicological interest.

Experimental

Equipment

The Tracor Northern F-100 intensified linear photodiode array detector (Tracor Northern Inc., WI, USA) consisted of a xenon pulsed-source optical unit in which selectable emission gratings are possible giving 150, 300 and 600 nm emission wavelength ranges. Slit widths are also selectable from 50 to 1000 μm, with a typical resolution of 3.6 nm given when using a 50 μm slit and 300 nm grating: excitation wavelength is governed by a manually adjusted grating capable of a wavelength range of 200–900 nm. Provided between the optical unit and the detector array is an image intensifier designed to amplify low level luminescence signals by means of a photocathode and microchannel plate. The detector comprises of a 512 element array cooled to −5°C to improve signal-to-noise levels.

The optical unit and detector are interfaced to an optical multichannel computing analyser by an RS 232C link. A 512 Kbyte resident data memory is provided with the capability of data storage on a 5.25-inch floppy disk drive. The main operating algorithms run in a lattice "C" environment with plotting routines in MS-DOS. Plotting was facilitated by a Model 7440A Hewlett–Packard (Waldbronn, FRG), "Colorpro", 8 pen plotter.

The liquid chromatographic system consisted of a dual reciprocating pump (LDC/Milton Roy, Model 3000; FL, USA) and a Rheodyne injection valve (Model 7125; Rheodyne, Berkeley, CA, USA) provided with a 50-μl loop.

LC procedure

Atenolol. A 200 × 4.6 mm i.d. stainless-steel column packed with 5-μm ODS-Hypersil (Shandon Southern Instruments, Cheshire, UK) was used, the mobile phase being methanol ≈10% v/v sulphuric acid with 0.166% w/v sodium *n*-octyl sulphate (40:60, v/v). A flow rate of 1.0 ml min^{-1} was used with an excitation wavelength of 270 nm. The HPLC method used is that recommended in a draft monograph from the British Pharmacopoeia Commission for atenolol and related substances.

Polynuclear aromatic hydrocarbons. A stainless steel column (100 × 4 mm) was packed with 3-μm ODS-Spherisorb (Phase Separations Ltd., Clwyd, UK). The mobile phase was methanol–water (70:30, v/v), the flow rate being 1.0 ml min^{-1} and injection volume 20 μl.

Reagents and materials

A British Pharmacopoeia Commission impurity reference standard was obtained containing the synthetic route precursors: 4-hydroxyphenylacetamide (PPA); *p,p*-[*N*-isopropyl-3,3-iminobis(2-hydroxypropoxy)]bis(phenylacetamide) (tertiary amine); and also containing degradation products: 1,3-bis(4-carbamoylmethoxyphenoxy)propan-2-ol (bis ether); *p*-2,3-dihydroxypropoxy-phenylacetamide (diol); 4-(2-hydroxy-3-isopropylaminopropoxy)phenylacetic acid (blocker acid). The individual impurities and the parent compound atenolol were dissolved in methanol (HPLC grade; Rathburn Chemicals, Walkerburn, UK) to give a solution of atenolol at 1 mg ml^{-1} in methanol and impurity levels less than 1.0% w/w. Standard solutions of the impurities were prepared as 10 μg ml^{-1} in methanol and diluted appropriately.

The polynuclear hydrocarbons consisted of carbazole, fluorene, anthracene and fluoranthene (Aldrich Chemical Co., Dorset, UK), prepared in methanol at concentrations of 8–100 ng ml^{-1}.

Results and Discussion

The model system of degradation products and synthetic route precursors of atenolol represents a classical problem in pharmaceutical analysis, involving the detection and quantitation of trace levels in the presence of major components. Moreover these assays can be further complicated when the methods are extended to pharmaceutical preparations and to samples of biomedical origin. In these cases interferences can accrue from the formulation matrix or, when metabolite detection is the aim, from the endogenous background.

LC has provided suitable methods for such assays and with luminescence detection

forms a powerful combination for problem solving where detection sensitivity of lumophores and discrimination against interference is a requirement [11].

With atenolol and its associated components the proposed pharmacopoeial monograph calls for an ion-pairing procedure at low pH. All peaks with retention values greater than that for the combined PPA/diol peak are measured with respect to their peak areas. The particular value of a multichannel luminescence detector in this assay of related impurities is apparent when viewing the isometric projection of (I_{em},λ_{em},t) data (Fig. 1). The rapid scanning capability combined with the flexibility of digital data capture and processing routines allow emission spectral information to be presented with digital background subtraction. In this respect the background signal arises from a number of sources including the image intensifier unit and the dark (or leakage) current, which is associated with the dissipation of capacitance across the photodiode array elements as a function of both light intensity and temperature.

Reduction in leakage current is achieved in the detector by cooling to −5°C. However, further reduction is possible by lowering the temperature. When examining trace components extrinsic noise levels can be attenuated in the LDA detector by a number of methods, which include ensemble averaging of sequential scans and the application of smoothing routines to improve signal-to-noise ratios.

Therefore, in the isometric projection for atenolol and its associated components background subtraction and data averaging have been completed with the projection rotated to allow observation of peaks obscured by the major component (Fig. 1). From the data it is possible to generate the most useful elution profiles for maximum detection sensitivities for each of the components (Fig. 2), and at any time point normalised emission spectra can be retrieved for identification by comparison with emission spectral libraries (Fig. 3).

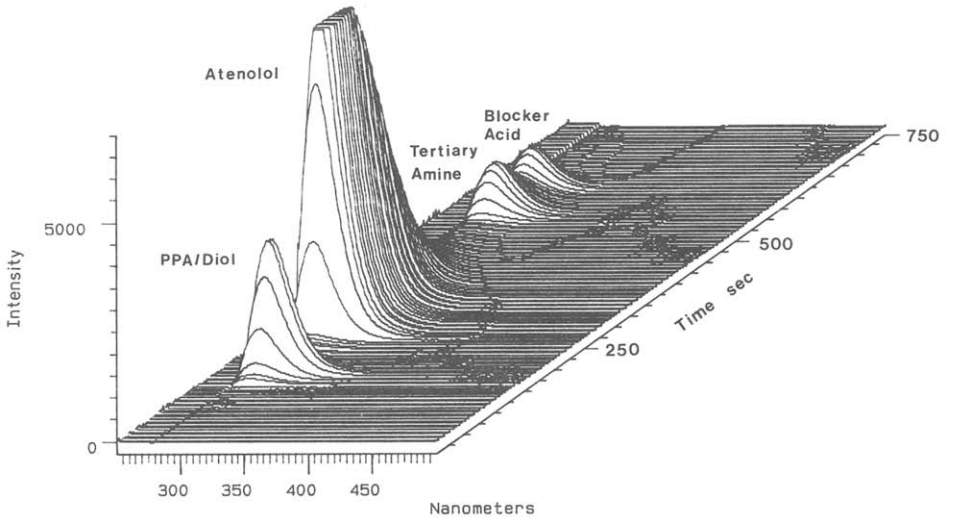

Figure 1
Isometric projection of (I_{em},λ_{em},t) data for atenolol, its major degradation products and synthetic route precursors 0.01–1.0 mg ml^{-1} in HPLC methanol. Data captured in 6 s intervals (10 scans) during elution with background subtraction. For chromatographic conditions, see text; injection volume 20 μl; intensifier gain 3.0; grating 300 nm; slit 250 nm; λ_{ex} 270 nm.

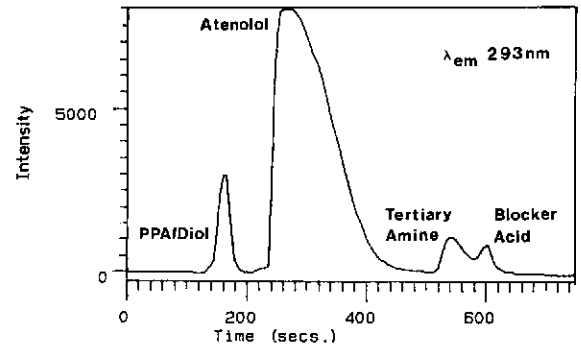

Figure 2
Chromatogram at an emission wavelength of 293 nm for atenolol, major degradation products and synthetic route precursors, λ_{ex} 270 nm. For chromatograph conditions, see text.

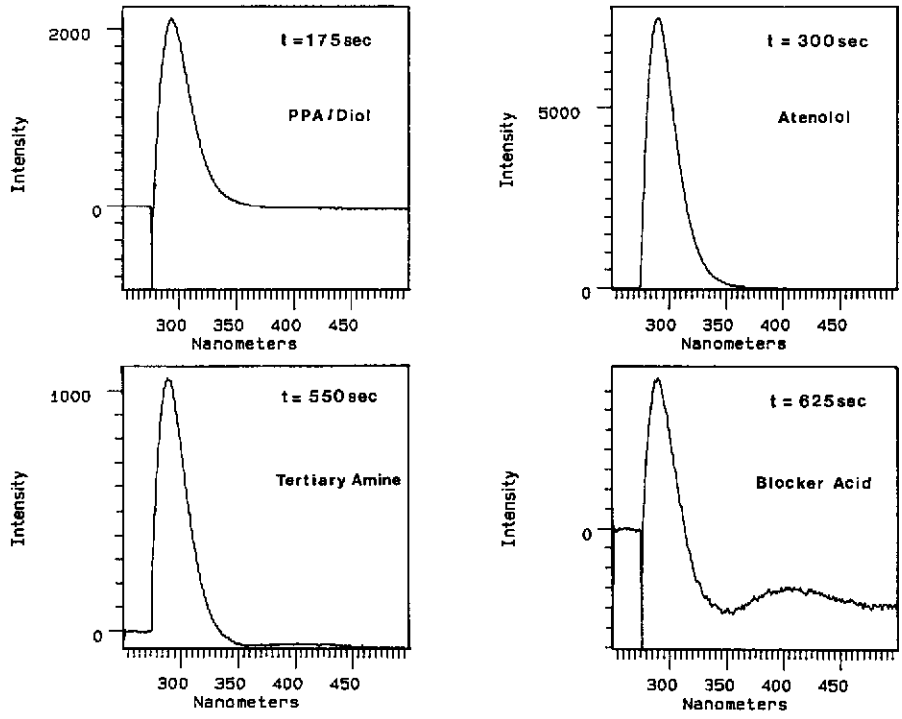

Figure 3
Normalised emission spectra of atenolol, major degradation products and synthetic precursors obtained by capturing spectra from the (I_{em},λ_{em},t) data matrix. See Fig. 1 for instrumental parameters.

A novel method of presenting the data matrix as a two-dimensional profile is to sum the data points relating to particular wavelengths to give the "total emission chromatogram" (Fig. 4). Although in this particular instance the profile does not give additional information, the method is generally useful for detecting trace components and has been very successful in absorptiometric detection for enhancing the visibility of extra impurity information [12].

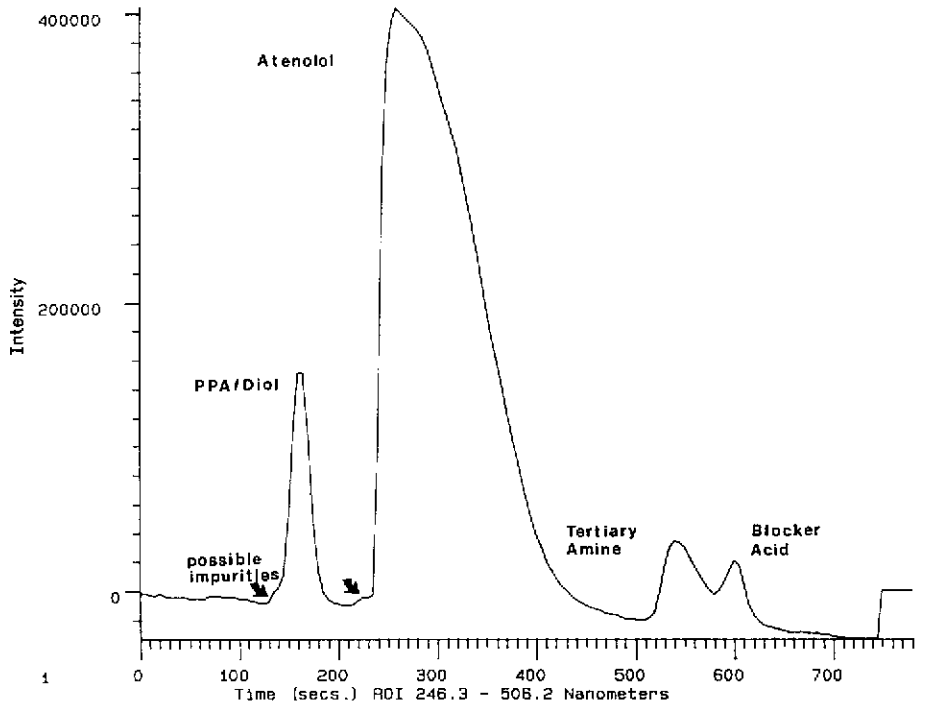

Figure 4
Total emission chromatogram from 250–500 nm for atenolol, major products and synthetic route precursors. For chromatographic conditions, see text; λ_{ex} 270 nm.

Although these graphical presentations of the emission intensity data matrix are of interest, the major significance of this technology, as in absorptiometric multichannel detection arises from the use of computer-aided techniques for assessing the homogeneity of a chromatographic peak [12]. One such method is the extraction of normalised emission spectra from the chromatographic profile, as impressively illustrated for the PNAHs in Fig. 5. Here interrogation of the apparently single peak recorded at λ_{em} = 380 nm from the (I_{em},λ_{em},t) matrix, indicates clearly the presence of three components.

An additional technique for purity validation which has not hitherto been reported in luminescence detection involves the generation of the fluorescence emission ratio ($K_{1,2}$) at two distinct wavelengths as a function of time:

$$K_{1,2} = \frac{I_{em}^{\lambda 1}(t)}{I_{em}^{\lambda 2}(t)} \text{ at } \lambda_{ex}$$

A pure peak is illustrated by the presence of a square-wave form across the peak profile. This is not the case with the example chosen to exemplify the method (Fig. 6) where the ratio of emission intensities at 450 and 380 m, respectively, plotted as a function of time indicates the non-homogeneity of the later eluting peak in the chromatograms.

The techniques so far illustrated are essentially used for qualitative validation of the integrity of a chromatographic peak. As discussed above, differentiation as a function of

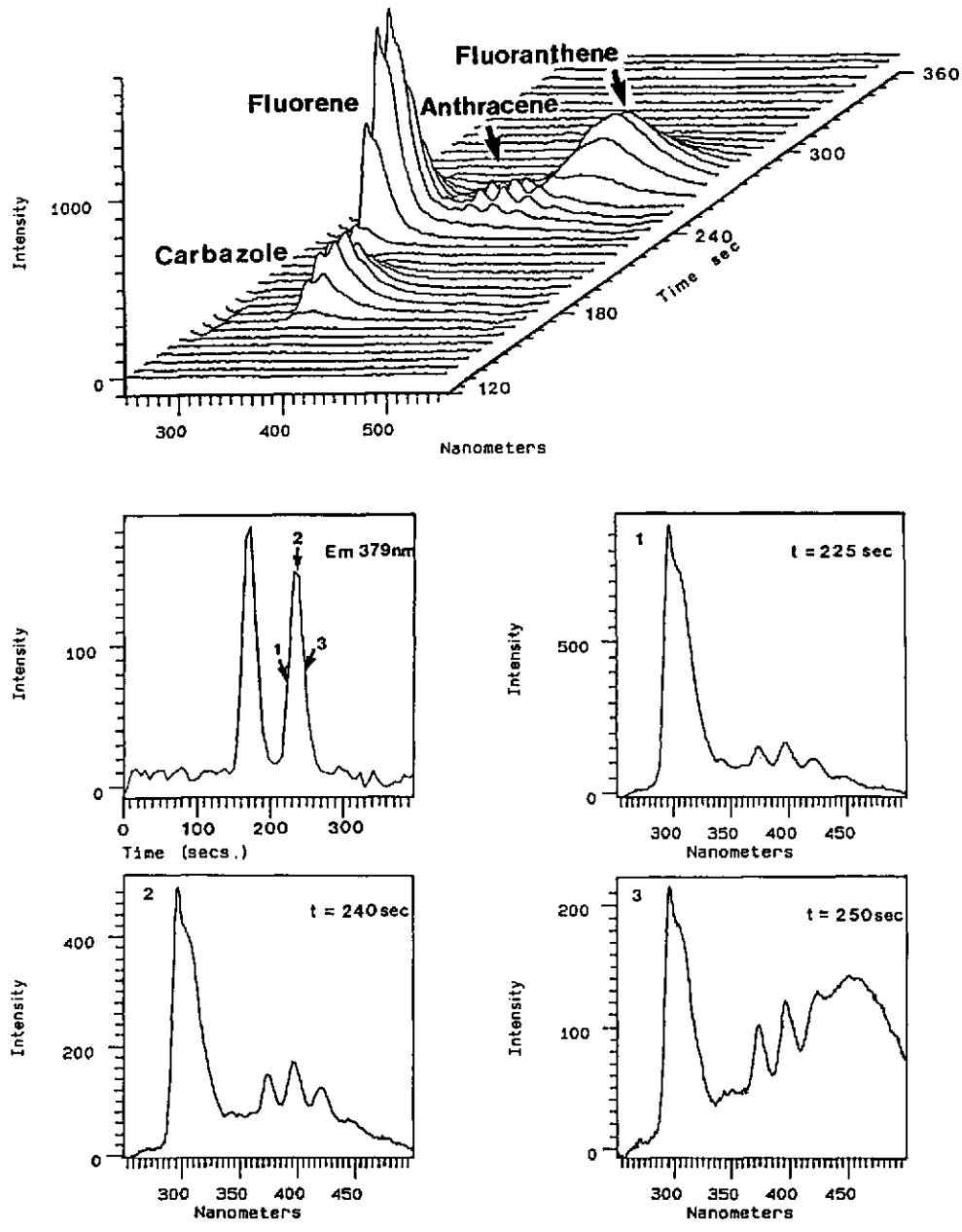

Figure 5
Isometric presentation of (I_{em},λ_{em},t) data for polynuclear hydrocarbons, carbazole, fluorene, anthracene and fluoranthene at concentrations of 8–100 ng ml^{-1} in methanol. Emission spectra captured at 6 s intervals (10 scans/interval) during elution with background subtraction. In the emission chromatogram at 379 nm, interrogation of the later eluting peak at positions 1–3 indicates the presence of non-homogeneity, as presented by the normalised spectra. For chromatographic conditions, see text; injection volume 20 μl; intensifier gain 4.8; grating 300 nm; slit 250 nm; λ_{ex} 250 nm.

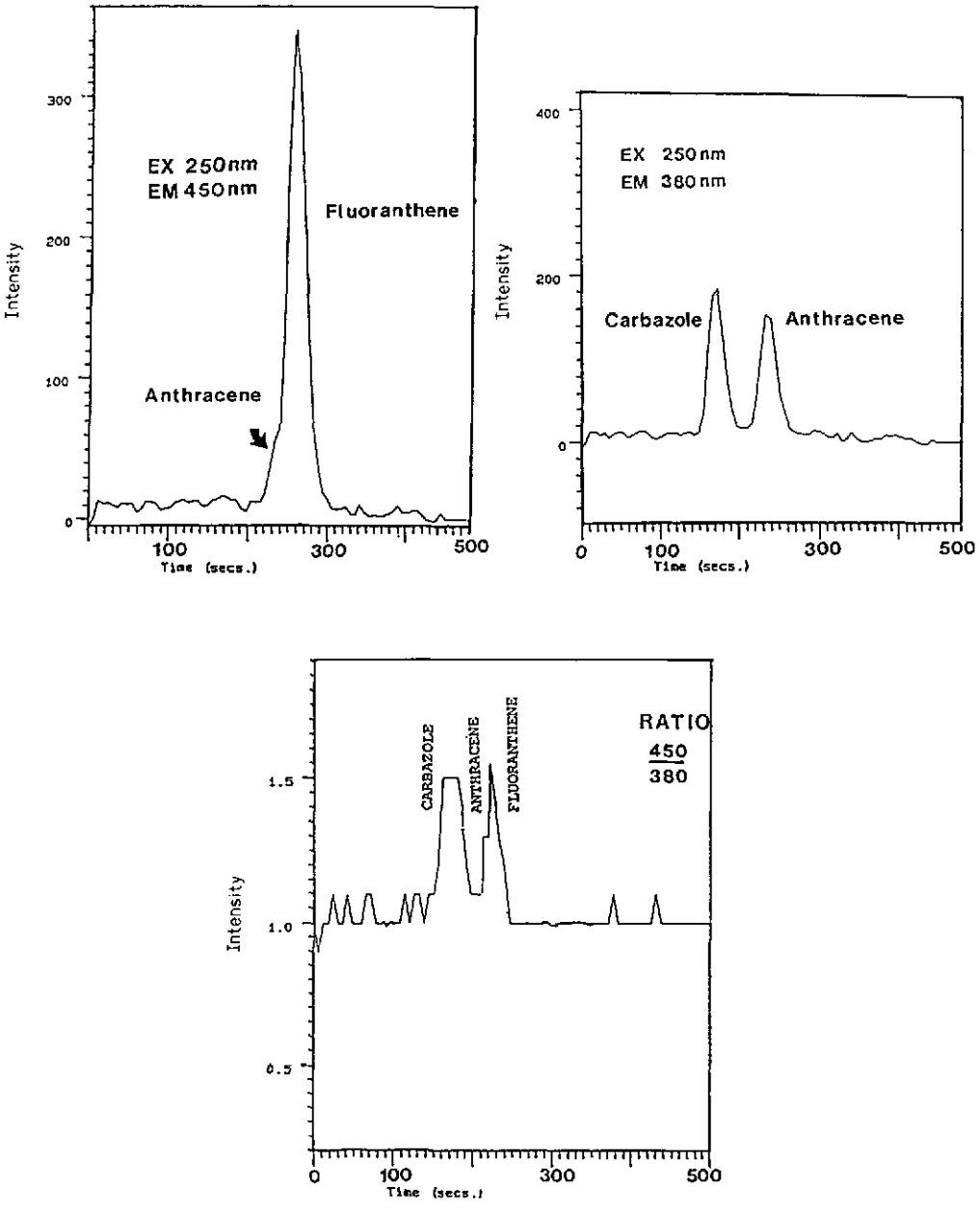

Figure 6
Conventional emission and emission ratio chromatograms of carbazole, anthracene and fluoranthrene at 450 and 380 nm, indicating the presence of fluoranthene in the anthracene peak of the ratio chromatogram. For chromatographic conditions, see text.

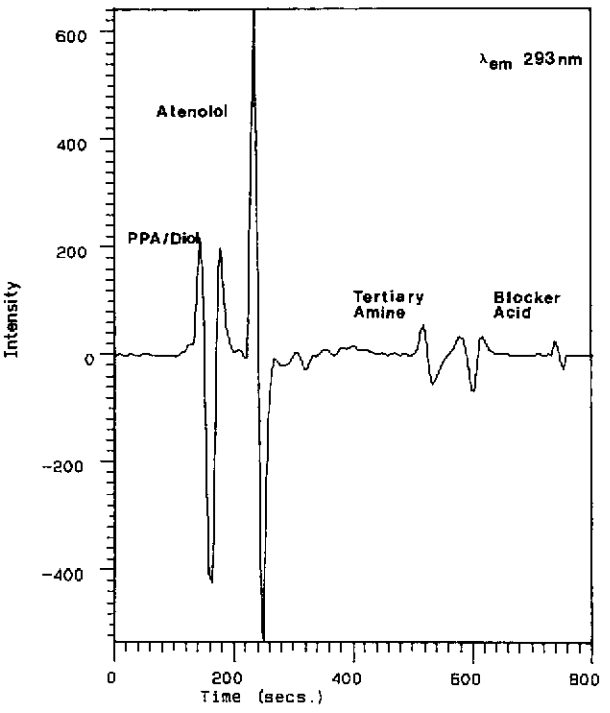

Figure 7
Second derivative emission chromatogram in the time domain of atenolol, degradation products and synthetic precursors, indicating the resolution of the tertiary amine and blocker acid. For instrumental parameters see Fig. 1 and for chromatographic conditions, see text.

time (dI/dt, $d^2I\ dt^2$) can give resolution of overlapping peaks provided that sufficient separation exists between them as in Fig. 7. In addition this technique can offer quantitative information in favourable cases [10].

In this example the band sharpening effects of the second derivative method provide resolution of the overlapping tertiary amine and blocker acid component peaks in the atenolol impurity profile. It should also be possible to generate the bipolar three dimensional derivative emission spectrochromatogram as recently discussed by the authors [12] and illustrated in multichannel UV detection [10] as a further aid to assessing chromatographic peak purity.

Conclusion

For the pharmaceutical analyst the intensified LDA luminescence detector provides a significant addition to the instrumentation available to aid detection of trace components in pharmaceutical and clinical samples. At present a number of digital techniques can be applied to aid solute identification and to assess chromatographic peak purity. These techniques are currently being extended in the authors' laboratories to include more advanced techniques such as spectral suppression [13] and multiple spectral suppression [14, 15]. These multivariate methods for UV multichannel detection are in principle likely to be equally applicable to luminescence detection.

It should also be possible to apply a number of advanced matrix-based techniques [16] to the detection and resolution of overlapping components thereby more efficiently exploiting the (I_{em},λ_{em},t) data.

In addition the development of detector technology based on the charge-coupled and charge-injection device could open up the possibility of relatively inexpensive acquisition of the emission–excitation matrix of luminescence data for advanced detection modes in LC.

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[*Received for review 26 October 1987*]