Journal of Chromatography, 238 (1982) 113–120
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,570

UNIVERSAL, MULTI-CHANNEL ULTRAVIOLET DETECTION IN THE PURITY ANALYSIS OF 2-ETHYL-3-(4-HYDROXYBENZOYL)INDOLIZINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received November 23rd, 1981)

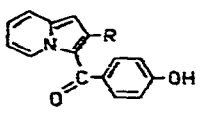
SUMMARY

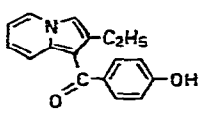
The use of a multi-channel UV–visible detector coupled with high-performance liquid chromatography in purity analysis is described. A discussion of some advantages and disadvantages in comparison with a conventional (single-wavelength) UV detector is given. Detection and identification limits for a particular contaminant of 2-ethyl-3-(4-hydroxybenzoyl)indolizine are determined. Sensitivities for both detectors with regard to the same contaminant are derived from the calibration curves.

INTRODUCTION

During our studies[1,2] on butoprozine, {4-[3-(dibutylamino)propoxy]phenyl-{(2-ethyl-3-indolizinyl)methanone, a new drug with anti-anginal properties, we found that a number of impurities could occur in the parent drug when no special precautions were taken during its synthesis.

When ^{13}C-labelled butoprozine was needed for drug metabolism studies, we again paid special attention to the purity, with emphasis, on the immediate butoprozine precursor in the synthetic route[3], 2-ethyl-3-(4-hydroxybenzoyl)indolizine (EHBI, I) since in large scale synthesis almost all impurities in butoprozine result from impurities in EHBI. These latter impurities can be separated more easily than the butoprozine impurities. In addition to the known impurities, other contaminants might be expected as the synthesis of the [^{13}C]material was not a normal large scale production.





	R
II	CH_3
I	CH_2CH_3

III

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In our laboratory both thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) are used routinely in purity control. Some compounds may go undetected when using column separation systems because of strong interaction between solute and stationary phase, so that these compounds are not eluted from the column. In TLC this is not a problem; compounds that interact strongly with the stationary phase appear as a spot at or near the start of the chromatogram. In addition, detection in general is more universal in TLC. Despite the disadvantages of HPLC in these respects, we have found this technique very useful in purity control because it allows the detection of trace amounts in the presence of a large amount of another substance. This can also apply when the substances are very similar.

In this paper we will describe some advantages and limitations of a multi-channel UV–visible detector as compared to a conventional UV detector in purity control by means of HPLC.

EXPERIMENTAL

Chemicals and reagents

[^{12}C]- and [^{13}C]EHBI were gifts from Labaz (Brussels, Belgium). Methanol was analytical grade (pro analysis) and obtained from E. Merck (Darmstadt, G.F.R.). Distilled water was used.

High-performance liquid chromatography

A M 6000 pump (Waters Assoc.) was used with a Model SF 770 variable-wavelength UV detector (Schoeffel), operated at 240, 280 or 380 nm, or with a HP 8450 A (Hewlett-Packard) multi-channel UV–visible detector equipped with a Model 178.32 QS quartz cell (Hellma) with 8-μl volume. Injections were made with a WISP 710 B autoinjector (Waters) using an injection volume of 25 or 100 μl.

In all experiments a stainless-steel column (150 × 4.6 mm I.D.) was used. The column was packed with Hypersil-ODS (mean particle size 5 μm, Chrompack) using a slurry in carbon tetrachloride–methanol (8:2). The mobile phase consisted of methanol–water (65:35, v/v). Before mixing the components were filtered through a 0.45-μm membrane filter (Schleicher and Schüll). The flow-rate was 1 ml/min.

The multi-channel detector measured an absorption spectrum (200–800 nm) every 7 sec. These spectra were stored on a tape cartridge using a dual tape drive Model HP 9875 A (Hewlett-Packard). Plotting of the chromatograms was done using a Model 9825 desk top computer (Hewlett-Packard) and a Model 9872 A plotter (Hewlett-Packard). The plotting program was obtained from Hewlett-Packard, but we adapted it in several ways to meet our requirements. Details have been given elsewhere[4].

RESULTS AND DISCUSSION

Detection

From results of other experiments we expected EHBI to be contaminated with the isomeric 1-(4-hydroxybenzoyl)-2-ethylindolizine (III) and with 2-methyl-3-(4-hydroxybenzoyl)indolizine (II). Small amounts of II and III could be separated from

each other and from large amounts of I by HPLC, but not by TLC, for which numerous systems were tried. The chromatograms at different wavelengths are given in Fig. 1; the small peaks b and c are caused by III and II respectively, while I gives d. Peak a was found to be composed of several compounds (see below).

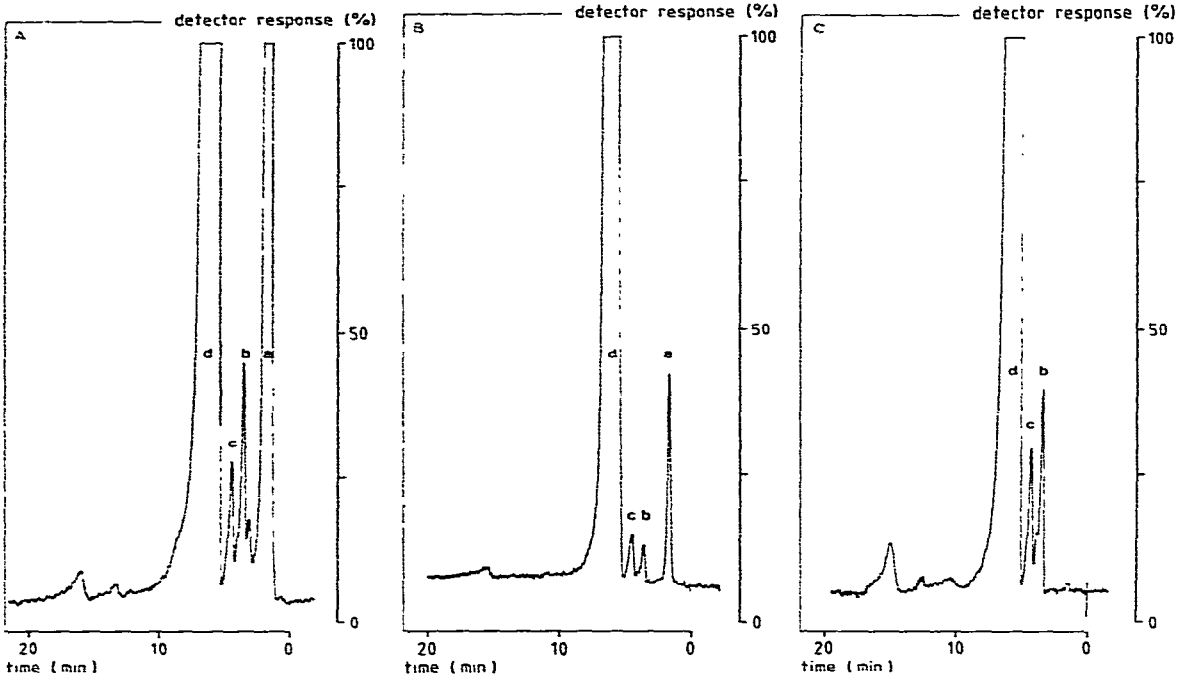

Fig. 1. Chromatograms of the crude [^{13}C]EHBI. Conventional UV detection at 240 (A), 280 (B) and 380 nm (C). Code: b = III; c = II; d = I.

As can be seen from Fig. 1, the selectivity of the conventional detector results in different chromatograms, depending on the wavelength selected. The fast eluting compounds in peak a are detected at 240 nm but not at 380 nm. Each individual wavelength required a new injection of the sample. In this way some information could be obtained on the presence of unknown compounds.

A detector that can cover a large range of wavelength, however, would provide more information, which in addition would be available much faster as only a single injection is needed. In this respect the multi-channel detector offers substantial advantages: in 1 sec the whole wavelength range between 200 and 800 nm is measured. This rapid measurement of the spectrum avoids the use of stop-flow conditions[5]. In cases where samples of unknown composition have to be analyzed, the stop-flow technique is useless anyhow for practical reasons: after every small time interval the flow has to be stopped in order to obtain an absorption spectrum. The amount of information produced by the multichannel detector is so large that data storage is necessary. After the chromatographic run the stored data can then be processed.

In Fig. 2 the absorbance–wavelength–time surface is shown, reconstructed from spectra taken with a 7-sec interval. Such a surface ("three-dimensional chromatogram") shows the presence of UV–visible active contaminants at a glance, together

with preliminary structural information. In the rear of the three-dimensional chromatogram EHBI causes the large mountain (d). The other ridges are due to contaminants. Although absorbances larger than 0.150 have not been plotted because of clarity, full spectral information is available on the tape; the UV–visible spectrum for each contaminant can be obtained. As there are small differences in the spectra between II and III the small ridges in front of I can readily be identified.

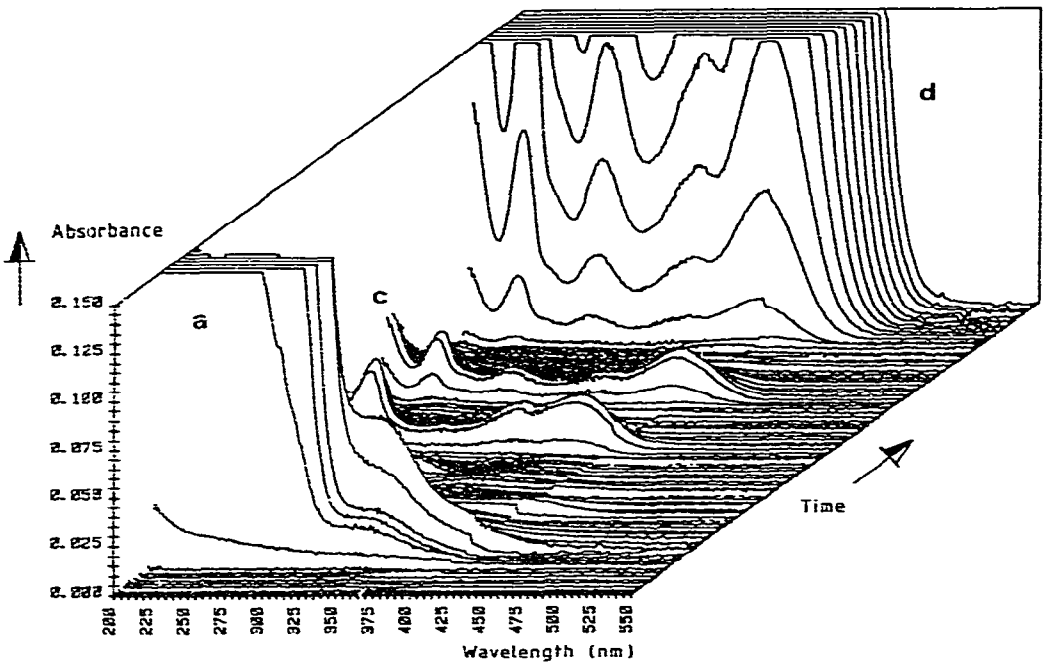

Fig. 2. Chromatogram of the crude [^{13}C]EHBI. Multi-channel UV detection.

The contaminants eluting in and just after the void volume show such a large absorbance that the surface behind cannot be observed. In order to obtain this information it is not necessary to repeat the measurement using more suitable conditions. The same data can be used to plot a new surface while omitting the interfering spectra (Fig. 3). An alternative is to plot the spectra in the reverse direction. In this way we can take a look at the rear of the front ridge (Fig. 4). It is obvious from this Figure that several compounds with different spectral properties are present.

Finally, in Fig. 5 the results obtained with material that has been purified by preparative HPLC[6] are shown. The amount injected was the same as in Fig. 2, so the much higher purity is readily seen.

Detection limits

As a basis we have taken the treatise on detection limits by Boumans[7]. This means that in the following discussion the net measures (net signal magnitudes) are used to make decisions. With the aid of a calibration line the minimum detectable amounts or concentrations are then calculated using these limiting signal magnitudes.

When we have to determine whether compounds other than the main com-

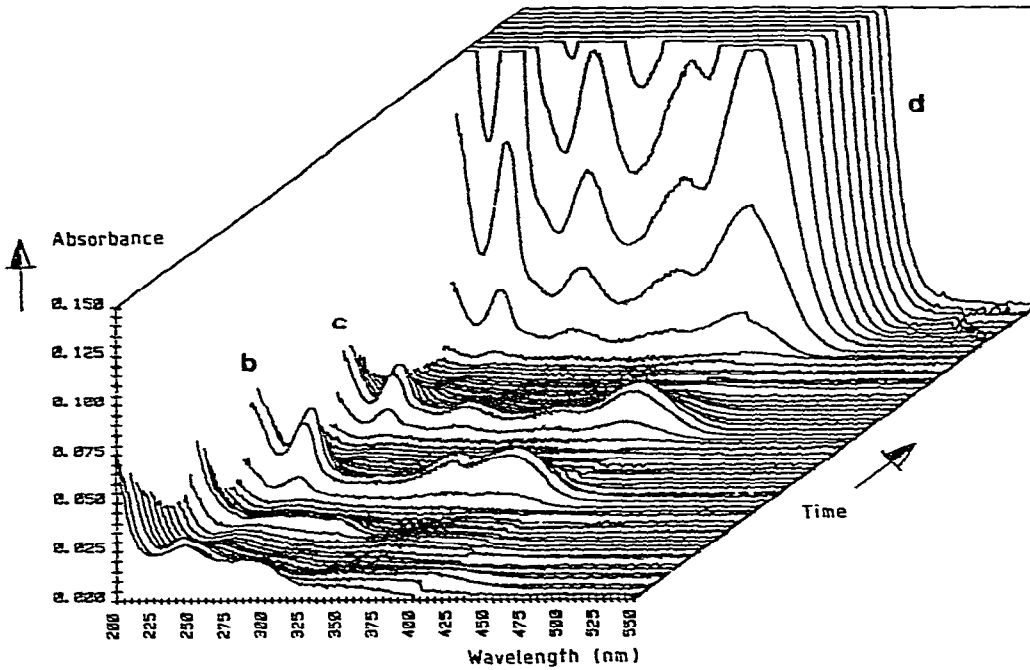

Fig. 3. The same chromatographic run as used in Fig. 2, but for a better view of the peaks eluting just after the front the first spectra have been omitted.

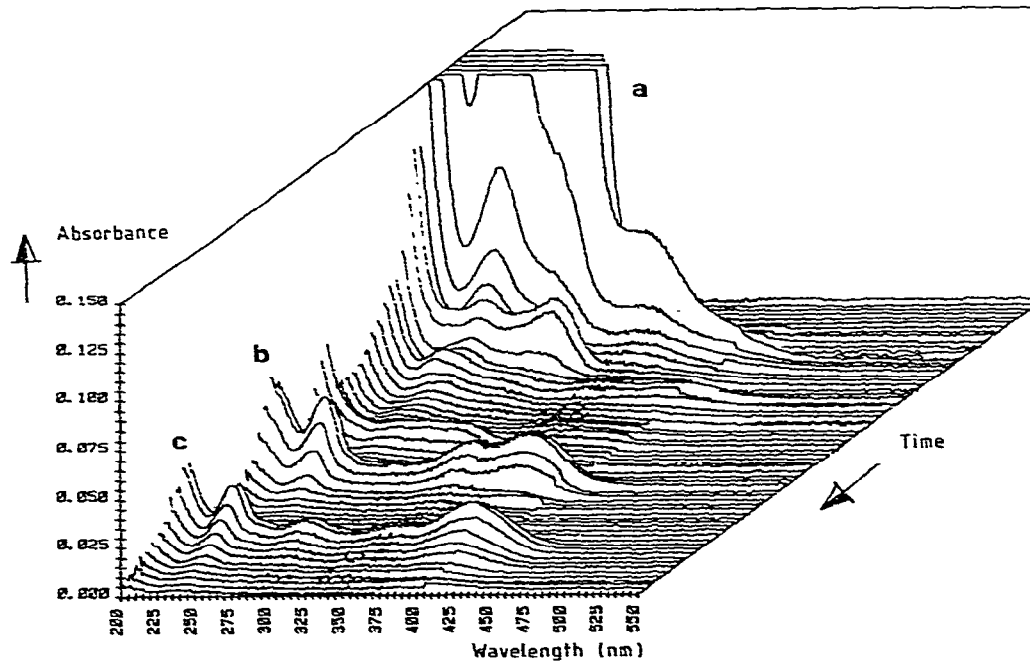

Fig. 4. The same chromatographic run as used in Fig. 2, but with the spectra plotted in reversed order (note the time axis), omitting the spectra resulting from EHBI.

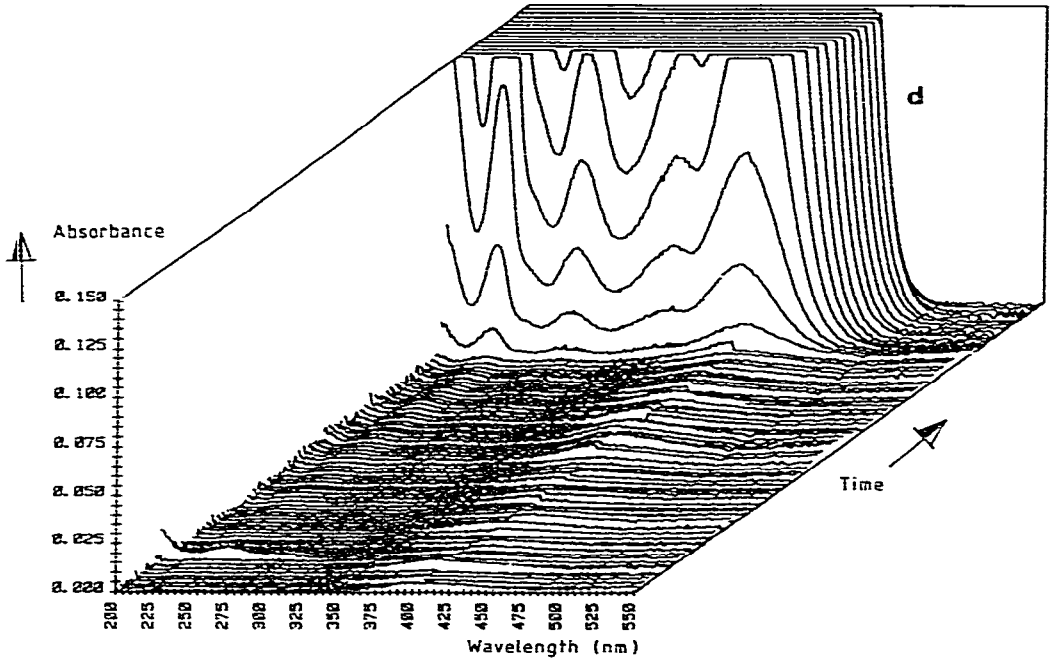

Fig. 5. Chromatogram of the purified [^{13}C]EHBI. Conditions as in Fig. 2.

pound are present, all we can do is to examine the chromatogram and decide if signals are present that cannot be attributed to the background or main compound. Such a decision requires a limiting signal, L_D, to be chosen. This is the smallest signal that is not attributed to background. The detection limit L_D is chosen in such a way that the risk, α, of fiding a background signal larger than L_D is small. If the background signals are normally distributed

$$L_D = k\sigma_B$$

where σ_B is the standard deviation related to the background signal distribution and k is the standard normal deviate. In general, the background fluctuations will be well known for isocratic elution: a reliable estimate, s_B, for σ_B is obtained from a large number of background measurements: $s_B \approx \sigma_B$. From a table of a (single sided) normal distribution it can be found that $L_D = 2.3\ \sigma_B$ for risk $\alpha = 1\%$. The detection limit depends on the background fluctuations only. The magnitude of these fluctuations vary somewhat over the wavelength range from 200 to 800 nm.

For unknown contaminants only a decision "detected" or "not detected" can be made. When contaminants are known and a calibration line is available, the limiting concentration or limiting amount can be calculated. However, for a sample giving a mean signal L_D the risk that a signal smaller than L_D will be produced is 50%. If a lower risk (β risk) for attributing a true signal to background is wanted, a larger limiting signal, L_I, must be chosen. For the identification limit L_I, we state

$$L_I = L_D + k\sigma_I$$

where σ_I is the standard deviation of the true signal distribution with mean L_I. As σ_I is usually estimated from a small number of observations the Student distribution has to be used:

$$L_I = L_D + t \cdot s_I$$

For compound II we determined the calibration function in the low mass range (0.5–40 ng) as

$$R = 0.225 \times 10^{-3} \times M - 0.029 \times 10^{-3}$$

where R = response, *i.e.*, the peak height (a.u.), and M = mass (ng) using the conventional detector (at 233 nm with time constant 0.5 sec).

For the multi-channel detector the calibration function found (at 233 nm with 1-sec interval) is:

$$R = 0.220 \times 10^{-3} \times M - 0.207 \times 10^{-3}$$

In both cases the same column, eluent and injection volume (100 μl) were used, so the same factors representing the dilution in the column[8] are included in the overall sensitivities.

Using these calibration curves the amounts (ng) corresponding to L_D and L_I can be calculated (risk α = 1%, risk β = 5%, t = 1.7):

	L_D	L_I
conventional:	0.000067 a.u. (0.4 ng)	0.000116 a.u. (0.6 ng)
multi-channel:	0.000383 a.u. (2.6 ng)	0.000667 a.u. (4.0 ng)

From the equations of the calibration curves it can be seen that the sensitivities (slopes of the lines) are almost equal. The noise level for the multi-channel detector, however, is much higher as can be seen from the values of L_D.

Although II could no longer be detected in the ^{13}C-material obtained after purification by preparative HPLC when using the multi-channel detector (Fig. 5), traces of II could be shown to be still present in the purified material when the conventional detector was used.

CONCLUSIONS

The multi-channel detector is exteremely useful for the screening of unknown compounds. An impression of the purity of a compound can be obtained in a short time as far UV–visible active contaminants are concerned. Although the multi-channel detector has a higher noise level than the conventional detector, this will be compensated to a large extent because the wavelength giving the highest sensitivity can be found easily with the former. In cases where the properties of contaminants are known, we prefer the use of a conventional detector tuned to the relevant contaminant because lower levels can be detected.

ACKNOWLEDGEMENT

We gratefully acknowledge Labaz S.A. for various gifts of materials and facilities.

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