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Note

Multichannel diode array UV–visible spectrophotometer as detector in screening for unknown butoprozine metabolites in dog bile by high-performance liquid chromatography

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In a recent paper[1] we described the applicability of gradient-elution reversed-phase high-performance liquid chromatography (HPLC) in the screening for unknown drug metabolites in dog bile after administration of radioactive butoprozine, a new anti-anginal drug. The structure of butoprozine and its UV-absorption spectrum are given in Fig. 1. The metabolites were detected by means of conventional UV detection and by counting of the radioactivity in the effluent from the UV detector. UV detection was employed in order to test a detection system in experiments in which only non-radioactive drugs are involved. However, in these investigations it was necessary to differentiate between the UV absorption of the metabolites and that of the background (endogenous bile components, eluent impurities, column bleed, etc.). Thus, we developed a method of recognizing the metabolites based on the impressive reproducibility of the separation system and comparison of continuous gradient chromatograms of dog bile before and after butoprozine administration.

Conventional UV detectors have an important limitation when used to characterize column effluents. In screening processes a single monitoring wavelength must be selected. Although this is usually done by taking into account the absorption spectrum of the parent drug, there is the risk that metabolites with an altered spectrum may go unnoticed at the selected wavelength. Thus, in order to be sure that all

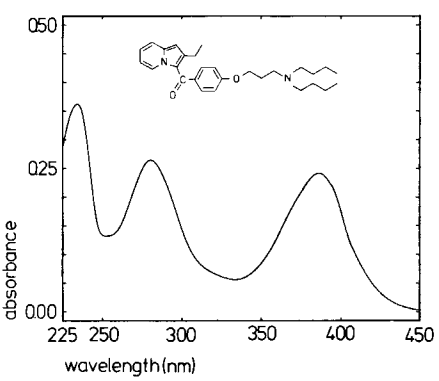

Fig. 1. Structure and UV spectrum of butoprozine.

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metabolites are being detected, a great many consecutive chromatographic runs at various wavelengths have to be carried out. Although stop-flow scanning may be of some help in overcoming this problem, it often appears to be impractical due to losses in resolution and reproducibility. In this area of research there is a need for fast scanning detectors[2,3] or detectors which can record complete spectra during a chromatographic run[4].

We now report the applicability of an universal multichannel diode array UV–visible spectrophotometer in combination with gradient-elution reversed-phase HPLC in screening for unknown butoprozine metabolites in non-radioactive "cold" dog bile.

EXPERIMENTAL

Compounds

Butoprozine was a gift from Labaz (Brussels, Belgium). Methanol and water were HPLC grade obtained from Baker (Deventer, The Netherlands). All other chemicals were analytical grade (pro-analysi) obtained from E. Merck (Darmstadt, G.F.R.).

Bile

Bile was obtained from Beagle dogs after cannulation of the biliary duct and was collected in ice-cooled tubes in the dark 1 h before (blank bile) and 8 h after intravenous administration of butoprozine (5 mg/kg). After the experiment the bile was deep-frozen (−20°C) and stored in the dark. Before the bile was used for the HPLC experiments it was subjected to a mild clean-up procedure. Part of the endogenous bile components were precipitated by adding two volumes of methanol to one volume of bile. Then, the supernatant was stored at 5°C in the dark for 25 days. Under these conditions no metabolite losses occurred but UV-absorbing endogenous bile components disappeared, either by precipitation or by decomposition[5].

HPLC

Fig. 2 shows the separation–detection set-up consisting of:

(1) Two eluent reservoirs, containing methanol and water (+0.05 *M* triethylamine, TEA), respectively. Before chromatography the water was led through a reversed-phase column (25 cm × 6.2 mm I.D.) packed with LiChrosorb RP-8,10 μm (Merck) to remove organic impurities. Elution took place at a flow-rate of 1 ml/min, starting with water containing 0.05 *M* TEA and then in a linear gradient mode methanol was added at a rate of 1% per minute up to 100 min when the final eluent composition was 100% methanol.

(2) A helium cylinder with a connection to the eluent bottles. To prevent air-bubbles in the low-pressure detector flow cells due to the mixing of the two eluent components[6,7], the water and methanol were saturated with helium before use. To maintain a helium atmosphere during elution, the helium was slowly bubbled through the eluents.

(3) Two M-45 pumps (Waters Assoc., Millford, MA, U.S.A.), as eluent mixing and delivery devices.

(4) A post-pump eluent mixing unit, necessary to reduce extra baseline drift

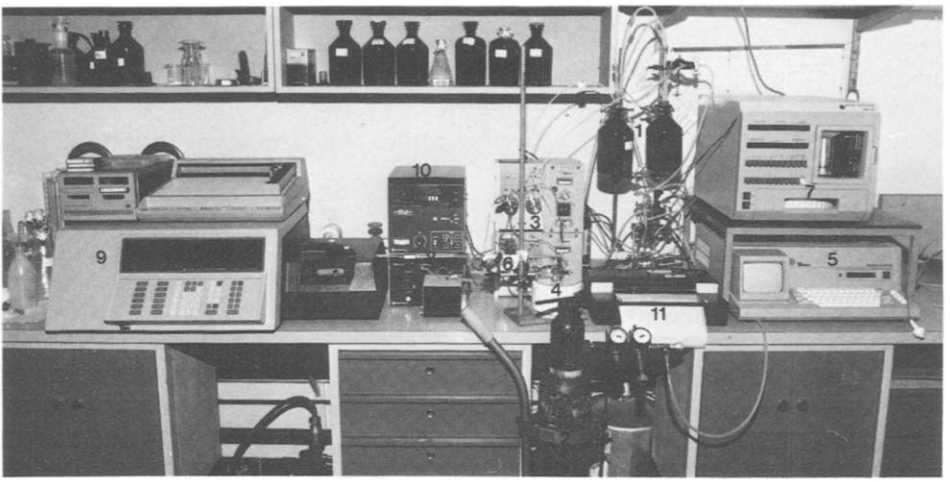

Fig. 2. The HPLC equipment constructed for the detector comparison experiment. The numbers 1 to 11 refer to the components of the set-up and are discussed in the text.

and baseline irregularity because the mixing capacity of the pumps is not optimal.

(5) A M-720 solvent programmer (Waters Assoc.).

(6) A M-7125 variable injection valve (Rheodyne). An 80-μl volume of bile supernatant was injected after the sample preparation.

(7) A WISP-710-B autoinjector (Waters Assoc.).

(8) A stainless-steel column (15 cm $\times$ 4.6 mm I.D.) packed with LiChrosorb RP-8, 5 μm (Merck) by means of a balanced density slurry method.

(9) A HP-8450-A multichannel diode array UV–visible spectrophotometer with a dual tape drive, tapes and plotter (Hewlett-Packard, Palo Alto, CA, U.S.A.). The balance between sample and reference cells, the latter containing methanol–water (50:50), was measured just before the chromatographic run and 30 and 70 min after the start of elution, respectively. This was done because of the special demands placed on this kind of gradient elution. Details will be given in a future paper.

(10) A SF-770 variable-wavelengt UV–visible detector (Schoeffel, NJ, U.S.A.) operated at 380 nm at an attenuation of 0.1 a.u.f.s. and connected in series with array 9.

(11) A BD-40 recorder (Kipp, Delft, The Netherlands) operated at a paper velocity of 0.5 cm/min and connected with detector 10.

All other experimental details were as described earlier[1] or are given, in the case of recording and plotting of the UV–visible spectra, elsewhere[8].

RESULTS AND DISCUSSION

The set-up described in the Experimental was chosen so as to perform a detailed comparison of the two detection systems. On-line measurement could be done without problems when using the helium procedure.

Representative parts of chromatograms of dog bile are shown in Fig. 3. The conventional chromatogram shows the absorption at 380 nm; the three-dimensional

one, which we prefer to call a spectro-chromatogram, depicts absorbance *versus* time over the range 225–450 nm as would seem to be relevant for butoprozine metabolites. For the latter chromatogram, spectra were taken every 8 sec, stored on tape during the run and then recalled for plotting after chromatography had been completed. Comparison of the two chromatograms clearly shows the dramatic gain in selectivity and information provided by the multichannel detector in a single run.

A direct recognition of butoprozine structural analogues against the background can be made immediately, based upon the absorption spectrum of the parent drug (metabolites 1–9). This is facilitated further when a spectro-chromatogram of blank bile is taken into account. The latter has not been reproduced, however, for simplicity. Recent structure elucidation studies have shown that the above metabolites have an intact aromatic system but that hydroxy and/or methoxy groups have been introduced. The peak designated 10 apparently contains a compound with a spectrum different from butoprozine and it thus goes undetected in the conventional chromatogram. Comparisons with chromatograms on blank bile clearly showed that peak 10 had to be a metabolite. Structure elucidation after isolation of the fraction then indicated that in this compound the indolizine moiety has been split off. So, use of the multichannel spectrophotometer represents an important improvement of metabolic screening of excretion liquids. This may be especially important for those metabolic studies in which radio-labelled drug administration cannot be performed, *e.g.*, in human beings.

Besides qualitative information, the spectro-chromatogram also provides greatly enhanced quantitative information, especially with regard to butoprozine-related structures. As is well known, substituents in the parent molecule may cause a shift in absorption maxima and minima with little change the form of the spectrum, therefore one does not know immediately how distant the peaks measured at a fixed wavelength (*e.g.*, 380 nm) are from their absorption maximum. Yet, from the spectro-chromatogram a semi-quantitative picture can readily be obtained. Also, note the changes in peak heights in, *e.g.*, compounds 2, 3, 8 and 9 relative to compound 4 and the parent compound.

A third major advantage of the multichannel diode array detector is that it gives much more information about possible peak overlaps, background interferences, etc., which is of particular importance in the isolation and structure elucidation of potential metabolites. Good illustrations of these phenomena are peak/fraction numbers 1 and 10, respectively. Fraction 1 is contaminated with a component exhibiting short-wavelength UV-absorption, whereas fraction 10 may be contaminated by a compound with a slightly shorter retention time. In these cases it would be necessary to revert to other chromatographic conditions or systems, otherwise interpretation of data from structure elucidation techniques such as mass spectrometry and nuclear magnetic resonance spectroscopy may be rather difficult. We found stepwise gradient elution to be quite useful[9]. The purity of eluting peaks may be checked further by looking at the absorbance ratio at two or more different wavelengths[8].

Finally, background interference in the short-wavelength region between 225 and 300 nm, which is frequently encountered in this kind of gradient elution with conventional detection[10], is not such a problem when using the multichannel detector. This is obviously due to the fact that the latter detector provides significantly enhanced spectral information so that the resulting spectro-chromatogram can more

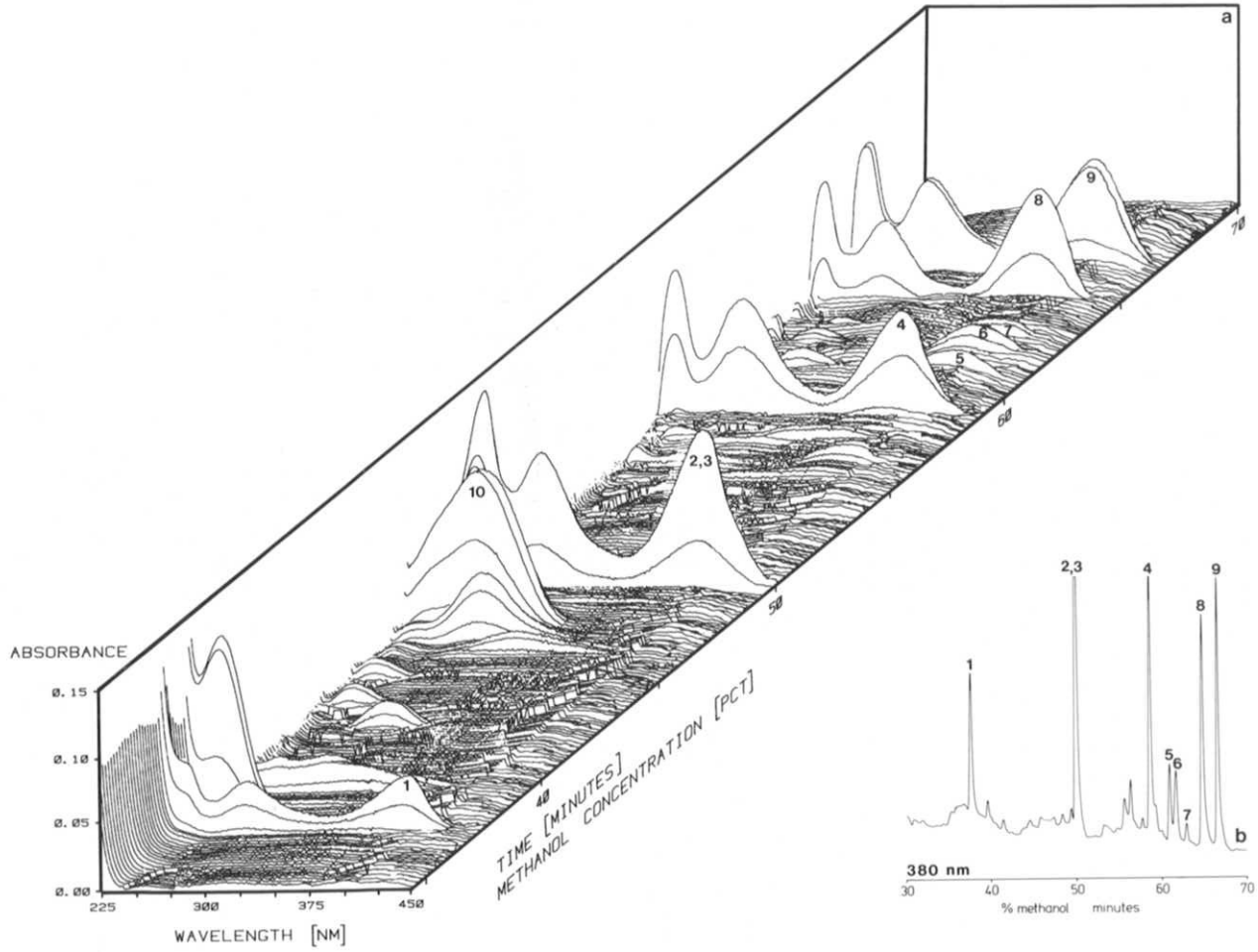

Fig. 3. (a) Spectro-chromatogram of the dog bile fraction obtained from 0 to 8 h after non-radioactive drug administration. (b) Conventional (one-wavelength) chromatogram of the same fraction as in (a), recorded on-line.

readily be interpreted, and is of particular relevance for compounds with absorption maxima only in this wavelength range.

The above results reflect our first experience of applications of the multichannel diode array detector. Other applications are presently being investigated. Although the detector is rather expensive we feel that it will become an important tool in the area of drug metabolic profiling as well as in other areas of bioanalytical research.

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