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ON-LINE DIODE ARRAY UV–VISIBLE SPECTROMETRY IN SCREENING FOR DRUGS AND DRUG METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The direct coupling of a multi-channel diode array UV–visible spectrophotometer to a powerful reversed-phase HPLC separation system is considered, especially for use in qualitative analysis, *e.g.*, screening/identification of drugs and drug metabolites. The approach is illustrated by the screening for metabolites of butoprozine and ticlopidine directly in human and rat bile.

INTRODUCTION

Recently a new generation of UV–visible spectrophotometers has been introduced in which the absorption spectrum of a compound is "pictured" within 1 sec by a multi-channel diode array system covering the range 200–800 nm. These detectors are capable of recording a series of spectra in the time and storing the data in their own memory and/or in external devices, such as magnetic tapes or disks, for later data handling and plotting. The potential of such an instrument as spectrophotometer has been discussed by James and Willis[1].

Being frequently involved in screening for unknown drugs and drug metabolites in biological materials, we felt that this diode array spectrophotometer (DAD) may also have great potential as an on-line detector for high-performance liquid chromatographic (HPLC) analysis (LC–DAD). In comparison with conventional single-wavelength detection it is evident that LC–DAD provides both multi-wavelength and spectral information in a single chromatographic run with the following advantages: saving of time, sample and eluent; smaller column leading; improved identification possibilities; rapid selection of optimal conditions for single-wavelength detection (*e.g.*, for quantitative purposes); possibilities for checking the purity of chromatographic peaks; rapid adaptation of the separation conditions to obtain better resolution.

In previous papers[2–5] we reported some preliminary experiences with LC–DAD in purity analysis and the detection of drug metabolites. In this paper we give a

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detailed account of the general potentials of LC–DAD in screening for unknown drugs and drug metabolites with special relevance to biological materials.

EXPERIMENTAL

Drugs and chemicals

Butoprozine and ticlopidine were gifts from Sanofi S.A. The other drugs were present in our Department for general reference purposes and were donated by various manufacturers (Bayer, Astra, Homburg, Interpharm, Hoffman-La Roche, Wyeth, Ciba-Geigy). All drugs were 99% chromatographically pure by HPLC and thin-layer chromatography (TLC), or had been purified previously to a chromatographically pure state. Individual solutions and a mixture of the drugs were prepared in methanol with concentrations of 100–300 μg/ml per substance.

For the chromatographic analyses, water was obtained fresh from a Milli-Q water purifier (Millipore, Bedford, MA, U.S.A.), methanol was of HPLC grade from Baker (Deventer, The Netherlands) and ammonia, as a basic modifier, was of pro analysi grade from Merck (Darmstadt, G.F.R.). All other chemicals were also obtained from Merck and were of pro analysi grade.

Bile

Human bile samples were obtained from a male patient, 57 years old and 65 kg in weight, who was having a biliary drain. Butoprozine was administered intravenously in a dose of 15 mg diluted in a Baxter glucose solution during 15 min.

Rat bile samples were obtained from adult Wistar rats (body weight *ca.* 300 g). Ticlopidine was given by i.v. injection in a dose of 25 mg/kg.

Bile was collected in fractions of 1 h in ice-cooled tubes in the dark via a bile cannule. After collection, the bile was deep-frozen ($-18°C$) until taken for analysis.

HPLC

The same separation–detection set-up as described earlier[4] was used. The detection unit is shown in more detail in Fig. 1. The equipment consisted of an SF-770 variable single-wavelength detector (Schoeffel, Westwood, NJ, U.S.A.) (A); an HP-8450A multi-channel diode array UV–visible spectrophotometer (Hewlett-Packard, Palo Alto, CA, U.S.A.) (B) equipped with an M-178.32 QS flow-through quartz cell with a cell volume of 8 μl (Hellma, Mühlheim Baden, G.F.R.) (C); an HP-9875A tape cartridge using a dual tape drive (Hewlett-Packard) (D); an HP-9872A graphics plotter (Hewlett-Packard) (E); an Apple II plus (64K) computer (Apple Computers, Cupertine, CA, U.S.A.) with a video display (F and G); a stainless-steel column (15 cm × 4.6 mm I.D.) (H) packed with LiChrosorb RP-8 (5 μm) (Merck) or Nucleosil C_{18} (5 μm) (Macherey, Nagel & Co., Düren, G.F.R.); a WISP-710B autoinjector (Waters Assoc., Milford, MA, U.S.A.); two M-45 pumps (Waters Assoc.); and an M-720 solvent programmer (Waters Assoc.).

The columns were packed by means of a balanced density slurry method specially developed for the ammonia elution system[6]. Gradient elution was performed with water (0.005 *M* ammonia) and methanol as eluent components, using linear and/or stepwise gradient programmes. Elution usually started with water (0.005 *M* ammonia) to which methanol was added, according to the desired programme. The

Fig. 1. UV–visible detection set-up used in the HPLC experiments.

final elution was usually effected with 100% methanol. Flow-rates were 1 ml/min. The eluent components water and methanol were saturated with helium before chromatographic use. During a run, a helium atmosphere was maintained in the eluent reservoirs.

RESULTS AND DISCUSSION

The spectrophotometer used as an HPLC detector was originally developed for UV–visible spectroscopic analysis only. By replacing the sample cell with a flow cell with a volume of 8 μl we could use it also as an HPLC detection system. When the spectra are stored in the memory of the instrument, consecutive spectra can be recorded with a time interval of 1 sec. However, because of the limited storage capacity, only 35 spectra can be stored in this way, which is usually not sufficient for a chromatographic run on a biological sample. Thus, for the latter purpose, the data must be transferred to an external storage device such as a tape or a disk, which takes at least 3 sec for one spectrum. Recording spectra with a minimum time interval of 3 sec was found to be more than adequate for our HPLC work. It will also be clear that,

because of the large amount of information generated by the detector, the data have to written into a memory device, as direct data plotting during analysis is not feasible under these conditions. During a run, a built-in monitor in the detector can provide either the full spectra that are being recorded or a portion thereof, or the chromatographic trace at a single, pre-selected wavelength. Comprehensive access to the stored data and plotting thereof, *e.g.*, in three-dimensional spectrochromatograms or in reconstructed, single-wavelength chromatograms is possible after the run.

In our experiments the diode array spectrophotometer was connected in series with a conventional single-wavelength detector as shown in Fig. 1, in order to compare the potentials of the two detectors[2,4]. It also had the advantage that we could now, during a run, display the absorption spectra of the eluting components via the monitor of the diode array detector and record the chromatogram at a single wavelength via the conventional detector. This set-up proved to be extremely useful when dealing with complex materials in relatively long runs. In this way the conventional chromatogram, recorded at a wavelength at which we anticipated that most of the compounds of interest would be detectable, served as a lead to recall relevant data from the memory after the run for further examination, manipulation and plotting.

In the following we describe three practical applications of LC–DAD in qualitative analyses.

Screening for butoprozine metabolites

Butoprozine is an indolizine derivative with anti-anginal properties[7,8]. As with all new drugs, the elucidation of its metabolic degradation pattern is important for a better understanding of its pharmacological and toxicological properties. The structure and UV spectrum of butoprozine are given in Fig. 2. In previous papers we described systematic approaches to the investigation of the three phases in drug metabolic profiling mainly based on conventional single-wavelength detection: (a) screening for unknown metabolites in the biological fluid of choice[9–12], (b) isolation

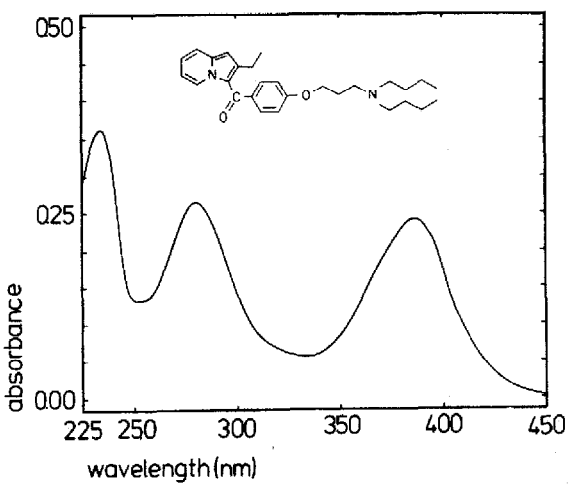

Fig. 2. Structure and UV spectrum of butoprozine. From ref. 4.

of the metabolites from the biological matrix[5,11] and (c) identification (structure elucidation) of the metabolites[11]. Separations in the screening and isolation phases were based on reversed-phase HPLC in both the isocratic and the gradient mode at alkaline pH established with amines or ammonia. Samples of excretory fluids such as bile and urine from different species were directly brought on to the column without extraction. In cases where a radioactive drug could be given the radioactivity of the eluent (measured on-line or off-line) provided a useful lead to the recognition of metabolites. However, in addition, UV detection at various wavelengths had to be carried out in order to obtain an insight in the UV-absorbing background of a metabolite peak and to develop screening methods for experiments in which only non-radioactive drug can be given. In a recent paper[11] we described an analytical approach to the recognition, isolation and identification of metabolites in human bile after administration of a non-radioactive drug. In these studies UV detection could only be carried out with conventional single-wavelength detection. With the DAD now being available we repeated the human bile investigations in order to compare the two UV approaches.

Fig. 3 shows the screening results represented in three spectrochromatograms taken with the DAD. Spectra were recorded with a time interval of 12 sec which resulted in 500 spectra of 400 absorption data points each in a complete run from water (0.005 *M* ammonia) to methanol (no ammonia) in 100 min, in a linear gradient. For reasons of time and simplicity only the relevant wavelength region 225–500 nm has been plotted (plotting of one complete spectrum from 200 to 800 nm takes about 50 sec); the region 500–800 nm was not of interest as none of the eluting compounds showed absorption there.

On the right is a spectrochromatogram of blank bile; the middle trace shows the bile situation during 0–6 h after administration of butoprozine. We call these chromatograms straight chromatograms because the sequence of the consecutive spectra corresponds to the time sequence. Comparison of these two runs shows readily (a) the presence of three main endogenous components (E_1, E_2 and E_3) in the blank and in treated bile, their concentrations changing during the time of the experiment, and (b) the presence of one main metabolite (M) in treated bile. This is in agreement with the single-wavelength experiments in which also one human main metabolite was found[11]. However, even though the plotting of the spectrochromatograms takes relatively long, LC–DAD provides a dramatic gain in time, by as much as 90% compared with the classical UV approach. Peak recognitions are based on retention time comparison (provided by the high reproducibility of the separation system) and spectra comparison (provided by the highly informative detection system). Especially the latter gives additional evidence about the character of the peaks in comparison with conventional detection. Also, the spectrochromatograms can give direct structural information, as is demonstrated in the UV spectrum of M. This metabolite has a similar spectrum to the parent drug but shows very different chromatographic behaviour (eluting with about 60% methanol instead of about 90% methanol for the parent drug). This indicates the introduction of a polar group(s) in an otherwise intact aromatic system in M. Structure elucidation has revealed the introduction of one hydroxy group at the 1-position of the butoprozine molecule[11].

With all the absorbance data available on a tape or disk, plotting of the data points as well as connecting them can be done in various ways, provided that the

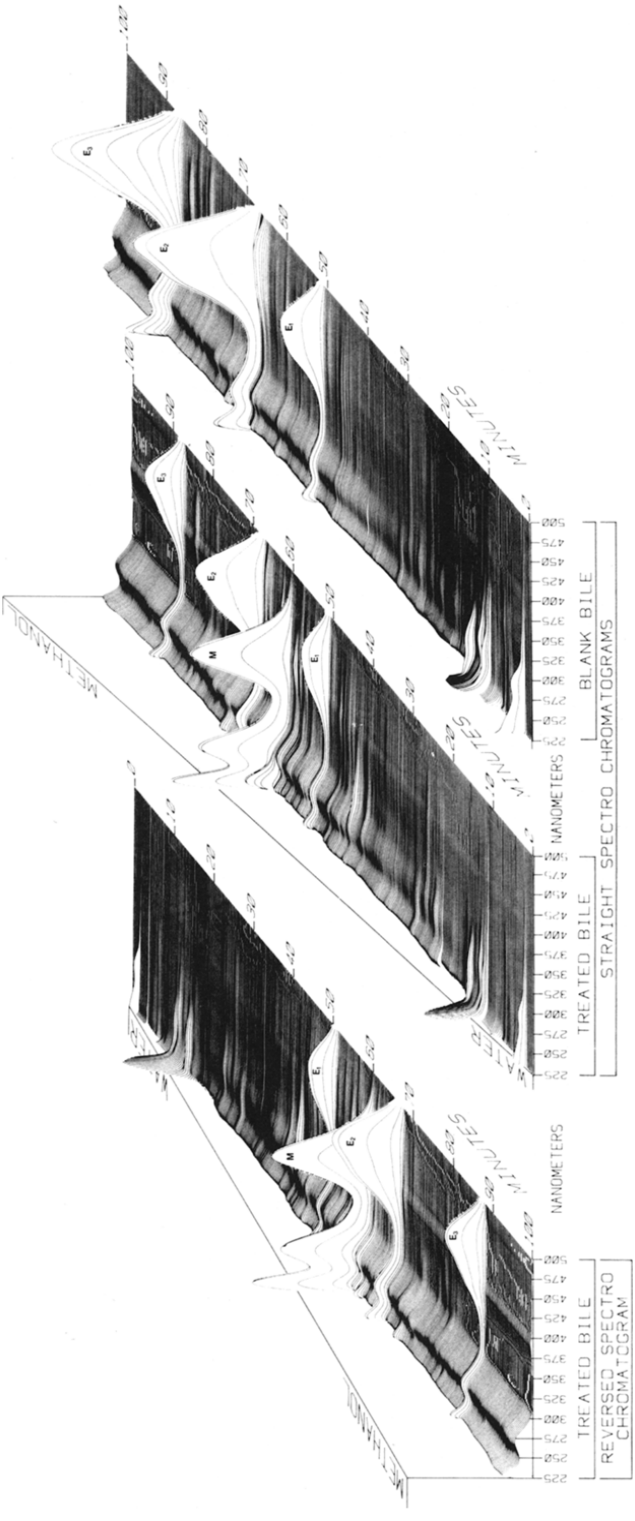

Fig. 3. Spectrochromatograms of blank human bile (−1 to 0 h before butoprozine administration) and treated human bile (0–6 h after butoprozine administration) for differentiation between metabolites and endogenous components. Conditions: column, 15 cm × 4.6 mm I.D.; packing, Nucleosil C_{18} (5 μm); eluent, 100% water (0.005 *M* ammonia) to 100% methanol (no ammonia) in 100 min; flow-rate, 1 ml/min; injection, 50 μl; absorbance, 0.0–1.0.

required computer programs are written for each purpose. Time, wavelength and absorbance axes can be varied in magnitude, direction and angle. A first demonstration is given in the left-hand spectrochromatogram in Fig. 3, where the time axis has been reversed so that we are "going back in time". This reversed spectrochromatogram allows us to see what is present behind a peak. A detailed study of the two treated chromatograms with respect to the blank chromatogram now shows the possible presence of other minor butoprozine metabolites just before and just after the main metabolite M. In order to obtain a better insight, this particular part of the run was plotted at a much higher sensitivity and with a greater distance between the spectra, as shown in Fig. 4. Now the presence of the minor metabolites a, b, c and d can be better observed. However, because we instructed the computer not to plot in the shadow of a peak, Figs. 3 and 4 still do not reveal what is present between M and E_2. To observe this, the spectra 41–50 were plotted (Fig. 5). Now the minor metabolite d can be observed better. These figures demonstrate that with the DAD a more detailed insight into the metabolic profiles can be obtained. In the earlier single-wavelength studies only the main metabolite could be recognized.

In the gradient runs also an increasing background in the wavelength region 225–310 nm can be observed when going from water to methanol. This is further illustrated in the eluent run shown in Fig. 6. It is possible to subtract such an eluent background from the blank and treated chromatograms by recording an eluent run before the analysis of the biological samples. However, it requires extra memory space and good run-to-run reproducibility of the chromatographic system. In our case, subtraction of the background in Figs. 4 and 5 confirmed the metabolic character of peaks a–d.

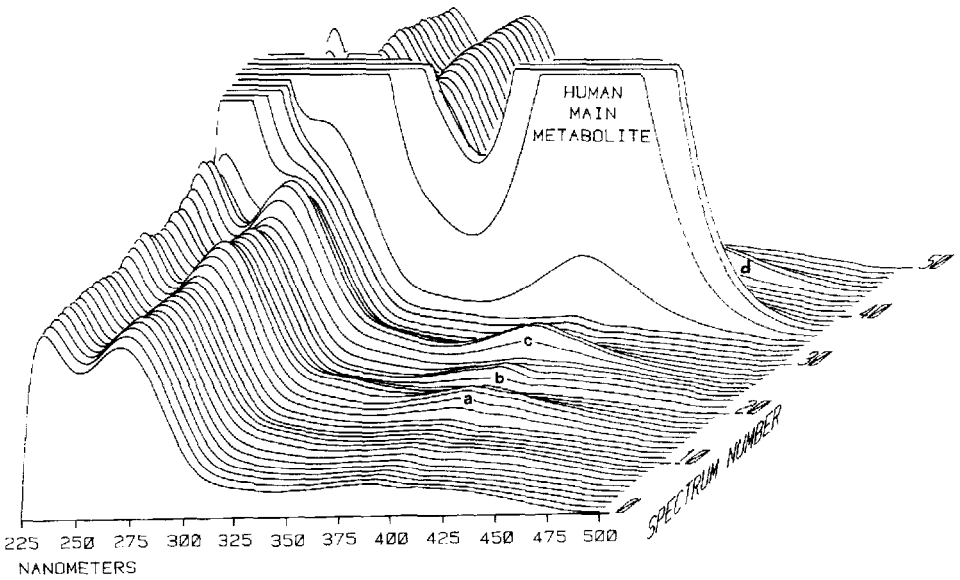

Fig. 4. Part of the treated straight spectrochromatogram in Fig. 3 for recognizing the minor metabolites (a, b, c and d). Absorbance, −0.01 to 0.2.

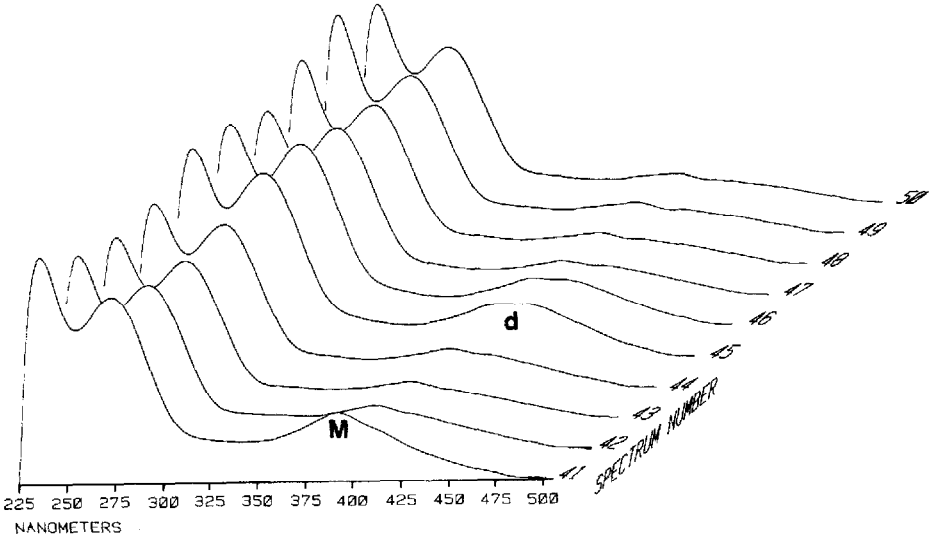

Fig. 5. Plots of spectra 41–50 in Fig. 4 just behind the human main metabolite for a complete view of minor metabolite d. Absorbance, −0.01 to 0.2.

Another way of manipulating the spectral data is demonstrated in Fig. 7, where conventional single-wavelength chromatograms have been reconstructed with an interval of 10 nm. In essence, it means connection of absorbance data points of the same wavelength and plotting in the conventional way at a sensitivity of choice. As

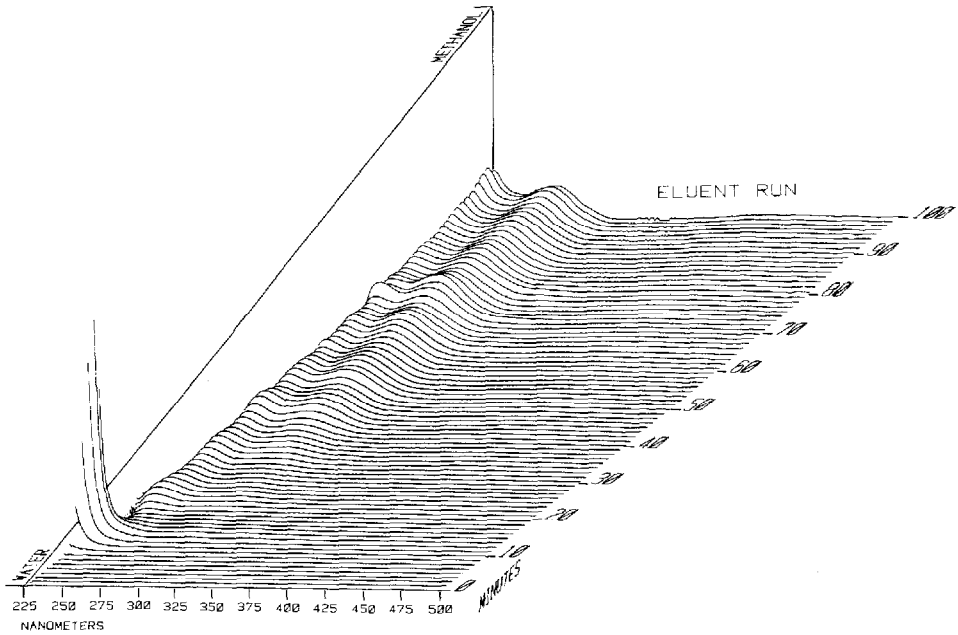

Fig. 6. Chromatographic run of water (0.005 *M* ammonia) to methanol to obtain more insight into the background. Conditions as in Fig. 3.

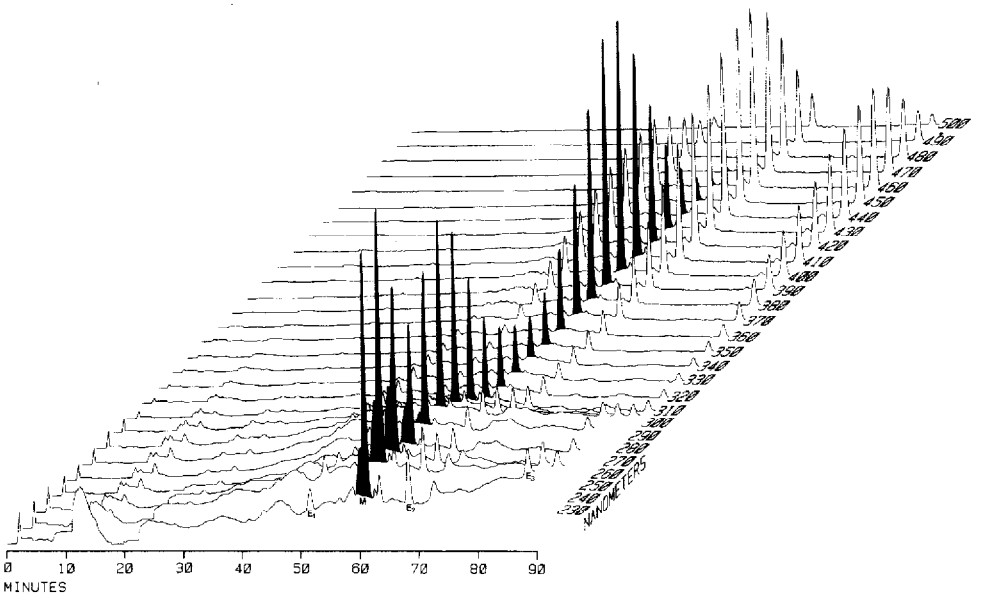

Fig. 7. Reconstructions of single-wavelength chromatograms from the treated bile data in Fig. 3. Attenuation, 1.0 a.u.f.s.

can be seen, the spectra of the main metabolite and the endogenous components are now represented in another way and at a different angle. This kind of plot especially facilitates the selection of a suitable wavelength for quantitation. The increase in the baseline from 225 to 310 nm in a gradient run can also be observed as in Fig. 3, but now in a more conventional way. From 300 to 500 nm M looks pure, but there clearly is a shoulder present, eluting just before M and absorbing between 230 and 290 nm. This shoulder can also be observed in the treated bile picture in Fig. 3 and may represent another metabolite as there is no peak indication in blank bile in this area. It should further be noted that this shoulder should be avoided when trying to isolate M for structure elucidation.

In Fig. 7 the sensitivity for plotting was 1.0 a.u.f.s. adapted to the main peaks. The same blank and treated runs were also followed by the conventional detector working at 400 nm at 0.2 a.u.f.s. The latter chromatograms are given in Fig. 8B and C, demonstrating the limitations of a conventional detector without a computer system. The wavelength and the attenuation are selected before the chromatographic run in the hope that a suitable result is obtained. Especially in screening analysis in which both qualitative and quantitative information about the compounds is not available, it is difficult to pre-select the optimal conditions, with the result that in most instances more runs must be carried out. With the data stored on tapes or disks the desired sensitivity can be chosen after the run. Fig. 8A shows a reconstruction of a chromatogram from the data recorded with the DAD and plotted on the same scale and with the same sensitivity as the other pictures in Fig. 8. A comparison between the pictures indicates that peak broadening in the detector set-up is negligible. Further, Fig. 8 demonstrates that the major human metabolite can be easily detected by

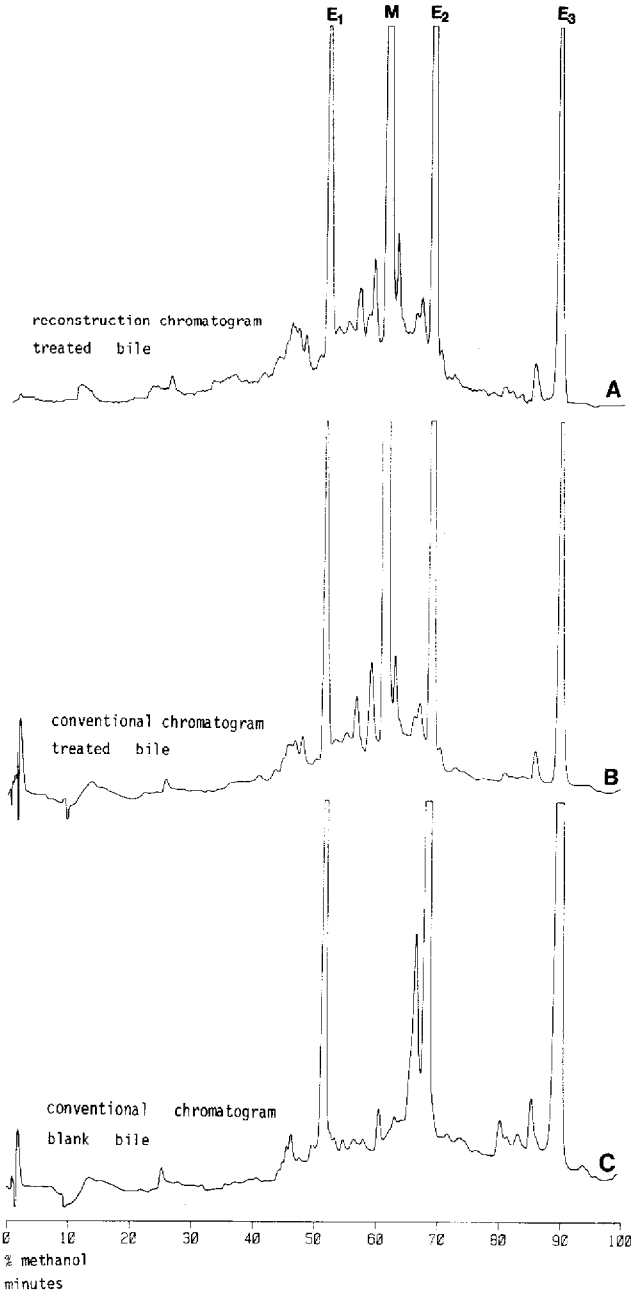

Fig. 8. Comparison of chromatograms obtained with the diode array detector (A) and with the single-wavelength detector (B) and C) recorded on-line. Conditions as in Fig. 3, except wavelength 400 nm and attenuation 0.2 a.u.f.s.

conventional detection but that it is virtually impossible to recognize the minor metabolites a–d in this way, even though the detector is being used at high sensitivity.

In the above application extensive use was made of spectrochromatograms.

This requires relatively long plotting times and time and effort are also lost on areas in the run where no compounds are eluted. Therefore, in the second application, we used the conventional detector to prepare a lead chromatogram at a suitable wavelength at which we can expect to recognize most if not all eluting compounds of interest. In addition, the drug used in the next application showed UV absorption only between 200 and 280 nm and was thus devoided of a characteristic absorption around 400 nm as was the case with butoprozine.

Screening for ticlopidine metabolites

Ticlopidine is a drug that reduces platelet aggregation. Its structure, UV spectrum and chromatographic behaviour in the general gradient with respect to butoprozine are shown in Fig. 9. From the UV spectrum of ticlopidine, 235 nm was chosen as initial wavelength for the conventional detector. At this wavelength we may expect to detect most metabolites with sufficient absorption and present in sufficient amount. The drug metabolic study was started on rat level after intravenous administration of ^{14}C-labelled ticlopidine. Radioactive measurements showed an excretion of 75 % in the bile and 25 % in the urine. In bile most radioactivity was found back in the first 2 h after drug administration. Therefore, blank bile (− 1 to 0 h) and treated bile (fractions 0–1 h and 1–2 h after drug administration) were taken for a first analytical study. In the chromatographic analysis of radioactive bile, on-line radioactivity detection was chosen as specific detection mode for detecting the separated metabolites containing the label. Also conventional UV detection was performed to gain more insight into the background, to detect possible metabolites without the label and to obtain a lead chromatogram for further DAD application.

In Fig. 10, three chromatograms at 235 nm are shown with the purpose of differentiating between ticlopidine metabolite peaks and endogenous bile peaks. Although it was possible to separate butoprozine metabolites without too much

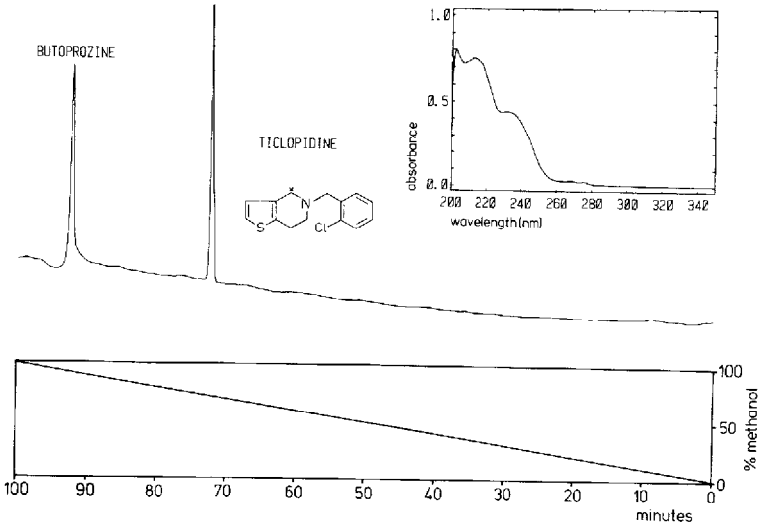

Fig. 9. Structure and UV spectrum of ticlopidine. Separation of a butoprozine–ticlopidine mixture in the general gradient. Conditions as in Fig. 3, except wavelength 235 nm and attenuation 0.5 a.u.f.s.

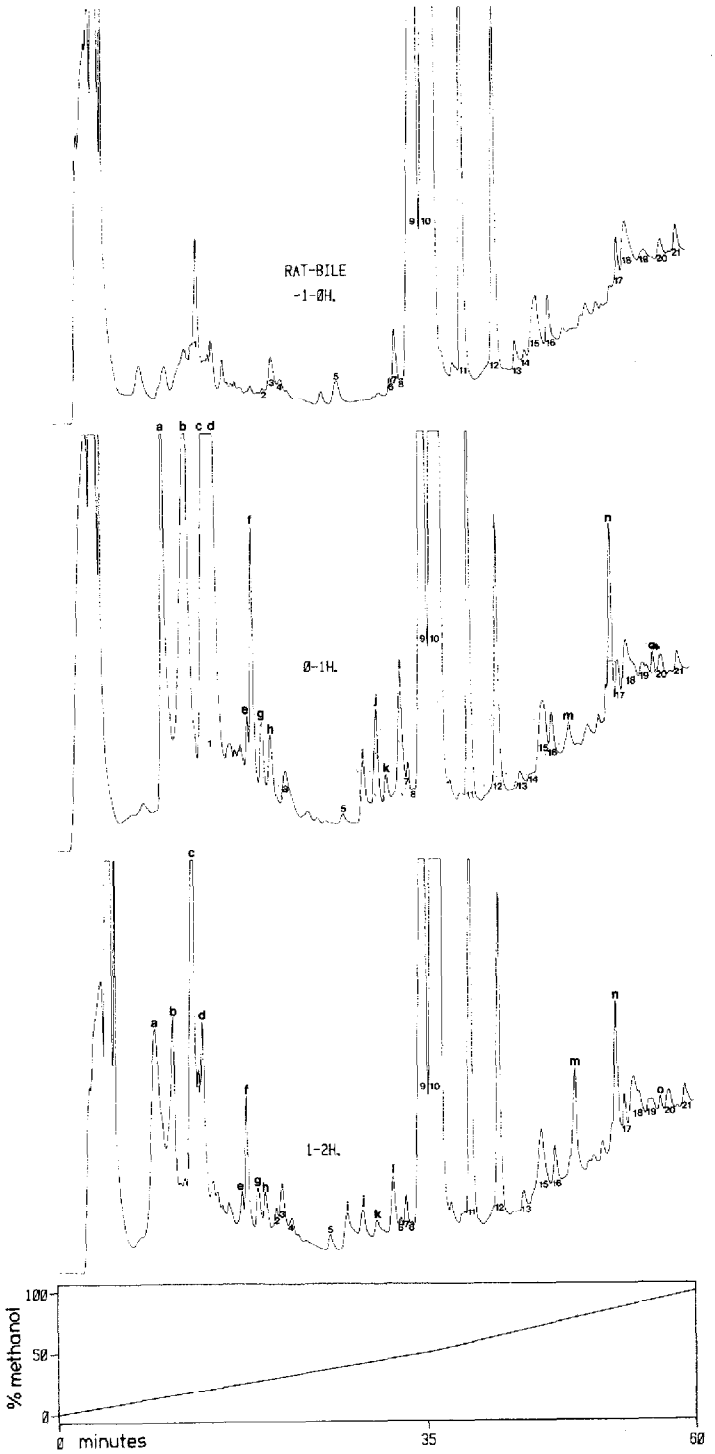

Fig. 10. Comparison of chromatograms recorded with the single-wavelength detector of rat bile, the hour before and the first and second hours after ticlopidine administration. Simultaneous recording of UV spectra took place with the diode array detector. Conditions as in Fig. 9, except the gradient profile.

interference from endogenous components in the general continuous gradient of 100 min, with ticlopidine most metabolites were much more polar, eluting in the first part of this gradient at lower methanol concentrations, concomitant with various endogenous compounds. Hence, the gradient profile was adapted to these separation demands as shown in Fig. 10. In order to recognize the metabolites against the background based on retention time comparison only, a high reproducibility between the blank and treated runs is required. In daily routine HPLC, maintenance of the reproducibility for longer periods of time is not always possible. In Fig. 10, the runs were recorded on different days with the result that an absolute reliable retention time comparison becomes difficult, also owing to the large number of peaks. For more evidence about character (metabolite or endogenous) and the correspondence of metabolites in different fractions, additional characterization is thus required and for that purpose we evaluated the LC–DAD system.

During the runs shown in Fig. 10, every 4 sec a spectrum was recorded, monitored and stored on tape. The spectra were numbered 0, 4, 8, 12, etc., thus corresponding to the time axis of the conventional chromatograms. After the run we screened the tapes for compounds absorbing at 235 nm and plotted their UV spectra by taking it at the top of the elution profile (or at a shoulder) with subtraction of the background absorbance. We then scanned the tapes for peaks not absorbing at 235 nm that seemed to be metabolite related. After such a spectral screening, peaks with similar retentions and UV properties were given the same symbol, 1, 2, 3, etc., for endogenous compounds and a, b, c, etc., for potential metabolites.

Four spectra of peaks obtained in this way are shown in Fig. 11. Spectra 1 and 2 were derived from peak 3 in blank bile and the first treated bile fraction, respectively. After retention time and spectra comparisons we may assume we are dealing with the same endogenous component. Spectrum 1 is given in its real form as recorded in the gradient run and also after a smoothing technique[13] carried out with the computer. Spectra 3 and 4 were recorded of peaks a and n, respectively, which are present only in treated bile, so that we may assume that they are metabolites. This was checked with radioactivity measurement of the respective peaks. Peak n contained radioactivity but peak a did not, indicating a metabolite without the label or a possible endogenous component formed during the course of animal experiments. In such a case, labeling of another part of the molecule is necessary. In this way most metabolites were localized and checked in the bile.

Although a UV spectrum may be compound-characteristic in terms of its maxima and overall shape, it provides little structural information. Nevertheless, in our work with butoprozine it could be concluded from the DAD approach that most metabolites in various species still had an intact indolizine moiety[4,11,12]. However, with ticlopidine we encountered numerous metabolites with a UV spectrum completely different from that of the parent compound. For elucidation of the structures of these metabolites, additional spectroscopic analysis is necessary with more powerful techniques such as mass and NMR spectroscopy.

Compared with the first application, the DAD approach used here may not look so spectacular. However, the gain in information from each run on using DAD was very large and also saved considerable time.

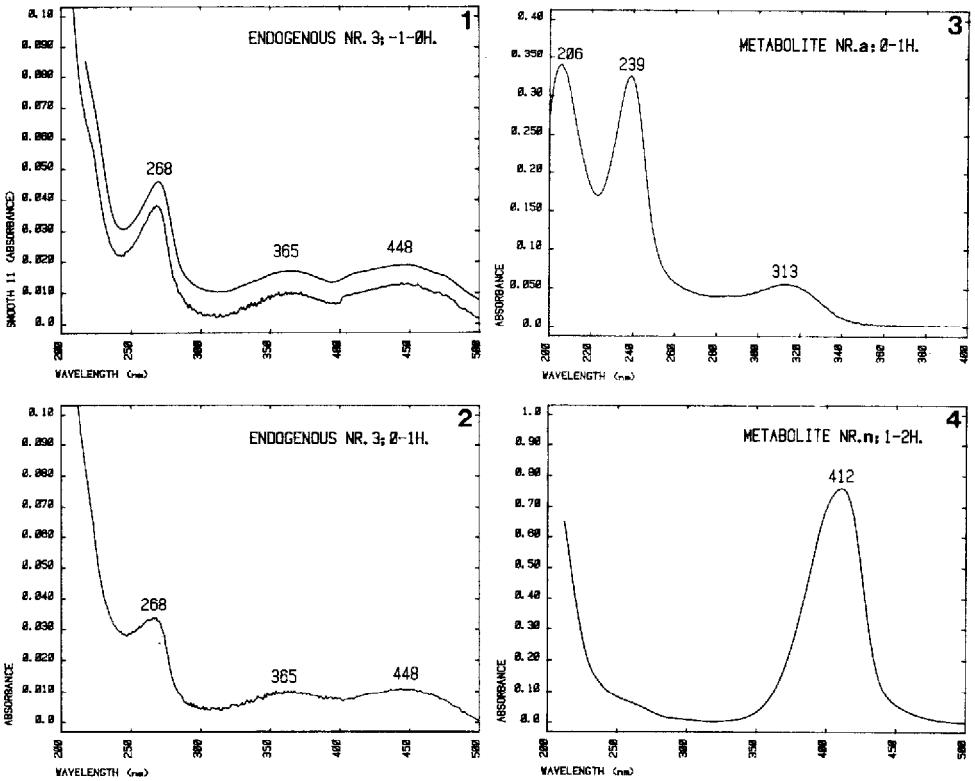

Fig. 11. Some UV spectra derived from the peaks in Fig. 10 by peak-top spectrum minus background.

Screening for drugs

There is a basic difference between screening/identification for drug metabolites and drugs. In drug metabolic profiling the metabolites are new biologically synthesized compounds. The only reference points are the structure and the properties of the parent drug from which they are being derived. Therefore, the screening is initially directed towards finding out how many metabolites have been formed, to see for how far the parent drug has been converted and if possible to obtain a relative quantitative picture about the drug and/or metabolite(s). Because many metabolic routes from the parent drug can be derived it is difficult to make exact predictions about the structure of the metabolites formed. To identify the metabolites, the screening must be followed by isolation and structural elucidation with spectroscopic techniques.

When starting a screening study on biological or other relevant materials for the presence of drugs, *e.g.*, in clinical or forensic toxicology, the number and identities of the drugs are also unknown. Contrary to the situation in metabolic profiling, however, the unknowns are not new compounds. Generally, reference compounds are available, from which we can compile a bank of reference data. When using a chromatographic system, the retention behaviour of the unknown compound can be checked against the data of the reference compounds in the data bank, thus present-

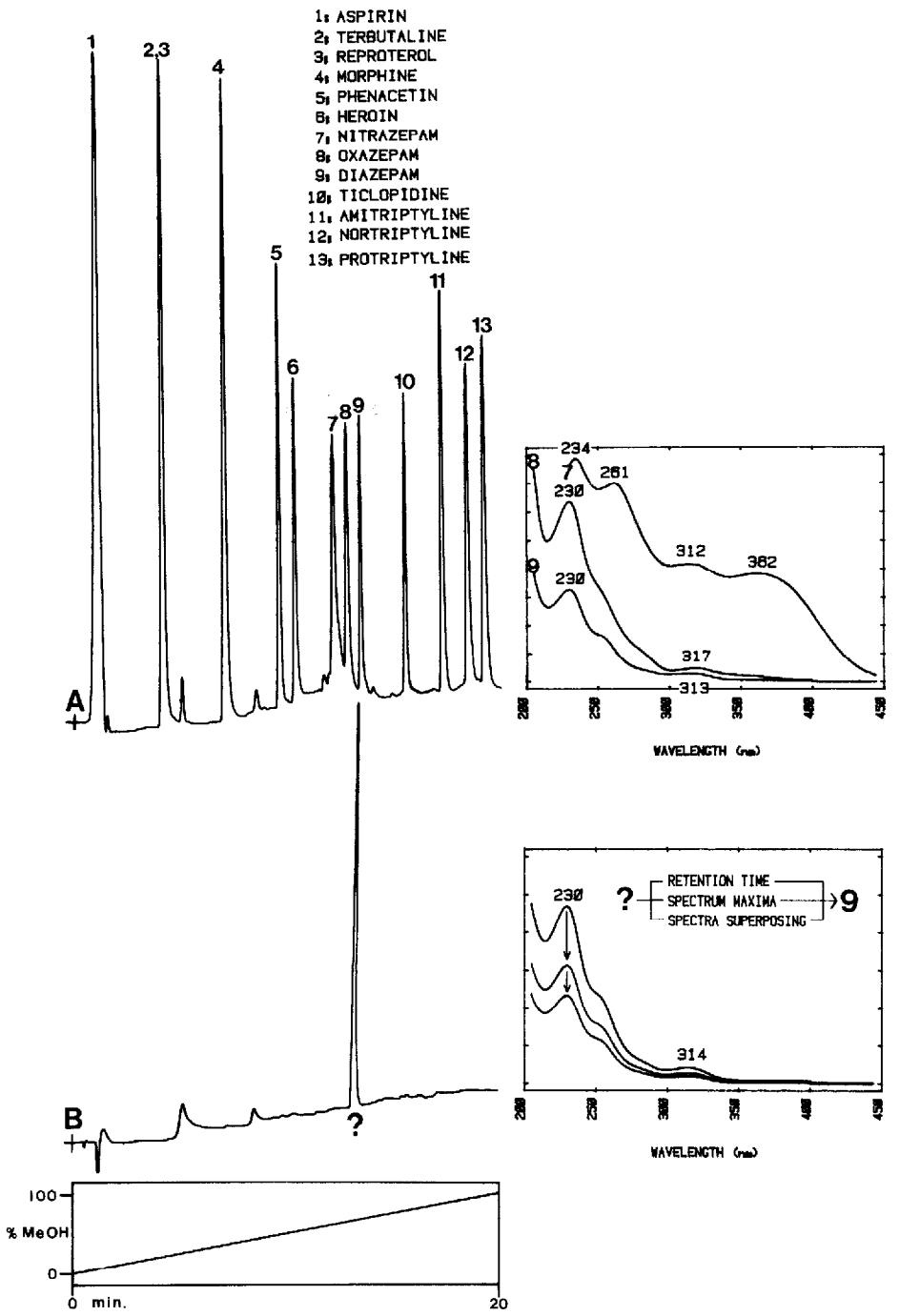

Fig. 12. Approach to the identification of drugs in biological fluids by comparisons of retention times and UV spectra. Conditions as in Fig. 9, except the gradient profile and packing, LiChrosorb RP-8 (5 μm). For further details see text.

ing a first possibility for identification. However, in view of the large number of drugs that have to be taken into account and the occurrence of intra- and inter-laboratory variations in the determination of the retention behaviour, a single retention time or R_F value usually is not sufficient to identify the compound under investigation. By using LC–DAD, the UV spectrum can serve as a second identification parameter which can be combined with the retention time parameter.

A preliminary application of this approach is depicted in Fig. 12. Here we used the HPLC system for rapid screening purposes, capable of dealing with a large variety of drugs, differing in character and polarity. More details about the separation system, such as kind of stationary phase, column packing procedures, gradient *versus* isocratic elution, kind of gradient profile and the basic modifier used and its concentration, will be given in a later paper[6].

Fig. 12A shows the separation of thirteen drugs in a linear gradient of 20 min. In this reference run, in addition to the determination of the retention time, recording of the UV spectrum also took place with the DAD. It should be noted that the spectra of the reference compounds in the data bank should be recorded at the eluent composition at which they elute in the chromatographic run. For three benzodiazepines (7, 8 and 9) such UV spectra are given in the upper insert after the chromatographic run A. Fig. 12B gives a chromatogram of a plasma sample after subtracting the endogenous background, showing a major unknown peak. Retention time comparison with the reference data allows the exclusion of drugs 1–6 and 10–13. Taking into account a certain spread in reproducibility, only compounds 7, 8 and 9 (and other drugs eluting in this region) are to be considered. For further identification, the UV spectrum was recorded in run B and plotted at different sensitivities after run B. Comparing the maxima with the reference maxima, nitrazepam (7) can be excluded. Differentiation between 8 and 9 is more difficult because they have very similar maxima. Now the overall shape of the spectra can be compared. Because this depends on concentration, the recorded spectrum of the unknown compound should be superimposed with reference spectra. With the DAD this is easy to perform. Now, it appeared that the unknown compound had the same spectrum as diazepam. Additional possibilities for confirming the identification are the generation of second or higher order derivative spectra with the DAD[14]. Of course, other chromatographic systems and/or mass spectrometry can also be considered.

Although this application represents a relatively simple case, it again illustrates the advantages of a high separation efficiency combined with highly informative detection.

CONCLUSIONS

This paper clearly shows the potential of LC–DAD especially in the area of qualitative analysis. Considering the detector unit of the HPLC equipment, the advantages with respect to conventional UV detection are evident. However, some limitations of the DAD used can be noted: first, the limited memory capacity for storing the spectral data of the instrument itself; second, the plotter technique, which is rather time consuming; and third, the limited possibilities for displaying the data during chromatographic analysis. With regard to the last aspect it is desirable that at least individual spectra plus the chromatographic trace at a pre-selected, fixed wave-

length can be displayed during the run. The ideal situation would be reached if a three-dimensional spectrochromatogram could be displayed.

It has also been shown that the information that can be obtained from a sample mixture is greatly enhanced when the spectrometer is coupled to a powerful HPLC separation unit. The system developed, based on reversed-phase HPLC at relative high pH established with ammonia, has proved to be both universal in chromatographing individual compounds with widely different structures and selective in the separation of complex mixtures, both a need in qualitative analysis.

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