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COMPUTER-AIDED STRATEGIES FOR ARCHIVE RETRIEVAL AND SENSITIVITY ENHANCEMENT IN THE IDENTIFICATION OF DRUGS BY PHOTODIODE ARRAY DETECTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An archive retrieval algorithm for high-performance liquid chromatography with UV detection (HPLC–UV) has been developed for the first time for the rapid identification of spectra acquired by rapid-scanning photodiode array detection in HPLC. The algorithm is based on a database of spectra (normalised with respect to area), inverse files of key spectral features, a selective search window, with parabolic weighting factors and least-squares comparison of test and retrieved spectra. The performance of the library search system is demonstrated with respect to a small library of solutes, including: cortisone acetate, ethynyl estradiol, ethisterone, progesterone; caffeine, theobromine, theophylline and 8-chlorotheophylline; morphine and diamorphine; and cycloserine.

Since photodiode array detection operates in the domains of both wavelength and time, to generate a matrix of (A,λ,t) data, the optimum conditions for sensitivity enhancement by ensemble averaging in these domains have been examined. At a given observation wavelength, increase in the detector bandwidth (or "diode bunching") yields a value of $\Delta\lambda$ which gives optimum sensitivity; this value is systematically related to the spectral bandwidth of the analyte. Sensitivity can also be optimised by varying the integration period in the time domain. Sensitivity can be further increased by combination of these instrumental optima, for which there is evidence of dependence on the particular instrument design. Response was found to be linear for detector bandwidths up to twice the optimum value. The comparative sensitivity of some commercially-available photodiode array detectors has been assessed relative to that of conventional detectors under strictly controlled conditions.

INTRODUCTION

The combination of high-performance liquid chromatography (HPLC) with rapid-scanning UV spectroscopy has led to the evolution of several new concepts for enhanced detection sensitivity and selectivity[1–8]. These multichannel spectrophotometers are conventionally based on the linear photodiode array detector and have found application in the analysis of drugs[5–8], metabolites[7–11] and environmental pollutants[12].

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The rapid rate of spectral acquisition during elution yields a large "spectrochromatogram" of (A,λ,t) data, so that special computer-aided techniques are required for data reduction, manipulation and presentation[4–6]. The digital techniques can be classified in three main groups:

(a) Methods drawn from classical analytical spectroscopy, including principal component analysis and archive retrieval techniques.

(b) Methods for data reduction and graphical presentation.

(c) Methods unique to the photodiode array detector itself.

In the first group, digital techniques drawn from analytical spectroscopy have been primarily developed to assess whether or not a chromatographic peak is homogeneous.These include the absorbance ratio[5,13] which suffers from lack of sensitivity[6]; and spectral suppression[6,14], which is based on difference spectroscopy and yields a difference function, which is linearly related to solute concentration.

Other digital techniques developed from, or by analogy with, analytical spectroscopy include the spectral deconvolution chromatogram, where all the spectral data are deconvoluted using standard reference spectra in a least-squares inverse matrix routine[1,6]. Principal component analysis[15,16] and matrix rank annihilation factor analysis[17] have also been proposed for the quantitative assessment of the probable number of components in a spectrochromatogram.

Also in this group, the second and higher derivative transformation has been proposed for the resolution enhancement of overlapping bands, both in the time domain and in the wavelength domain[3]. In the second derivative chromatogram the function d^2R/dt^2 (where R represents the response of any HPLC detector) is plotted against elution time in order to improve the apparent resolution of overlapping peaks. This approach, which can be applied at any convenient wavelength, has been proposed as a test for peak homogeneity[3,6,11] and as a criterion for automatic method development[18], with the proviso that it does not apply to peaks which exactly coincide. In the wavelength domain, second and higher derivative spectra ($d^2A/d\lambda^2$, ...) have been shown to yield more characteristic profiles for qualitative comparison[3,5,7] and for archive retrieval purposes.

In establishing a library of spectra for use in characterising solutes separated under well-defined HPLC conditions, it is essential to ensure the purity of the candidate reference compounds. This can be achieved by applying one or more of these digital techniques for assessing peak homogeneity. A number of methods have been proposed for archive retrieval in spectroscopy[19]. Of these, one of the most readily implemented is the method based on "inverse files" of key spectral features and least squares comparison of the observed spectrum with the retrieved candidate spectra. The value of any spectral archive is, however, conditioned by the solvent system and the operating parameters employed, as discussed in the present work.

In the second group of digital methods, data reduction and graphical presentation techniques for the photodiode array detector in HPLC are now well-established[4]. The "spectrochromatogram" can be conveniently presented as an isometric plot of (A,λ,t) at any convenient viewing angle[6–8]. Alternatively, all the data can be viewed simultaneously "from above" as a contour plot, where concentric, isoabsorptive contours are used to map the data in the (λ,t) plane, as recently proposed for studies on drug metabolites and endogenous amino acids[11,20,21]. Colour-coding has also been proposed in order to simplify interpretation of the contour plots, where,

for example, the most strongly absorptive features can be coded red, with the use of up to eight colours to code the lower contour levels[20,21]. Data reduction modes where the (A,λ,t), spectrochromatogram is interrogated to present an individual spectrum for each peak, and a chromatogram at one or more wavelengths, are readily implemented with contemporary equipment.

Finally, one feature of the photodiode array detector element itself, which can be readily exploited, is to combine adjacent diode outputs under computer control. If, for example, the outputs of all photodiodes in the UV range are summed, then a total absorbance chromatogram is obtained, which is analogous to the total ion current technique in mass spectrometry[11]. This can give a rapid overview of all the solutes separated.

Recently, the present Authors have shown that by "bunching" adjacent photodiodes together, the signal-to-noise ratio (SNR) can be improved[11]. This might be expected from consideration of the noise characteristics of the detector, since the noise level decreases in proportion to the square root of the number of datum points averaged[1]. For the metabolite norzimeldine it was also found that on increasing the detector bandwidth, maximum SNR increase was observed at a specific detector bandwidth, beyond which the sensitivity progressively decreased[11]. This latter observation implies that for a given solute there may exist an optimum "wavelength window" for highest sensitivity in monitoring the elution profile, and that deterioration in sensitivity may be expected to occur when the low absorptivity "wings" of a spectral band are sampled. The hypothesis can be advanced that the detector bandwidth for maximum SNR performance should be related in some systematic way to the shape and spectral bandwidth of the analyte in question.

By analogy, it would not be unreasonable to suggest that in the time domain, an optimum observation "time window" exists, giving highest SNR, and that this is systematically related to the chromatographic bandwidth of the peak, under defined conditions of spectral detector bandwidth. It might also be expected that it should be feasible to maximise the overall sensitivity of the photodiode array detector by combining the optimum criteria found in the two domains of wavelength and time.

Since sensitivity is a critical factor in the use of HPLC for detection of drugs, impurities, drug metabolites, endogenous materials and environmental residues, the sensitivity of some commercially-available photodiode array detectors and conventional variable-wavelength detectors has been critically examined. In the present work, the relationship between photodiode array detection sensitivity and spectral bandwidth of the detector has been established for a variety of compounds with differing spectral bandwidth and shape. The influence of the detector "time window" on detection sensitivity and on chromatographic performance has been studied. The conditions for optimising detection sensitivity with respect to both the wavelength and the time domains have also been examined.

An algorithm for establishing and searching an archive of spectra, acquired by a photodiode array detector, has been developed for the first time and its performance assessed for the qualitative characterisation of solutes separated by HPLC.

EXPERIMENTAL

Reagents and materials

Methanol and acetonitrile (HPLC grade, Rathburn Chemicals, Walkerburn, U.K.) were used as received. Eluents were prepared with glass-distilled water and filtered through Millipore 0.45-μm filters in an all-glass apparatus, before degassing for 10 min in an ultrasonic bath under reduced pressure.

The following drugs were used as received: 8-chlorotheophylline, cortisone acetate, cycloserine, ethynyl estradiol, ethisterone, progesterone and theophylline (Sigma, St. Louis, MO, U.S.A.); caffeine and theobromine (BDH, Poole, U.K.); diamorphine hydrochloride and morphine sulphate (MacFarlan Smith, Edinburgh, U.K.).

Standard solutions of each drug were prepared in eluent system 1 [acetonitrile–water (70:30, v/v)], except as indicated, in concentrations appropriate to permit measurement of the SNR data: cycloserine, 20.3 μg/ml (in water); diamorphine HCl, 8.0 μg/ml; ethynyl estradiol, 9.8 μg/ml; morphine sulphate, 4.3 μg/ml; theophylline, 27.4 μg/ml (in water). A standard three-component mixture of cortisone acetate (1.1 μg/ml), ethisterone (1.1 μg/ml) and progesterone (1.7 μg/ml) was prepared in acetonitrile–water (70:30, v/v). In order to establish the spectral archive, concentrations approximately ten-fold higher were employed. Standard solutions for UV-spectroscopy were prepared in the same solvents, in concentrations which gave an absorbance between 0.2–1.0 absorbance units (a.u.) for the peak of interest.

All samples of drugs were weighed on a Cahn Model C29 electromicrobalance (Cahn, CA, U.S.A.).

Equipment

The modular liquid chromatograph consisted of a constant-flow LC pump, this being either the LKB 2150 (LKB, Bromma, Sweden) or the Waters Model 510 (Waters Assoc., Milford, MA, U.S.A.), together with a Rheodyne injection valve (Model 7125, Berkeley, CA, U.S.A.) provided with a 100-μl loop. Two column and eluent systems were employed.

System 1 was used for the measurement of SNR and comprised a 300 × 3.9 mm I.D. stainless-steel column, slurry-packed with 10-μm μBondapak C_{18} material (Waters Assoc.), with acetonitrile–water (70:30, v/v) as eluent at 1.5 ml/min.

System 2 was used for establishing the spectral archive under dynamic flow conditions, and comprised a 100 × 5 mm I.D. stainless-steel column, slurry-packed with 5-μm ODS-Hypersil (Shandon-Southern Instruments, Cheshire, U.K.), with acetonitrile–water (50:50, v/v) as eluent at 1.5 ml/min.

The column temperature of system 1 was maintained at 25 ± 0.1°C by furnishing the column with a thermostatted jacket (Jones Chromatography, Llanbradach, U.K.).

UV-spectra were recorded with a Perkin-Elmer Lambda 5 spectrophotometer (Perkin-Elmer, Beaconsfield, U.K.) and a matched pair of 1.0-cm silica cells.

Detector systems

Three optical multichannel detector systems were employed. System I comprised a Hewlett-Packard Model HP-1040A LC detector (Hewlett-Packard, Wald-

bronn, F.R.G.), equipped with a 4.5-μl quartz flow-cell. This detector was interfaced with the HP-85 microcomputer, equipped with input/output, plotter/printer, mass storage, matrix and advanced programming ROMs, 16-kbyte additional memory and an HP-IB IEEE-488 interface. A graphics plotter (HP-7470A) and a dual 5¼ in. flexible-disk drive (HP-82901M) were connected to the HP-85 microcomputer by means of the HP-IB interface.

Detector System II comprised the optical and data units of the LKB Model 2140 Rapid Spectral Detector System (LKB Produkter AB, Bromma, Sweden), interfaced by means of modified RS 232C with the IBM personal computer, equipped with dual 5¼ in. disk drives and a Canon A-1210 ink-jet eight-colour printer (Canon, New York, U.S.A.). The flow-cell volume was 5 μl.

Detector System III comprised the Pye-Unicam Model 4021 Multichannel UV–Vis Detector (Pye-Unicam, Cambridge, U.K.) equipped with a potentiometric recorder. The flow-cell volume was 8 μl.

Detector system IV comprised a Pye-Unicam Model LC–UV variable-wavelength detector, supplied with a 8-μl flow-cell and potentiometric recorder.

Detector system V comprised a Waters Model 451 variable-wavelength detector (Waters Assoc.), supplied with a 14-μl flow-cell and potentiometric recorder.

For detector systems I and II the software on disks supplied by the manufacturers was employed. Detector system III was used as a "stand-alone" unit, with its own software. The capability of detector system I was extended by developing an archive retrieval routine, as discussed below.

Methods

Archive retrieval. Detector system I was employed for archive retrieval work, using a "stand-alone" software module developed by the authors. After injection of a 20-μl standard, a spectrum was captured at the peak maximum under dynamic flow conditions for the drugs listed above.

The performance of the archive retrieval algorithm was examined for this small library of spectra by attempting to identify "test" compounds, whose spectra were captured under similar flow conditions.

The spectral archive system developed for detector I was based essentially on an algorithm described by Zupan[19]. In this method the key spectral features of a reference compound separated by HPLC are located after any necessary Savitzky–Golay smoothing[22]. The list of peak maxima and minima can be edited to eliminate any spurious signals, before storing each feature in the appropriate "inverse file" (Fig. 1). One inverse file is established for each nominal wavelength in the spectral range required.

With the particular detector used for this work (system I), an inverse file can be set up for each diode in the range 190–600 nm. The nominal resolution, r, in this case is 2 nm per diode. The spectral reference number is indexed into those inverse files, which correspond to one of the key features using a negative prefix to denote a minimum. The spectrum is normalised to the area under the curve and then stored under its index number. An index file is also established, in which the name of the compound, the index number and a list of maxima and minima are stored.

The archive retrieval method operates by locating the maxima and minima in the unknown sample spectrum, smoothed as appropriate. Then inverse files corre-

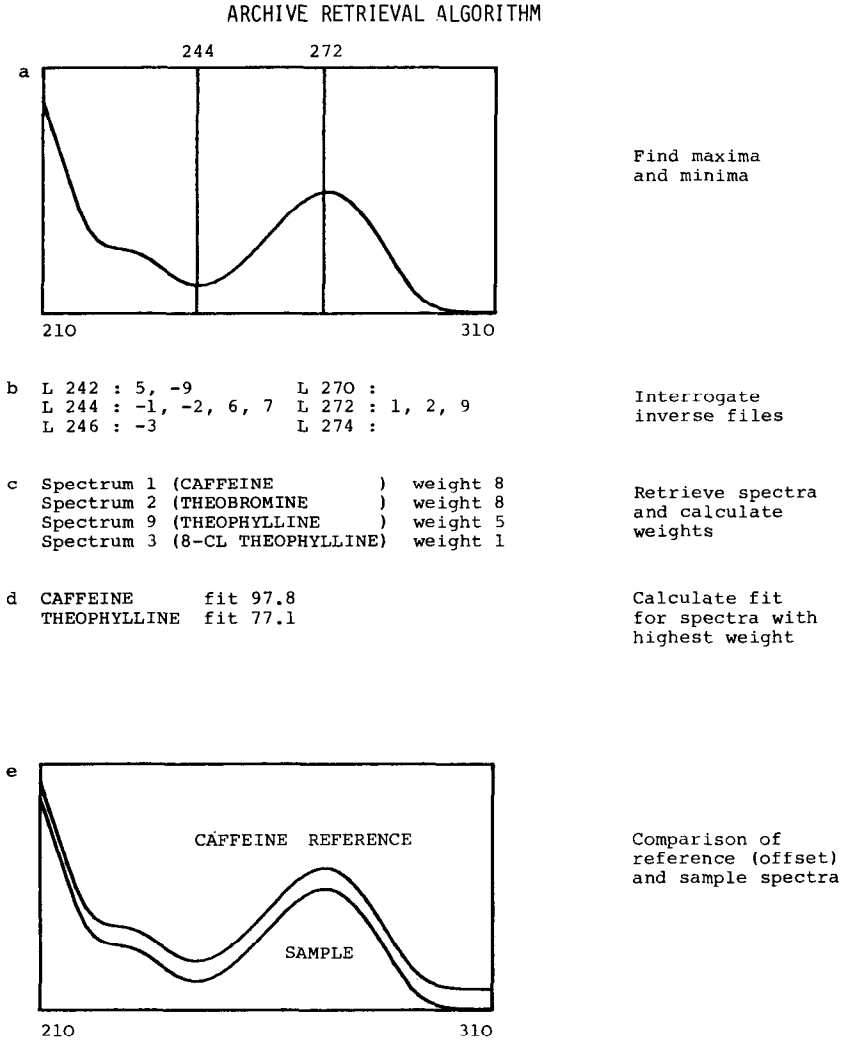

Fig. 1. Archive retrieval algorithm for HPLC–UV detection. a, peak maxima and minima are located for unknown spectrum at 244 nm and 272 nm; b, inverse files of maxima and minima (denoted by negative sign) are interrogated within a search window of ±2 nm; c, spectra (normalised with respect to area) are retrieved, excluding spectra of opposite sign; d, parabolic weights are assigned to each candidate spectrum (see text); e, sample spectrum is compared with retrieved spectra using a least squares fit parameter, scaled 0–100; e, visual comparison of best retrieved spectrum (caffeine; offset spectrum) with original sample spectrum. For chromatographic conditions, see text.

sponding to each feature are called up (Fig. 1). The inverse files within a search window, $\Delta\lambda = \pm 2$ nm (*i.e.* ± 1 diode), are also interrogated to allow for any uncertainty in the location of the feature, as may occur for example with very broad maxima and minima (Fig. 1).

A short-list of candidate spectra is then set up and a weight, w, assigned to each spectrum. This weight is determined both by the number, n, of inverse files

which refer to the candidate spectrum, and by the proximity of the inverse file wavelength retrieved, λ_i, to the wavelength of the feature observed, λ_s, in the sample spectrum:

$$w = \sum^{n} \left[1 + \frac{0.5\,\Delta\lambda - |\,\lambda_i - \lambda_s\,|}{r} \right]^2$$

where $\Delta\lambda$ is the width (in nm) of the search window and r is the resolution in nm per diode. This expression assigns the highest weight to the wavelength at the centre of the search window; weights then diminish parabolically towards the window limits. For example, if $\Delta\lambda = 4$ nm and $r = 2$ nm, the weights across the wavelength window become: 1, 4, 1.

For each of the retrieved candidate spectra with the *highest* weight value, a fit parameter is calculated, based on the root mean square of the difference between the normalised candidate spectrum and the sample spectrum. This fit parameter is scaled so that a perfect match is assigned a value of 100. If required, the fit parameter is then calculated for each of the candidate spectra with lower weight values. The candidate spectra are then listed in rank order.

Some considerable care is required in establishing the spectral archive. Several concentrations should be used in order to locate the maxima and minima of a spectrum accurately, especially in cases where the ratio of absorptivities is high. Clearly, in the case of spectral capture during elution in HPLC, a series of concentrations can be readily obtained by sampling different points in the peak profile. A critical aspect of the process of building and using a spectral archive is that precisely similar experimental conditions should be used for reference and sample compounds. This applies both to the HPLC separation conditions (column, eluent, flow-rate and temperature), and to the parameters selected for acquiring the spectral information (acquisition rate, reference wavelength and reference bandwidth, spectral range). Moreover, the rate of change of concentration, and therefore absorbance, should be slow relative to the spectral acquisition rate in order to avoid spectral distortion.

Detection limits. For individual detectors the detection limits were estimated by injecting three 100-μl samples of a three-component mixture of cortisone acetate, ethisterone and progesterone. Chromatographic system 1 was employed and a suitably sensitive absorbance scale selected. The photodiode array detectors I–III were used under their normal "default" conditions of detector bandwidth, integration time and, if specified, reference wavelength.

The peak-to-peak noise was sampled at the following points in each chromatogram and the average of these data used in calculating the detection limits: 35, 70, 140, 200 and 270 sec. The detection limit for each compound was calculated as the quantity (in ng) which gave a response equal to twice the average peak-to-peak noise level.

Sensitivity experiments. The relationship of the SNR with detector bandwidth (DBW) on the one hand, and with integration time (Δt) or "time-window" on the other hand, was established for a number of drugs, using detector system II with chromatographic system 1, a suitable absorbance scale sensitivity and a sample size of 100 μl. Three separate injections were made for each experiment.

The effect of varying the detector bandwidth was examined using a constant integration time of 0.2 sec and sampling the elution profile with a "step-time" of 1.0 sec. Each chromatogram was evaluated at the principal λ_{max} for a solute by using the IBM-PC to set up a series of values of DBW from 4 to 60 nm. An individual chromatogram was then plotted for each value of DBW and for each of the three injections corresponding to each compound examined. In some cases the SNR relationship with DBW was studied in more detail by examining small increments in DBW around the maximum.

The average peak-to-peak noise level for each chromatogram was estimated by measurement at specific points, as discussed above. The average SNR was then calculated, using the peak height data and the average noise data for all three chromatograms of a compound at a specified value of DBW.

In all, eight compounds of differing *spectral* bandwidth were examined in this way: cortisone acetate, cycloserine, diamorphine, ethisterone, ethynyl estradiol, morphine sulphate, progesterone and theophylline.

The effect on SNR of varying the integration time, Δt, was examined using the minimum DBW (4 nm) at the principal wavelength (240 nm) for the solutes in the standard three-component mixture of steroids: cortisone, ethisterone and progesterone. At each of the values of Δt examined, three injections were made. The "step-time" (*i.e.* sampling interval) was set to the same value as the integration time, Δt: 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 2.0 and 5.0 sec. The average peak-to-peak noise, calculated as above, was used to calculate the SNR from the peak height data for each compound at the specified values of Δt, in each of the three chromatograms.

Linearity experiments. The effect of the detector bandwidth on calibration linearity was examined using detector system II with chromatographic system 1, with a three-component steroid mixture as sample, diluted to give six standards over a ten-fold range of concentrations. Each was injected in duplicate (20 μl) at 0.04 absorbance units full scale (a.u.f.s.). DBW values selected were: default (4 nm), optimum SNR (12 nm) and sub-optimal (24 nm). Regression analysis was applied to data in the usual manner.

RESULTS AND DISCUSSION

Archive retrieval

The new rapid-scanning detector technology for HPLC generates such large quantities of spectral and chromatographic data that computer-aided data reduction and library searching becomes a necessity. Although spectral libraries are well established in mass spectrometry (MS) and in Fourier transform infrared spectroscopy (FTIR) combined with gas–liquid chromatography, they have not been widely exploited in the context of HPLC.

The implementation of archive retrieval algorithms in rapid-scanning LC detectors is reported here for the first time. Until recently, such detectors were not routinely available. A further reason for the lack of exploitation of electronic spectra may be their relatively low information content, compared with MS or FTIR data. This limitation on the utility of library searching is made more serious by the fact that electronic spectra are notoriously susceptible to modification by solvent conditions; this applies especially to ionisable compounds.

In the present work, therefore, a single column-eluent system was employed, together with a small library of compounds, some of them closely related, in order to exemplify the principles of one approach to designing an archive retrieval system for rapid-scanning detection in HPLC.

A number of archive retrieval systems have been described[19,23–25]. These systems differ in the choice of two important design parameters: (a) the storage format in the reference library; and (b) the criterion used for comparison during the retrieval process. Since the aim of the present work was to establish a basis for spectral identification rather than the more complex requirement of an interpretative library search system, it was considered that an archive retrieval algorithm based on the relatively simple "inverse file" structure, supported by an archive of files of normalised spectra, should be adequate for the purpose.

The key element in the search algorithm is the comparison metric. The inverse files are interrogated with respect to the set of parameters which describe the sample spectrum. The total weight assigned to each candidate spectrum takes account of the proximity of the features in the retrieved spectrum relative to the features observed in the sample spectrum, in that the weight is scaled parabolically (Fig. 1c). In the example search cited, where the test sample injected was caffeine, both caffeine and theobromine (spectra 1 and 2 respectively) are assigned the same weight of 8, since their maxima and minima exactly coincide (Fig. 1). The closely related methylxanthine, theophylline (9), also displays these two features, but is assigned a weight of 5, because the minimum is displaced to the lower wavelength limit of the search window (Fig. 1). Similarly, 8-chlorotheophylline (spectrum 3) is assigned the lower weight of 1, since the only feature in common with the test spectrum is a minimum, displaced to the higher wavelength limit of the search window (Fig. 1).

The rank order of the spectra retrieved is determined by a fit parameter based on the quadratic sum of the differences between the candidate and the sample spectra. Thus, the apparently identical spectra of caffeine and theobromine can be readily discriminated by this fit parameter, scaled for convenience from 0–100, whose values are found to be 97.8 and 77.1 respectively. Other spectra of lower weight are found to have lower fit parameter values of 69.9 (theophylline) and 53.1 (8-chlorotheophylline).

A further example relates to spectra of steroidal compounds with closely similar molecular structure. Cortisone acetate (5), ethisterone (6) and progesterone (7) present a particular challenge to the archive retrieval algorithm, since they are characterised in UV by a single maximum near 240 nm. The minimum in these cases is represented by tailing of the spectrum to zero. If progesterone is injected as a test sample, the library search assigns the highest weights to spectra 6 and 7 (Fig. 2), discriminating against cortisone (spectrum 5), which has a peak maximum at the lower wavelength limit (242 nm) of the search window. The fit parameters for ethisterone and for progesterone are found to be 96.0 and 97.0, respectively, so that progesterone is correctly identified in this case, albeit with difficulty. The closely similar cortisone acetate spectrum is readily rejected through its fit parameter of 70.0 (Fig. 2).

In the converse experiment, ethisterone as a test sample was found to be correctly discriminated from progesterone, the fit parameters being 98.2 and 96.3, respectively. Cortisone acetate was rejected with a fit parameter of 70.1. However, it

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File          STER 2

Spectrum      16
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Find

Peak       1 λ 244
Valley     2 λ 282

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Search

  Peak   1 in spec 5   weight 1
  Peak   1 in spec 6   weight 4
  Peak   1 in spec 7   weight 4

2 spectra with weight 4

Rank  Compound          Fit
  1   PROGESTERON       97.0
  2   ETHISTERONE       96.0

1 spec with weight 1

Rank  Compound          Fit
  3   CORTISONE         70.0
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Fig. 2. Library search for progesterone as test sample, which is correctly identified on the basis of a single peak maximum and the least-squares comparison metric.

was found to be essential in these examples to set the absorbance zero baseline for the test spectrum correctly in the preliminary stage of the search. Since only one feature is available to the retrieval algorithm, the criterion for discrimination rests primarily on the fit parameter, which was found to be sensitive to baseline imbalance.

The archive retrieval system for LC–UV could clearly be improved by incorporating a suite of inverse files based on retention time (or relative retention time), used as a coarse screen prior to interrogation of the spectral database. However, it is probable that a suitably wide "time window" would be required to accommodate variations in chromatographic conditions. Moreover, further investigation would be necessary in order to establish appropriate wavelength windows to take account of any change in spectral shape caused by variations in eluent composition affecting polarity, pH or ionic strength.

Although the present report describes experiments on a limited library of spectra acquired and searched under standardised chromatographic conditions, the principles of the archive retrieval system developed should be generally applicable to the large spectral libraries required for practical purposes. The system performed reason-

ably well, despite the close similarity of the spectra in the two groups of compounds examined. Any subtle differences in the spectral profile can be more readily discriminated in the derivative domain[3]. Further work in progress will exploit the more highly characteristic profiles observed in the first and second derivative spectra ($dA/d\lambda$, $d^2A/d\lambda^2$), obtained by transformation of spectra acquired by rapid-scanning HPLC detection, as described in earlier reports[5,6].

Sensitivity experiments

The three-dimensional (A,λ,t) data set acquired by the rapid-scanning detector (Fig. 3) permits SNR to be improved by ensemble averaging both in the wavelength and the time domains. An increase in detector bandwidth (DBW), $\Delta\lambda$, sometimes referred to as "diode bunching", is represented by a vertical section through the contour plane (λ,t) (Fig. 4), symmetrically disposed about the nominal observation wavelength of 240 nm. In complementary manner, an increase in the "wavelength window" or integration time, Δt, is represented by a horizontal section through the contour plane (Fig. 4).

The effect of increasing the DBW using detector II under standardised con-

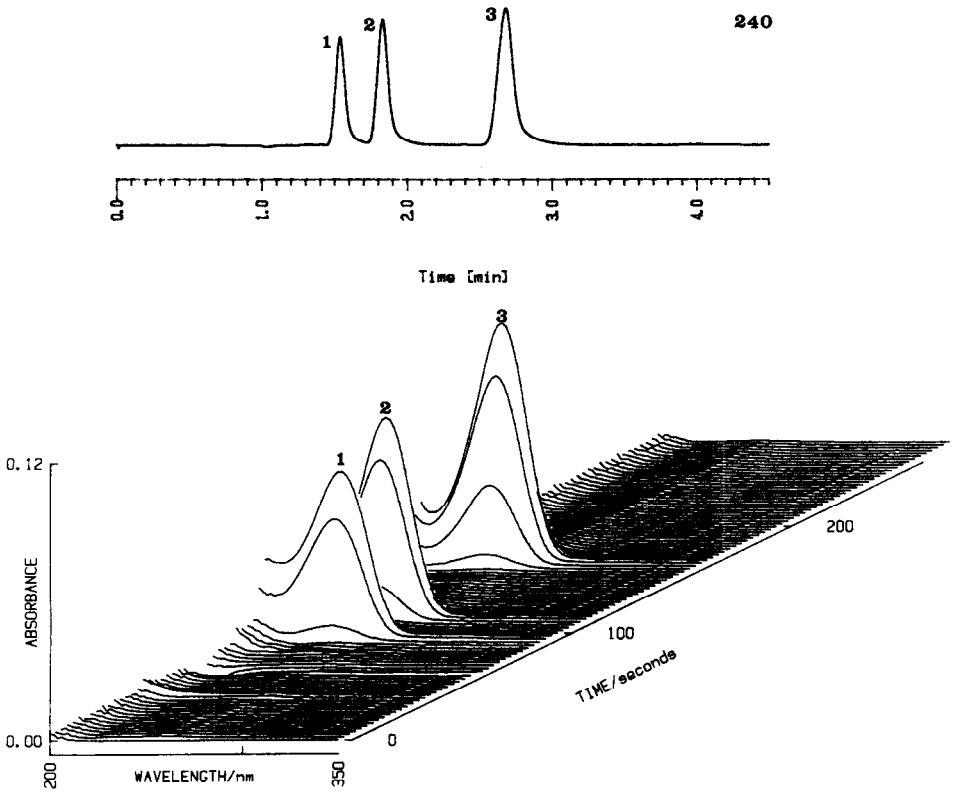


Fig. 3. Pseudo-isomeric (A,λ,t) presentation of steroid spectra captured at 1-sec intervals during elution, together with the conventional chromatogram at 240 nm. For chromatographic conditions, see text. Conditions for detector system I: injection volume, 100 μl; reference wavelength, 500 nm, with bandwidth 100 nm; detection wavelength 240 nm and bandwidth 4 nm (for chromatogram); sensitivity, 0.012 a.u.f.s. 1, Cortisone acetate (21.6 μg/ml); 2, ethisterone (21.7 μg/ml); 3, progesterone (33.6 μg/ml).

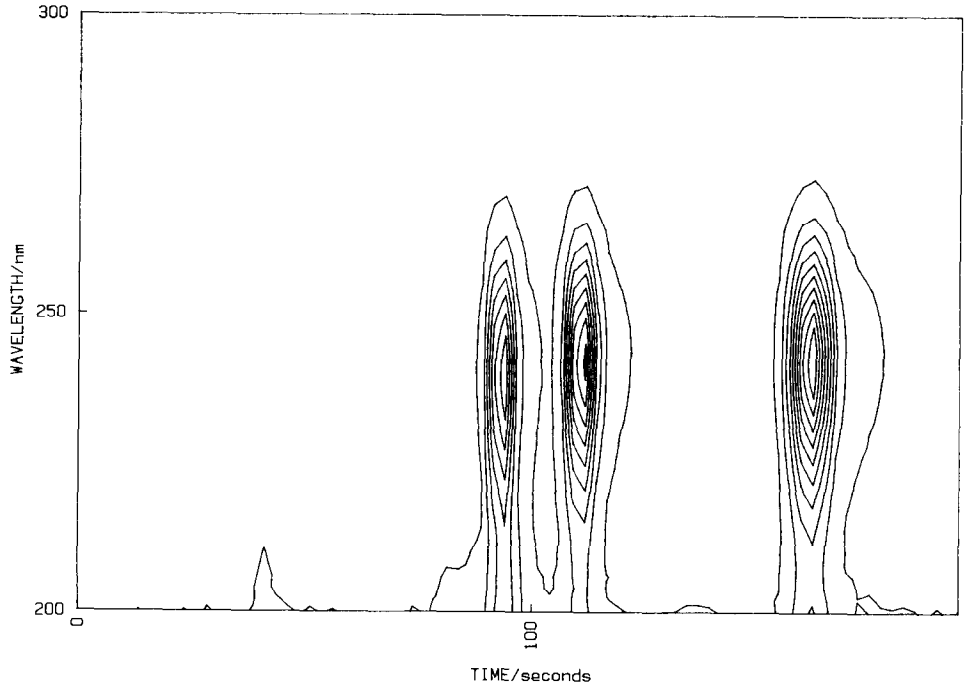


Fig. 4. Contour plot of steroids, illustrating the optimum detection wavelength. The effect of increasing the spectral bandwidth ("diode bunching") at the nominal detection wavelength, 240 nm, and of increasing the integration time are denoted by $\Delta\lambda$ and Δt, respectively. Chromatographic conditions and conditions for detector system I: as in Fig. 3; contour interval, 0.01 a.u. 1, Cortisone acetate; 2, ethisterone; 3, progesterone.

ditions, with a constant integration time of 0.2 sec, is illustrated for ethynyl estradiol, whose UV spectrum shows a well-defined aborption maximum at 280 nm, with a spectral bandwidth (SBW) at half-height of 24 nm (Fig. 5). As the DBW at 280 nm is increased from the "default" value (equivalent to 4 nm, but coded "0" in the present work for discussion purposes), the baseline noise level decreases perceptibly (Fig. 6). However, because this effect is accompanied by a decrease in signal intensity, the net effect is that the resultant SNR passes through a maximum at 10 nm, before falling progressively (Figs. 6 and 7). In this particular case, an overall four-fold improvement in SNR was observed at the optimum DBW. This was confirmed by conducting three separate experiments at 18 points over a window of 80 nm (280 ± 40 nm) for ethynyl estradiol under the specified conditions (Fig. 7). The mean ratio of DBW:SBW for ethynyl estradiol was found to be 0.42 (n = 3).

A total of eight solutes was examined in this manner, with spectral bandwidths varying from 16–36 nm. On the basis of triplicate measurement of signal, noise and SNR at each of 18 values of the DBW, the optimum value for DBW was established for each compound (Table I). The average value of the ratio DBW:SBW was 0.42 for this group of test solutes [relative standard deviation (R.S.D.) = 15%; n = 8], as illustrated in Table I. The enhancement of SNR at the optimum DBW varied from a factor of *ca.* 1.5 for morphine and theophylline, to *ca.* 2 for cortisone, cycloserine, diamorphine, ethisterone and progesterone. Ethynyl estradiol appeared to be an ex-

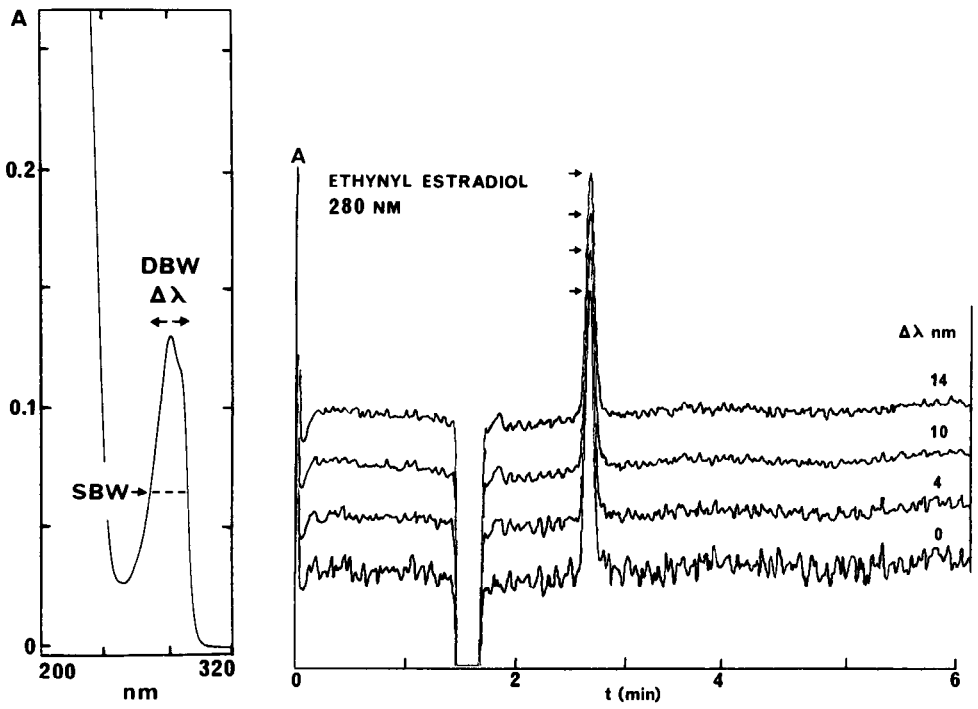


Fig. 5. UV-absorption spectrum of ethynyl estradiol (19.5 μg/ml) in acetonitrile–water (50:50, v/v). SBW, spectral bandwidth at half-height; DBW, detector bandwidth.

Fig. 6. Chromatogram of ethynyl estradiol (9.77 μg/ml) plotted at 280 nm after processing by IBM-PC microcomputer to give different values of spectral bandwidth ($\Delta\lambda$), from default conditions (denoted by 0) to 14 nm. Instrumental conditions for detector system II: injection volume, 100 μl; integration time, 0.2 sec; sampling step interval, 1.0 sec; sensitivity, 0.020 a.u.f.s. For chromatographic conditions, see text.

ception in this respect. Under these conditions, therefore, the optimum bandwidth found for detector system II was equivalent to about half the spectral bandwidth of the analyte.

Preliminary studies on a similar range of test solutes indicated that the optimum DBW for detector system I corresponded to a bandwidth ratio of *ca.* 1. This observation may well reflect the fact that the nominal resolution of the two detector systems differs by a factor of 2, being 1 nm per diode for detector system II and 2 nm per diode for detector system I. Earlier work on norzimeldine with detector I also indicated a pronounced optimum in the DBW value, although in this case the bandwidth ratio was *ca.* 0.45 for an enhancement in SNR of 56%[11]. Care should be taken in interpreting data of this kind, however, since the spectral bandwidth values are often difficult to estimate and do not in themselves reflect any fine structural features in individual spectra.

Optimisation of SNR in the time domain was examined with detector system II at 240 nm, using the default value of DBW (4 nm) and various values of the integration time from 0.4–5.0 sec (the sampling "step" interval being set to the same value as integration time) (Fig. 8). The SNR for the three-steroid mixture increased

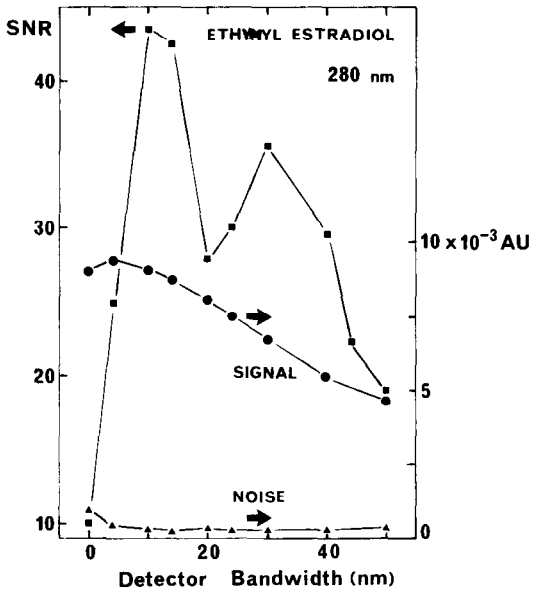


Fig. 7. Relationship of signal, noise and SNR with detector bandwidth for ethynyl estradiol at an observation wavelength of 280 nm. For chromatographic conditions, see text. Instrumental conditions for detector system II, as in Fig. 6.

by a factor of 1.2–1.6 at the optimum value of Δt, which was *ca.* 1 sec. Under these conditions, the baseline noise improved perceptibly (Fig. 9). Although it would be expected that the optimum Δt value should be related to the *chromatographic* bandwidth, this could not be established in the present work, since the peakwidths were closely similar (Fig. 9).

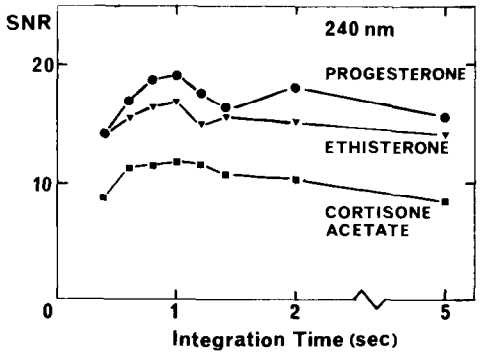
TABLE I

RELATIONSHIP OF OPTIMUM DETECTOR BANDWIDTH TO SPECTRAL BANDWIDTH

Detector bandwidth (DBW) observed for optimum signal-to-noise ratio is expressed as a ratio to spectral bandwidth (SBW), based on the mean of three independent estimates with detector system II (instrumental conditions and chromatographic conditions as in text). Nominal detection wavelengths employed were: morphine sulphate, 210 nm; diamorphine hydrochloride, 273 nm; ethynyl estradiol, 280 nm; cortisone acetate, ethisterone and progesterone, 240 nm; theophylline, 270 nm; cycloserine, 225 nm.

	DBW (nm)	*SBW (nm)*	*Ratio*
Morphine	9	16	0.56
Diamorphine	12	22	0.55
Ethynyl estradiol	10	24	0.42
Ethisterone	12	29	0.41
Theophylline	14	30	0.47
Progesterone	12	33	0.36
Cortisone acetate	12	36	0.33
Cycloserine	14	36	0.39
$\bar{x}$			0.42
R.S.D.			15%

When the optima for DBW and Δt are combined, a further small enhancement in sensitivity can be achieved, to give an overall SNR improvement for the three-steroid mixture by a factor of 2–3 with detector system II. A similar enhancement was observed in the sensitivity of detector system I using the combined optima, although the relationship of the optimum DBW and Δt values with spectral and chromatographic parameters appeared to differ systematically from that for detector system II, as discussed above.



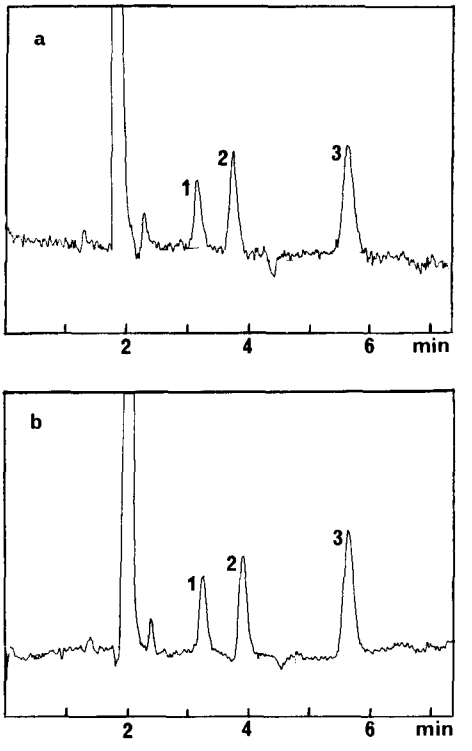


Fig. 8. Relationship of SNR with integration time for cortisone acetate, ethisterone and progesterone at 240 nm. For chromatographic conditions and instrumental conditions for detector system II, see Fig. 6.

Fig. 9. Effect of integration time on the chromatogram of steroids detected at 240 nm. a, Integration time 0.4 sec and sampling step interval, 0.4 sec; b, integration time 1.0 sec and sampling step interval, 1.0 sec. For chromatographic conditions and instrumental conditions for detector system II, see Fig. 6. 1, Cortisone acetate (1.08 μg/ml); 2, ethisterone (1.08 μg/ml); 3, progesterone, 1.68 μg/ml.

Linearity experiments

The data in Table II summarise duplicate experiments on each of three steroids, with six values of concentration in a ten-fold range, for three values of DBW at 240 nm: default bandwidth (4 nm), optimum DBW (12 nm) and sub-optimal DBW (24 nm). The default value of integration time, 0.2 sec, was used with a sampling "step" interval of 1.0 sec at a sensitivity of 0.04 a.u.f.s. It is seen that as DBW increases from the default value of 4 nm, the gradient decreases by 10–15% for two of the

steroids examined. In the case of progesterone, the gradient remains essentially constant up to the optimum DBW, and then falls by about 2%. These data confirm the observation that peak height falls progressively as DBW is increased beyond the default value (Figs. 6 and 7). However, the regression linearity does not appear to be affected by the values of DBW employed in these experiments.

It can therefore be concluded that for these relatively broad compounds, quantitation can be satisfactorily performed at the optimum sensitivity value for DBW of 12 nm, at least up to 0.04 a.u.f.s. The detector linearity should, however, be checked as a standard procedure for the analyte of interest at the optimum value for DBW up to the absorbance limit required.

Detection limits

For a given chromatographic system, the detection limits are determined primarily by the overall noise level in the detector output. The two principal sources of noise are pump-induced flow noise and detector noise. In photodiode array detectors, the various sources of noise include random noise, lamp drift and fixed-pattern noise[1]. The latter arises from switching "glitches", dark-current variation and sensitivity variations across the array of photodiode resolution elements. In the single-cell rapid-scanning detectors employed in the present work, fixed-pattern noise is usually corrected by recording reference values for the dark-current and sensitivity of each diode in the array at the start of a run. By contrast, when using the dual-cell multichannel spectrophotometer used in earlier work reported from this laboratory (HP 8450A)[5–7], the status of dark-current and diode sensitivity reference values can be updated continuously at 200 msec intervals.

The detection limits for each of the five detector systems employed were measured at 240 nm by triplicate injection of the three-component mixture of steroids,

TABLE II

REGRESSION DATA FOR STEROIDS AT DIFFERENT DETECTOR BANDWIDTHS

Regression statistics were calculated from duplicate measurements at each of six concentrations within the specified range: *M*, gradient; *C*, intercept; *R*, correlation coefficient. Instrumental conditions for detector system II: detection wavelength, 240 nm; sensitivity, 0.040 a.u.f.s.; other conditions as in text. For chromatographic conditions, see text.

	Range (μg/ml)		*N*	*DBW (nm)*	*M*	*C*	*R*
	Low	*High*					
Cortisone acetate	1.080	10.80	6	4	10.8	1.47	0.9978
				12	10.4	1.46	0.9987
				24	9.73	1.04	0.9983
Ethisterone	1.083	10.83	6	4	13.4	−2.44	0.9989
				12	12.5	1.52	0.9973
				24	11.4	1.07	0.9993
Progesterone	1.677	16.77	6	4	8.58	0.50	0.9960
				12	8.64	−0.22	0.9992
				24	8.40	−1.37	0.9893

at a suitable dilution (Table III). The same combination of pump, column, injector and eluent was employed for each of the detector systems examined. The photodiode array detectors were used with the normal default conditions (Table III). For comparison purposes, a regular variable-wavelength single-channel detector (IV) was used, and also a detector with a flow-cell whose geometry was designed to permit very sensitive absorbance measurements (V).

The data summarised in Table III confirm that detector system V was indeed more sensitive than the regular detector (IV) by a factor of *ca.* 4 (range 2.5–5.8). Photodiode array systems II and III compared reasonable well with the regular detector IV. Detector system I, however, was found to be more sensitive than systems II and III by a factor of *ca.* 4 (range 2.3–5) for the compounds examined. Relative to detector system V, however, detector I was *ca.* 1.5 times less sensitive, under normal default conditions (Table III).

If detector systems I and II were optimised in both the wavelength and the time domains, however, their sensitivity increased by 29 and 36% respectively, to give on-column detection limits for the three steroids respectively as follows (n = 3): (system I) 0.43, 0.64 and 0.69 ng; (system II) 1.36, 1.43 and 3.33 ng. When using these optimised conditions, the sensitivity of the photodiode array detector I was found to be comparable with that for the single-channel detector system V. It should perhaps be noted that the swept-volume of detector V is 14 μl, compared with the smaller cell volumes of the other detectors examined. This may be significant in those cases where very high-resolution separations are achieved, as for example in bioanalytical studies where interference from endogenous peaks presents a problem[21]. The relatively high sensitivity of photodiode array detector I may be attributable in part to a combination of the noise suppression achieved when a reference wavelength is used, as previously reported[7], and software manipulation algorithms for data smoothing during or after acquisition.

TABLE III

DETECTION LIMITS FOR PHOTODIODE ARRAY AND CONVENTIONAL DETECTORS

Detection limits expressed as ng injected on-column for SNR = 2, are the mean of three determinations. Injection volume, 100 μl of a mixture of steroids dissolved in eluent: cortisone acetate, 0.0540 μg/ml; ethisterone, 0.0452 μg/ml; progesterone, 0.0836 μg/ml. Nominal wavelength for each detector, 240 nm; photodiode array detectors were used at the nominal default values of bandwidth and integration time. Specific instrumental conditions employed for detectors were as follows: system I: sensitivity, 1.0 ma.u.f.s.; reference wavelength 500 nm at 100 nm bandwidth; system II: sensitivity, 5.0 ma.u.f.s.; sampling "step" interval, 1.0 sec; system III: sensitivity, 10 ma.u.f.s.; system IV: sensitivity, 5.0 ma.u.f.s.; time constant, 1.0 sec; system V: sensitivity, 1.0 ma.u.f.s.; time constant, no. 5. For chromatographic conditions, see text.

No.	*Compound*	*Detection limit (ng)*				
		Photodiode array			*Single-channel*	
		I	*II*	*III*	*IV*	*V*
1	Cortisone	0.70	3.51	2.39	2.07	0.36
2	Ethisterone	0.82	1.85	2.04	1.31	0.26
3	Progesterone	0.93	4.38	5.57	2.85	1.16

With the rapid development of efficient narrow-bore column systems, flow-cell geometries one order of magnitude smaller in volume have been developed for conventional single-channel detectors. The difficulties is designing suitable flow-cells for the application of rapid-scanning technology to narrrow-bore HPLC present a major challenge to manufacturers of chromatographic equipment. There are strong indications, however, that this challenge will soon be met, so that the remarkable flexibility of rapid-scanning detector technology can be applied as a practical strategy for data acquisition, transformation and archive retrieval in the application of narrow-bore HPLC to problems of pharmaceutical and biomedical interest.

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