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Journal of Pharmaceutical and Biomedical Analysis
27 (2002) 191–200

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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# Flow injection analysis with multiple on-line spectroscopic analysis (UV, IR, $^1$H-NMR and MS)

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Received 26 May 2001; accepted 3 July 2001

**Abstract**

Studies on the capabilities of flow injection analysis (FIA) combined with on-line characterisation of model compounds via a combination of diode array UV, $^1$H-NMR, FT-IR spectroscopy and mass spectrometry are described. Using this combination of spectrometers enabled the on-flow collection of UV, $^1$H-NMR, IR and MS for a range of model compounds. Samples were introduced into the system as solutions in deuterium oxide in concentrations ranging from 1.4 to 8.4 mg ml$^{-1}$. A sample volume of 100 μl was used for FIA at a flow rate of 1 ml min$^{-1}$. From these studies a practical working quantity of ca. 140 μg/sample of analyte was determined which provided characteristic spectra. 

*Keywords:* Flow injection analysis; Multiple hyphenation; Spectroscopic characterisation

## 1. Introduction

The determination of the structure of unknowns, or the confirmation of structure of compounds thought to be present in a sample, is an important and often time consuming, task for analytical chemists in the pharmaceutical and other industries. The effect of combinatorial synthesis and the use of chemical libraries for high throughput screening, etc. has greatly added to this burden. We, together with a number of other groups, have been attracted by the potential of multiple hyphenation ('hypernation') of several spectroscopies in a single system as a means of speeding up this type of analysis, enabling comprehensive spectroscopic information to be obtained in a single analysis. This has led to the use of systems in which chromatography has been linked to nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) to provide HPLC–NMR–MS analysis of complex mixtures (e.g. see Refs. [1–9], reviewed in Ref. [10]). More recently we have investigated systems

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0731-7085/02/$ - see front matter 
PII: S0731-7085(01)00534-9

that also enabled infrared (IR) spectroscopy to be performed on analytes, with spectra obtained either off-line (with on-line collection of peaks) [11,12] or directly on-line [13–15]. As part of this work we constructed a flow injection analysis (FIA) system that enabled the on flow collection of UV-diode array (DAD), IR, $^{1}$H-NMR and time of flight (TOF) MS [13]. This prototype was by no means fully optimised but, nevertheless, provided us with valuable insights into the potential value of such systems and enabled a comprehensive set of spectroscopic data to be obtained on each of the model analytes. Here, we describe studies on a variant of this theme aimed at further characterising and optimising hypernated systems and introducing a degree of automation.

## 2. Experimental

### 2.1. Reagents

The compounds employed in this investigation were paracetamol (acetaminophen), caffeine, *p*-aminobenzoic acid, α-hydroxyhippuric acid, propranolol, *p*-aminohippuric acid, 4-aminoantipyrine, 4-dimethylaminoantipyrine, antipyrine and hippuric acid (Sigma, Poole UK, Fluka, Gillingham, UK and Aldrich, Gillingham, UK). Samples were dissolved, at concentrations of between 1.4 and 8.4 mg ml$^{-1}$ (see text) in deuterium oxide ($D_2O$) containing 0.1% (deutero)formic acid.

The FIA system (Fig. 1) consisted of a Constametric 3200 HPLC pump (Laboratory Data Control, Stone, Staffs, UK) which delivered $D_2O$:0.1% (deutero)formic acid at 1 ml min$^{-1}$. Typically 100 μl of sample were introduced, with 6 min between samples, into the flowing stream of $D_2O$ via a Perkin–Elmer ISS 200 autosampler (Perkin–Elmer, Bucks, UK) fitted with a 200 μl sample loop.

From the autosampler the sample went via 25 cm of 0.020 in. i.d. PEEK tubing to a splitter from which 60% of the flow was then directed, via 110 cm of 0.020 in. i.d. PEEK tubing, to a Bio-Rad model FTS3000 Excaliber series Fourier transform (FT) IR spectrometer (Cambridge, MA, USA) fitted with a Spectra Tech (Stamford, CT, USA) Micro Circle ATR (attenuated total reflectance) high pressure stainless steel flow cell of 25 μl volume fitted with a zinc selenide ATR crystal. The spectrometer compartment containing the flow cell was purged with dry nitrogen to minimise any interference on the collected spectra by water vapour in the IR beam. Any residual water vapour in the collected data was subtracted using a water vapour reference spectrum. Spectra were acquired with the kinetics software, enabling Gram Schmidt reconstruction of the on-flow data, collecting 57 scans per spectrum (10 s acquisition time) with a sensitive mercury cadmium telluride (MCT) liquid nitrogen cooled detector. The spectra were acquired at 8 cm$^{-1}$ spectral resolution. The sample was ratioed against a background spectrum of the flowing solvent through the cell prior to injection of the sample solution, thus automatically subtracting out the solvent spectrum from the sample spectra.

Simultaneously the remainder of the flow (40%) from the splitter was directed to a Micromass Platform single quadrupole mass spectrometer (Micromass, UK, Wythenshawe, UK) via 100 cm of 0.010 in. i.d. PEEK tubing. Mass spectra were acquired over a mass range of 115–650 Da. Prior to the sample entering the ion source of the mass spectrometer the flow was mixed with a make up flow of 90:10 methanol/water, introduced via a t-piece at 0.5 ml min$^{-1}$. This make-up flow was used to provide a backpressure to ensure that the bulk of the flow was directed to the IR, and thence to the UV and NMR spectrometers. Positive ion spectra were recorded with a cone voltage of 25 V, a scan time of 0.9 s and an inter scan delay of 0.1 s. The multiplier was set to 400 V.

The outlet of the FT-IR flow cell the flow was connected to a Bruker DAD detector (Bruker, Coventry, UK) via 30 cm of 0.010 in. i.d. PEEK tubing. UV spectra were collected over the wavelength range 188–1000 nm. From the UV detector the solvent stream was connected to the NMR via 280 cm of 0.01 in. i.d. PEEK tubing.

All of the instrumentation described above was located outside the 5 Gauss line of the stray magnetic field generated by the 500 MHz NMR spectrometer.

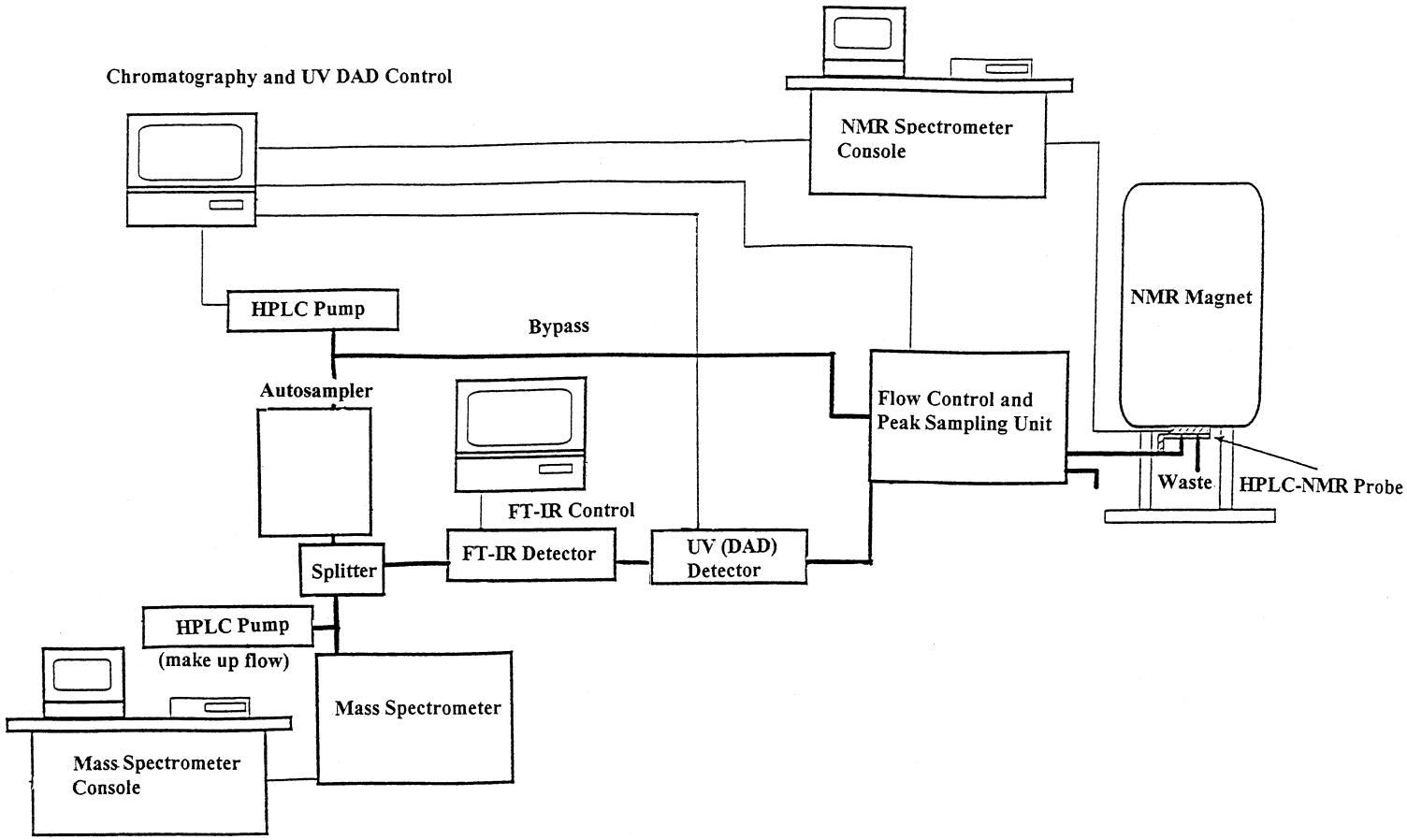


Fig. 1. The experimental layout of the various spectrometers used in this FIA system.

NMR spectra were acquired using a Bruker DRX-500 NMR spectrometer. On-flow $^1$H-NMR detection was carried out in the pseudo-2-dimensional (pseudo-2D) mode at 500.13 MHz using a flow-through probe of 3 mm i.d. with a cell volume of 60 μl. Typically, 16 scans/FIDs (free induction decays) per increment were acquired into 8 K data points each with a spectral width of 8278 Hz. Spectra were acquired using the NOESYPRESAT pulse sequence (Bruker Spectrospin, UK) in order to suppress residual water resonances. 90° pulses were used with an acquisition time of 0.5 s, a relaxation delay of 0.7 s and a mixing time of 100 ms.

Further details concerning the layout of this system are shown in Fig. 1.

## 3. Results and discussion

### 3.1. Instrumental layout

The instrumental layout (Fig. 1) employed in the present study differed somewhat from that previously used for our prototype FIA–IR–UV–NMR–MS system [13]. Thus, the present system incorporated an autosampler in place of the manual injection valve previously used, enabling automatic sample introduction and hence unattended operation. In addition this system was slightly less complex than the prototype as a result of the ability of the latest version of software used with the NMR spectrometer (for chromatography control) to acquire data directly from a UV-DAD. This eliminated the need for both a variable wavelength UV detector to monitor the flow to the NMR probe and a separate UV-DAD to obtain UV spectra. In a further modification of the instrumental layout we positioned the MS directly after the autosampler rather than splitting the flow later in the system at the point where the flow was spilt to the NMR spectrometer. This ensured that mass spectra were obtained early in the process of spectral acquisition. This layout should afford the possibility (not investigated in this study) of using mass directed analysis to

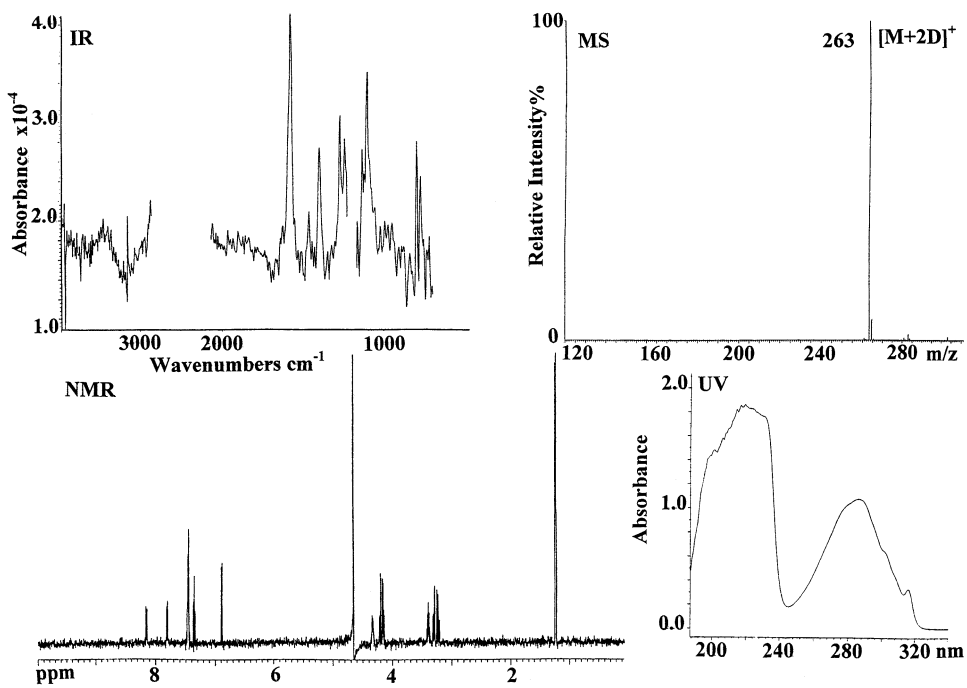


Fig. 2. The IR, UV, $^1$H-NMR and MS data obtained for propranolol (215 μg injected in 100 μl) using the FIA system shown in Fig. 1.

decide whether or not to perform stopped-flow NMR studies, e.g. in order to obtain two-dimensional NMR spectra, etc., should such information be required for structure elucidation. Such a possibility, which could potentially easily be automated, might be of benefit where the actual and expected masses of the analyte did not agree, or where the spectrum contained unexpected ions from, e.g. a potential impurity, thereby triggering the need for full characterisation. Alternatively, rather than stopping the flow during a run, such compounds could be diverted to the peak sampling unit. This device contains loops in which the sample could be stored until the end of the run, and then automatically sent to the NMR flow probe for characterisation, in this way maximising the throughput of the system.

As with our prototype system we do not claim that the current configuration is fully optimised and have no doubt that further refinements would enable a more efficient arrangement, with an improved performance, to be devised. However, irrespective of any inherent deficiencies this instrumental set-up was sufficient for the investigations described below.

### 3.2. Performance

Clearly a major consideration for this type of work is to define the minimum quantity of material needed for analysis. In general, depending upon the chromophore and ability of the compound to ionise under the conditions used for MS, it is to be expected that UV and MS detection will be more sensitive than either IR or NMR. Thus, the quantities of material analysed here were chosen to reflect the needs of the latter spectrometers. For practical purposes we have defined the minimum quantity of material needed as the amount that enables a diagnostic spectrum to be acquired on a sample, i.e. that permits a compound's characterisation rather than merely its detection. This will, inevitably, be compound dependent. For example, in the case of caffeine, the minimum amount of material required for $^{1}$H-NMR detection under these conditions, based on the three-methyl signals seen for this compound between 3 and 4 ppm, was in the region of 10 μg. However, at this concentration the signal for the single proton at ca. 7.6 ppm was undetectable. In order to obtain a full spectrum ca. 50 μg was required. In the case of propranolol, a more realistic model for most drugs, the spectrum is complex due to spin–spin coupling and ca. 70 μg of material was required for an adequate $^{1}$H-NMR spectrum. With this as the minimum we opted to use at least twice that amount of compound to ensure that adequate spectra were obtained. Thus, the range of concentrations used for compounds in the model library examined here was from 140 μg in the case of paracetamol up to 840 μg for antipyrine (this compound was also analysed at 210 μg injected). In our previous FIA investigations quantities of sample of between 1 and 2 mg/compound were analysed [13]. The exact amounts for each of the compounds used in the model library are given in the caption to Fig. 4. A sample volume of 100 μl was used on-flow at 1 ml min$^{-1}$.

### 3.3. Application to a model library

For the analysis of the model library, samples were automatically injected at intervals of 6 min. This time interval between samples was chosen to ensure that there was minimal carry over and careful examination of the spectra obtained in these studies showed no evidence for cross contamination. This result provides a theoretical capacity of 240 samples per day for this system. However, as carry over was not investigated further this is clearly the minimum number of samples that could be characterised, and it might well be possible to decrease the interval between samples. Any increase in the sample capacity would obviously be of value in making more efficient use of this array of spectrometers.

Given the comparatively high (in MS and UV terms) concentrations of analytes used in this study the detection of the model compounds was not particularly testing for the either the UV-DAD or the single quadrupole mass spectrometer employed here. Thus all ten compounds in the model library gave the expected UV spectra (e.g. see the examples provided in Figs. 2 and 3). In addition the majority of the test analytes gave the

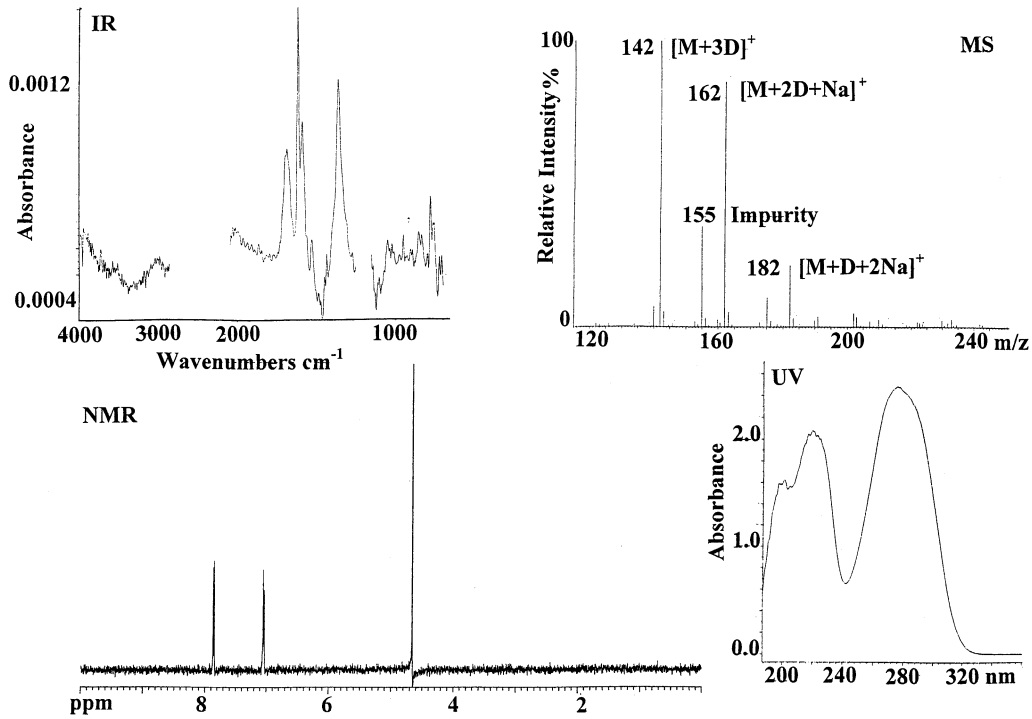


Fig. 3. The IR, UV, $^1$H-NMR and MS data obtained for *p*-aminobenzoic acid (185 μg injected in 100 μl) using the FIA system shown in Fig. 1. The MS data show in addition to the $[M + 3D]^+$, $[M + 2D + Na]^+$ and $[M + D + 2Na]^+$ a prominent ion at $m/z$ 155 indicating the possible presence of an unknown impurity. Examination of the $^1$H-NMR spectrum, however, shows only the presence of signals for *p*-aminobenzoic acid indicating that the impurity forms only a very minor component of the sample.

expected deuterated $[M + D]^+$ ions. In some cases $[M + Na + D]^+$ were also observed. Typical results, for propranolol (including IR, UV and NMR spectra), are shown in Fig. 2. The result for *p*-aminobenzoic acid showed that this sample also appeared to contain an impurity ($m/z$ 155) however, in this on flow experiment the evidence from other techniques, particularly $^1$H-NMR spectroscopy, suggests that this must have been due to the presence of a relatively minor component. The MS, UV, IR and NMR spectral data for *p*-aminobenzoic acid are shown in Fig. 3. Another interesting example was provided by α-hydroxyhippuric acid where the dominant ion was observed at $m/z$ 248. This ion was not due to the presence of an impurity, but represented an adduct of the analyte with formate present in the $D_2O$ (as with *p*-aminobenzoic acid the NMR, IR and UV spectra were consistent with those of standards). In some cases the analytes overloaded the MS and adduct ions of the type $[2M + D]^+$ or $[2M + Na]^+$ were observed. Such results highlight the effect of overloading the MS and show the need for care in setting the split ratio between the MS and the other instruments in the system. In any future refinements of this system this area will receive greater attention.

The FT-IR spectra obtained for the model library provided good quality, diagnostic, spectra for all of the test analytes. This is reflected in the results of the library searches, where the two criteria required for confidence in the result are that the top hit has a high percentage match and that there is a large difference between the first and second hits. In these experiments the best spectral match in each case was to the target

analyte. In general the top match was greater than 90% for the bulk of the compounds examined here. Thus in the case of caffeine the match was 91%, with the nearest alternative at 70%, giving a high degree of confidence in the result. However, some care must be taken using library searches because, as might be anticipated the FT-IR spectra of, e.g. antipyrine and its analogues 4-aminoantipyrine and 4-dimethylamino antipyrine share many common features. Thus, the top match for 4-aminoantipyrine was 80%, but the second match from the spectral library was quite close (78%) for the structural analogue 4-hydroxyantipyine, making the result less clear cut. However, antipyrine itself was easily distinguished from the above analogues giving a library search match of 98%, with dimethylamino-, 4-amino- and another analogue (present in the spectral library) 4-hydroxyantipyrine at 73, 72 and 70%, respectively, to give a fairly unambiguous identification.

The on-flow FT-IR spectra obtained for all of the compounds in the model library are illustrated in Fig. 4.

Similarly on flow $^{1}$H-NMR spectra were obtained for all of the test compounds as illustrated by the on flow pseudo-2D $^{1}$H-NMR spectrum obtained following the injection of all ten test compounds shown in Fig. 5. This figure also shows the on-flow $^{1}$H-NMR spectra extracted from the pseudo-2D experiment for all of the individual compounds. In all cases diagnostic $^{1}$H-NMR spectra were obtained that showed all of the expected structural features for each of the test compounds.

Quite clearly therefore, the amounts of material employed in this investigation were sufficient for the purposes of obtaining a complete set of spec-

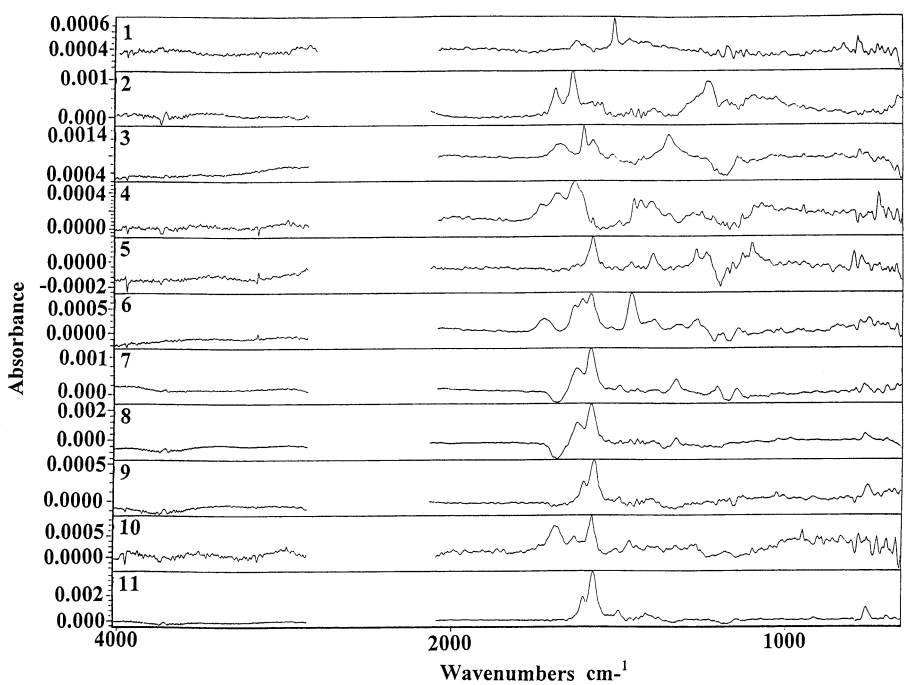


Fig. 4. The FT-IR spectra obtained using the FIA system illustrated in Fig. 1. Key: (1) paracetamol (140 μg), (2) caffeine (165 μg), (3) *p*-aminobenzoic acid (185 μg), (4) α-hydroxyhippuric acid (200 μg), (5) propranolol (215 μg), (6) *p*-aminohippuric acid (270 μg), (7) 4-aminoantipyrine (295 μg), (8) 4-dimethylaminoantipyrine (545 μg), (9) antipyrine (840 μg), (10) hippuric acid (365 μg) and (11) antipyrine (210 μg).

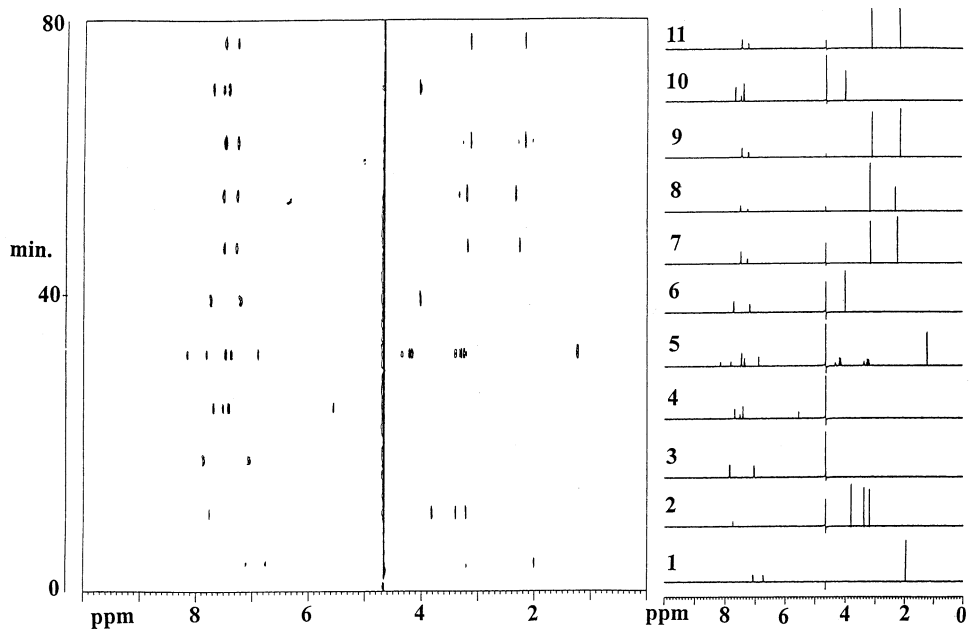


Fig. 5. A pseudo-2D NMR chromatogram of model library, showing spectra extracted from the individual peaks. Key as for Fig. 4.

troscopic data for each compound. Whilst a range of concentrations were used these investigations suggest that ca. 150–200 μg of compound represented a practical working range. However, this is unlikely to represent the minimum that would be required in a fully optimised system. Thus, the Gram Schmidt reconstruction of the IR data (not shown) indicated that the peaks were ca. 1 min. wide, implying a significant dispersion of the sample before FT-IR. As FT-IR spectra were acquired every 10 s across the peak, giving on average 6–7 IR spectra per compound, clearly less material could have been used if band broadening could have been reduced. The UV flow profile (monitored at 254 nm) for the first five injections of the model library are illustrated in Fig. 6, showing that by the time that the analytes had entered the UV-DAD flow cell the peaks, though symmetrical, had undergone further band broadening and were about 2 min wide. Peaks entering the NMR flow cell were ca. 2.1 min wide. When the peak for propranolol entering the NMR flow probe was examined incrementally it was possible to acquire three perfectly acceptable $^{1}$H-NMR spectra for this compound, as illustrated in Fig. 6. Thus for all of these spectrometers the concentrations on which the spectra were actually obtained were much lower than the nominal amounts injected. Thus, it seems likely that further optimisation of the split, to ensure that the bulk of the flow was directed to the IR and NMR spectrometers, combined with measures to ensure that band broadening was minimised (i.e. shorter lengths of tubing, of narrower bore), would provide much greater sensitivity than the present system. Sensitivity could also perhaps have been enhanced by employing lower flow rates to increase the residence time of the peaks in the IR and NMR spectrometers, although this would be likely to increase band broadening. However, employing a flow rate of 0.5 ml min$^{-1}$ enabled $^{1}$H-NMR spectra for 10 μg of caffeine to be obtained (results not shown), but the use of this lower flow rate would obviously have been at the expense of throughput.

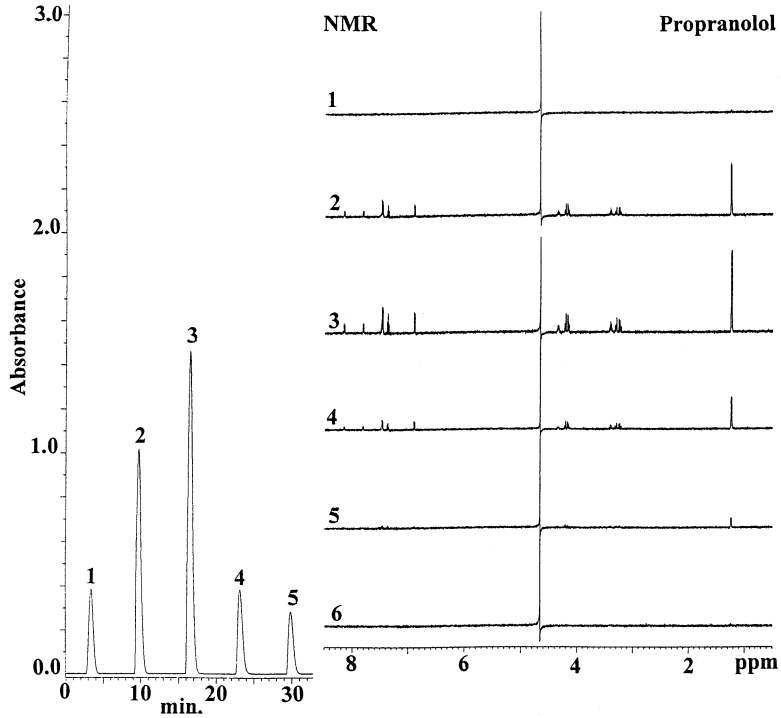


Fig. 6. The UV-flow profile for compounds **1**–**5** of the model library showing the $^{1}$H-NMR spectra obtained for propranolol (peak 5). The spectra are numbered **1**–**5** from the beginning of the propranolol peak.

## 4. Conclusions

The system employed here represents a simplified version of our previous instrumental set-up, combined with a degree of automation via an autosampler, allowing unattended sample introduction and data acquisition. Under the conditions employed here the acquisition of a full set of spectroscopic data was readily achieved with 140 μg of propranolol. These results provide a further demonstration that there are no particular experimental difficulties to be overcome in combining all of these spectrometers into a single hypernated system. In addition, continuing developments by manufacturers in the integration of instrument control and data acquisition are facilitating this process. Further studies will be directed towards increasing sensitivity by optimising the system to reduce band broadening.

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