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# Flow injection spectroscopic analysis of model drugs using on-line UV-diode array, FT-infrared and $^1$H-nuclear magnetic resonance spectroscopy and time-of-flight mass spectrometry

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A prototype flow injection analysis (FIA) system for the characterisation of compounds *via* a combination of diode array UV, $^1$H NMR, FT-IR spectroscopy and time-of-flight (TOF) mass spectrometry has been investigated using a number of pharmaceuticals and related compounds as model compounds. This combination of spectrometers allowed the on-flow collection of UV, $^1$H NMR, IR and mass spectra together with atomic composition data, enabling almost complete structural characterisation to be performed. Practical detection limits with the current system were in the region of 50 μg, however, the use of state of the art spectrometers would result in a significant reduction in the amount of material required.

## Introduction

The characterisation of substances by spectroscopy to prove identity or determine structure remains an essential feature of modern pharmaceutical chemistry. There is an urgent need to be able to perform such work rapidly and efficiently as a result of the vastly increased use of large 'libraries' of compounds for high throughput screening. Correspondingly the increased numbers of compounds synthesised as a result of the widespread adoption of combinatorial chemistry as a means of increasing the number of structures available for testing has placed a similar burden on the production of structure purity data. We have therefore been interested in the possibilities for accomplishing such characterisation *via* the multiple hyphenation of the required spectroscopies in order to provide comprehensive spectroscopic information in a single analysis. Thus we, together with a number of other groups, have been actively investigating the potential of chromatography linked jointly to nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) for complex mixture analysis [*e.g.*, see refs. 1–9, reviewed in ref. 10]. Indeed this technology has now matured to the extent that it is now possible to purchase an integrated HPLC-NMR-MS system from one manufacturer (Bruker). However, whilst NMR and MS data are often sufficient to confirm identity, or to determine the structure of unknowns, other spectroscopies are available that also provide useful structural information. In particular, infrared (IR) spectroscopy can provide direct evidence for structural features each as, *e.g.*, carbonyl or nitrile groups. We have, therefore, also investigated size exclusion chromatography-NMR combined with on-line collection of peaks *via* a dedicated interface for subsequent FT-IR spectroscopy.[11] More recently this system was extended by the addition of MS[12] enabling the collection of NMR and mass spectra on-line with off-line FT-IR of the peaks of interest.

The experience gained with these preliminary experiments, whereby chromatography has been linked to several spectroscopic detectors in a single run, has encouraged us to construct a flow injection analysis system for the characterisation of compounds from, *e.g.*, chemical libraries. Here results obtained when this prototype system, which consisted of a UV-diode array (DAD) detector, an FT-IR spectrometer, an NMR spectrometer and a time-of-flight (TOF) MS, was applied to a selection of model compounds are described.

## Experimental section

### Reagents

The compounds employed in this investigation were, ibuprofen, flurbiprofen, naproxen, indomethacin and atenolol (Sigma, Poole, Dorset, UK), antipyrine (Fluka, Gillingham, Dorset, UK), 4-hydroxyantipyrine and salicylic acid (Aldrich, Gillingham, Dorset, UK), nor-antipyrine (Janssen Chimica, Geel, Belgium) and propranolol (AstraZeneca Pharmaceuticals, Alderley Park, Cheshire, UK). Samples were dissolved in deuterium oxide ($D_2O$) at varying concentrations to give samples containing between 0.25 and 100 mg ml$^{-1}$.

The FIA system (Fig. 1) consisted of a Bruker LC22 pump (Bruker, Coventry, UK) which delivered $D_2O$ at 1 ml min$^{-1}$. Typically 200 μl of sample were introduced into the flowing steam *via* a model 7125 Rheodyne injector (Rheodyne, Cotati, CA, USA) fitted with a 200 μl sample loop.

From the injector the flow was directed to a Varian 9065 UV-diode array detector (Varian UK Ltd., Walton-on-Thames, Surrey, UK) *via* 30 cm of 0.005" id PEEK (polyethyl ether ketone) tubing in order to obtain UV spectra. UV data was collected over the wavelength range 190–360 nm, using the Star Chromatography Workstation, Version 4.0 (Varian), and analysed for spectral information using Polyview Version 2.0 (Varian).

Following DAD the flow went *via* 110 cm of 0.005" id PEEK tubing to a Bio-Rad (Cambridge, MA, USA) FT-IR model 375C spectrometer fitted with a Spectra Tech (Stamford, CT, USA) Macro Circle Cell ATR (attenuated total reflectance) stainless-steel flow cell of 400 μl volume fitted with a zinc selenide ATR

crystal. Spectra were acquired with the kinetics software collecting 20 scans per spectrum (5 s acquisition time) with a sensitive MCT (mercury cadmium telluride) 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.

Following FT-IR the flow entered, *via* 150 cm of 0.005" id PEEK tubing, a Bischoff Lambda 1000 UV detector (Bruker) set at 254 nm after which the solvent stream was split 95 : 5 with 5% of the flow being directed to the mass spectrometer *via* 250 cm of 0.007" id PEEK tubing, and the remainder to the NMR *via* 280 cm of 0.01" id PEEK tubing. Further details concerning the layout of this system are shown in Fig. 1.

Mass spectra were acquired on a Micromass LCT time-of-flight (TOF) mass spectrometer (Micromass, Manchester, UK) using orthogonal acceleration electrospray ionisation (ESI) with a Z Spray source. The nebuliser gas flow was set to 85 l $h^{-1}$ and the desolvation gas to 973 l $h^{-1}$. Spectra were acquired in either positive or negative ion mode with a capillary voltage of 3.2 kV and a cone voltage of 25 V. The source temperature was set to 120 °C and the desolvation temp to 350 °C. The pusher cycle time was 50 μs with 0.9 s acquisitions and an interacquisition delay of 0.1 s. The mass range was 100–900 Da. Dichlofenac, 295.0152 Da, was used to provide a lock mass for the spectrometer in negative ion mode and leucine enkephalin, 556.2771 Da, for positive ion work. This was introduced *via* a T-piece at 0.5 ml $min^{-1}$ and a concentration of approximately 5 ng $ml^{-1}$; the exact concentration being adjusted to give approximately 300–500 counts signal intensity to allow good accurate mass confirmation.

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

NMR spectra were acquired using a Bruker DRX-500 NMR spectrometer. On-flow $^1H$ NMR detection was carried out in the pseudo-2D mode at 500.13 MHz using a flow-through probe of 4 mm id with a cell volume of 120 μl. Typically, 4 scans/FIDs per increment were acquired into 4k data points each with a spectral width of 8278 Hz. Spectra were acquired using the NOESYPRESAT pulse sequence (Bruker Spectrospin, Coventry, UK) in order to suppress residual ACN contained in the pump/PEEK capillaries. 90° pulses were used with an acquisition time of 0.25 s, a relaxation delay of 1 s and a mixing time of 100 ms.

When stopped flow analysis was performed the NOESY-PRESAT pulse sequence was also used for double solvent suppression. FIDs were collected into 16k data points over a spectral width of 8278 Hz, resulting in an acquisition time of 0.99 s. A relaxation delay of 2 s and a mixing time of 100 ms were used.

## Results and discussion

### Instrumental layout

The instrument layout shown in Fig. 1 for this prototype appeared to be the most appropriate position to site each of the spectrometers, and was arrived at by trial and error. Undoubtedly with more work it might prove to be possible to obtain different configurations with potentially improved performance. The inability of the NMR flow probe used in this work to tolerate back pressures did, however, limit the options available to us. More modern flow probes are capable of withstanding sufficient back pressure as to enable, *e.g.*, the mass spectrometer to be sited in-line with the NMR if desired.

The configuration finally adopted provided UV spectra *via* the DAD detector as the initial step followed immediately by FT-IR using a 400 μl flow cell for ATR detection. The UV detector placed immediately in-line with the FT-IR enabled an evaluation of the degree of band broadening resulting from the use of the large flow cell in the FT-IR and was also used to calibrate the time taken by the analyte to reach the flow probe of the NMR spectrometer from the splitter. Thus on emerging from the UV detector the flow was immediately split with 5% of the flow directed to the TOF-MS and the remainder to the NMR. This layout of instrumentation proved reliable and robust for the purposes of the experiments described here.

### Limits of detection

Clearly the different spectrometers employed in this system provide a very wide range of sensitivities, with TOF-MS capable of providing information on substances present in the flowing stream at concentrations in the pg range whilst both the FT-IR and NMR instruments used here required (in the on-flow mode) quantities of *ca.* 50–100 μg for good spectra, with UV-DAD falling in between MS and NMR detection limits (depending upon the compounds studied). Given that the

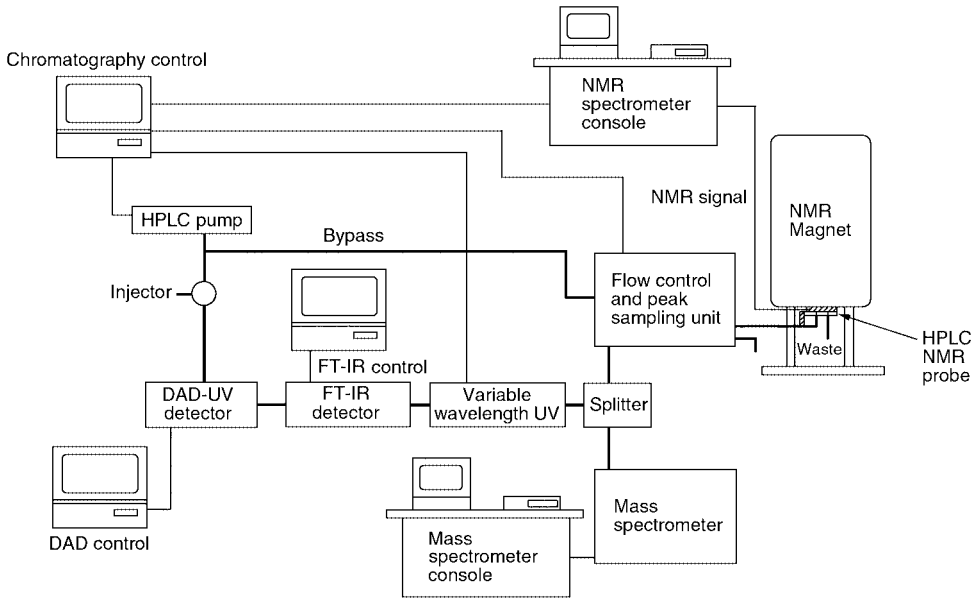


**Fig. 1** The experimental layout of the various spectrometers used in this FIA system.

sensitivities of the IR and NMR spectrometers were the limiting factors for this device we therefore examined the minimum system requirements that would provide complete on-flow spectroscopic characterisation (UV, IR, $^1$H NMR, TOF-MS with accurate mass determination for atomic composition). In order to determine the system requirements 200 μl aliquots of antipyrine, 4-hydroxyantipyrine and salicylic acid were injected into the FIA system over a wide range of concentrations (see below) at a flow rate of 1 ml min$^{-1}$.

In the case of antipyrine, the concentrations examined ranged from 250 μg ml$^{-1}$ up to 100 mg ml$^{-1}$ corresponding to 50 μg to 20 mg injected. For 4-hydroxyantipyrine and salicylic acid the highest concentration used was 25 mg ml$^{-1}$ (5 mg injected). For both TOF-MS and UV-DAD even the smallest quantities injected (50 μg) represented a considerable excess over the amount actually required to obtain spectra which therefore had to be obtained from the trailing edge of the plug of analyte as it flowed through the system. Indeed, in future studies steps will be taken to further dilute the sample, once it has emerged from the splitter, to within the working range of these spectrometers. This will also require the relocation of the UV-DAD out of the direct flow from the injector and place it in-line with the MS. Even so the UV spectra obtained from these analytes were quite adequate for the purposes of compound identification. Similarly, TOF-MS data obtained from the trailing edge of the peak of analyte, once allowance had been made for deuterium exchange, proved to be quite suitable for providing the required accurate molecular mass, and thence atomic composition data.

The results obtained from this experiment showed that both FT-IR and NMR spectra could be obtained on-flow with as little as 50 μg of material in a 200 μl injection. Thus Fig. 2 shows that FT-IR spectra obtained for antipyrine over the whole range, from 250 μg ml$^{-1}$ to 100 mg ml$^{-1}$. As these spectra show there is little to choose between the FT-IR spectra of samples containing between 100 and 1 mg ml$^{-1}$ (the latter corresponding to 200 μg injected). Even the result obtained with the 250 μg ml$^{-1}$ (50 μg injected) sample shows all of the essential features of the IR spectrum of antipyrine. With the use of an improved FT-IR flow cell design and a reduced flow rate of, *e.g.*, 0.5 or 0.25 ml min$^{-1}$ there is no doubt that sensitivity could be improved further.

The equivalent $^1$H NMR and TOF-MS spectra for the 250 μg ml$^{-1}$ antipyrine sample are shown in Fig. 3a and b (with the UV spectrum shown inset to the NMR spectrum) and, as with the FT-IR spectrum, are perfectly adequate for the purposes of identification *via* FIA analysis.

The arguments used above concerning the use of reduced flow rates to enhance sensitivity thereby enabling the FT-IR spectra to be improved apply equally to the NMR experiments described here. In addition, the results obtained here were the result of on-flow studies and a further increase in sensitivity could be achieved in the case of the NMR by using the stopped-flow mode. Indeed stopped-flow NMR spectroscopy on a sample of 50 μg of antipyrine in this system gave excellent spectra after only 64 scans (data not shown). The use of stopped-flow techniques would also permit two-dimensional NMR spectra to be obtained if required.

These conclusions regarding the effective sensitivity of the combination for antipyrine were confirmed by the data obtained on 4-hydroxyantipyrine and salicyclic acid which gave essentially the same results (data not shown).

## Application to a model library

Having demonstrated that the required spectra could be obtained from as little as 50 μg of material for the remaining FIA experiments 200 μl aliquots of samples containing between 5 and 10 mg ml$^{-1}$ (*i.e.*, between 1 and 2 mg injected) were used. The on-flow pseudo-2-dimensional $^1$H NMR spectrum obtained

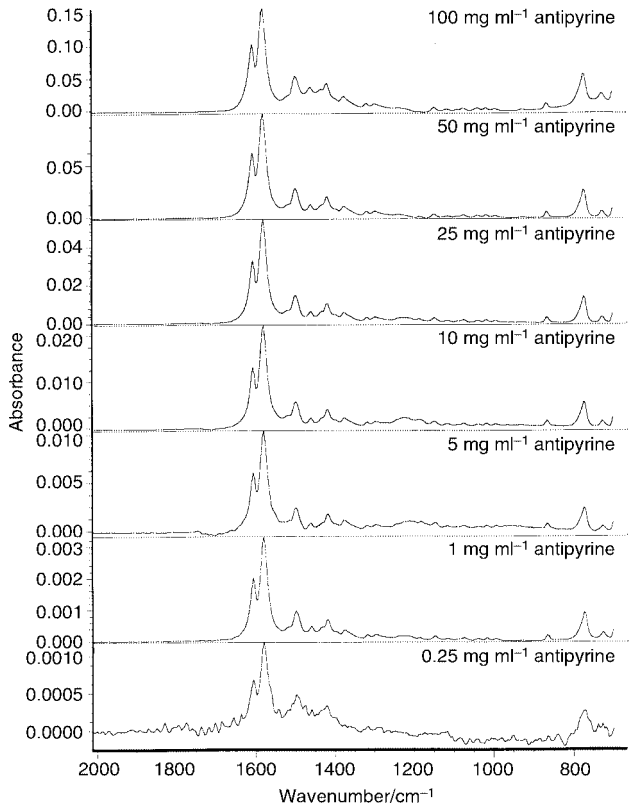


**Fig. 2** On-flow FT-IR spectra obtained for antipyrine for 100 mg ml$^{-1}$ down to 250 μg ml$^{-1}$.

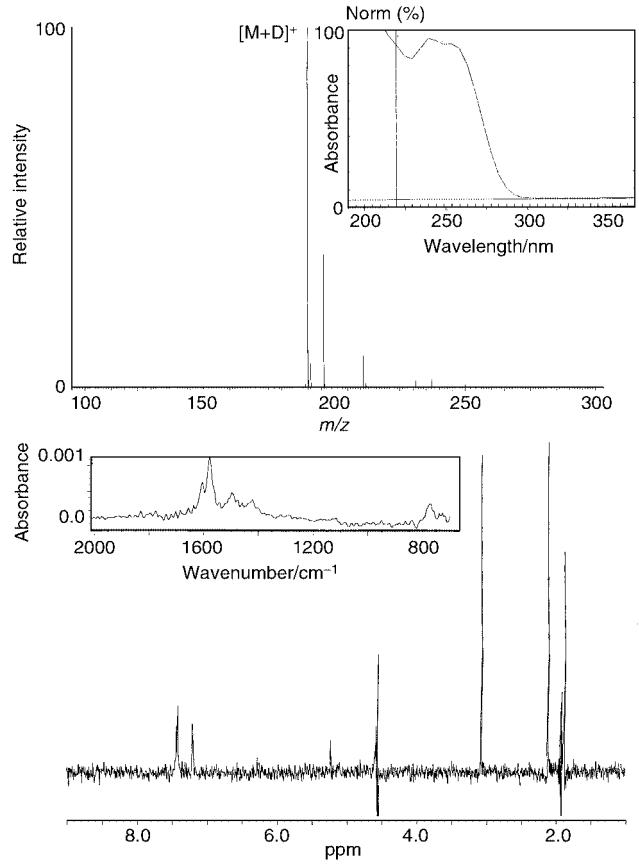


**Fig. 3** The TOF-MS (upper), $^1$H NMR (lower), DAD-UV (inset to TOF-mass spectrum) and FT-IR (inset to NMR spectrum) spectra obtained on-flow for the 250 μg ml$^{-1}$ sample of antipyrine.

following the injection of six model compounds of the type typically found in compound collections is shown in Fig. 4, together with the on-flow $^1$H NMR spectra extracted from the pseudo-2-D experiment for the individual compounds. In all cases this concentration of analyte provided sufficient material to provide $^1$H NMR spectra that showed all of the expected structural features. Similarly, good FT-IR and UV data were obtained for all of the compounds (Fig. 5). In the case of the TOF-MS, excellent results for molecular mass and atomic composition were obtained for the acidic non-steroidal anti-inflammatory drugs used as model analytes (Fig. 6a–d) using negative ionisation. Thus, the calculated molecular formulae for ibuprofen, flurbiprofen, naproxen and indomethacin were $C_{13}H_{18}O_2$, $C_{15}H_{13}O_2F$, $C_{14}H_{14}O_3$ and $C_{19}H_{16}ClNO_4$, respectively (for actual and found mass data see insets to Fig. 6). However, under the same conditions no results were obtained by TOF-MS for the two basic β-blockers which required re-analysis in positive ionisation mode (Fig. 6e–f) yielding the expected molecular formulae of $C_{16}H_{21}NO_2$ and $C_{14}H_{22}N_2O_3$ for propranolol and atenolol, respectively (mass data inset to Fig. 6). Future developments in the TOF instrument will enable it to be switched between positive and negative ionisation within the run, which will eliminate this problem, but this feature was not available for use with the current system.

Although only a prototype, the results presented here demonstrate that there are no insurmountable difficulties in combining all of these spectrometers in a single system, and if it were possible to use state of the art (commercially available) instrumentation sensitivities in the low microgram region should be possible. Given that both the TOF-MS and UV-DAD spectrometers were clearly not challenged in terms of sensitivity in these studies it is clear that further increases in overall sensitivity will depend upon advances in FT-IR and NMR flow probe technology. In the case of NMR, when combined with capillary separation techniques the ability to obtain spectra on sub-microgram and nanogram quantities has already been

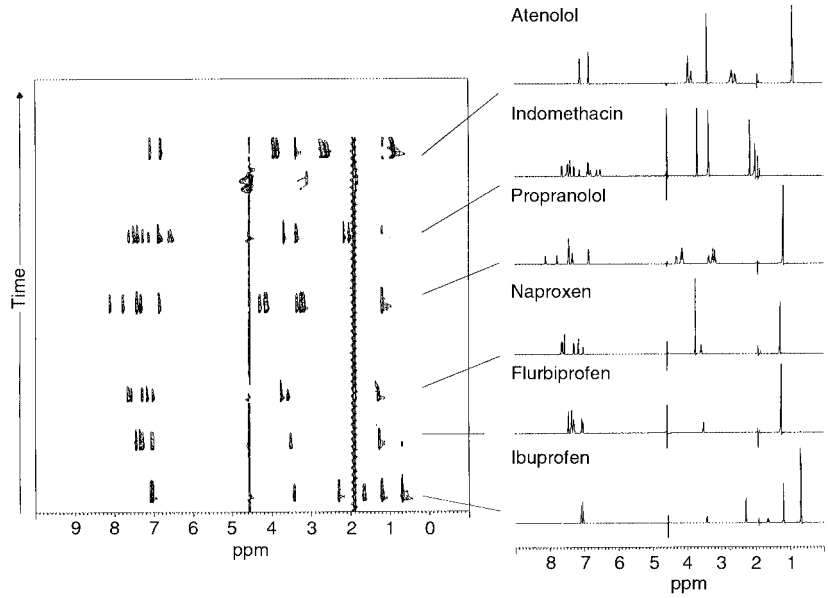


**Fig. 4** A pseudo-2-D NMR chromatogram of model library, showing spectra extracted from the individual peaks.

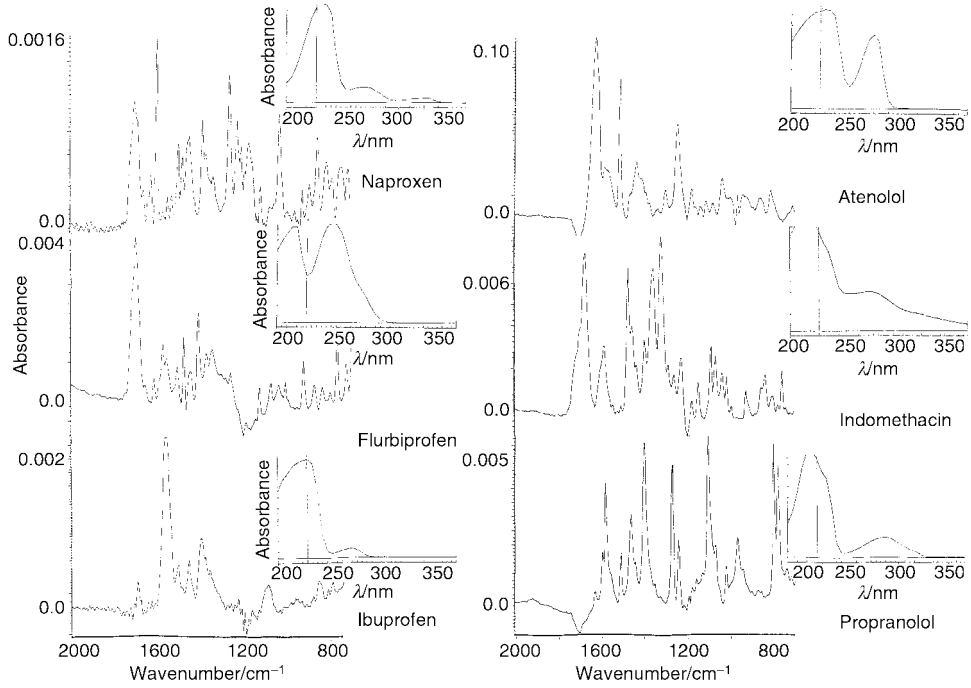


**Fig. 5** IR and UV (inset) spectra obtained for the model library shown in Fig. 4.

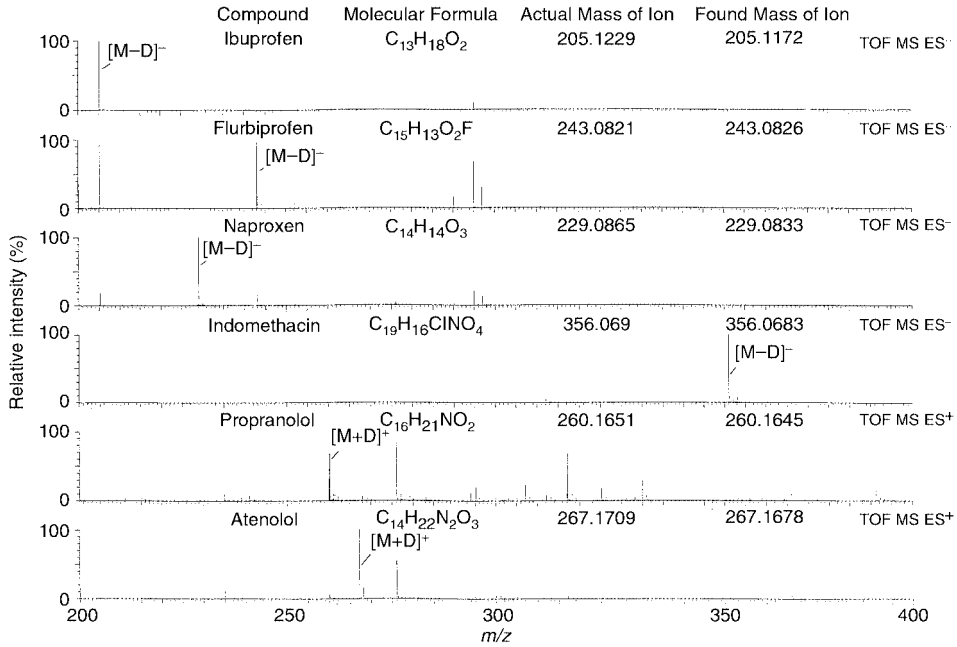


**Fig. 6** TOF-MS spectra obtained for the model library shown in Fig. 4. The spectra for the non-steroidal anti-inflammatory drugs ibuprofen, flurbiprofen, naproxen and indomethacin were acquired in negative ion mode, which proved to be unsuitable for propranolol and atenolol for which spectra were acquired in positive ion mode in a subsequent run.

demonstrated,[13–16] and cryogenically cooled probes also hold out the possibility of significant gains in sensitivity. Further enhancements to the system can be envisaged including the automation of the sample injections, *etc.* In addition, as the bulk of the sample is subjected to non-destructive analysis by NMR there is no reason why, in an automated system, it could not either be returned to the receptacle from which it had been sampled (*e.g.*, a 96 well plate) for reuse or taken directly into some further test system to determine, *e.g.*, biological activity, in a high throughput screen.

It seems to us therefore that, in the not too distant future, it will be possible to obtain all of the required spectra on a few nanograms of material in a multiply hypenated system such as this. However, as noted in 1980 by Hirschfeld in a perceptive review of the area of hyphenation, in which he briefly discussed the possibility of IR/UV/NMR/MS, 'feasibility is an insufficient test for whether the job needs doing in the first place'.[17] The fact that we have now achieved this degree of hyphenation still leaves the practical question of whether such a system would offer significant advantages over injecting the sample into each spectrometer individually.

## Conclusions

Whilst by no means an optimised system, the results presented here clearly demonstrate that it is possible to build and operate a flow injection system capable of providing UV, IR, NMR and MS spectra together with atomic composition based on accurate mass determination. Such a 'total organic analysis device' could thus be used to provide a wealth of information that should enable unequivocal confirmation of structure. No doubt further instrumentation (*i.e.*, chiroptical detectors) could also be included to provide an even more complete means of characterisation. An obvious further step is to combine this system with a chromatographic separation to enable the analysis of complex mixtures to be undertaken, and the results of such studies will be reported in future publications.

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