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UPLC/MS for the identification of β-blockers

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Abstract

The β-blockers Oxprenolol, Metoprolol, Acebutolol, Atenolol, Propranolol, Pindolol, and Alprenolol were analysed by both UPLC/MS and HPLC/MS using mobile phases containing acetonitrile, TFA and either H_2O or D_2O. UPLC gave superior separation performance and the quality of the mass spectra were at least as good as those from HPLC.

Keywords: UPLC; UPLC/MS; Structural elucidation using deuterium exchange

1. Introduction

1.1. HPLC and impurity identification

HPLC is a standard technique for the assessment of the purity of new pharmaceuticals in development. For example HPLC is used to evaluate the quality of the active pharmaceutical substance during the development of the synthetic route, and to assess the stability of the drug substance in trial formulations. The identification of any impurities generated during synthesis or on stability testing, and which have been separated by HPLC, is undertaken by spectroscopic techniques such as MS and NMR. Because of the low levels of impurities typically encountered during the development of pharmaceuticals, HPLC/MS is normally the first technique used for identification. NMR is a less sensitive technique and so unless the impurity is present at a high level preparative chromatography and isolation is normally the best approach. A wide range of MS techniques are routinely used such as accurate mass measurement, MS/MS, and deuterium exchange [1–4]. Deuterium exchange can give important chemical information such as distinguishing between different functional groups. Protons in certain chemical groups (e.g. in alcohol and amine groups) can exchange much more readily than others (e.g. in aliphatic groups) with protons in the surrounding solvent. If the surrounding solvent contains exchangeable deuterium then this provides a way of replacing the protons in reactive chemical groups with deuterium. Thus if HPLC/MS is performed with an acetonitrile/water based mobile phase and then repeated with an acetonitrile/deuterium oxide based mobile phase, mass shifts can be detected which assist in structural elucidation of the analyte molecules. This deuterium exchange approach can therefore help to identify impurities separated by HPLC. For example metabolites in which hydroxylation has occurred can be distinguished from those where oxidation has occurred at a nitrogen or sulphur atom [5,6].

1.2. UPLC and UPLC/MS

Ultra performance liquid chromatography (UPLC) is a new HPLC technique in which the use of high operating pressures (up to 1000 bar as opposed to the 400 bar maximum of HPLC), enables columns packed with sub 2 μm particles to be operated at high linear velocities. UPLC means that high peak capacities [7,8] and high resolving power can be generated along with short separation times. Because of the performance of UPLC the technique is being linked to MS and is being used in metabolism studies where the ability to rapidly separate and identify a large number of analytes in a sample is at a premium [8–10].

Because of the separation benefits of UPLC in purity assessment we decided to investigate the potential of UPLC/MS in impurity identification. For UPLC to be accepted as a routine tool in the purity assessment of new pharmaceutical development compounds analysts need to be sure that impurities separated can be characterised and identified to the same extent that they can by conventional HPLC based techniques. Because of this need to

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explore the equivalence of the two approaches UPLC/MS studies were performed in parallel with HPLC/MS studies in order to compare the quality of the separation and mass spectral data obtained.

1.3. Analysis of β-blockers

A series of β-blockers was used for this work as they are a well-known and characterised group of pharmaceutical compounds. Several MS and HPLC/MS studies of β-blockers have been published covering the use of deuterium exchange and other experiments to determine fragmentation pathways [11,12]. For further information on the use of chromatographic and MS techniques for the analysis of β-blockers the reader is referred to works such as the following [13,14].

2. Experimental

The β-blocker samples were obtained from Sigma–Aldrich (Gillingham, UK). Individual stock solutions of each β-blocker were prepared at about 1 mg ml^{-1} using 40% acetonitrile/60% water. These stock solutions were used to prepare the sample solutions containing all the β-blockers.

2.1. UPLC

UPLC was performed using an Acquity system from Waters (Elstree, UK) and 100 mm columns with an i.d. of 2.1 mm packed with 1.7 μm Acquity C_{18} BEH particles (Elstree, UK). Water was de-ionised using a Millipore system (Waters, Elstree, UK). Acetonitrile and tri-fluoro acetic acid (TFA) were from Fisher Scientific (Loughborough, UK). The A solvent was 0.1% TFA in water (v/v) and the B solvent was 0.1% TFA in acetonitrile (v/v). The gradient programme ran from 20% B to 50% B in 10 min at a flow rate of 0.5 ml min^{-1}. With the deuterium exchange experiments the A solvent was 0.1% TFA dissolved in 99.9% D_2O (Fluorochem, Glossop, UK). The column was thermo-stated at 35 °C. UV absorbance data were collected at 270 nm with a bandwidth of 10.8 nm and a data collection rate of 10 Hz was employed. An injection volume of 5 μl was employed.

2.2. HPLC

HPLC was performed using an Agilent 1100 binary system (Agilent, Stockport, UK). A 150 mm column with an i.d. of 3.0 mm packed with 3.5 μm X-Terra MS C_{18} (Waters, Elstree, UK) was used. The HPLC mobile phases and operating conditions were the same as those used for UPLC.

2.3. MS

MS experiments were carried out on a Micromass ZQ single quadrupole instrument (Manchester, UK). The instrument was operated in +ve electrospray mode using cone voltages of 30 and 50 V with a scan range of 150–500 amu and cycle times of 1 s. The source temperature was 120 °C, the de-solvation temperature 350 °C, the cone gas flow 50 dm^3 h^{-1} and the de-solvation gas flow 400 dm^3 h^{-1}.

2.4. NMR

Proton NMR experiments were carried out using a Bruker DRX500 (Coventry, UK) with water suppression for the H_2O based solvent system. Pindolol solutions at about 1 mg ml^{-1} were prepared using either 0.1% TFA in D_2O, or 0.1% TFA in 90% H_2O and 10% D_2O. The D_2O was used to provide the lock signal.

3. Results and discussion

3.1. Comparison of UPLC and HPLC for the separation of β-blockers

A series of β-blocker samples was prepared for this work. The structures of the β-blockers are shown in Fig. 1 along with their molecular masses. The main sample had all of the β-blockers present at similar concentrations (about 0.1 mg/ml) to each other in order to give similar UV response at 270 nm. Propranolol and Pindolol, which have extended aromatic systems, were present at lower concentrations because of their stronger UV absorbance. Other samples had Oxprenolol present at a concentration of about 0.1 mg/ml, and the other β-blockers present at relative concentrations of about 0.5 and 0.1% to represent the low-level impurities which might be present in a pure sample of a pharmaceutical drug substance.

The β-blocker mixture was separated by both UPLC and HPLC using the same mobile phase start and end conditions and gradient time. The conditions given were not fully optimised but were found to give a satisfactory separation for analytes with a range of hydrophobicities within a short analysis time. The UPLC separation was obtained from a 100 mm × 2.1 mm column packed with 1.7 μm BEH C_{18} particles and the HPLC separation from a 150 mm × 3.0 mm column packed with 3.5 μm X-Terra MS C_{18} particles. The HPLC stationary phase was chosen in order to have a similar chemistry to that used with UPLC. Flow rates of 0.5 ml min^{-1} (UPLC) and 1.0 ml min^{-1} (HPLC) were chosen so as to give similar mobile phase linear velocities. With UPLC the operating pressure at the start of the gradient was 670 bar and with HPLC 330 bar. The separations of the main β-blocker mixture using UPLC and HPLC are shown in Figs. 2 and 3, respectively. In both cases the elution order was Atenolol, Pindolol, Acebutolol, Metoprolol, Oxprenolol, Propranolol, and Alprenolol. In this sample all of the β-blockers are present at similar concentrations.

From Figs. 2 and 3 it can be seen that UPLC gave a similar separation selectivity to HPLC but with somewhat sharper peaks and a better resolution of Propranolol and Alprenolol. The relative performance of the UPLC and HPLC was compared by measuring the peak capacities of the two systems. Peak capacity was determined by dividing the gradient time by the peak width at baseline, with the baseline width obtained by multiplying the width at half height by the factor 4/2.35. With UPLC the mea-

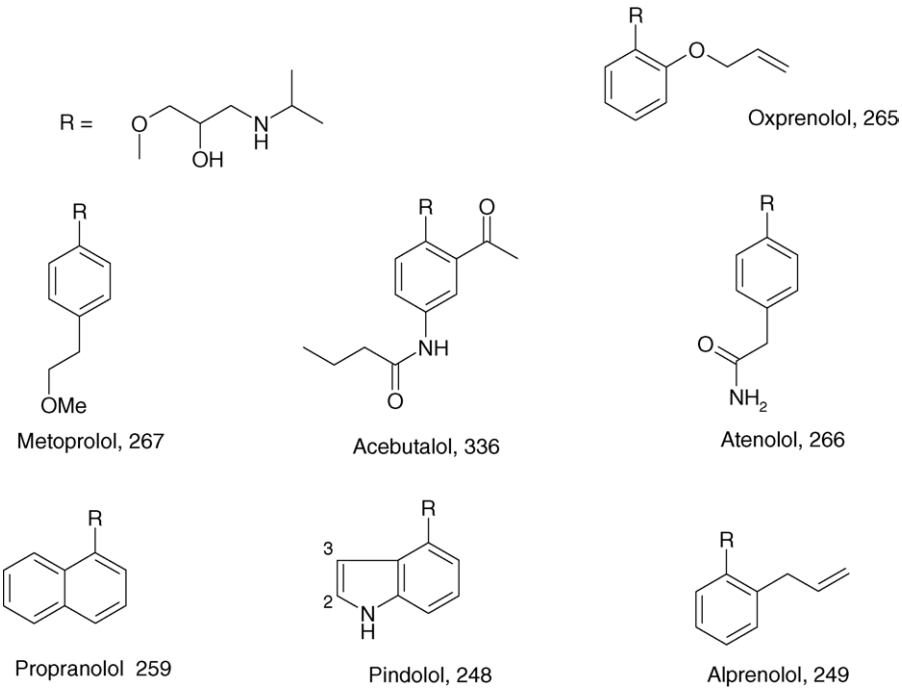

Fig. 1. The structures of the β-blockers Oxprenolol, Metoprolol, Acebutolol, Atenolol, Propranolol, Pindolol, and Alprenolol.

sured peak capacities were all significantly above those obtained from HPLC. For example Acebutolol and Metoprolol gave peak capacity values of 189 and 160, respectively, by UPLC whereas the values obtained by HPLC with a flow rate of 1.0 ml min^{-1} were both 76. In subsequent experiments HPLC (see below) a mobile phase flow rate of 0.5 ml min^{-1} was used and the peak capacities measured from Acebutolol and Metoprolol were 52 and 56, respectively. Thus UPLC generated significantly higher peak capacities than HPLC even at the same mobile phase linear velocity.

3.2. Comparison of UPLC/MS and HPLC/MS with aqueous mobile phase

HPLC/MS was performed using flow rates of both 1.0 and 0.5 ml min^{-1} with all the mobile phase passing into the mass spectrometer. The higher flow rate gave the same linear velocity as with UPLC. A comparison of the results, however, showed that the MS response was significantly weaker at the higher HPLC flow rate, with the maximum total ion current (TIC) for Oxprenolol dropping from 5.7×10^7 counts to 1.3×10^7. The

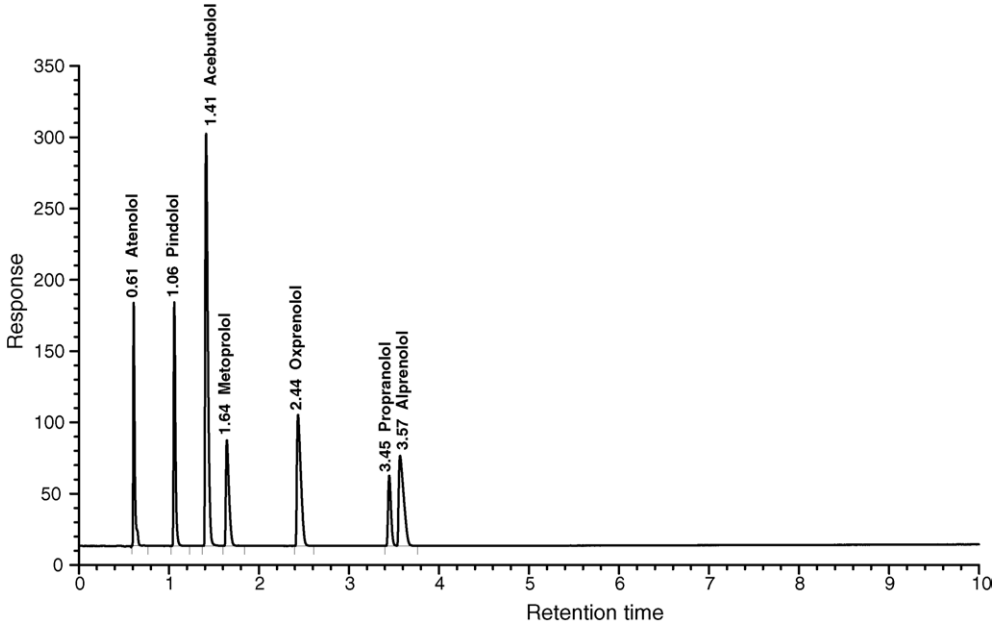

Fig. 2. UPLC chromatogram of the β-blockers.

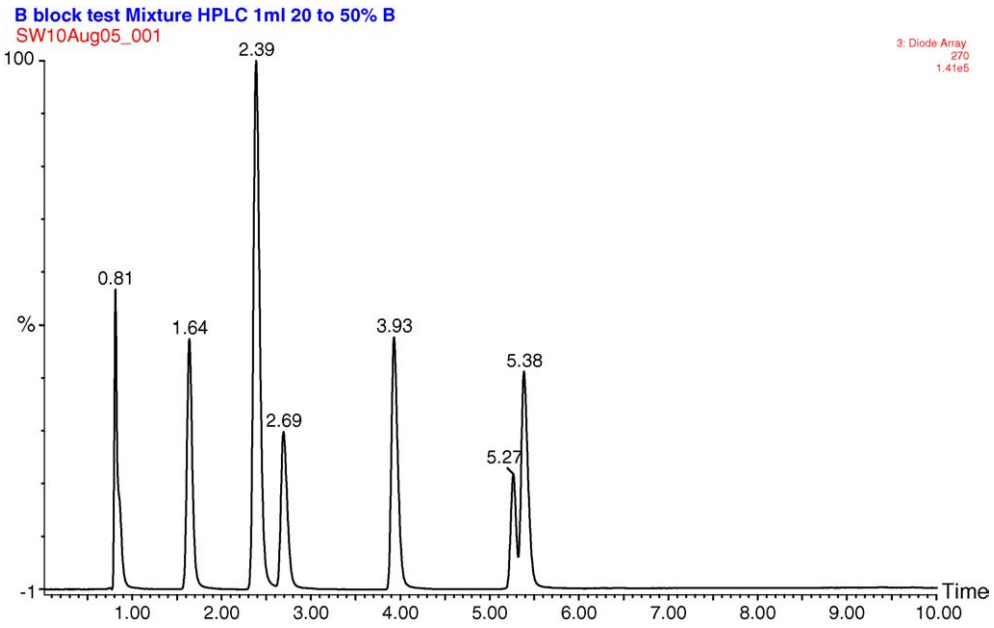

Fig. 3. HPLC chromatogram of the β-blockers.

lower HPLC/MS response at higher flow rate also resulted in a significant increase in the level of background noise in the mass spectra obtained for the individual β-blockers. It is possible to use a flow splitter after the UV detector and before the MS but this was found to cause additional band broadening and so some loss of chromatographic integrity. Because of the loss of MS sensitivity at higher HPLC flow rates the UPLC/MS and HPLC/MS data given below are all obtained using the same flow rate of 0.5 ml min^{-1}.

MS data were collected at cone voltages of 50 and 30 V in order to obtain fragmentation information as well as the mass of the protonated molecular ion. The UPLC/MS and HPLC/MS experiments were carried out on subsequent days using the same MS conditions. The objective was to try and determine whether the choice of the separation approach had any impact on the quality of the mass spectra obtained.

Fig. 4 shows the mass spectra of the individual β-blockers separated by UPLC and using a cone voltage of 30 V. The sample contains Oxprenolol at a concentration of about 0.1 mg/ml and the other β-blockers at a relative concentration of about 0.5%. The spectra of the β-blockers are displayed according to their elution order with that of Atenolol at the bottom and that of Alprenolol at the top. From Fig. 4 it can be seen that at the lower cone voltage the main signals are due to the protonated molecular ions of the β-blockers (MH^+). For example Alprenolol gives a protonated molecular ion with an m/z value of 250. From Fig. 4 it can also be seen that the quality of the spectra are good in terms of the signals and background noise. At the higher cone voltage some fragments were obtained and they are discussed below along with the results of the deuterium exchange work.

Comparison with results from HPLC/MS showed that similar quality mass spectra were obtained from both UPLC/MS and HPLC/MS. Fig. 5 for example shows the spectra obtained for Acebutolol by UPLC/MS and HPLC/MS, respectively. From Fig. 5 it can be seen that the two spectra are of a similar quality in terms of signal intensity and noise.

3.3. Deuterium exchange in UPLC/MS and HPLC/MS

As deuterium exchange can be a useful tool in gaining chemical information about low-level impurities using HPLC/MS, the same approach was evaluated with UPLC/MS. The β-blocker samples were used for this work and UPLC/MS and HPLC/MS experiments were performed on subsequent days using the same single quadrupole mass spectrometer and with the same operating conditions. The data from the two sets of experiments were examined to compare sensitivity and spectral quality. UPLC has a potential benefit over HPLC for deuterium exchange work as the narrower column diameter, 2.1 mm as compared to the 3.0 or 4.6 mm that are standard with HPLC, implies a lower consumption of an expensive solvent. The performance comparison is important to ensure that data of the same standard can be obtained.

3.3.1. Spectra of the molecular ions

UPLC/MS and HPLC/MS data were obtained using cone voltages of both 30 and 50 V. The use of the lower cone voltage is intended to produce the deuterated equivalent of the protonated molecular ions discussed above. Fig. 6 shows the TICs of the 0.1% β-blocker sample obtained with a cone voltage of 30 V from UPLC/MS and HPLC/MS, respectively. From Fig. 6 it can be seen that UPLC/MS gave a TIC with a much better signal to noise ratio than was obtained by HPLC/MS, with the peaks due to the low-level impurities being much easier to locate in the background signal. The reason for the better sensitivity of UPLC/MS with D_2O in the mobile phase is not known but it was observed at both high and low cone voltages and with all samples. Fig. 7 shows the mass spectra for Acebutolol obtained

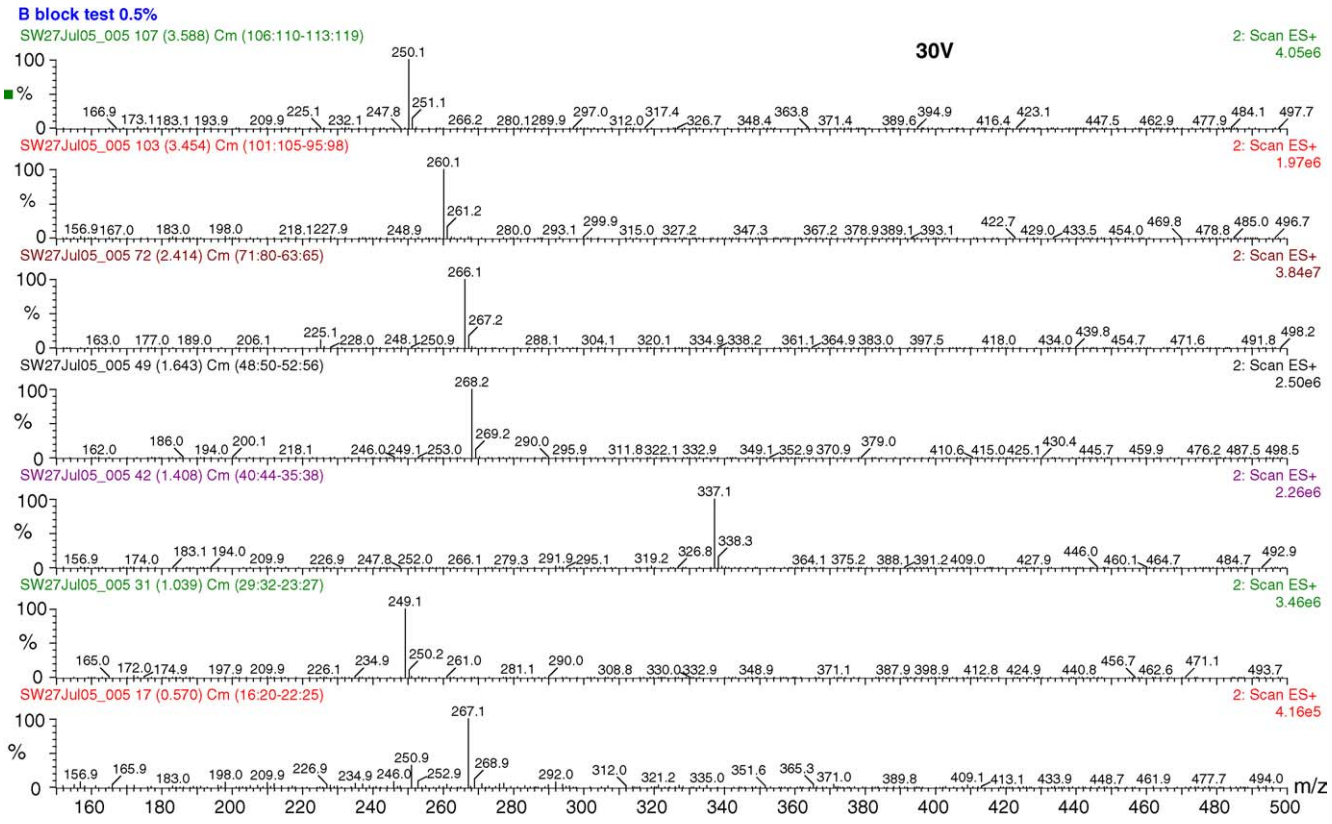

Fig. 4. Low fragmentation voltage mass spectra of the β-blockers separated by UPLC.

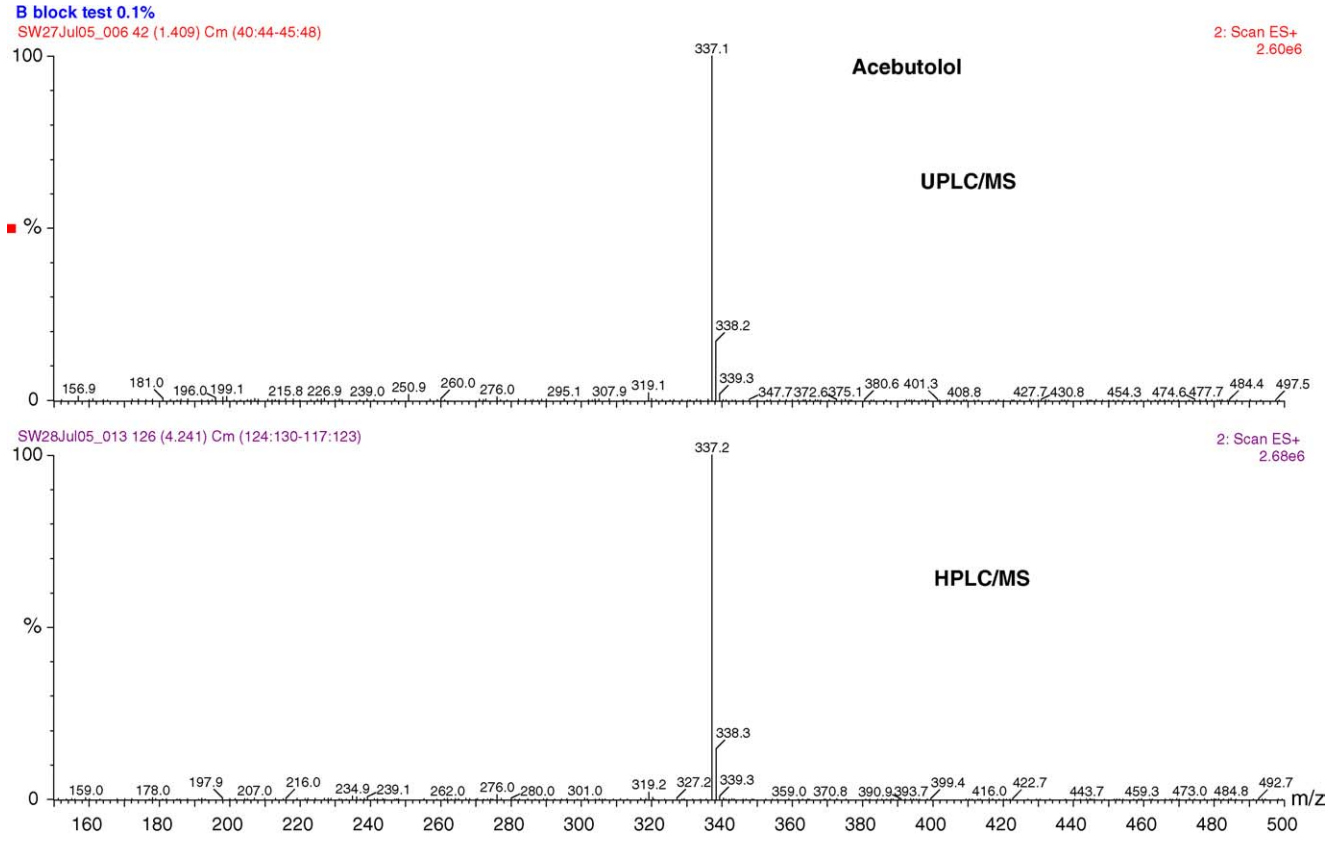

Fig. 5. Mass spectra of Acebutolol obtained by UPLC/MS and HPLC/MS.

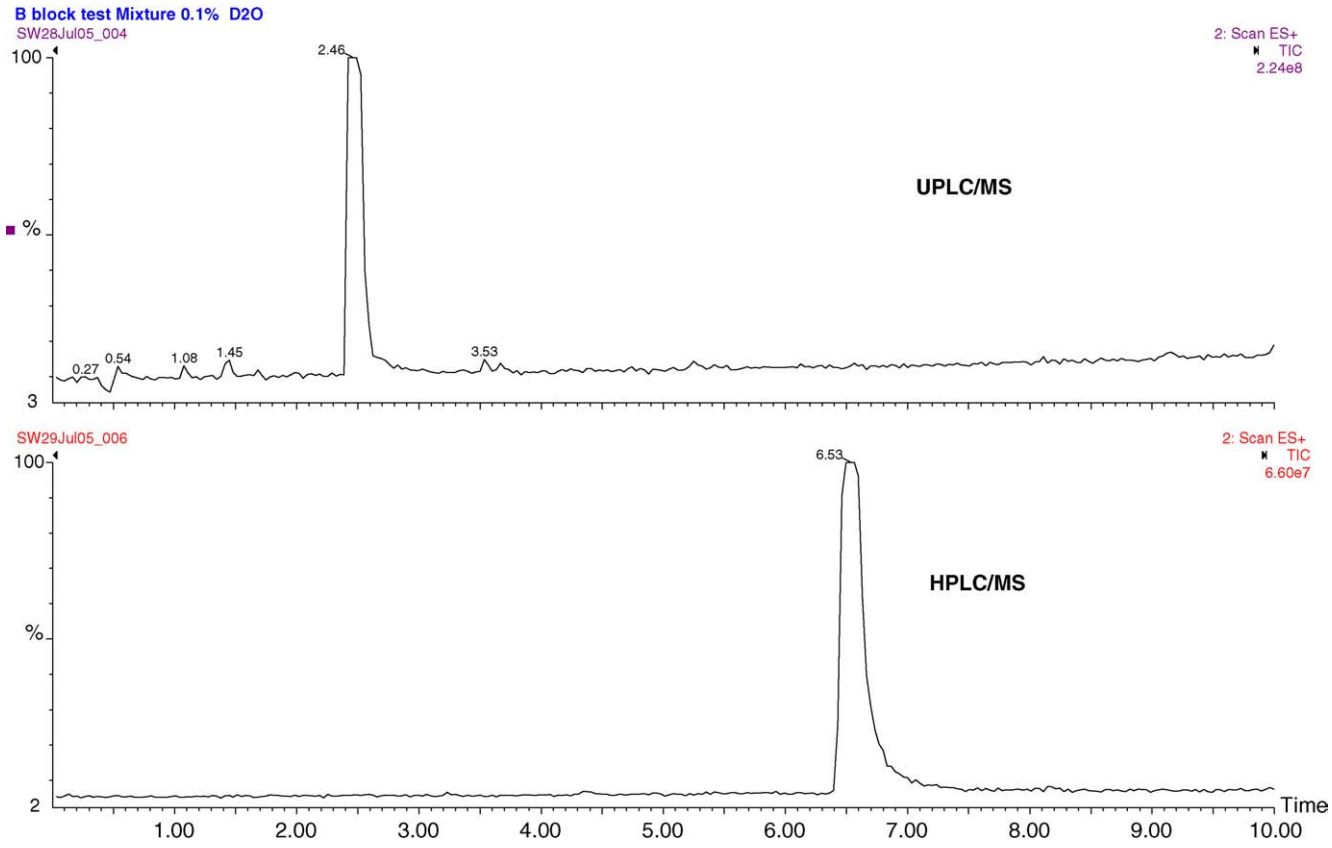

Fig. 6. TICs of the β-blockers obtained by UPLC/MS and HPLC/MS, and using deuterium oxide in the mobile phase.

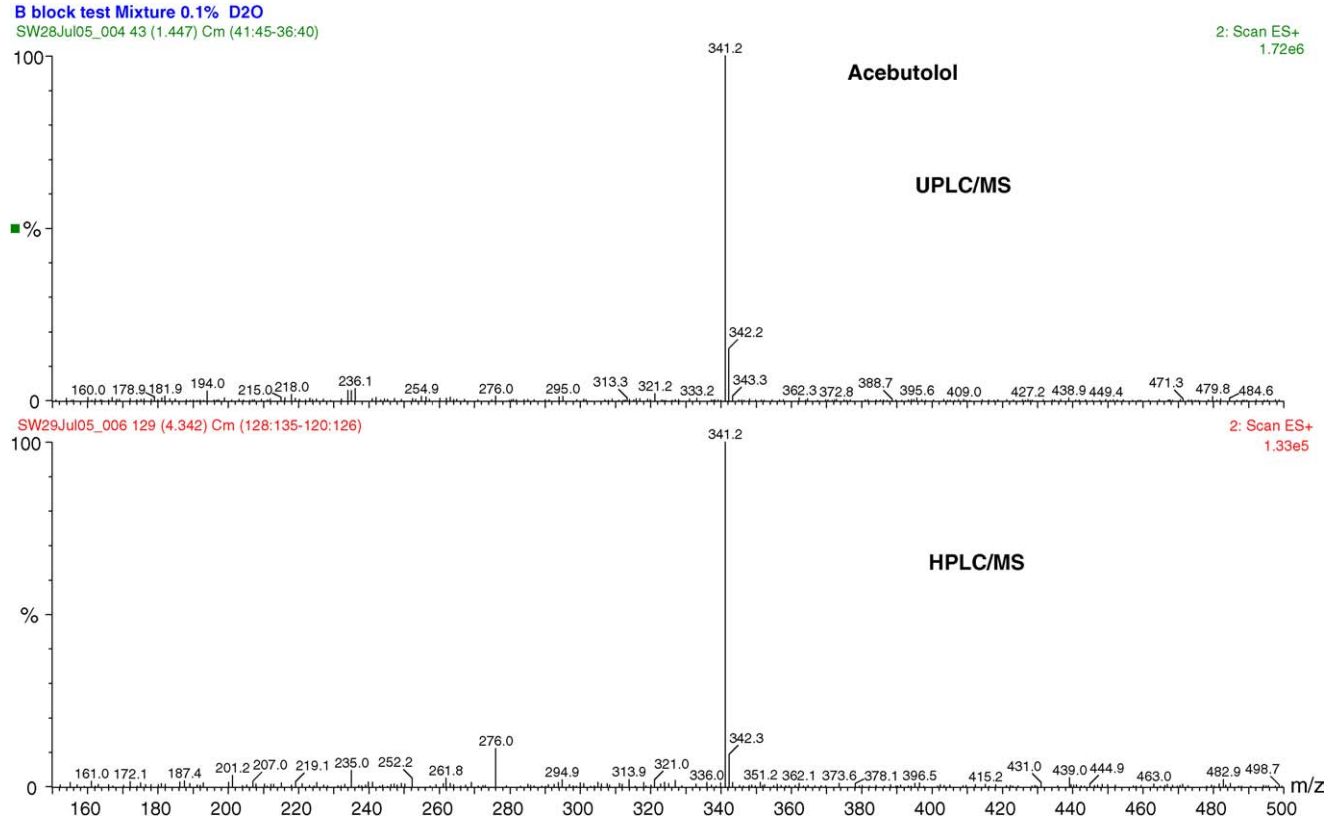

Fig. 7. Mass spectra of Acebutolol obtained by UPLC/MS and HPLC/MS, and using deuterium oxide in the mobile phase.

by UPLC/MS and HPLC/MS, respectively. As expected from the higher sensitivity seen in the TIC traces shown in Fig. 6, the UPLC/MS spectrum in Fig. 7 shows a better signal to noise ratio than that from HPLC/MS. The difference is particularly seen in the greater level of high mass noise seen with the HPLC/MS spectrum.

3.3.2. Mass shifts observed with the use of D_2O

The UPLC mass spectra obtained using D_2O in the mobile phase were compared with those obtained from H_2O in order to confirm the number of readily exchangeable protons. A comparison with the mass spectra of Acebutolol obtained by using H_2O as the mobile phase (Fig. 5) with that obtained by using D_2O as the mobile phase (Fig. 7) shows a mass shift of +4 with the deuterated solvent system. The shift in mass arises because of the alcohol and secondary amine protons in the amino alcohol side chain, the amide proton in the side chain at the 4 position, and the change from a protonated to a deuterated molecular ion (i.e. $M'D^+$ instead of MH^+). With Acebutolol deuterium exchange is complete and no trace of partially deuterated species could be seen.

Fig. 8 shows the mass spectra obtained for all of the β-blockers using the same experimental conditions as those which were used for Fig. 4, except that D_2O was used instead of H_2O in the mobile phase. A comparison of Figs. 8 and 4 shows that for most of the β-blockers the mass shifts from those of the protonated molecular ions are those expected from the structures shown in Fig. 1. Alprenolol, Propranolol, Oxprenolol, and Metoprolol all show a mass shift of +3, as expected from the amino alcohol side chain and the switch from a molecular ion with H^+ to one with D^+. In each case the exchange reaction has gone to completion with very little trace of partially deuterated species. Acebutolol shows a mass shift of +4 as discussed above. The exceptions to the expected behaviour are Atenolol and Pindolol where the spectra are not as simple as might be expected at first.

3.3.3. Mass shifts observed with Atenolol and Pindolol

3.3.3.1. Atenolol MS data. The spectrum of Atenolol in the D_2O mobile phase has a peak at *m/z* 272 which is a mass shift of +5 on that of the protonated molecular ion with *m/z* 267. The mass shift of +5 is as expected from the following: the amino alcohol side chain; the amide chain in the 4 position; and a protonated molecular ion. In addition however the Atenolol spectrum in D_2O has several peaks which do not seem to correspond to those seen in H_2O. The reason for this spectral complexity is not known but it may be due to noise, as the signal seen with Atenolol is only about 10% of the intensity seen with the other β-blockers. At higher concentrations of Atenolol the peak at *m/z* 272 is much more intense than the other *m/z* values.

3.3.3.2. Pindolol MS data. The spectrum of Pindolol in D_2O shows peaks with *m/z* values of 253 and 254 in the ratio of about 2:1. These *m/z* values correspond to mass shifts of +4 and +5 relative to that of the protonated molecular ion at *m/z* 249. A mass shift of +4 is consistent with the pattern described above

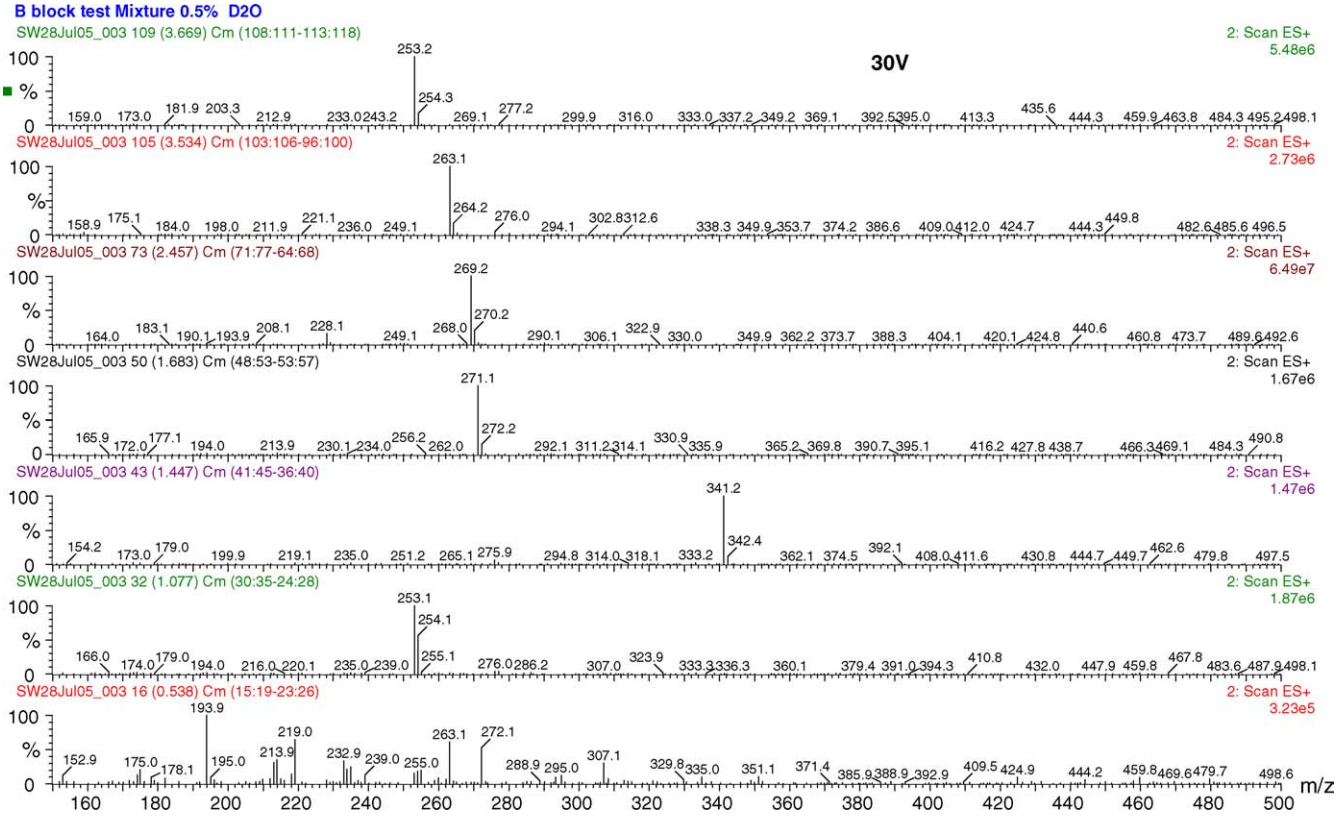

Fig. 8. Low fragmentation voltage mass spectra of the β-blockers separated by UPLC, and using deuterium oxide in the mobile phase.

(amino alcohol side chain and protonated molecular ion), and the expected exchange from the hydrogen bound to the nitrogen in the indole ring. The mass shift of +5 implies that a second exchange is also occurring in the indole ring. The fact that two peaks are seen implies that the deuterium exchange reaction has not gone to completion, presumably because of slow kinetics. In the experiments described here samples were dissolved in acetonitrile and H_2O so exchange can only begin once the analytes have been mixed with the mobile phase at injection, and exchange stops when the analytes become de-solvated upon entering the mass spectrometer. The hypothesis that the kinetics of exchange for the second indole proton are slow was tested in two ways: by using a higher temperature to increase the rate of reaction, and by examining the impact of a longer reaction time.

The reaction temperature for deuterium exchange was increased by changing the temperature of the UPLC column from 35 to 60 °C. This temperature increase made only a small difference to the chromatography with retention time for Pindolol dropping from 1.08 to 0.91 min. Fig. 9 shows the mass spectra for Pindolol obtained using column temperatures of 35 and 60 °C, respectively. From Fig. 9 it is seen that the ratio of the intensities of the *m*/*z* 253 to *m*/*z* 254 peaks has changed from about 2:1 at 35 °C to about 1:3 at 60 °C, i.e. the exchange reaction has gone from about 33% completion to about 75% completion.

The impact of a longer time for deuterium exchange was investigated by comparing the spectrum obtained from UPLC/MS with that from HPLC/MS obtained with the same column temperature of 35 °C. With HPLC the retention time of Pindolol was about 3.3 min as opposed to 0.91 min for UPLC. The longer time on column with HPLC means that there is a longer period of time for deuterium exchange to occur. The impact of this longer exchange time in HPLC is seen in the HPLC/MS spectrum of Pindolol with the peaks at *m*/*z* 253 and *m*/*z* 254 having a ratio of about 5:7, i.e. the exchange reaction has gone to about 58% completion.

Thus both sets of data show that Pindolol differs from the other β-blockers in that it has an aromatic proton bound to a carbon atom which is capable of exchange, but that the reaction kinetics are much slower than those seen with the other protons. Deuterium exchange reactions which are slow on the timescale of an HPLC separation may also be observed in systems where there are many exchangeable protons. For example with the peptide substance P, which has 23 exchangeable protons, several minutes at room temperature was required before the final equilibrium distribution was reached [15].

3.3.3.3. Pindolol NMR data. The position of the slow exchanging indole ring proton was determined by ^1H NMR studies using either D_2O or H_2O as solvents along with 0.1% TFA. The NMR spectra of Pindolol were recorded at 500 MHz. In H_2O two broad singlets were observed in the aromatic part of the spectrum at about 6.6 and 7.3 ppm and these are due to the protons in the 3 and 2 positions in the indole ring (Fig. 1). The broadness of these signals results from the small splitting constant. In D_2O the 6.6 ppm signal disappears and the 7.3 ppm signal becomes

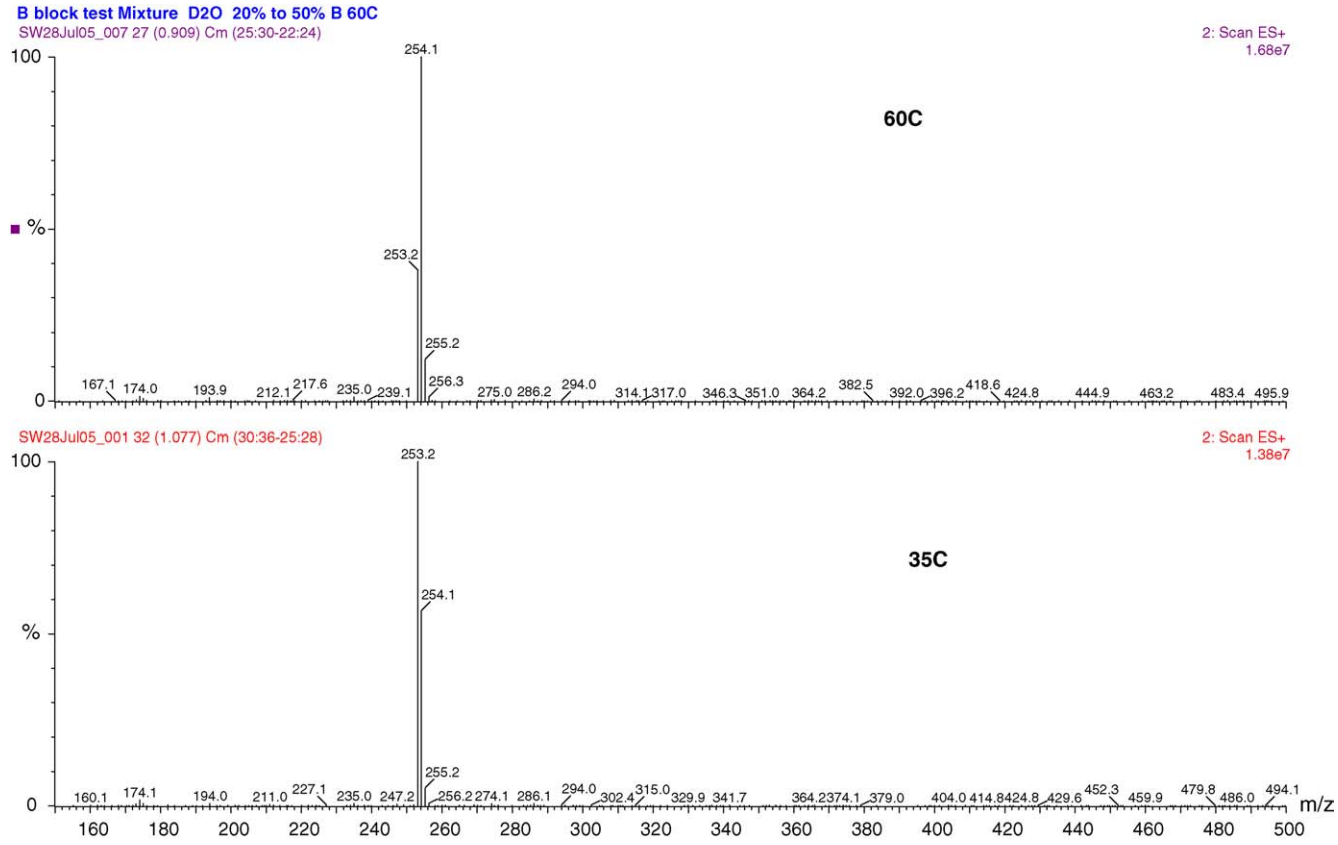

Fig. 9. Mass spectra of Pindolol obtained using deuterium oxide in the mobile phase and column temperatures of 60 and 35 °C.

much sharper, indicating that the proton in the 3 position has exchanged with the solvent.

The ^{1}H NMR studies demonstrate that slow deuterium exchange occurs with the proton at position 3 on the indole ring. With indoles position 3 is known to be activated to electrophilic substitution (e.g. nitration). Preferential electrophilic substitution at the indole 3 position can be rationalised either by examining the canonical structures which describe the transition state [16], or by a molecular orbital approach looking at the frontier electron population of indole in the ground state [17].

3.3.4. Fragmentation and structure confirmation

The use of 50 V as a cone voltage leads to fragmentation of the protonated molecular ions. The fragments produced can normally be related to the structure of the analyte by commonly observed processes such as the loss of stable neutral molecules from the protonated molecular ion. The mass shifts observed with the use of D_2O helps to identify the neutral molecules lost and the remaining fragment, and so help to confirm the identity of known analytes and unknowns such as related substances.

The mass spectra of the β-blockers in the 0.5% sample which have been separated by UPLC using H_2O and D_2O in the mobile phase are shown in Figs. 10 and 11, respectively. The spectra shown in Figs. 10 and 11 are consistent with fragmentation processes observed with Propranolol and indicate the presence of the amino alcohol side chain [12]. The spectra of many of the β-blockers show a mass loss of 42 for both H_2O and D_2O containing mobile phases. The lack of a mass shift between the two sets of spectra indicate that the species lost from the protonated molecular ion does not contain any readily exchangeable protons. This observation is consistent with the loss of 42 being due to the loss of propene from the molecular ion. In contrast to this all of the β-blockers show a loss of 77 in the H_2O based solvent and a loss of 80 in the D_2O based mobile phases. The loss of a mass with an odd number indicates that the component(s) lost contain a nitrogen atom and the mass shift of +3 between the two solvents indicates the presence of three exchangeable protons. These observations are consistent with the loss of both water and isopropylamine from the protonated molecular ion. Acebutolol shows a loss of *m*/*z* 18 in the mass spectrum from the H_2O based mobile phase and a loss of *m*/*z* 20 in the mass spectrum from the D_2O based mobile phase. These mass losses are consistent with the loss of H_2O and D_2O, respectively, from the protonated and deuterated molecular ions.

4. Performance of UPLC/MS and HPLC/MS in routine operation

An important aspect of the comparison of a newer analytical approach with more established techniques is the performance in routine operation. Such a comparison of UPLC/MS with HPLC/MS is beyond the scope of this work, but in a recent study we have found UPLC with UV detection to give good performance in the assessment of the purity of a number of AstraZeneca development compounds [18]. UPLC/UV gave satisfactory sensitivity, linearity of response with concentration, standard recovery, retention time precision, and peak area precision for impurities at a range of concentrations. UPLC/UV

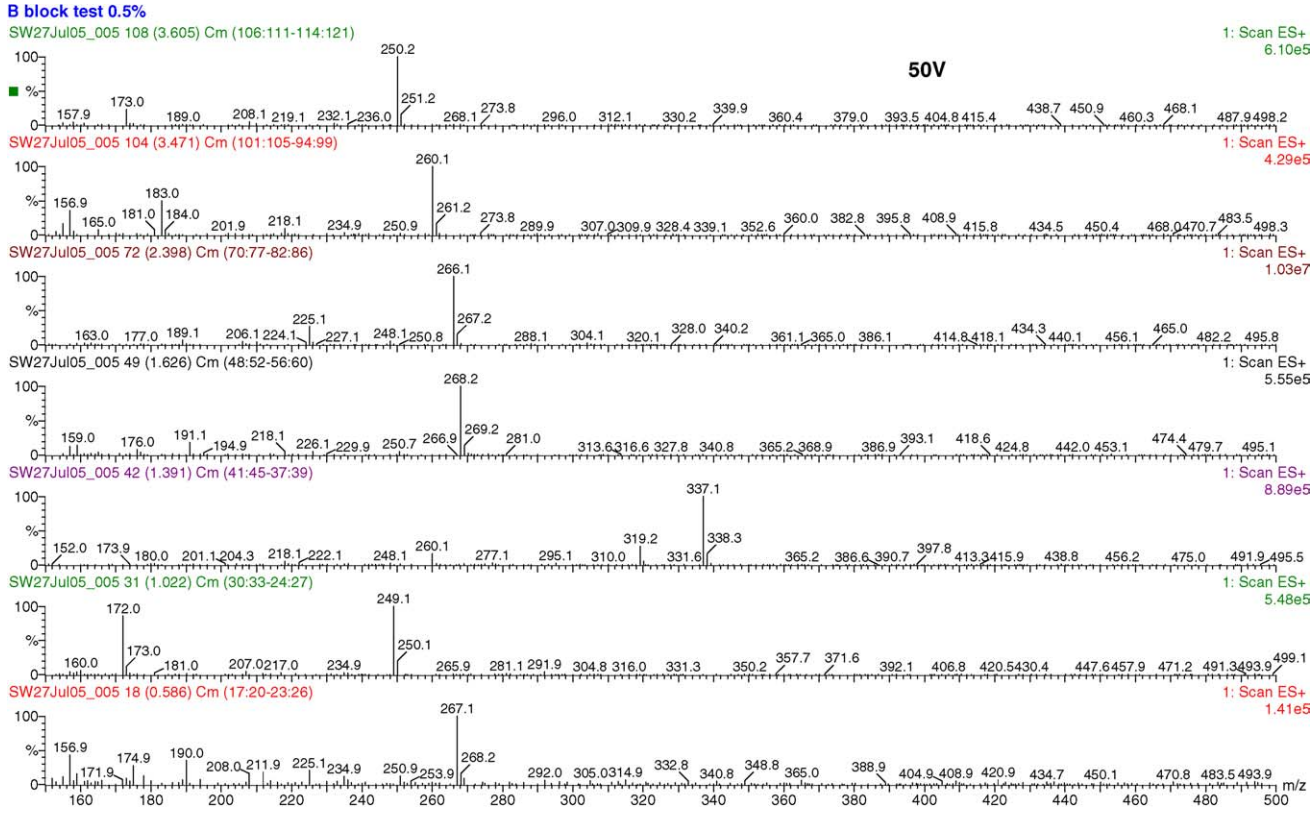

Fig. 10. High fragmentation voltage mass spectra of the β-blockers separated by UPLC, and using water in the mobile phase.

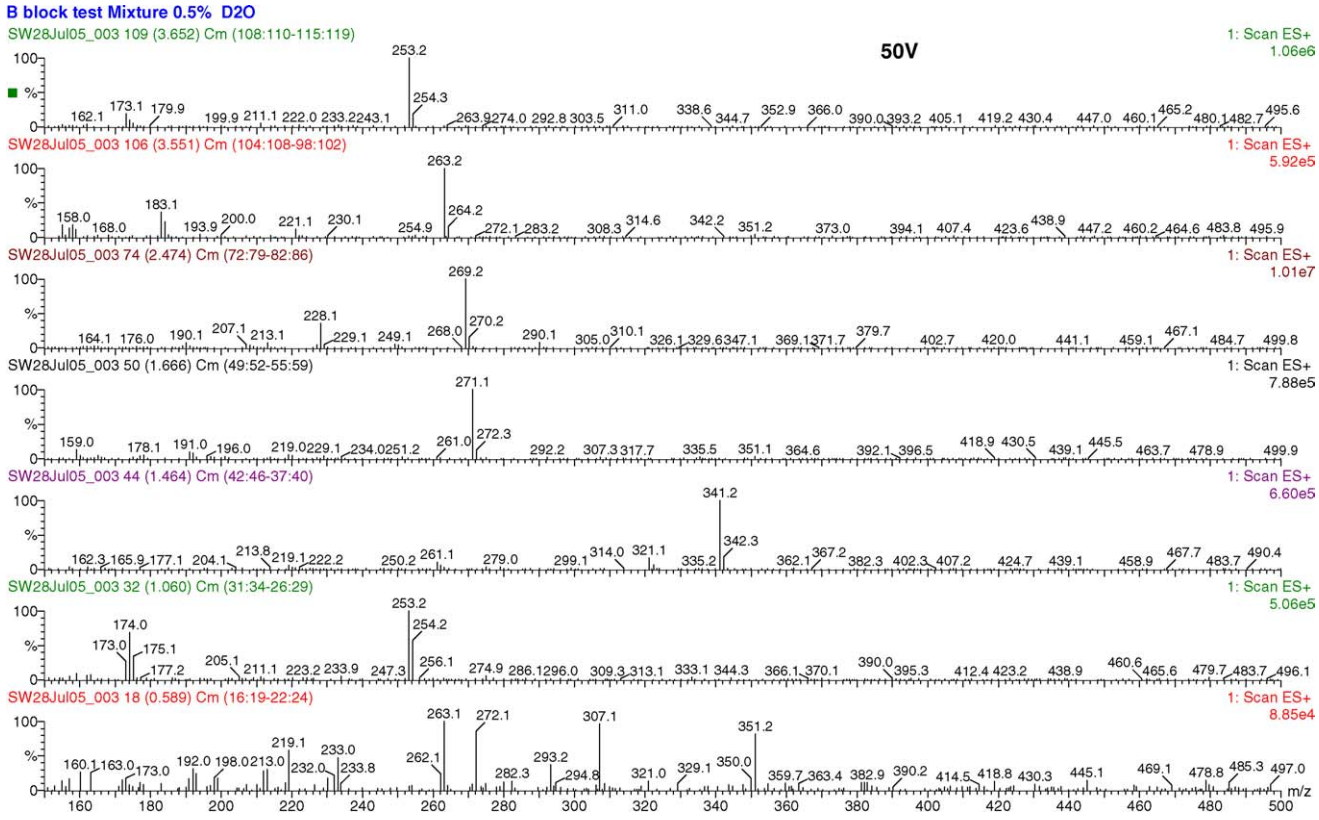

Fig. 11. High fragmentation voltage mass spectra of the β-blockers separated by UPLC, and using deuterium oxide in the mobile phase.

analysis also gave similar impurity levels to those which had been found by HPLC/UV.

5. Summary and conclusions

A series of β-blockers was analysed using both HPLC/MS and UPLC/MS using mobile phases containing acetonitrile and TFA and either H_2O or D_2O. The use of UPLC gave both faster analysis and a higher peak capacity. The spectra obtained from UPLC/MS were as good as those from HPLC/MS and showed better signal/noise performance with the D_2O containing mobile phases.

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Gary Sharman for performing the NMR experiments on Pindolol.

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