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Evaluation of the use of UPLC-TOFMS with simultaneous [^{14}C]-radioflow detection for drug metabolite profiling: Application to propranolol metabolites in rat urine

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ABSTRACT

The non-selective β-adrenergic receptor antagonist propranolol [1-(isopropylamino)-3-(1-naphthoxy)-2-propanol] is metabolised extensively *in vivo*. Enumerating and identifying the many metabolites that result from multiple biotransformations provides a considerable analytical challenge, greatly aided by efficient chromatography coupled to sensitive mass spectrometric detection. Here the use of the newly introduced high-resolution technique of "ultra performance liquid chromatography" (UPLC) linked to quadrupole time-of-flight mass spectrometry (TOFMS) with simultaneous [^{14}C]-radioflow detection was applied to rapid metabolite profiling. [^{14}C]-propranolol, dosed intraperitoneally to rat at 25 mg kg^{-1} and 200 μCi kg^{-1} was used as a model compound for this evaluation. Some 14 metabolites were detected in the urine by this technique including a number of conjugated metabolites such as sulphates, several isobaric glucuronides and two novel di-glucuronides.

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1. Introduction

Rapid and effective, high resolution, methods for metabolite profiling, detection and identification remain a key focus of drug metabolism groups in both industry and academia. Currently the workhorse for this type of investigation is liquid chromatography coupled to mass spectrometry (LC–MS) and there is no doubt that this represents an efficient and effective methodology for this type of work. However, there is also no doubt that improved chromatographic resolution would be of significant benefit in cases where metabolism is complex, giving rise to many different metabolites covering a range of concentrations. Recently, ultra performance liquid chromatography (UPLC) has been introduced with increased separation performance compared to conventional HPLC [1,2]. UPLC employs chromatographic separations based on a sub 2 μm stationary phase operated at high pressures to give flow rates of ca. 0.6–1.0 mL min^{-1}. One of the major advantages of this improvement in separation that UPLC affords is that chromatographic runtime can often be greatly reduced, giving a considerable saving in both instrument and analyst time; of value to those involved in high-throughput applications in drug discovery and development [3,4]. UPLC (in conjunction with mass spectrometry) has also found applications in metabonomics/metabolomics studies, where the need to separate efficiently the components of complex biological matrices prior to spectroscopic examination is important for efficient biomarker discovery [5–8]. Independent of the application, UPLC can increase sensitivity over that offered by HPLC by improving chromatographic peak characteristics.

However, the chromatographic advantages offered by UPLC may be limited in certain circumstances where the resolution of the detector is relatively low as a result of the requirement, e.g., to use large flow cells for sensitivity. In order to investigate the utility of UPLC for studying drug metabolism in association with on-line radiochemical detection we have evaluated UPLC-time-of-flight mass spectrometry (TOFMS) with simultaneous [^{14}C]-radiometric detection for the analysis of urine samples obtained in the course of a study on the drug propranolol.

Propranolol [1-(isopropylamino)-3-(1-naphthoxy)-2-propanol] (Fig. 1) is a non-selective β-adrenergic receptor antagonist used in the relief of numerous cardiovascular disorders including hypertension, angina pectoris and cardiac arrhythmia [9]. The *in vivo* fate of propranolol is complex and has been the subject of numerous [10–33] investigations in many species and has been reviewed [34].

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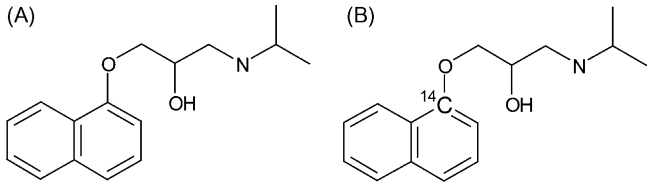

Fig. 1. Propranolol (A) and [^{14}C]-propranolol (B), showing the location of the radiolabel incorporated to allow radioprofiling in this study.

The rich nature of the metabolite profiles arising because of this metabolic complexity was therefore attractive as a test for the use of UPLC in drug metabolism.

2. Experimental

2.1. Test compounds

DL-Propranolol (Lot 105H0652) was purchased from Sigma (Sigma, St. Louis, MO, USA). ^{3}H labelled DL-propranolol (Lot 024K9420/21, labelled in the 4-position of the napthalene ring) was also obtained from Sigma. [^{14}C]-labelled DL-propranolol (batch M45520/34R, labelled in the 1-position of the napthalene ring, Fig. 1) was synthesised at AstraZeneca (Macclesfield, UK).

2.2. Chemicals

Acetonitrile and water were of HPLC grade and obtained from Sigma–Aldrich (Poole, Dorset, UK) along with leucine enkephalin (>97%) and formic acid (~98%). Ultima-Gold™ and Ultima-Flow™, were purchased from PerkinElmer, UK.

2.3. Animal experiments

Three male Wistar-derived rats (AP rat, Rodent Breeding Unit, AstraZeneca, UK) each received a 25 mg kg^{-1} intraperitoneal administration of radiolabelled DL-propranolol in 0.9% (w/v) saline, with a dose volume of 2 mL kg^{-1}. The dose incorporated 50 mCi kg^{-1} of [^{3}H]-DL-propranolol*; 200 mCi kg^{-1} [^{14}C]-DL-propranolol and unlabelled DL-propranolol, such that the animals received approximately 250 μCi kg^{-1} in total. The dose solution was prepared on the day of dosing, vortex mixed, and was stirred continuously prior to and during the administration procedure. (*The ^{3}H label was incorporated in the dose to evaluate the effect of dual radiolabelling on scintillation counter quench curve calibration—this label was not considered in the present work where only [^{14}C]-detection was performed).

After dosing, excreta and cage washings were collected until 120 h post dose, at which point the animals were killed by Fluothane™ (AstraZeneca) inhalation and the carcasses retained for analysis. All samples were analysed for radioactive content and the proportion of the dose excreted in the urine and faeces, or retained in the carcass, determined.

2.4. UPLC-TOFMS-[^{14}C]-radioflow separations

Chromatographic separations were performed on a 2.1 mm × 100 mm Acquity UPLC™ BEH C_{18} column (1.7 μm particle size), using a Waters® Acquity UPLC™ system (Waters, Watford, UK) comprising a binary solvent manager, automatic sample hander, column oven and managed using Empower software. A zero-dead-volume T-piece was used to divide the eluent flow (600 μL min^{-1}), with some 150 μL min^{-1} directed to the mass spectrometer, and 450 μL min^{-1} to the radioflow detector cell.

For chromatography 0.1% (v/v) formic acid in water (solvent A) and 0.1% formic acid (v/v) in acetonitrile (solvent B) were used to provide a solvent gradient. Three different chromatographic gradients were evaluated in this work, with total run times of 18, 33 and 60 min—summarised in Table 1. For the 18 min runs the column conditions were maintained at 100% A over 0–1 min, and raised using a linearly changing gradient to 20% B at 7 min and then 40% B at 15 min. For the 33 min runs the column conditions were maintained at 100% A over 0–1 min, and raised using a linearly changing gradient to 20% B at 15 min and then 40% B at 20 min. For the 60 min runs the column conditions were maintained at 100% A over 0–1 min, and raised using a linearly changing gradient to 20% B at 50 min and then 40% B at 55 min. In all cases, the column was then washed with 90% B to remove any remaining material and then returned to the starting conditions to re-equilibrate. The flow rate was maintained at 600 μL min^{-1}. For each chromatographic separation, 20 μL of urine was injected onto the column at the starting gradient conditions. To minimise the carry-over of sample material from consecutive sample injections, the injection needle and sample loop were washed with 90% B between chromatographic runs.

Mass spectrometry was performed using a Micromass® Q-TOF™ Micro (Waters) equipped with an electrospray ionisation source operating in positive ion mode and controlled using Waters® MassLynx™ software (Waters). A LockSpray™ interface was used to provide accurate mass measurements, referenced to leucine enkephalin (200 pg μL^{-1}, in 50:50 ACN:H_2O, 0.1% formic acid) supplied at 10 μL min^{-1} by a Waters Alliance® 2795XC HPLC pump, giving an $[M+H]^+$ ion at m/z 556.2771. The source was set at 120 °C with a cone gas flow of 10 L h^{-1}. The desolvation gas was maintained at 320 °C and the nebulization gas flow set at 350 L h^{-1}. The capillary voltage was set at 3.5 kV. During the chromatographic run, centroid mass spectra of the UPLC eluent were acquired in the m/z range 70–900 as the average of 10 scans. The inter-scan delay was set at 0.1 s. A contact closure was used to synchronise data acquisition in MassLynx™ and sample injection. Acquisition was halted during the 'wash' period of the gradient run.

Radiochemical metabolite profiling was carried out on a Packard FLO-ONE® Radiomatic 500TR series flow scintillation analyser (Packard Instruments, Chicago, USA) using a 100 μL liquid scintillant cell. Ultima-Flow™ scintillant (PerkinElmer Life Sciences, Boston, USA) was delivered by the integrated pump via a T-piece mixer to give a 3:1 scintillant:eluent flow composition prior to entry to the analyser cell. ^{14}C radiochemical and UV data was collected and analysed using FLO-ONE® v.3.65 software (Packard).

Prior to the main metabolite profiling, as described above, UPLC-TOFMS-[^{14}C]-radioflow separations on a 0–6 h urine sample were made using several sizes of radioflow cell (50, 100, 500 and 1000 μL) to determine the optimum cell size for use in this type of work.

Table 1
Gradient conditions for UPLC separation of urine used in this study

Solvent composition		Time (min)		
%A (water + 0.1% formic acid)	%B (ACN + 0.1% formic acid)	A '18 min'	B '33 min'	C '60 min'
100	0	0	0	0
100	0	1	1	1
80	20	7	15	50
60	40	15	20	55
10	90	16	30	57
10	90	17	31	58
100	0	17.1	31.1	58.1
100	0	18	33	60

To investigate the effect of chromatographic runtime on detector resolution a 50 μL flow cell was used to profile the eluent alongside the mass spectrometer as described above. The gradient used for the metabolite profiling in this study (18 min) was compared with two similarly stepped, but longer gradients (33 and 60 min). The 50 μL cell was used for these separations as it provided an adequate limit of detection and, of the cells available, was the most able to deal with the improved chromatographic resolution provided by UPLC.

3. Results

3.1. Excretion of radioactivity

The overall recovery of the [^{14}C]-radiolabel was greater than 90% for the five days of the study (data not shown). The majority of the dose was recovered over the first 24 h with ca. 47% in the urine and ca. 37% in the faeces. These results are similar are similar to previously published observations [24]. As the bulk of the dose was excreted via the urine in the first 24 h after administration, the 0–6 h urine was selected for metabolite profiling by UPLC-TOFMS.

3.2. Suitability of radioflow cells for UPLC

A feature of UPLC is the very high chromatographic efficiency of the system, which generally results in very sharp analyte peaks. However, some of this greater separation efficiency is lost in radioflow analysis as a result of the need to mix the eluent with scintillant for the detection of radioactivity. To minimise band broadening clearly the smallest possible flow cell should be used, but this results in much lower sensitivity of detection for metabolites. In this study a number of different radioflow cells were investigated, ranging in volume from 50 to 1000 μL. It rapidly became clear that whilst the larger flow cell offered greater sensitivity the band broadening introduced by using these high volume flow cells resulted in the loss of many of the benefits of the short run times and increased resolution provided by UPLC (data not shown). The result of the evaluation of the use of the various flow cell formats was that the 50 and 100 μL flow cells provided the best compromise between sensitivity and peak shape. However, even with the 50 μL flow cell it became clear from the subsequent MS studies that peak overlap occurred in the radioflow cell for some metabolites, resulting in lower observed resolution compared to the MS data. A comparison of the data for the 18, 33 and 60 min

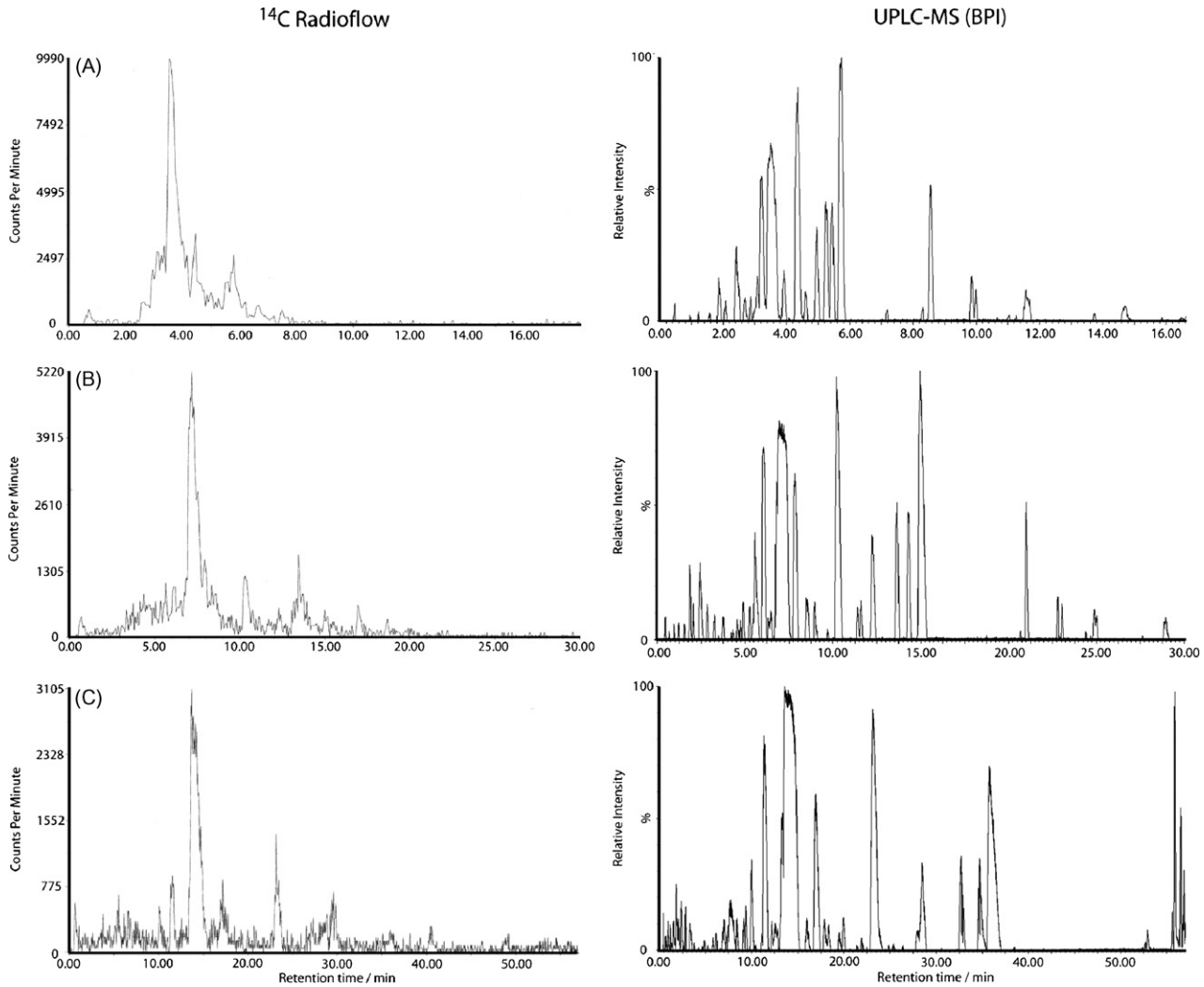

Fig. 2. Comparison of radioflow trace and mass spectral BPI for stepped gradient chromatographic conditions of three lengths: 18 min (A), 33 min (B) and 60 min (C).

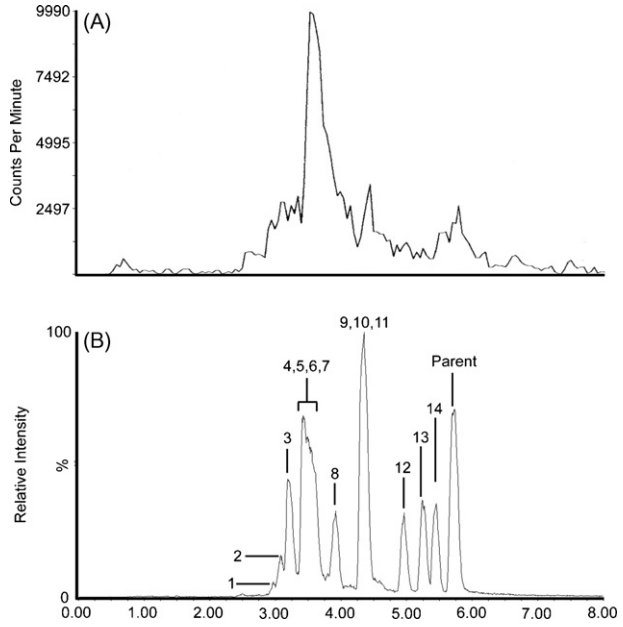

Fig. 3. ^{14}C radioflow chromatogram (A) and positive ion base peak intensity trace (B) of a 0–6 h urine sample following intraperitoneal dosing with [^{14}C]-propranolol at 25 mg kg^{-1} and 200 μCi kg^{-1}.

separations are shown in Fig. 2. It can be seen that UPLC resolution, represented by the base peak ion (BPI) trace shows good separation of peaks for all gradient times. Increasing runtime (i.e. using shallower gradients) moderately increased the resolution of the peaks in the BPI but resulted in those detected in the radiochromatogram becoming much better resolved, with only the 60 min run providing clear separation of radiolabelled components. Indeed, the number of clearly resolved peaks in the radiochromatogram increased with run time such that the 60 min gradient shows 11 well resolved peaks (Fig. 2). This degree of separation was not seen for the 18 min run, where the degree of overlap was such that only five main peaks were observed, with the smaller peaks lost. This result is not surprising, but emphasises the fact that peak broadening caused by the radioflow cell is the limiting factor when attempting to reduce chromatographic runtime.

Despite the radioflow cell being a limiting factor in the balance of runtime optimisation and peak resolution when used with UPLC separation, there are still advantages to the use of radioflow detection in this type of study. Not the least of these is that it provides quantitative data, which mass spectrometry cannot without the use of authentic standards and specific calibration data. In the present work, even using short runtimes, the radioflow trace was also useful in guiding more focused searching of the mass spectral dataset. If only radioprofiling is required without MS detection then fraction collection probably provides the only current option for exploiting the separation efficiency of UPLC, as explored by Dear et al. [35].

3.3. Metabolic profiling by UPLC-TOFMS with radioflow detection

As indicated above, prior to investigating the metabolic fate of propranolol chromatographic method development was performed to define the gradient run time and optimum radioflow cell size. For subsequent metabolite characterisation with UPLC-ToF/MS the 18 min gradient with the 50 μL radioflow cell was employed. Under these conditions [^{14}C]-propranolol had a retention time of 5.71 min. A UPLC-TOFMS BPI trace acquired in positive electrospray ionisation mode for a 0–6 h urine sample is shown in Fig. 3, together with the radioflow trace, using a 50 μL flow cell, of the same chromatographic run.

The peaks in the radiochromatogram indicated the presence of numerous (>10) metabolites of [^{14}C]–propranolol with a small amount of unchanged parent eluting at 5.71 min (confirmed by reference to the parent compound retention time) and positive ion accurate mass data (*m/z* 260.1642; −3.5 ppm). Peak alignment between MS and radiochemical traces allowed interrogation of the acquired TOFMS data to be guided by the peaks in the radiochromatogram. The mass spectral properties – and putative identities – of the metabolites are considered below in elution order (see also Table 2). Where isobaric species were detected eluting at a number of retention times these are discussed with the first eluting metabolite.

A small proportion of the detected radioactivity was observed in the radiochromatogram as peaks with relatively short retention times (up to 2 min), including the solvent front (Fig. 3). The complexity of the mass chromatogram at these early elution times meant that peaks present in the radiochromatogram could not be assigned to peaks in the UPLC-TOFMS traces with certainty. Manual searching though the mass spectra obtained during the first 2 min of the separation was confounded by co-elution of numerous endogenous compounds and no assignments were made for these early eluting components.

The first metabolite for which a mass spectrum was obtained (Metabolite 1), eluting at a retention time of 2.98 min, had a molecular ion at *m/z* 468.19 in positive ESI, and was isobaric with two other metabolites which eluted at 3.08 and 3.42 min, respectively (Metabolites 1, 2 and 5, Fig. 3). A spectrum for Metabolite 1 is shown in Fig. 4, and essentially the same spectrum was seen for all three of these isobaric metabolites, with the neutral loss of 176 Da indicat-

Table 2
. Propranolol metabolites identified by UPLC-TOFMS with simultaneous [^{14}C]-radiometric detection using an 18 minute gradient

M	Retention Time	+ve mode m/z	Deviation (ppm)	Molecular Formula	Metabolite	Reference
P	5.71	260.1642	-3.5	$C_{16}H_{21}NO_2$	Parent	-
1	2.98	468.1895	5.3	$C_{22}H_{29}NO_{10}$	Dihydroxypropranolol glucuronide	[13]
2	3.08	468.1885	3.2	$C_{22}H_{29}NO_{10}$	Dihydroxypropranolol glucuronide	[13]
3	3.20	452.1901	-4.4	$C_{22}H_{29}NO_9$	Hydroxypropranolol glucuronide	[33]
4	3.33	421.1805	1.9	$C_{21}H_{28}N_2O_5S$	*N*-acetylcysteinyl propranolol	[11]
5	3.42	468.1910	8.5	$C_{22}H_{29}NO_{10}$	Dihydroxypropranolol glucuronide	[13]
6	3.49	644.2176	-2.3	$C_{28}H_{37}NO_{16}$	Dihydroxypropranolol diglucuronide	-
7	3.52	452.1920	-0.2	$C_{22}H_{29}NO_9$	Hydroxypropranolol glucuronide	[33]
8	3.92	421.1806	2.1	$C_{21}H_{28}N_2O_5S$	*N*-acetylcysteinyl propranolol	[11]
9	4.32	276.1606	2.2	$C_{16}H_{21}NO_3$	Hydroxypropranolol	[33]
10	4.35	292.1566	5.8	$C_{16}H_{21}NO_4$	Dihydroxypropranolol	[13]
11	4.35	452.1931	2.2	$C_{22}H_{29}NO_9$	Hydroxypropranolol glucuronide	[33]
12	4.96	356.1158	-2.8	$C_{16}H_{21}NO_6S$	Hydroxypropranolol sulfate.	[15]
13	5.23	436.1953	-4.1	$C_{22}H_{29}NO_8$	Propranolol glucuronide	[33]
14	5.45	612.2294	0.3	$C_{28}H_{37}NO_{14}$	Propranolol diglucuronide	-

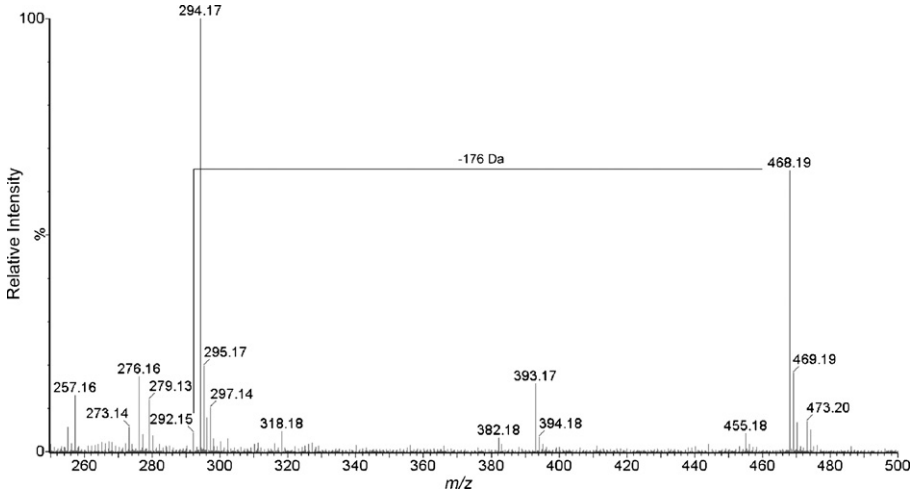

Fig. 4. Mass spectrum of Metabolite 1, eluting at 2.98 min in the 18 min gradient run. Metabolite 1 was isobaric with Metabolites 2 and 5, which exhibited similar mass spectral profiles. The neutral loss of a 176 Da glucuronide fragment is indicated.

ing the presence of a glucuronide moiety. From the accurate mass data an empirical formula of $C_{22}H_{29}NO_{10}$ was assigned (Metabolite 1: *m*/*z* 468.1895; 5.3 ppm, Metabolite 2: *m*/*z* 468.1885; 3.2 ppm, Metabolite 5: *m*/*z* 468.1910; 8.5 ppm) indicating a likely identity for these compounds as isomeric, dihydroxylated, glucuronidated metabolites of propranolol. The dosed compound was a racemate and the resulting glucuronides may be diastereoisomers allowing for a complex mixture of both positional and enantiomeric forms of these metabolites. Isolation and further investigation using NMR spectroscopy would be required for unequivocal identification of these compounds. Dihydroxylated metabolites of propranolol have previously been observed following dosing to both rat and man [13]. These were identified following glucuronidase/sulphatase incubation and then derivatisation with TMS and subsequent analysis by GC–MS for metabolite identification. A high percentage of the dihydroxylated metabolites encountered in this early study were conjugated with either glucuronide or sulphate, but the methods employed did not allow the extent of conjugation to each to be determined.

Three metabolites (peaks 3, 7 and 11), eluting at 3.20, 3.52 and 4.35 min, respectively were detected in positive ESI with accurate mass measurements (Metabolite 3: *m*/*z* 452.1901; −4.4 ppm, Metabolite 7: *m*/*z* 452.1920; −0.2 ppm, Metabolite 11: *m*/*z* 452.1931; 2.2 ppm) that suggested they shared an empirical formula of $C_{22}H_{29}NO_9$. Spectra of these metabolites clearly showed the loss of 176 Da from the molecular ion, leaving a hydroxypropranolol fragment ion. Isomeric hydroxypropranolol glucuronides have previously been reported as urinary metabolites following dosing to rat [14,33]. The very high intensity of the signal in the UPLC-TOFMS trace for the peak at 3.52 min, combined with the large peak in the radioprofile at this retention time, indicates that this is the major metabolite (Metabolite 7). As with the dihydroxyglucuronides 1, 2 and 5 described above both positional and diasteroisomers may be represented by metabolites 3, 7 and 11. The major peak seen in the present study is, based on these previous investigations, most likely the 4-hydroxy isomer.

Two isobaric metabolites (peaks 4 and 8), eluted at 3.33 and 3.92 min respectively. Accurate mass measurements (Metabolite 4: *m*/*z* 421.1805; 1.9 ppm, Metabolite 8: *m*/*z* 421.1806; 2.1 ppm) suggested a molecular formula of $C_{21}H_{28}N_2O_5S$, consistent with the presence of *N*-acetylcysteinyl conjugates of propranolol. Methylthionaphthyl metabolites of propranolol have previously been detected in the urine of orally dose rats, dogs and humans, indicating the possible involvement of glutathione conjugation as a viable

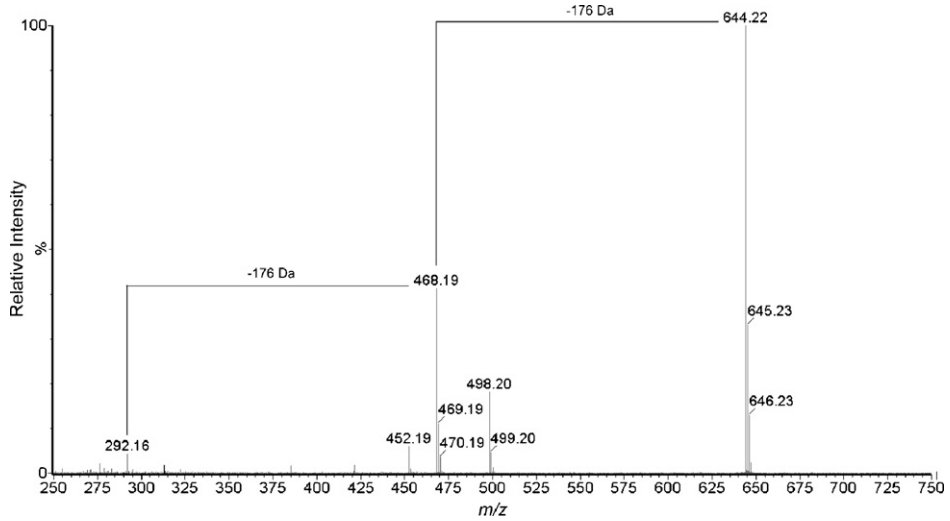

Fig. 5. Mass spectrum of Metabolite 6, eluting at 3.49 min in the 18 min gradient run. The two sequential neutral losses of 176 Da glucuronide fragments are indicated.

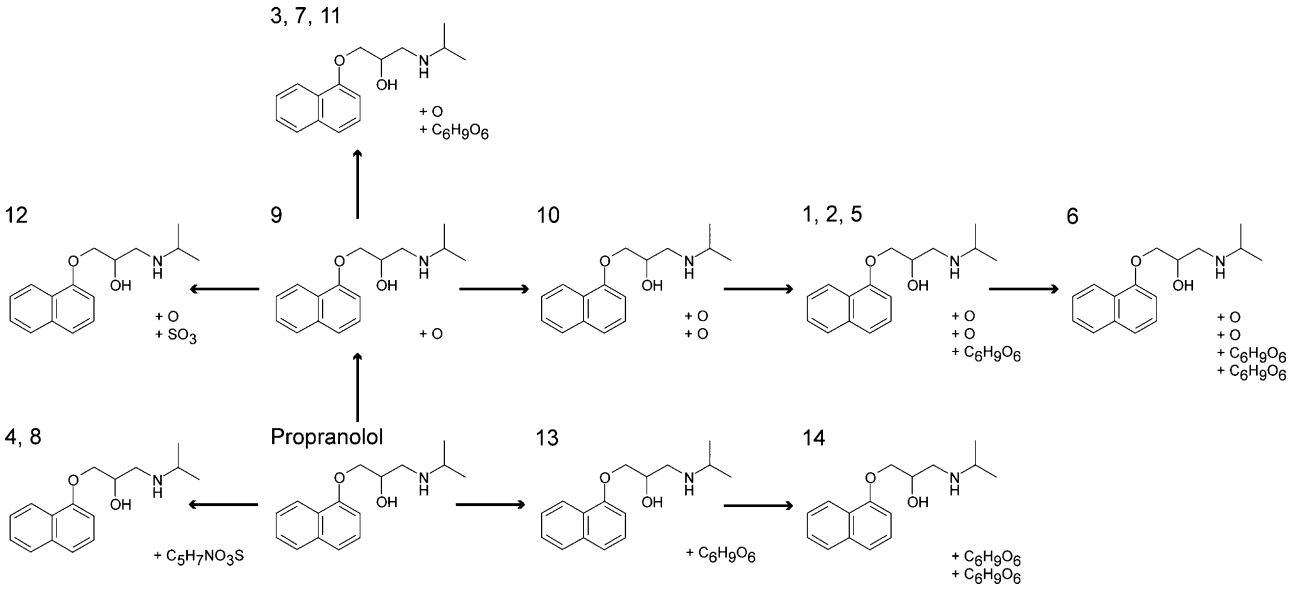

Fig. 6. Metabolism scheme showing the metabolites identified in this study. For several of the metabolites, isomeric forms were detected. Metabolite numbers correspond to ascending order of elution; further details are found in Table 2.

metabolic route. Additionally, glutathione adducts of propranolol have been shown to form *in vitro* [11].

We also detected an apparent double glucuronide, Metabolite 6, eluting at 3.49 min. Accurate mass measurements suggested a molecular formula of $C_{28}H_{37}NO_{16}$ (m/z 644.2176; −2.3 ppm). Mass spectra showed two losses of 176 Da to m/z 468.1910 and 352 Da to m/z 292.1566. Although visible in the 18 min run, the improved separation of the 60 min run (Fig. 2) gave cleaner mass spectral data (Fig. 5). These data are consistent with the metabolite being a dihydroxypropranolol diglucuronide metabolite, which has not previously been reported.

The minor Metabolite 9, which eluted at 4.32 min had an m/z value of 276.1606 in positive ion mode, suggesting an empirical formula of $C_{16}H_{21}NO_3$. This corresponds to a mono-hydroxylated metabolite of propranolol, most likely the 4-hydroxy isomer previously reported in numerous *in vivo* studies.

Similarly, the positive ion mass spectral data for peak 10, eluting at 4.38 min with a molecular ion of m/z 292.1566 (corresponding to an empirical formula of $C_{16}H_{21}NO_4$) (5.8 ppm) was probably an isomer of dihydroxypropranolol. Talaat and Nelson [13] previously reported the presence of 5 isomers of dihydroxypropranolol *in vivo* following administration of propranolol to rat.

Metabolite 12, eluted at 4.96 min with an m/z value of 356.1150 corresponding to a hydroxypropranolol sulphate with an empirical formula $C_{16}H_{21}NO_6S$ (−2.8 ppm). This type of metabolite has previously been observed in urine following *in vivo* studies in man and dog [15], but not in rat.

Metabolite 13, eluting at 5.23 min had positive ESI molecular ion at m/z 436.1953, suggesting a molecular formula of $C_{22}H_{29}NO_8$ (−4.1 ppm). In addition, the mass spectra showed a neutral loss of 176 Da, corresponding to loss of glucuronic acid, to give propranolol. A direct glucuronide of propranolol has previously been observed by Bargar et al. [33].

Metabolite 14, eluting at 5.45 min had a parent ion with an m/z of 612.2294 in positive ion mode, and showed a fragment ion at m/z 436.1949, suggesting that it may be propranolol diglucuronide ($C_{28}H_{37}NO_{14}$, 0.3 ppm). Most likely, both the hydroxyl group and secondary amine group have become glucuronidated to give this previously unreported metabolite of propranolol.

On the basis of the mass spectral data obtained here the metabolic fate of propranolol in rat is summarised in Fig. 6.

4. Conclusions

In agreement with previous studies propranolol was shown to undergo extensive metabolism to a large number of metabolites. The use of UPLC in combination with both MS and radioprofiling has been successfully demonstrated for the detection and characterisation of a wide range of metabolites in rat urine. Indeed, 14 metabolites of propranolol were detected and characterised in this study including possible novel diglucuronidated and dihydroxydiglucuronide metabolites. However, the need for a sufficiently large radioflow cell to enable sensitive radioactivity detection remains a source of considerable peak broadening. Clearly, to fully exploit the advantages provided by UPLC with this type of detection will require further developments in radioflow cell design if the benefits to metabolite quantification provided by on-line analysis are to be obtained. In the absence of improvements in on-line radioflow cells, either longer chromatographic run times will be required, negating the high resolution of UPLC, or fraction collection and off-line radioactivity determination will have to be used, eliminating the advantages of speed provided by the technique.

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