Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 1065–1077
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

HPLC–NMR with severe column overloading: Fast-track metabolite identification in urine and bile samples from rat and dog treated with [^{14}C]-ZD6126

E.M. Lenz*, R.A. D'Souza, A.C. Jordan, C.D. King, S.M. Smith, P.J. Phillips, A.D. McCormick, D.W. Roberts

Department of Drug Metabolism and Pharmacokinetics, AstraZeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK

Received 7 July 2006; received in revised form 4 September 2006; accepted 4 September 2006
Available online 9 October 2006

Abstract

The subject of this study was the determination of the major urinary and biliary metabolites of [^{14}C]-ZD6126 following *i.v.* administration to female and male bile duct cannulated rats at 10 mg/kg and 20 mg/kg, respectively, and male bile duct cannulated dogs at 6 mg/kg by HPLC–NMR spectroscopy.

ZD6126 is a phosphorylated pro-drug, which is rapidly hydrolysed to the active metabolite, ZD6126 phenol. The results presented here demonstrate that [^{14}C]-ZD6126 phenol is subsequently metabolised extensively by male dogs and both, male and female rats.

Recovery of the dose in bile and urine was determined utilising the radiolabel, revealing biliary excretion as the major route of excretion (93%) in dog, with the majority of the radioactivity recovered in both biofluids in the first 6 h.

In the rat, greater than 92% recovery was obtained within the first 24 h. The major route of excretion was *via* the bile 51–93% within the first 12 h. The administered phosphorylated pro-drug was not observed in any of the excreta samples.

Metabolite profiles of bile and urine samples were determined by high performance liquid chromatography with radiochemical detection (HPLC–RAD), which revealed a number of radiolabelled components in each of the biofluids. The individual metabolites were subsequently identified by HPLC–NMR spectroscopy and HPLC–MS.

In the male dog, the major component in urine and bile was the [^{14}C]-ZD6126 phenol glucuronide, which accounted for 3% and 77% of the dose, respectively. [^{14}C]-ZD6126 phenol was observed in urine at 1% of dose, but was not observed in bile. A sulphate conjugate of demethylated [^{14}C]-ZD6126 phenol was identified in bile by HPLC–NMR and confirmed by HPLC–MS.

In the rat, the bile contained two major radiolabelled components. One was identified as the [^{14}C]-ZD6126 phenol glucuronide, the other as a glucuronide conjugate of demethylated [^{14}C]-ZD6126 phenol.

However, a marked difference in the proportions of these two components was observed between male and female rats, either due to a sex difference in metabolism or a difference in dose level. The glucuronide conjugate of the demethylated [^{14}C]-ZD6126 phenol was present at higher concentration in the bile of male rats (4–34%), while the phenol glucuronide was present at higher concentration in the bile of female rats (8–70%) over a 0–6 h collection period.

A third component was only observed in the bile samples (0–6 h and 6–12 h) of male rats. This was identified as being the same sulphate conjugate of demethylated [^{14}C]-ZD6126 phenol as the one observed in dog bile.

The rat urines contained two main metabolites in greatly varying concentrations, namely the demethylated [^{14}C]-ZD6126 phenol glucuronide and the glucuronide of [^{14}C]-ZD6126 phenol. Again, the differences in relative amounts between male and female rats were observed, the major metabolite in the urines from male rats being the demethylated [^{14}C]-ZD6126 phenol (0–17% in 0–24 h), whilst the phenol glucuronide, accounting for 0.5–50% of the dose over 0–24 h, was the major metabolite in females.

* Corresponding author. Tel.: +44 1625 514653; fax: +44 1625 230614.
E-mail address: Eva.Lenz@astrazeneca.com (E.M. Lenz).

0731-7085/$ – see front matter
doi:10.1016/j.jpba.2006.09.010

Methanolic extracts of the pooled biofluid samples were submitted for HPLC–NMR for the quick identification of the major metabolites. Following a single injection of the equivalent of 6–28 ml of the biofluids directly onto the HPLC-column with minimal sample preparation, the metabolites could be largely successfully isolated. Despite severe column overloading, the major metabolites of [^{14}C]-ZD6126 could be positively identified, and the results are presented in this paper.

Keywords: Vascular targeting agent; ZD6126; Metabolism identification; Fast-track HPLC–NMR

1. Introduction

ZD6126 has been developed for the treatment of human solid tumours at AstraZeneca, UK. It is a tumour-selective vascular targeting agent (VTA) which has shown activity in a wide range of preclinical tumour models, such as colorectal, lung, breast and prostate tumours grown as xenografts in nude mice [1,2,13]. Vascular targeting agents, such as ZD6126, disrupt the vascular network, crucial to the survival of and growth of solid tumours, by impairing blood vessel integrity, leading to vessel shut-down and consequently tumour necrosis [1–14]. VTAs for the treatment of cancer are expected to show the greatest therapeutic benefit as part of combined modality regimens. Preclinical studies have shown the enhancement of the effects of conventional chemotherapy agents, radiation, hyperthermia, radioimmunotherapy and antiangiogenic agents [7–10].

Structurally, it is a derivative of the tubulin-binding agent, colchicine. The water-soluble pro-drug, ZD6126, a phosphate ester, is rapidly hydrolysed *in vivo* to yield the active component, ZD6126 phenol, as shown in Fig. 1a.

The present study was designed to provide information on the recovery and metabolism of radiolabelled [^{14}C]-ZD6126 following a single *i.v.* administration at 10 mg/kg and 20 mg/kg to female and male bile cannulated rats, respectively, and to male bile cannulated dogs at 6 mg/kg. Recovery and metabolite profiles were established utilising the ^{14}C-label, and identification of some of the major metabolites was achieved by HPLC–MS. The samples were then submitted for fast-track metabolite identification by HPLC–NMR.

The hyphenation of HPLC with NMR (and other spectroscopic techniques) has enabled the rapid and efficient structural identification of metabolites (e.g. [16–18]), by eliminating the need for prior isolation and sample clean up, as also shown in this study. However, in this study, in order to satisfy detection limit requirements and to reduce analysis time (in view of meeting tight deadlines), the bulk of the pooled samples were injected onto the HPLC-column for the analysis in a single run. Sample preparation simply comprised freeze-drying and MeOH-extraction of the samples, in order to void them off the high salt content. Above all, with the instrumentation available at the time of analysis, this method provided a distinct analytical challenge as sample volumes as large as 6 ml and 28 ml (for MeOH-extracted bile and urine, respectively) were analysed directly by HPLC–NMR. The HPLC-method was adjusted to ensure maximum retention of the bulk freeze-dried samples. Chromatographic retention did not appear to deteriorate following seven injections of bile and urine onto the same column. The results from the metabolite identification are discussed.

2. Methods and materials

2.1. Compounds

ZD6126, (5*S*)-5-(acetylamino)-9,10-[^{14}C],11-trimethoxy-6,7-dihydro-5*H*-dibenzo[*a*,*c*]-cycloheptoten-3-yl-dihydrogen-phosphate, was developed at AstraZeneca Pharmaceuticals (Macclesfield, UK). For the metabolite identification radiolabelled [^{14}C]-ZD6126 was used, with a specific activity of 69.2 μCi/mg and 3.65 μCi/mg for rats and dog, respectively, and a radiochemical purity of >97%. The compound was stored at −80 °C in the dark.

The reference compounds, the glucuronide conjugate of ZD6126 phenol and the 10-desmethyl ZD6126 phenol, were supplied and characterised by AstraZeneca, France, and Isotope Chemistry, Alderley Park, respectively.

The mobile phase constituents were all obtained from Fluorochem (Derby, UK), consisting of deuterium oxide (D_2O, 99.9% purity), formic acid-d_2 (98% purity) and acetonitrile (ACN, pestanal grade, supplied *via* Riedl-de-Haën).

2.2. Animal dosing

Dog. Three male Beagle dogs, *ca*. 9–18 months old at dosing, with body weights 13–16.2 kg at time of dosing were surgically prepared with indwelling bile duct and duodenal cannulae.

Following a suitable recovery period, each animal received a single *i.v.* slow bolus dose of [^{14}C]-ZD6126 *via* the cephalic vein of 6 mg/kg at a dose volume of 5 ml/kg in 0.9% phosphate buffered saline (pH adjusted with 0.1 M sodium carbonate). It appears that polyethylene glycol (PEG) was accidentally introduced into the dosing solution. The animals were allowed to acclimatise for 24 h before dosing. Water was available *ad libitum*, and approximately 400 g of food was offered daily.

For the duration of the study period, the animals were housed in single metabolism cages designed for the separate collection of faeces, urine and bile.

Faeces and urine were collected pre-dose, at 0–24 h and at 24–48 h. Bile was collected pre-dose, then 6 h, 12 h, 24 h and 48 h after dosing. Both, urine and bile were collected over solid carbon dioxide. Aqueous cagewash was collected daily and pooled over the 0–48 h collection period.

Rat. Four male and six female Alpk:APSD rats, aged between 7 and 12 weeks and weighing between 187 g and 247 g at the time of dosing, were supplied by AstraZeneca's rodent breeding unit. Animals were surgically fitted with bile duct cannuli. Each animal received a single *i.v.* slow bolus dose of [^{14}C]-ZD6126 at either 20 mg (males) or 10 mg (females)/kg at a dose volume of 5 ml/kg in 0.9% phosphate buffered saline (pH

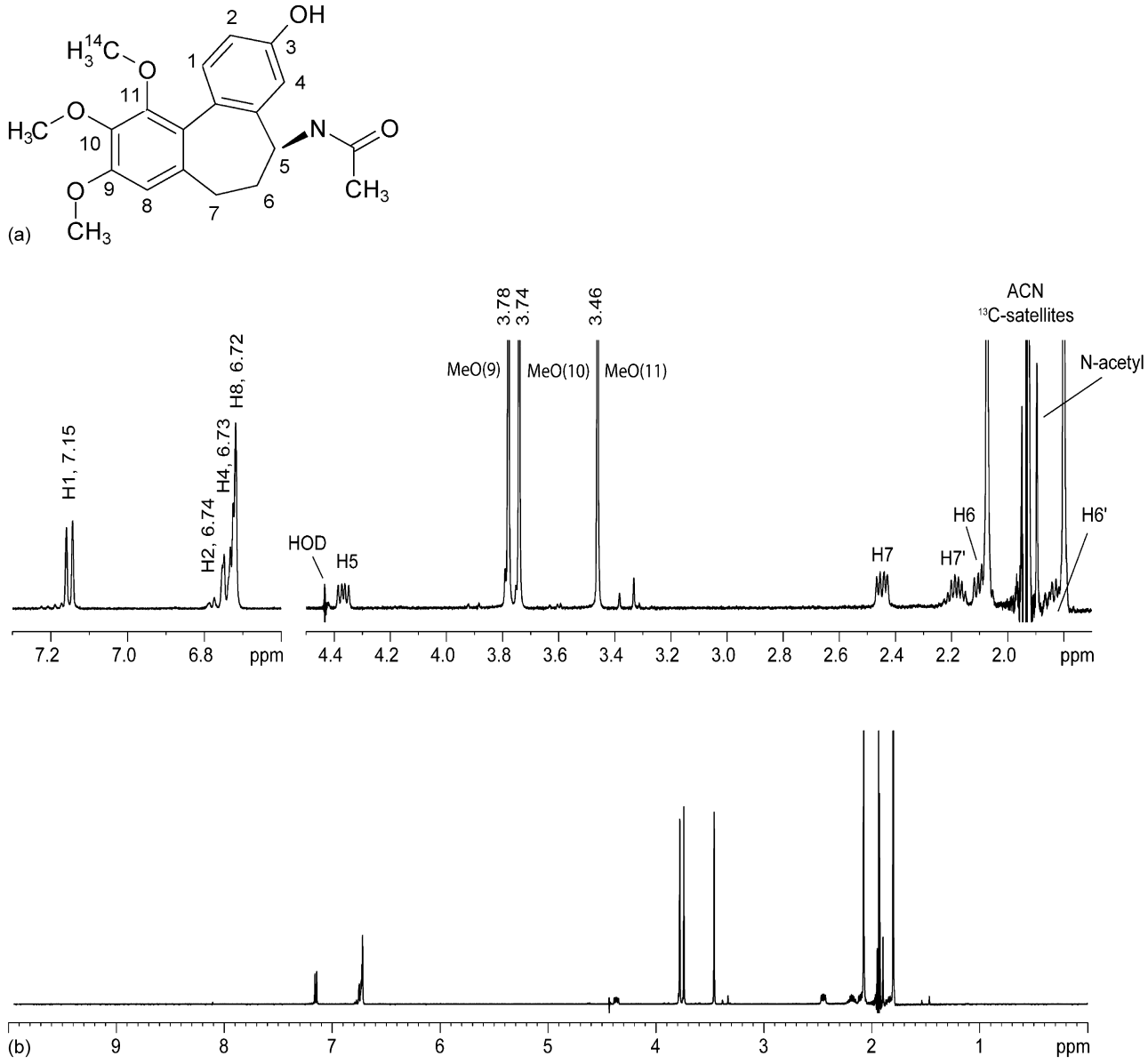

Fig. 1. (a) The structure of ZD6126 phenol. The numerical labels correspond to the assignments as listed in Table 6. (b) The stop-flow ^{1}H NMR spectrum of [^{14}C]-ZD6126 phenol. Shown are the whole spectrum (bottom) with expansions of the aromatic region and the aliphatic region. 1 mg/ml injected, t_R = ca. 41 min. *Key*: HOD, residual water.

adjusted with 0.1 M sodium carbonate). It appears that PEG was accidentally introduced into the dosing solution. The measured specific activity of the *i.v.* formulated material was 32.27 μCi/mg and 17.5 μCi/mg for the 10 mg/kg and 20 mg/kg dose groups, respectively. Prior to dosing, the animals were housed individually in metabolism cages designed for the separate collection of urine, faeces and bile. The animals were allowed to acclimatise for 24 h before dosing. Food and water were available *ad libitum* throughout the experiment. Faeces and urine were collected pre-dose, at 0–24 h and at 24–48 h. Bile was collected at 6 h, 12 h, 24 h and 48 h after dosing. Both urine and bile were collected over solid carbon dioxide. Aqueous cagewash was collected daily and pooled over 0–48 h collection period.

The samples were then stored at −20 °C until analysed. Before analysis the urine samples were centrifuged at 3000 rpm at 4 °C to remove solid debris (particles of food, etc.) and the urinary volume and pH were determined.

2.3. Radioactivity measurements

All urine, bile and cagewash samples were collected and analysed for total radioactivity by liquid scintillation counting (LSC). Faeces, homogenised in water, were oxidised prior to LSC. The details of sample preparation and analysis are not discussed in this paper.

2.4. Determination of [^{14}C]-metabolite patterns in urine, bile and faeces by HPLC–RAD

Weighed aliquots of urines were freeze-dried, reconstituted in MeOH, centrifuged and freeze-dried, before

reconstitution in ACN:water (20:80, v/v) prior to HPLC-analysis.

Aliquots of bile samples were analysed by direct injection onto the HPLC-column, following centrifugation.

Selected faecal samples were extracted twice with ACN. Any lipid material was removed by initial extraction with hexane. The ACN fractions were combined and evaporated to dryness. The samples were then reconstituted in ACN:water (20:80, v/v) prior to HPLC-analysis.

Metabolite patterns were established by HPLC on a Phenomenex Prodigy ODS3 (250 mm × 4.6 mm, with a guard column, Optimize Technologies, OPTI-GUARD, 1 mm, C_{18}). The UV-detector was a Shimadzu UV-VIS detector SPD-10A and the radiodetector a Packard Radio-matic Flo-one Beta-500TR series, with a flow-cell of 500 μl.

The mobile phase conditions were as follows. An isocratic flow was employed for 25 min with ACN:water (22:78, v/v), both containing formic acid (0.1%, v/v). The ACN concentration was then raised in two linear gradients, first from 22% to 33% over 15 min, then from 33% to 80% over the next 10 min. The column temperature was held at 45 °C and the flow-rate was 0.75 ml/min.

2.5. HPLC–MS analysis of urine and bile

Selected urine and bile samples were analysed by HPLC–MS using the same HPLC conditions as stated above. Positive electron spray mass spectra were recorded using a Micromass ZQ single quadrupole mass spectrometer.

2.6. Sample preparation for HPLC–NMR analysis

Sample volumes comprised, typically 3–6 ml for bile and 6–28 ml for urines (see Table 6). In order to comply with HPLC–NMR detection limits and to ensure positive identification of the metabolites by stop-flow ^{1}H NMR spectroscopy with minimum sample preparation requirements, the equivalents of the total sample volume per collection period were injected onto the HPLC-column.

The urine and bile samples were simply freeze-dried, reconstituted in MeOH (to a total volume of 2–10 ml, depending on biofluid volume) to void them off excessive salt content, centrifuged to remove particulate matter (3000 rpm/5 min) and the resultant supernatant evaporated to dryness under nitrogen, before re-freeze-drying. The samples were then reconstituted in approximately 400–800 μl D_2O:ACN (50:50, v/v), containing 1% formic acid-d_2, and centrifuged, prior to injection onto the HPLC-column for direct ^{1}H HPLC–NMR analysis.

2.7. ^{1}H HPLC–NMR spectroscopy

The mobile phase consisted of D_2O and ACN, each solvent buffered with 0.1% formic acid-d_2. The HPLC-method and column were identical to the HPLC-details stated above/earlier, using a flow-rate of 1 ml/min. In order ensure retention of the large sample volumes of the urine and bile extracts loaded onto the column, an introductory isocratic phase with 0% ACN for 5 min or 10 min was employed, depending on biofluid volume injected.

HPLC–NMR analysis was performed on an integrated Bruker HPLC–NMR system consisting of a Bruker LC22 pump and Diode Array Detector, and a Bruker DRX500 spectrometer, operating at 500.13 MHz ^{1}H resonance frequency. The NMR spectrometer was equipped with a dual tunable ^{1}H/^{19}F LC flow-probe (3 mm i.d., 60 μl active volume). A Berthold LB507A HPLC radioactivity monitor was additionally interfaced (prior to the DAD, λ = 258 nm) for ^{14}C detection, equipped with solid flow-cell (volume of 150 μl). The NMR analysis was driven by the XWIN-NMR software (Version 2.6) and the HPLC-analysis by HYSTAR 2.1.

HPLC–NMR analyses were carried out in the stop-flow mode, halting the chromatographic separation on a UV- or ^{14}C-peak of interest. Manual time-slicing was employed, where deemed appropriate.

Typically, spectra were recorded at 30 °C in order to avoid fluctuations in (ambient) temperature. Usually, ^{1}H NMR spectra were measured with approximately 124–1024 scans (depending on concentration of the analyte) into 32*k* data points over a spectral width of 8012.82 Hz, which resulted in an acquisition time of 2 s. Double solvent suppression was achieved *via* the NOESYPRESAT pulse sequence (Bruker Biospin Ltd.) in which the residual water in the D_2O and the organic solvent peak were irradiated during the relaxation delay of 1.7 s and during the mixing time of 100 ms. Spectra were referenced to internal ACN at δ^1H 1.92.

Table 1
Recoveries of radioactivity following a single intravenous dose of [^{14}C]-ZD6126 to male bile duct cannulated dogs

Sample	Collection period (h)	Recovery (% administrated dose)		
		Dog 1	Dog 2	Dog 3
Urine	0–48	56.18	6.17	12.41
Bile	0–48	32.84	93.32	54.46
Faeces	0–48	1.23	0.93	29.70
Cagewash	0–48	0.83	0.16	1.24
Total	0–48	91.08	100.58	97.81

3. Results and discussion

3.1. Individual recovery data for the dog

A quantitative recovery of the dose was achieved (>99%), with the majority recovered in the first 6 h post dose. The major route of excretion was *via* bile (93%) [15], with the remaining dose (6%) recovered in the urine, and to a lesser extent in the faeces (<1%) (Table 1). Although samples from three dogs were collected and analysed for total radioactivity (Table 1), the samples from dogs 1 and 3 were omitted from metabolite identification by HPLC–NMR as both animals had extreme liver enzyme readings (clinical chemistry and haematology).

Table 2
HPLC profiles of metabolites in excreta following a single intravenous dose of [^{14}C]-ZD6126 to male bile duct cannulated dogs

Sample	t_R (min)	M1	M2	M3	Proposed identification	Protonated molecular ion
Urine (0–24 h)	∼26	47	3	7	Phenol glucuronide	*m/z* 534
	∼45	2	1	3	Phenol	*m/z* 358
Bile (0–6 h)	∼24	26	77	47	Phenol glucuronide	*m/z* 534
Faeces (0–24 h)	∼21	NS	NS	2	ND	NA
	∼45	NS	NS	24	Phenol	NA

Results expressed as % dose. The proposed identification is based on retention time (t_R) comparison of authentic standards and HPLC–MS. *Key*: M, male dog; ND, not determined; NS, insufficient radioactivity for analysis; NA, peak not identified by HPLC–MS.

3.2. Metabolite profiling by HPLC–RAD and HPLC–MS for dog excreta

Urine and bile samples from dog were analysed by HPLC–RAD and HPLC–MS (data not shown). The results of which are summarised in Table 2. The main urinary metabolites were tentatively identified as [^{14}C]-ZD6126 phenol glucuronide and [^{14}C]-ZD6126 phenol by HPLC–MS (protonated molecular ions at *m/z* 534 and 358, respectively) and co-chromatography with authentic standards. The metabolites accounted for 3% and 1% of the dose over a 0–24 h collection period, respectively.

The major component in the bile was tentatively identified as the [^{14}C]-ZD6126 phenol glucuronide, which accounted for 77% of the dose. This was characterised by HPLC–MS (protonated molecular ion at *m/z* 534) and co-chromatography with the reference standard. The bile additionally contained four minor metabolites, which were not identified at this point.

3.3. Individual recovery data for the rat

The individual and mean recovery data following intravenous administration of [^{14}C]-ZD6126 to male and female bile duct cannulated rats are presented in Table 3. There was no marked difference in the excretion patterns of total radioactivity between male and female animals at different dose levels, although a high degree of inter-animal variability was observed. Furthermore, the data for animals M3 and F11, although included in this table for completeness, are considered unreliable as problems were encountered during dosing.

A quantitative recovery of the dose was achieved (>92%), with the majority recovered in the first 24 h post dose. The major route of excretion was *via* bile within the first 12 h (50–90% in male rats and 15–88% in female rats). The remaining dose was mainly recovered in the first 0–24 h urine samples (2–37% in male rats and 5–64% in female rats). Faecal excretion accounted for approximately 1–4% over 48 h in both sexes.

3.4. Metabolite profiling by HPLC–RAD and HPLC–MS of rat excreta

Urine samples (0–24 h) and bile samples (0–6 h for both male and female and 6–12 h for male rats) were analysed by HPLC–RAD and HPLC–MS. The proportions of radioactivity associated with each of the major radiolabelled components are summarised in Tables 4 and 5.

Table 3
Recoveries of radioactivity following a single intravenous dose of [^{14}C]-ZD6126 to male rats at 20 mg/kg and female rats at 10 mg/kg

Sample	Time (h)	M1	M2	M3[a]	M4	Mean	S.E.	F7	F8	F9	F10	F11[a]	F12	Mean	S.E.
Urine	0–24	2.24	35.0	36.8	3.94	13.7	10.6	19.4	4.53	22.6	7.19	64.3	6.33	10.8	4.27
	24–48	0.12	0.25	0.67	0.07	0.15	0.05	1.01	0.12	0.52	0.39	4.46	0.07	0.07	0.17
Total		2.36	35.2	37.5	4.01	13.9	10.7	20.4	4.65	23.1	7.58	65.8	6.40	12.4	3.86
Bile	0–6	86.1	34.0	24.3	82.5	67.5	16.8	65.8	83.8	46.0	80.9	9.73	84.0	72.1	7.33
	6–12	3.61	22.1	26.1	8.09	11.3	5.57	5.73	3.97	6.31	3.81	5.58	4.00	4.76	0.52
	12–24	0.81	1.45	2.01	2.09	1.45	0.37	1.27	1.23	2.27	1.08	2.02	1.55	1.48	0.21
	24–48	0.33	0.35	0.56	0.47	0.38	0.04	0.51	0.18	2.05	0.37	0.78	0.28	0.68	0.35
Total		90.8	57.9	53.0	93.2	80.6	11.4	73.3	89.2	56.7	86.2	18.1	89.9	79.0	6.33
Faeces	0–24	2.37	1.47	3.70	1.15	1.66	0.37	0.79	2.72	2.71	2.80	1.85	1.86	2.18	0.39
	24–48	0.41	0.85	0.72	0.30	0.52	0.17	0.59	0.15	1.09	0.12	0.90	0.26	0.44	0.18
Total		2.78	2.32	4.42	1.45	2.18	0.39	1.38	2.87	3.80	2.92	2.75	2.11	2.62	0.41
Cagewash	0–48	1.50	1.95	3.01	0.57	1.34	0.41	1.42	0.74	5.45	1.02	5.32	0.38	1.80	0.93
Total recovery		97.4	97.4	97.9	99.2	98.0	0.61	96.6	97.4	89.0	97.7	91.9	98.7	95.9	1.75

The results are expressed as % administered dose.
[a] M3 and F11 were excluded from the mean calculation.

Table 4
HPLC profiles of metabolites in rat bile (0–6 h, and selected 6–12 h[a] samples) obtained following a single intravenous dose of [^{14}C]-ZD6126 to male (M) and female (F) bile duct cannulated rats

Peak	t_R (min)	M1	M2	M3	M4	M2[a]	M3[a]	F7	F8	F9	F10	F11	F12	Proposed identification	Protonated molecular ion
1	~3.1	ND	ND	ND	ND	0.6	ND	ND	ND	ND	ND	ND	ND	NA	NA
2	~6.3	6	0.3	0.4	9	0.2	0.3	1	2	0.5	1	0.1	1	NA	NA
3	~8.6	29	4	7	34	2	6	4	7	3	6	ND	6	Demethylated phenol glucuronide	*m/z* 520
6	~27.4	33	19	13	25	4	4	57	68	39	68	8	70	Phenol glucuronide	*m/z* 534
7	~31.5	7	8	1	6	13	12	ND	ND	ND	ND	ND	ND	Demethylated phenol sulphate	*m/z* 424
8	~45.0	ND	ND	ND	ND	ND	ND	0.5	2	0.5	1	0.2	2	Phenol	*m/z* 358

Results expressed as % dose. The proposed identification is based on retention time (t_R) comparison of authentic standards and HPLC–MS. ND: not detected; NA: peak not identified by HPLC–MS.
[a] Selected 6–12 h bile samples from male rats.

Inter-animal variability and a marked difference in the relative proportions between the male and female rats were observed in the metabolite excretion pattern, either due to the differences in dose levels or a sex difference in metabolism. The major biliary metabolites, such as the glucuronide conjugate of demethylated [^{14}C]-ZD6126 phenol accounted for 4–34% of the total dose in males over 0–6 h (c.f. 0.3–7% in females), while the [^{14}C]-ZD6126 phenol glucuronide was present in higher concentration in the bile of female rats (8–70%, c.f. 13–33% in males). A third metabolite was observed in the bile and urine of male rats, accounting for 1–8% in the 0–6 h bile, 12–13% in the 6–12 h bile and 0–7% in the 0–24 h urine samples, but was not detected in the bile or urine samples of female rats. This metabolite was tentatively identified by HPLC–MS as a sulphate conjugate of demethylated [^{14}C]-ZD6126 phenol.

The urine (0–24 h, 27 ml) and bile (0–6 h, 3 ml) samples from dog 2 were prepared for HPLC–NMR as stated earlier.

The rat urine and bile samples from male animals (M1–M4) and female animals (F7–F12) were combined prior to HPLC–NMR analyses. The bile pools consisted of 3 ml and 6 ml for 0–6 h male and female rats, respectively, and 6 ml for 6–12 h male rats. The 0–24 h urine pools comprised 24 ml and 28 ml for male and female rats, respectively. The samples were then prepared for HPLC–NMR as stated earlier.

The pooling of the samples was carried out for numerous reasons, such as to average the observed inter-individual variability in metabolite excretion, to satisfy the detection limit requirements of HPLC–NMR, especially since the extraction efficiencies were not assessed prior to injection, and most of all, owing to a lack of resource and the tight deadlines encountered.

3.5. Metabolite profiling by ^{1}H HPLC–NMR spectroscopy

Metabolite identification by direct stop-flow HPLC–NMR was requested to aid identification of the major [^{14}C]-ZD6126 metabolites. It allowed positive characterisation of a series of metabolites in the urine and bile samples of dog and rat in a single analytical run.

The chemical shifts of the metabolites contained in the pooled urine and bile samples of rat and dog are listed in Table 6, and some representative examples are discussed in more detail. Due to severe column overloading and slight adjustments to the HPLC conditions to ensure retention of the large volumes of biofluids injected, the chromatographic retention times are not compatible to the ones observed during the HPLC–RAD metabolite profiling experiments. In the bile samples, a consistent change in the elution order of the metabolites has been observed, presumably as a consequence of column overloading.

Table 5
HPLC profiles of metabolites in rat urine (0–24 h) obtained following a single intravenous dose of [^{14}C]-ZD6126 to male (M) and female (F) bile duct cannulated rats

Peak	t_R (min)	M1	M2	M3	M4	F7	F8	F9	F10	F11	F12	Proposed identification	Protonated molecular ion
1	~3.1	ND	ND	0.9	0.6	0.4	ND	ND	ND	ND	ND	ND	NA
2	~6.3	0.2	3	6	0.5	0.6	ND	0.8	ND	1	ND	ND	NA
3	~8.6	ND	11	17	0.3	1	ND	2	ND	5	ND	Demethylated phenol glucuronide	*m/z* 520
4	~20.2	0.2	0.6	1	0.5	ND	ND	ND	ND	ND	ND	Demethylated phenol	*m/z* 344
5	~22.4	ND	ND	ND	ND	0.7	0.3	0.6	0.6	0.8	0.6	Demethylated phenol	*m/z* 344
6	~27.4	ND	3	2	ND	10	0.5	14	0.6	50	0.5	Phenol glucuronide	*m/z* 534
7	~31.5	ND	7	3	ND	ND	ND	ND	ND	ND	ND	Demethylated phenol sulphate	*m/z* 424
8	~45.0	1	2	2	1	5	3	3	4	3	4	Phenol	*m/z* 358

Results expressed as % dose. The proposed identification is based on retention time (t_R) comparison of authentic standards and LC–MS. *Key*: ND, not detected; NA, peak not identified by HPLC–MS. *Note*. The demethylated metabolites with t_R = *ca.* 20.2 min (in males) and 22.4 min (in females) are positional isomers, as previously observed in human liver microsomes [14]. The earlier eluting metabolite is suggested to be the 10-desmethyl phenol.

Table 6
List of chemical shifts (δ^1H) of synthetic standards and metabolites of [^{14}C]-ZD6126

Listing of resonances	H1	H2	H4	H8	gluc^{H1}	MeO^{9}	MeO^{10}	MeO^{11}	Proposed metabolite ID
Standards									
[^{14}C]-ZD6126 phenol	7.15	6.74	6.73	6.72	N/A	3.78	3.74	3.46	
ZD6126 phenol glucuronide	7.27	7.00	6.96	6.76	5.02	3.79	3.75	3.47	
10-Desmethyl-ZD6126 phenol	7.19	6.75	6.73	6.69	N/A	3.78	–	3.33	
Samples (volume equivalents injected/ml)									
D.m.u.0–24 h (27 ml)	7.27	7.00	6.96	6.76	5.02	3.79	3.75	3.47	Phenol glucuronide
D.m.b.0–6 h (3 ml)	7.21	6.78	6.74	7.08	N/A	3.83	–	3.47	Desmethyl sulphate
	7.26	7.00	6.96	6.75	5.02	3.79	3.75	3.47	Phenol glucuronide
R.m.b.0–6 h (3 ml)	7.21	6.78	6.73	7.08	N/A	3.83	–	3.48	Desmethyl sulphate
	7.25	6.99	6.95	6.59	5.02	–	3.75	3.48	Desmethyl glucuronide
	7.25	6.98	6.95	6.74	5.02	3.79	3.75	3.47	Phenol glucuronide
R.m.b.6–12 h (6 ml)	7.20	6.77	6.74	7.08	N/A	3.83	–	3.47	Desmethyl sulphate
	7.26	6.99	6.95	6.59	5.02	–	3.75	3.48	Desmethyl glucuronide
	7.26	6.99	6.95	6.74	5.02	3.79	3.75	3.48	Phenol glucuronide
R.f.b.0–6 h (6 ml)	7.26	7.00	7.26	6.59	5.02	–	3.75	3.48	Desmethyl glucuronide
	7.26	6.99	6.95	6.74	5.02	3.78	3.74	3.48	Phenol glucuronide
R.m.u.0–24 h (24 ml)	Overlapped				5.02	3.79	3.75	3.48	Phenol glucuronide
R.f.u.0–24 h (28 ml)	7.26	6.99	6.96	6.74	5.02	3.78	3.75	3.47	Phenol glucuronide

Key: D, dog; R, rat; m, male; f, female; b, bile; u, urine; gluc, glucuronic acid. Superscript H1: glucuronide anomeric proton; 9, 10, 11: denote positions of the MeO– groups.

This change in elution order has, however, since been observed in subsequent analyses utilising the same chromatographic methods [12].

3.6. [^{14}C]-ZD6126 phenol

The stop-flow ^{1}H NMR spectrum of the [^{14}C]-ZD6126 phenol is shown in Fig. 1, with the diagnostic signals listed in Table 6. The compound contains three diagnostic methoxy-groups which resonated as singlets (s) at $\delta 1_H$ 3.78 (MeO– at C9), $\delta 1_H$ 3.74 (MeO– at C10) and $\delta 1_H$ 3.46 ppm (MeO– at C11). The methoxy-group at position 11 is considered to be metabolically stable. The aromatic protons gave rise to a doublet (d, $^3J = 8.5$ Hz) at $\delta 1_H$ 7.15 for H1, a doublet-of-doublets (dd, $^3J = 8.5$ Hz and $^4J = 2.4$ Hz) at $\delta 1_H$ 6.74 for H2 and the associated doublet (d, $^4J = 2.4$ Hz) at $\delta 1_H$ 6.73 for H4. The proton, labelled H8, of the tri-methoxylated phenyl ring resonated as a singlet at $\delta 1_H$ 6.72.

The *N*-acetyl methyl, represented by a singlet at $\delta 1_H$ 1.91, was partially overlapped with the acetonitrile peak (at $\delta 1_H$ 1.92, with its satellites at $\delta 1_H$ 1.8 and $\delta 1_H$ 2.08) of the mobile phase, and hence, was also severely attenuated. The residual cycloheptane protons resonated at $\delta 1_H$ 4.37 (a doublet-of-doublets) for H5, and at $\delta 1_H$ 2.43 (dd) and $\delta 1_H$ 2.19 ppm (a multiplet) for H7 and H7′, respectively. The multiplets for H6 and H6′ at $\delta 1_H$ 2.09 and $\delta 1_H$ 1.8 ppm, however, were severely overlapped with the ACN satellite peaks. Although the *N*-acetyl and cycloheptane signals were largely obscured by the presence of the ACN peak throughout the analyses, this did not prevent positive identification of the [^{14}C]-ZD6126 metabolites in the pooled urine and bile samples.

Similarly, the synthetic standards, the ZD6126 phenol glucuronide and the 10-desmethyl-ZD6126 phenol, were analysed by stop-flow ^{1}H NMR spectroscopy with the chemical shifts of the diagnostic signals listed in Table 6.

3.7. [^{14}C]-ZD6126 metabolites

3.7.1. Dog urine

The UV-chromatogram and ^{14}C-radiotrace following the injection of the methanolic extract of 27 ml of urine from dog 2 (0–24 h collection) are shown in Fig. 2a.

The bulk of the endogenous metabolites eluted within the first 20 min of the separation. However, the strong UV-peak at $t_R = 29.19$ min could be identified as the glucuronide conjugate of [^{14}C]-ZD6126 phenol. The corresponding stop-flow ^{1}H NMR spectrum of this peak shows the characteristic anomeric proton H1$_{gluc}$ at $\delta 1_H$ 5.02 ($^3J = 8$ Hz) indicative of an ether-glucuronide (Fig. 2b). The glucuronic acid proton H4$_{gluc}$ gave a doublet at $\delta 1_H$ 3.98 whilst the protons for H2-4$_{gluc}$ gave rise to overlapped multiplets at $\delta 1_H$ 3.45–3.6. The residual aromatic protons ($\delta 1_H$ 7.27 and $\delta 1_H$ 7.0, for H1 and H2, respectively, $\delta 1_H$ 6.96 for H4 and $\delta 1_H$ 6.76 for H8), and the methoxy-groups at $\delta 1_H$ 3.79, $\delta 1_H$ 3.75 and $\delta 1_H$ 3.47 (for MeO– at C9, C10 and C11, respectively) were preserved. Even the signals from the cyclopentane ring could be identified at $\delta 1_H$ 4.38, $\delta 1_H$ 2.48 and $\delta 1_H$ 2.2 for H5, H7 and H7′, respectively.

The corresponding HPLC–RAD metabolite profiling experiment of the 0–24 h urine from dog 2 showed two major metabolite peaks (data not shown, Table 2), namely, the phenol glucuronide ($t_R = ca.$ 26 min) and the phenol (*ca.* 45 min)

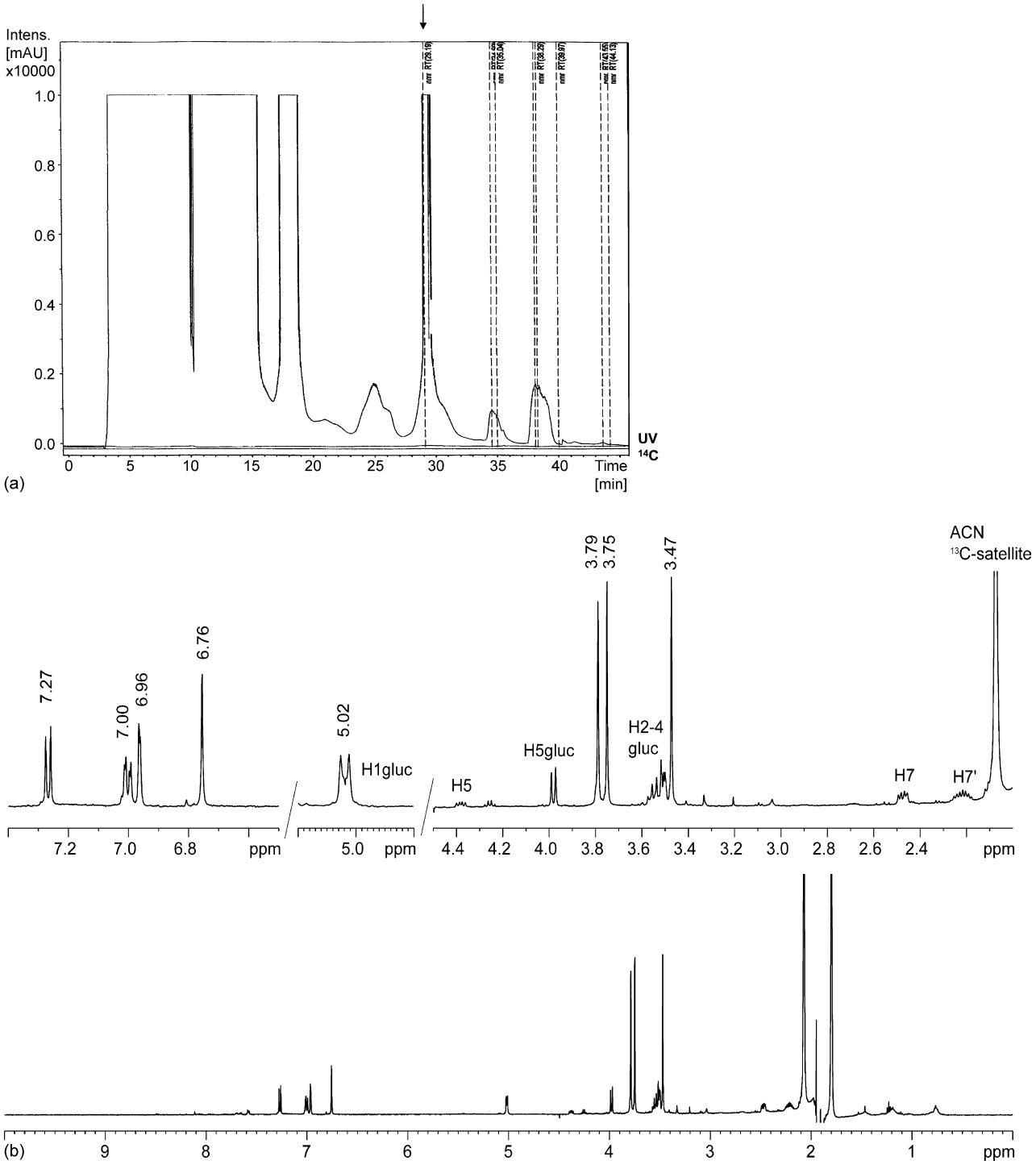

Fig. 2. (a) The UV- and ^{14}C-trace of the separation of the methanolic extract of the dog urine sample from dog 2 (0–24 h collection, 27 ml urinary volume), injected in 800 μl of 50:50 D_2O:ACN (acidified with 1% formic acid-d_2). The arrow indicates the peak of interest eluting at 29.19 min. (b) The stop-flow ^{1}H NMR spectrum of the [^{14}C]-ZD6126 metabolite (t_R = 29.19 min) contained in the urine from dog 2 (0–24 h collection). Shown are the whole spectrum (bottom) and expansions of the aromatic and aliphatic regions of interest.

(representing 3% and 1% of the total dose, respectively). The [^{14}C]-ZD6126 phenol was easily identified by comparison of retention times and HPLC–MS data, hence identification by HPLC–NMR was not required throughout the study.

3.7.2. Dog bile

The UV-chromatogram and ^{14}C-radiotrace representing the separation of the 0–6 h bile sample from dog 2 (0–6 h collection) are shown in Fig. 3a. Stop-flow ^{1}H NMR was carried out on the earlier eluting peaks (e.g. 16.33 min and 18.83 min), but

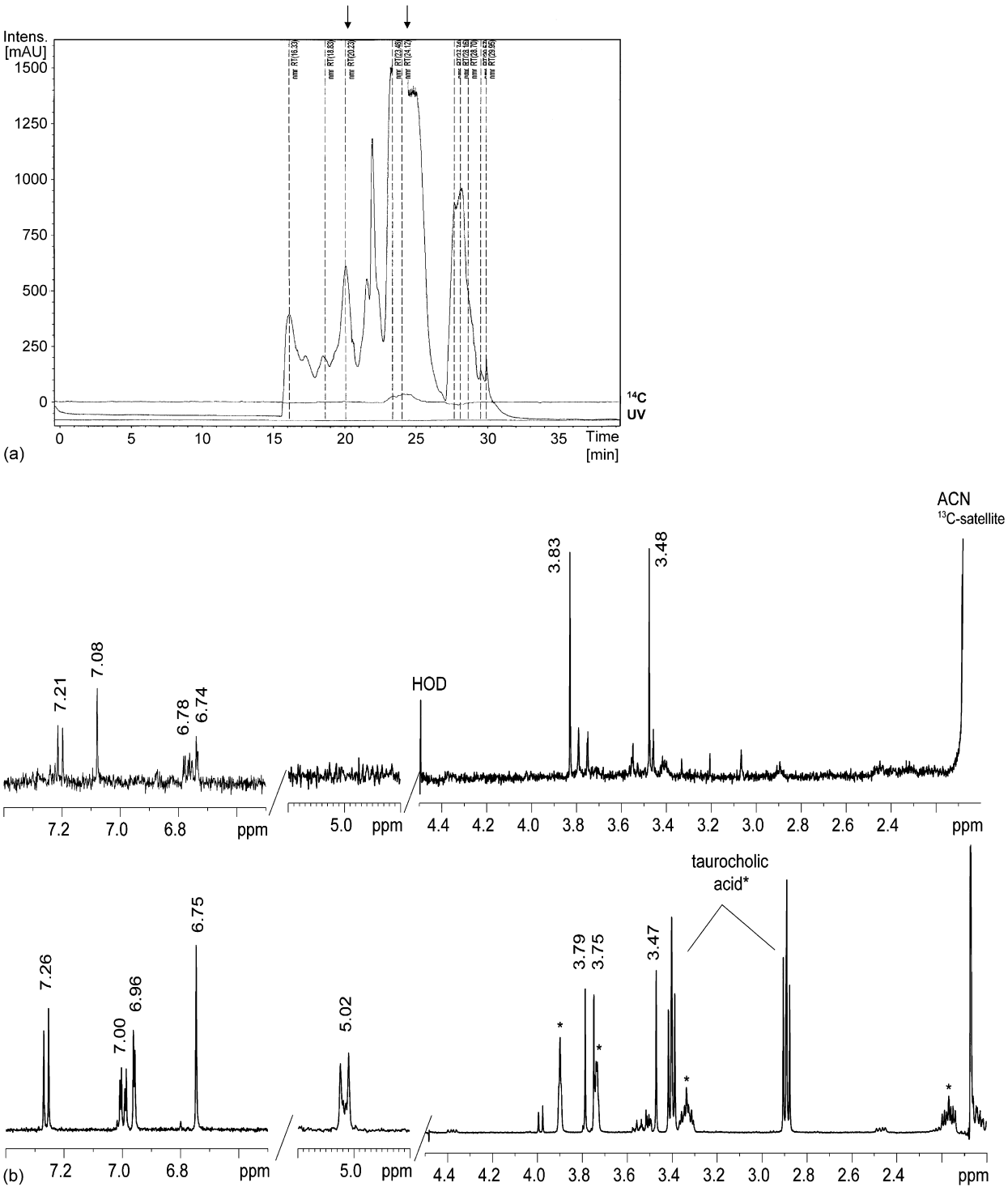

Fig. 3. (a) The UV- and ^{14}C-trace of the separation of the methanolic extract of the dog bile sample from dog 2 (0–6 h collection, 3 ml volume), injected in 300 μl of 50:50 D_2O:ACN (acidified with 1% formic acid-d_2). The arrows indicate the peaks of interest eluting at 20.23 min and 24.12 min. (b) The stop-flow ^{1}H NMR spectra of the [^{14}C]-ZD6126 metabolites contained in the bile from dog 2 (0–6 h collection) eluting at 20.23 min (top) and 24.12 min (bottom). Shown are the expansions of the aromatic and aliphatic regions of interest.

the fractions contained mainly PEG, the dosing vehicle contaminant.

The stop-flow ^{1}H NMR spectrum of the UV-peak at $t_R = 20.23$ min (Fig. 3b (top)) revealed an unexpected minor desmethyl-[^{14}C]-ZD6126 phenol metabolite, which was collected and subsequently identified as a sulphate conjugate by HPLC–MS. The stop-flow ^{1}H NMR spectrum showed a clear loss of one of the methoxy-signals, with the residual methoxy-signals experiencing a slight chemical shift change, observed at $\delta 1_H$ 3.83 and $\delta 1_H$ 3.48, compared to the chemical shifts of [^{14}C]-ZD6126 phenol or the phenol glucuronide observed in the dog urine. Similarly, a chemical shift change was experienced

by the aromatic protons, which were observed at $\delta 1_H$ 7.21 and 6.78 (for H1 and H2, respectively) and at $\delta 1_H$ 6.74 (for H4). The largest shift was experienced by H8, which resonated at $\delta 1_H$ 7.08, supporting a structural modification on the methoxylated aromatic ring. There was no evidence of glucuronide conjugation in the NMR spectrum, indicating either simple demethylation or conjugation with an NMR silent substituent, such as a sulphate group.

The chemical shifts of this metabolite did not match with those of the synthetic 10-desmethyl-ZD6126 phenol (chemical shifts listed in Table 6), suggesting either loss of the methyl-group at position 9 (as the radiolabel in position 11 was preserved throughout) and/or conjugation with an NMR silent moiety such as a sulphate group. Sulphate conjugation was subsequently confirmed by HPLC–MS, detecting a molecular ion $[M+H]^+$ of *m/z* 424, compared to that of the [^{14}C]-ZD6126 phenol with $[M+H]^+$ *m/z* 358. The exact site of demethylation and subsequent conjugation, could, however, not be determined in this experiment or in later work [12,14]. Although a sulphate ZD6126 standard (conjugation at position 3) was synthesised in subsequent work, it was found not to match any of the *in vitro* and *in vivo* metabolites observed [12,14]. Owing to the large chemical shift change of H8 and the residual methoxy-group (either at C9 or C10), sulphate conjugation on the demethylated aromatic ring rather than at the hydroxyl-group in position 3 remains a possibility.

The second stronger metabolite (t_R = 24.12 min) as shown in Fig. 3b (bottom), was identified as the glucuronide conjugate of [^{14}C]-ZD6126. Despite co-elution with taurocholic acid, the anomeric proton $H1_{gluc}$ at $\delta 1_H$ 5.02 (^3J = 8 Hz) could be clearly detected, together with the residual glucuronic acid protons ($H5_{gluc}$, $H2\text{-}4_{gluc}$). Similarly to the dog urine metabolite, the three methoxy signals at $\delta 1_H$ 3.79, $\delta 1_H$ 3.75 and $\delta 1_H$ 3.47 were preserved, as well as the aromatic protons at $\delta 1_H$ 7.26, $\delta 1_H$ 7.0 and $\delta 1_H$ 6.96 (for H1, H2, and H4 respectively), and at $\delta 1_H$ 6.75 (for H8).

Taurocholic acid could be identified by the characteristic taurine-triplets at $\delta 1_H$ 2.88 and $\delta 1_H$ 3.42, and the methyl singlets representing C18 (at $\delta 1_H$ 0.6) and C19 ($\delta 1_H$ 0.8) and the C21 doublet resonating at $\delta 1_H$ 0.88.

The corresponding HPLC–RAD-trace of the 0–6 h bile in the metabolite profiling experiment showed one major peak at *ca.* 23 min (data not shown), which was assigned as the phenol glucuronide and confirmed by HPLC–MS. The sulphate conjugate was not initially identified by HPLC–MS, although the radiotrace contained four minor metabolites. The minor metabolites were not investigated in detail at the time, and hence, are not listed in Table 2.

3.7.3. Rat bile (male)

The methanolic extracts of the pooled 0–6 h and 6–12 h bile samples (3 ml and 6 ml for the 0–6 h and 6–12 h collections, respectively) were reconstituted in either 300 or 400 μl (for 0–6 h or 6–12 h, respectively) of D_2O:ACN (50:50, v/v acidified with 1% formic acid-d_2) prior to injection onto the HPLC-column.

The UV-chromatogram and ^{14}C-radiotrace representing the separation of the 6–12 h pooled bile sample is shown in Fig. 4a. Several attempts have been made to identify early eluting UV peaks partly out of curiosity and to avoid undetected elution of the [^{14}C]-ZD6126 metabolite peaks. The ^{14}C-response was considered unreliable as it lacked resolution in view of the large amounts injected and the large volume of the radio-flow-cell. However, the earlier eluting metabolites either comprised endogenous biliary metabolites, such as tyrosine or phenylalanine (data not shown), or the dosing vehicle contaminant, PEG, which could be continuously detected during the analysis for approximately 20 min.

The 0–6 h and 6–12 h bile samples contained the same three metabolites, namely, the demethylated phenol sulphate, the demethylated phenol glucuronide and the phenol glucuronide. As observed previously in the dog bile and subsequent work [12], the elution order had changed in both cases compared to the HPLC-DAD metabolite profiling experiment, presumably due to overloading of the HPLC-column. The spectra from the pooled 6–12 h collection are discussed here in more detail, while the chemical shifts of the 0–6 h biliary metabolites are simply listed in Table 6.

3.7.3.1. Metabolite 1. The stop-flow ^{1}H NMR spectrum of the UV-peak at t_R = 17.88 min (Fig. 4b (top)) revealed a desmethyl-[^{14}C]-ZD6126 phenol metabolite. The stop-flow ^{1}H NMR spectrum showed a clear loss of one of the methoxy-signals, with the residual methoxy-signals resonating at $\delta 1_H$ 3.83 and $\delta 1_H$ 3.47. This was identical to the metabolite (t_R = 20.23 min, Fig. 2b) observed in dog bile. Similarly, the aromatic protons were observed at $\delta 1_H$ 7.20 and 6.77 (for H1 and H2, respectively) and at $\delta 1_H$ 6.74 (for H4). The largest shift was again observed for H8, which resonated at $\delta 1_H$ 7.08, supporting a structural modification on the methoxylated aromatic ring. There was again no evidence of glucuronide conjugation in the NMR spectrum, indicating either simple demethylation or conjugation with an NMR silent substituent, such as a sulphate group. The spectrum still contained small signals from residual PEG.

Based on the NMR characteristics, this metabolite was identical to the sulphate conjugate observed in dog bile. It was again confirmed by HPLC–MS as a sulphate conjugate, detecting a molecular ion $[M+H]^+$ of *m/z* 424, compared to that of the [^{14}C]-ZD6126 phenol with $[M+H]^+$ *m/z* 358. However, as for the dog bile desmethyl sulphate conjugate, the exact site of demethylation and conjugation could not be determined [12,14].

3.7.3.2. Metabolite 2. Metabolites 1 and 2 appeared to co-elute in the chromatographic fraction captured at 19.28 min (Fig. 4b (middle)). Despite contamination with metabolite 1, the stop-flow ^{1}H NMR spectrum showed metabolite 2 as demethylated, with the residual methoxy-signals at $\delta 1_H$ 3.75 and $\delta 1_H$ 3.48 and the aromatic signals at $\delta 1_H$ 7.26 (for H1), $\delta 1_H$ 6.99 (for H2), $\delta 1_H$ 6.95 (for H4) and $\delta 1_H$ 6.59 (for H8). Additionally, metabolite 2 was glucuronidated, with the characteristic anomeric proton $H1_{gluc}$ resonating at $\delta 1_H$ 5.02 (^3J = 8 Hz), and the residual glucuronic acid protons $H2\text{-}5_{gluc}$ clearly present. Metabolite 2 was, hence, identified as the demethylated glucuronide conjugate of [^{14}C]-ZD6126 phenol. Comparison of the chemical shifts with those of the glucuronide standard (see Table 6) and work carried out subsequently [12,14] suggests position 9 as

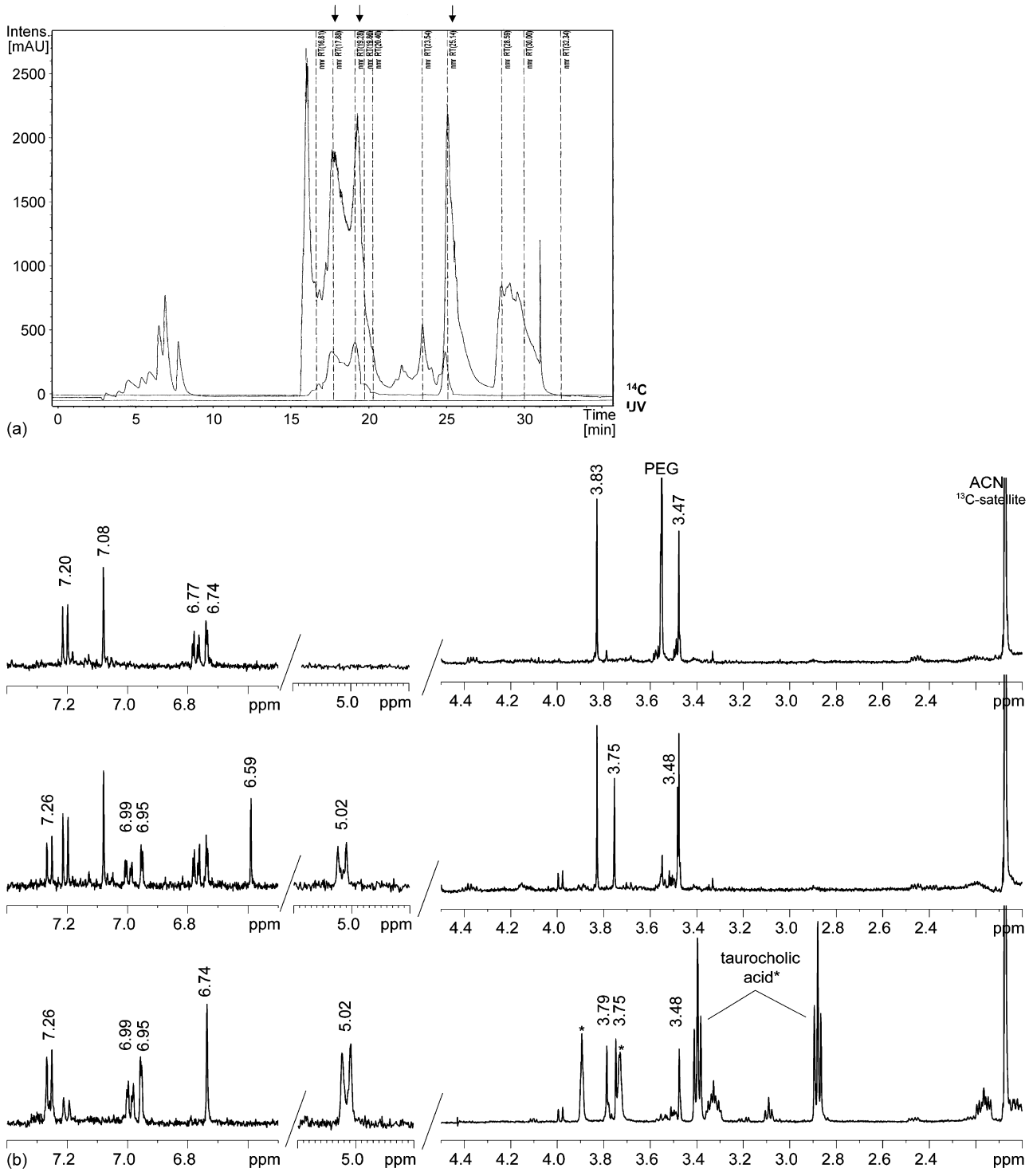

Fig. 4. (a) The UV- and ^{14}C-trace of the separation of the methanolic extract of the pooled bile samples from male rats (6–12 h collection, 6 ml total volume), injected in 400 μl of 50:50 D_2O:ACN (acidified with 1% formic acid-d_2). The arrows indicate the peaks of interest eluting at 17.88 min, 19.28 min and 25.14 min. (b) The stop-flow ^{1}H NMR spectra of the [^{14}C]-ZD6126 metabolites contained in the pooled bile from male rats collected over 6–12 h (total volume = 6 ml) eluting at 17.88 min (top), 19.28 min (middle) and 25.14 min (bottom). Shown are the expansions of the aromatic and aliphatic regions of interest.

the likely site for demethylation and position 3 as the site of conjugation.

Manual time-slicing was employed in an attempt to fully separate the co-eluting metabolites in the 0–6 h and 6–12 h bile samples. In the 0–6 h bile sample, metabolite 2 was successfully resolved from metabolite 1 (stop-flow ^{1}H NMR data not shown, but chemical shifts listed in Table 6). However, on this occasion, co-elution with taurocholic acid was observed, which did not prevent the assignment of the resonances. The methoxy signals could be confirmed at $\delta 1_H$ 3.75 and $\delta 1_H$ 3.48, together

with the aromatic signals at $\delta 1_H$ 7.25 (for H1), $\delta 1_H$ 6.99 (for H2), $\delta 1_H$ 6.95 (for H4), and at $\delta 1_H$ 6.59 (for H8). The anomeric proton $H1_{gluc}$ was present at $\delta 1_H$ 5.02 ($^3J = 8$ Hz).

3.7.3.3. Metabolite 3. The third major metabolite ($t_R = 25.14$ min) as shown in Fig. 4b (bottom), was identified as the glucuronide conjugate of [^{14}C]-ZD6126 phenol. Despite co-elution with taurocholic acid (identified by the characteristic taurine-triplets at $\delta 1_H$ 2.9 and $\delta 1_H$ 3.42, and the methyl groups C18, C19 and C21 at $\delta 1_H$ 0.6, $\delta 1_H$ 0.8 and $\delta 1_H$ 0.88, respectively), the anomeric proton $H1_{gluc}$ at $\delta 1_H$ 5.02 ($^3J = 8$ Hz) could be clearly detected, together the three methoxy signals at $\delta 1_H$ 3.79, $\delta 1_H$ 3.75 and $\delta 1_H$ 3.48. The aromatic protons resonated at $\delta 1_H$ 7.26, $\delta 1_H$ 6.99 and $\delta 1_H$ 6.95 (for H1, H2, and H4 respectively), and at $\delta 1_H$ 6.74 (for H8).

3.7.4. Rat bile (female)

The methanolic extract of the pooled 0–6 h bile samples (6 ml total volume) from female rats were reconstituted in 400 μl of D_2O:ACN (50:50, v/v, acidified with 1% formic acid-d_2) prior to injection onto the HPLC-column. The UV-chromatogram, ^{14}C-trace and the individual stop-flow ^{1}H NMR spectra are not shown, but the metabolites are discussed and the chemical shifts summarised in Table 6.

The first eluting [^{14}C]-ZD6126-related metabolite in the chromatographic fraction ($t_R = 19.27$ min), co-eluted with taurocholic acid and tryptophan, two endogenous biliary metabolites. However, the signals of interest could still be positively assigned. The [^{14}C]-ZD6126-related metabolite captured in this chromatographic fraction was clearly demethylated, showing the residual methoxy-groups at $\delta 1_H$ 3.75 and $\delta 1_H$ 3.48. and glucuronidated, owing to the distinct doublet from the anomeric proton $H1_{gluc}$ at $\delta 1_H$ 5.02 ($^3J = 8$ Hz). Although there was severe overlap in the aromatic region due to the presence of the tryptophan signals (doublets at $\delta 1_H$ 7.58 and $\delta 1_H$ 7.4 and triplets at $\delta 1_H$ 7.14 and $\delta 1_H$ 7.05), the aromatic signals from the postulated demethylated [^{14}C]-ZD6126 phenol glucuronide metabolite could still be identified. The aromatic protons resonated at $\delta 1_H$ 7.26 (for H1), $\delta 1_H$ 7.0 (for H2) and at $\delta 1_H$ 6.95 (for H4), with the singlet for H8 at $\delta 1_H$ 6.59. The chemical shifts of this metabolite were identical to those of the demethylated glucuronide conjugate observed in the bile from male rats. Thus, it is suggested to be the same 9-desmethyl phenol glucuronide.

Manual time-slicing, stopping the separation at $t_R = 19.77$, resolved the taurocholic acid, but did not achieve the separation of the tryptophan and demethylated [^{14}C]-ZD6126 phenol glucuronide.

The later eluting metabolite ($t_R = 21.22$ min) could be assigned with confidence as the glucuronide conjugate of [^{14}C]-ZD6126 phenol, clearly displaying the signals from the three methoxy-groups at $\delta 1_H$ 3.78, $\delta 1_H$ 3.74 and $\delta 1_H$ 3.48. The aromatic signals resonated at $\delta 1_H$ 7.26 (for H1), $\delta 1_H$ 6.99 (for H2), $\delta 1_H$ 6.95 (for H4) and $\delta 1_H$ 6.74 (for H8). The anomeric proton signal was at the characteristic chemical shift of $\delta 1_H$ 5.02 ($^3J = 8$ Hz). Co-elution with taurocholic acid did again not perturb signal assignment.

3.7.5. Rat urine (male and female)

The individual 0–24 h urine samples from male and female rats were pooled (yielding a total volume of 24 ml and 28 ml, respectively), freeze-dried, extracted into MeOH, blown dried, re-freeze-dried and reconstituted in 800 μl (for male) or 1 ml (for female) aliquots of 50:50 D_2O:ACN (acidified with 1% formic acid-d_2) prior to injection onto the HPLC-column. The UV-chromatogram, the associated ^{14}C-radiotrace and the individual stop-flow ^{1}H NMR spectra are not shown, but the metabolites are again discussed and the chemical shifts summarised in Table 6.

The main urinary metabolites in the 0–24 h urines were suggested to be the glucuronide conjugate of [^{14}C]-ZD6126 phenol in female rats or the demethylated phenol glucuronide in males (see Table 4), together with the minor demethylated phenols in both male and female and the demethylated sulphate conjugate in males.

The identification of the earlier eluting metabolite peaks (e.g. 15–17 min) in the UV-chromatograms and ^{14}C-radiotraces proved difficult as they were was either severely obscured by co-eluting endogenous metabolites present in high concentrations, such as hippurate (at approximately 17.6 min) and/or the dosing vehicle contaminant, PEG, which appeared to bleed off the column until approximately 24 min (data not shown). It was those two contaminants, which severely obscured the assignment of the demethylated glucuronide conjugate in the urine from male rats. Furthermore, the known low concentrations of the postulated demethylated phenol sulphate conjugate in the urines from male rats (Table 4, 0–7% of dose) and of the demethylated phenols (Table 4, 0.2–1% of the dose in male and female) impaired detection in the urines.

However, the later eluting metabolite, namely, the [^{14}C]-ZD6126 phenol glucuronide, could be successfully identified in each of the urine pools. The stop-flow ^{1}H NMR spectrum of the peak at 23.79 min (in male rats) and 24.25 min (in female rats) clearly showed the anomeric proton $H1_{gluc}$ at $\delta 1_H$ 5.02 ($^3J = 8$ Hz), together with the three diagnostic methoxy-groups at $\delta 1_H$ 3.79, $\delta 1_H$ 3.75, and $\delta 1_H$ 3.47 (as listed in Table 6). The aromatic signals were clearly identifiable in the pooled urine sample from the female rats: ($\delta 1_H$ 7.26 (for H1), $\delta 1_H$ 6.99 (for H2), $\delta 1_H$ 6.96 (for H4) and $\delta 1_H$ 6.74 (for H8)), but were partly obscured in the urines from male rats, for which this metabolite only accounted for 4% of the dose (c.f. 0.5–14% in females, see Table 4).

4. Summary/conclusions

Successful drug metabolite identification generally relies on the production of isolated compounds. Hyphenated techniques such as HPLC–NMR and the recently developed HPLC–SPE–NMR have largely facilitated the preparative isolation of the analytes in question from their complex biological matrices [16–22]. However, despite the technical advances, co-elution, especially with endogenous metabolites or other contaminants can still be a problem. In fact, even low resolution techniques such off-line solid-phase extraction (SPE) have been shown to yield fractions of sufficient quality [23], and recently, a crude biofluid extraction method has been developed which has

shown to allow successful and very rapid metabolism identification by NMR with minimal sample preparation [24]. Most of all, not every laboratory is equipped with the latest commercially available hyphenated equipment such as HPLC–SPE–NMR, as has been the case at the time of analysis in this study, and when time and resource are restricted, then the fast-track crude HPLC–NMR approach, as demonstrated in this study might be suitable.

Despite the lack of sample clean-up or selective metabolite extraction by off-line solid-phase extraction chromatography prior to the HPLC-analyses, the majority of the metabolites could be positively identified. Surprisingly, despite severe column overloading, the biofluid constituents and drug metabolites retained, and in most cases eluted with minor cross contamination. Seven successive injections were made with biofluid volumes as large as 6 ml (for bile) and 28 ml (for urine) yet, the chromatography did not deteriorate markedly.

Most problems were encountered with the detection of early eluting [^{14}C]-ZD6126 metabolites, especially, in the urine samples. The presence of the UV-silent dosing vehicle contaminant, PEG, which appeared to bleed off the column for up to 24 min, and the co-elution of endogenous metabolites such as hippuric acid perturbed the assignment of some [^{14}C]-ZD6126 metabolites, such as the early eluting desmethyl phenol metabolites and its glucuronide and sulphate conjugates. The low concentrations of some of these metabolites, together with the lack of chromatographic resolution (especially, with a radiodetector-flow-cell of 150 μl interfaced prior to the UV-detector) complicated their identification further.

In the bile samples, the volumes injected onto the columns were smaller (3–6 ml), hence, the UV-chromatograms (and ^{14}C-traces) showed better resolution and allowed more successful peak-picking/selection. In the rat bile, all major [^{14}C]-ZD6126-related metabolites could be positively identified, and the analysis of the dog bile even revealed the identity of an unexpected minor metabolite, a desmethyl phenol sulphate conjugate.

Confirmation of the metabolite identities could be achieved by NMR signal assignment, especially demethylation and/or conjugation with glucuronic acid, further aided by the comparison of the chemical shifts with those of synthetic standards. The input of the results produced by HPLC–MS is of course invaluable. The absolute configuration of the demethylated sulphate conjugate, however, remains obscure. The conjugate is NMR silent, and although the metabolite could be confirmed by HPLC–MS, the sites of demethylation (at position 9 or 10) and conjugation (at position 3, 9 or 10) could not be.

In summary, this work confirmed biliary excretion as the major route of elimination [15]. The metabolism in rat and dog comprised glucuronidation in both species, gender, and all biofluids. Demethylation followed by glucuronidation was observed in both, male and female rats, while demethylation followed by sulphation was only observed in the male rats and dog.

References

[1] D.C. Blakey, F.R. Westwood, M. Walker, G.D. Hughes, P.D. Davis, S.E. Ashton, A.J. Ryan, Clin. Cancer Res. 8 (2002) 1974–1983.
[2] H. Goto, S. Yano, H. Zhang, Y. Matsumori, H. Ogawa, D.C. Blakey, S. Sone, Cancer Res. 62 (2002) 3711–3715.
[3] P.D. Davis, G.J. Dougherty, D.C. Blakey, S.M. Galbraith, G.M. Tozer, A.L. Holder, M.A. Naylor, J. Nolan, M.R. Stratford, D.J. Chaplin, S.A. Hill, Cancer Res. 62 (2002) 7247–7253.
[4] M.F. McCarty, A. Takeda, O. Stoeltzing, W. Liu, F. Fan, N. Reinmuth, M. Akagi, C. Bucana, P.F. Mansfield, A. Ryan, L.M. Ellis, Br. J. Cancer 90 (2004) 705–711.
[5] G. Micheletti, M. Poli, P. Borsotti, M. Martinelli, B. Imberti, G. Taraboletti, R. Giavazzi, Cancer Res. 63 (2003) 1534–1537.
[6] S.A. Radema, L.V. Beerepoot, P.O. Witteveen, M.F. Gebbink, C. Wheeler, E.E. Voest, Proc. Am. Soc. Clin. Oncol. 21 (2002) 439.
[7] M.R. Horsman, R. Murata, Int. J. Radiat. Oncol. Biol. Phys. 57 (2003) 1047–1055.
[8] D.W. Siemann, E. Mercer, S. Lepler, A.M. Rojiani, Int. J. Cancer 99 (2002) 1–6.
[9] D.W. Siemann, A.M. Rojiani, Int. J. Radiat. Oncol. Biol. Phys. 53 (2002) 164–171.
[10] P.E. Thorpe, Clin. Cancer Res. 10 (2004) 415–427.
[11] J. Soltau, J. Drevs, I. Drugs 7 (2004) 380–387.
[12] I. Judson, M. Scurr, D.W. Roberts, E.A. Partridge, R.A. D'Souza, S. Tan, in preparation.
[13] R.A. D'Souza, E.A. Partridge, D.W. Roberts, S. Ashton, A. Ryan, F.R. Westwood, A.B. Patterson, Z. Wilson, C.C. Thurrell, Xenobiotica, submitted for publication.
[14] A.D. McCormick, D.L. Slamon, E.M. Lenz, C. King, P.J. Phillips, D. McKillop, D.W. Roberts, Xenobiotica, submitted for publication.
[15] A.L. Hunter, C.D. Klaassen, J. Pharmacol. Exp. Ther. 192 (1974) 605–617.
[16] J.C. Lindon, J.K. Nicholson, I.D. Wilson, J. Chromatogr. B 748 (2000) 233–258.
[17] M. Tugnait, E.M. Lenz, M. Hofman, M. Spraul, I.D. Wilson, J.C. Lindon, J.K. Nicholson, J. Pharm. Biomed. Anal. 30 (2003) 1561–1574.
[18] U.G. Sidelmann, I. Bjornsdottir, J.P. Shockcor, S.H. Hansen, J.C. Lindon, J.K. Nicholson, J. Pharm. Biomed. Anal. 24 (2001) 569–579.
[19] A.J. Simpson, L.H. Tseng, M.J. Simpson, M. Spraul, U. Braumann, W.L. Kingery, B.P. Kelleher, M.H.B. Hayes, Analyst 129 (2004) 1216–1222.
[20] V. Exarchou, Y.C. Fiamegos, T. Van Beek, C. Nanaos, J. Vervoort, J. Chromatogr. A 112 (2006) 293–302.
[21] A. Pukalskas, T. Van Beek, P. de Waard, J. Chromatogr. A 1074 (2005) 81–88.
[22] M. Godejohann, L.H. Tseng, U. Braumann, J. Fuchser, M. Spraul, J. Chromatogr. A 1058 (2004) 191–196.
[23] E.M. Lenz, I.D. Wilson, B. Wright, E.A. Partridge, C.T. Rodgers, P.R. Haycock, J.C. Lindon, J.K. Nicholson, J. Pharm. Biomed. Anal. 28 (1) (2002) 31–43.
[24] U. Gerhard, S. Thomas, R. Mortishire-Smith, J. Pharm. Biomed. Anal. 32 (2003) 532–538.