RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. **14**, 2377–2384 (2000)

Directly coupled liquid chromatography with inductively coupled plasma mass spectrometry and orthogonal acceleration time-of-flight mass spectrometry for the identification of drug metabolites in urine: application to diclofenac using chlorine and sulfur detection

Olivia Corcoran[1], Jeremy K. Nicholson[1], Eva M. Lenz[2], Fadi Abou-Shakra[3], Jose Castro-Perez[3], Ashley B. Sage[3] and Ian D. Wilson[2]*

[1]Biological Chemistry, Imperial College of Science, Technology and Medicine, Sir Alexander Fleming Building, South Kensington, London SW7 2AZ, UK
[2]Drug Metabolism and Pharmacokinetics, AstraZeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK
[3]Micromass UK Ltd, Floats Rd, Wythenshawe, Manchester M23 9LZ, UK

We report the application of high-performance liquid chromatography (HPLC) linked to inductively coupled plasma mass spectrometry (ICPMS) and orthogonal acceleration time-of-flight mass spectrometry (oa-TOFMS) for the identification of phase I and II urinary metabolites of diclofenac. The metabolites were separated by reversed-phase HPLC monitored with a UV diode array detector (UV-DAD) after which 90% of the eluent was directed to an ICPMS source, with the remainder going to an oa-TOF mass spectrometer. Compounds containing ^{35}Cl, ^{37}Cl and ^{32}S were detected specifically using ICPMS and identified by oa-TOFMS. The metabolites detected and identified in this way included glucuronic acid and sulfate conjugates, mono- and dihydroxylated and free diclofenac. In addition a previously unreported *in vivo* metabolite, an *N*-acetylcysteinyl conjugate of diclofenac, was also characterised. This is the first application of the combination of HPLC/UV-DAD/ICPMS/oa-TOFMS for the investigation of the metabolic fate of chlorinated xenobiotics by direct biofluid analysis. **Copyright © 2001 John Wiley & Sons, Ltd.**

Received 4 September 2000; Revised 24 October 2000; Accepted 25 October 2000

Conventional methods for studying the metabolic fate of xenobiotics need selective detection of metabolites in complex biofluids, which generally requires the use of radiolabelling. Otherwise, studies performed in the absence of a suitable tracer always leave open the possibility that important metabolites may be missed, especially if they are significantly different in structure to the parent. To circumvent some of these problems we have successfully used techniques such as high field NMR for drug metabolites containing suitable NMR handles: ^{1}H, ^{2}H, ^{31}P, ^{13}C, ^{15}N or ^{19}F.[1–3] However, for xenobiotics which lack suitable NMR-accessible labels but contain heteroatoms such as eg. ^{79}Br, ^{81}Br, we have shown that HPLC/ICPMS provides a rapid and sensitive method for the detection and quantification of metabolites.[4] HPLC/ICPMS may also be applied to ^{35}Cl-, ^{37}Cl- and ^{32}S-containing compounds. Such element specific atomic mass spectrometry combined with molecular mass spectrometry via oa-TOFMS offers a powerful approach to metabolite detection, identification and quantification. Here, we show the potential of HPLC/UV-DAD/ICPMS/oa-TOFMS for the simultaneous, selective detection and identification of the phase I and II metabolites of chlorine-containing drugs, exemplified by use of the non-steroidal anti-inflammatory drug (NSAID) diclofenac, (*o*-(2,6-dichlorophenyl)aminophenylacetic acid). The sodium salt of diclofenac is the most frequently prescribed NSAID in the world for rheumatic disease, minor surgery, postpartum pain and dysmenorrhoea.[5,6] However, it causes mild liver damage in up to 15% of patients and severe hepatotoxicity in a few susceptible individuals.[7] Although the toxic mechanisms remain controversial, recent evidence implicates the reactive acyl glucuronide metabolite of diclofenac which can adduct with critical hepatic transporter proteins, which in turn leads to impaired bile acid secretion and reduced bile flow that may precede classical hepatotoxicity.[8] In the present study we have applied HPLC/UV-DAD/ICPMS/oa-TOFMS to study the urinary excretion and metabolic fate of diclofenac.

*Correspondence to: I. D. Wilson, Drug Metabolism and Pharmacokinetics, AstraZeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK.
E-mail: ian.wilson@astrazeneca.com

EXPERIMENTAL

Chemicals and sample preparation

Diclofenac sodium salt was purchased from Fluorochem Ltd (Old Glossop, UK) and was used as received. Solvents for chromatography were of HPLC grade and were obtained from Merck (Poole, UK). Male Sprague-Dawley rats (n = 3, 200–300 g) were acclimatised individually in plastic metabolism cages for 24 h prior to dosing and permitted free

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Table 1. Gradient conditions for separation of diclofenac metabolites

Time (min)	% 0.01 M Ammonium formate (pH 7.0)	% Methanol	Flow mL/min	Ramp
0	95	5	1	Linear
20	40	60	1	Linear
22	0	100	1	Linear

Table 2. Instrument operating conditions for ICPMS

Cooling gas:	13.57 L/min	Plasma power:	1160 W
Plasma gas:	0.59 L/min	Acquisition mode:	SIR
Nebuliser gas:	0.98 L/min	Dwell time:	200 ms
Helium gas:	1 mL/min	Masses monitored:	32, 35, 37 u
Hydrogen gas:	4.0 mL/min	Total analysis time:	50 min

access to food and water throughout the study. Diclofenac was dosed orally at 30 mg kg^{-1} in ethanol/water, at a concentration of 50 g mL^{-1}. Urine was collected over CO_2 for the period 0–8 h post dose. After thawing, the samples were centrifuged at 13 k rpm for 10 min to remove any solid debris. A 2 mL aliquot of urine was freeze-dried and reconstituted in 1 mL 0.01 M ammonium formate, pH 7, to which 150 µL MeOH was added. The sample was then centrifuged and 50 µL injections were made onto the HPLC column.

Analysis of metabolites in urine

Reversed-phase HPLC was performed using an Alliance 2690 HPLC system (Waters, Milford, MA, USA) with a UV-DAD coupled to a Platform ICP mass spectrometer (Micromass, Manchester, UK). In the configuration used here, the mass spectrometers were linked in parallel with ca. 90% of the flow from the UV-DAD directed to the ICP mass spectrometer and the remaining 10% to the oa-TOF mass spectrometer. The coupling of the UV-DAD to the two mass spectrometers was effected by simply connecting the outlet from the UV-DAD to a T-piece using a short length of PEEK tubing (5 cm of 0.007′ i.d) and thence directly to the nebuliser inlet of the ICPMS using a further 15 cm of PEEK, and to the inlet of the oa-TOFMS with 1.5 m of the same tubing (Upchurch Scientific, WA, USA). The chromatographic separation was carried out using a Symmetry C18 4.6 × 250 mm column (Waters, USA) with elution using a reversed-phase gradient method based on 0.01 M ammonium formate (pH 7) and methanol, as summarised in Table 1.

ICPMS

The Platform ICP system uses a hexapole collision/reaction cell based ICP mass spectrometer for simultaneous measurement of the ^{35}Cl, ^{37}Cl and ^{32}S isotopes. In order to avoid excessive carbon build-up on the cones of the instrument, the nebuliser gas was mixed to 5% v/v oxygen using an onboard mass flow controller. MassLynx™ software was used for instrument control, data acquisition and data handling. The flow of the nebuliser gases and the operating and acquisition conditions of the ICP mass spectrometer are shown in Table 2.

oa-TOFMS

In conjunction with ICPMS data, the acquisition of full-scan electrospray data was carried out using a Micromass LCT oa-TOF mass spectrometer (Micromass UK, Manchester, UK) equipped with a Z-Spray ion source. Prior to

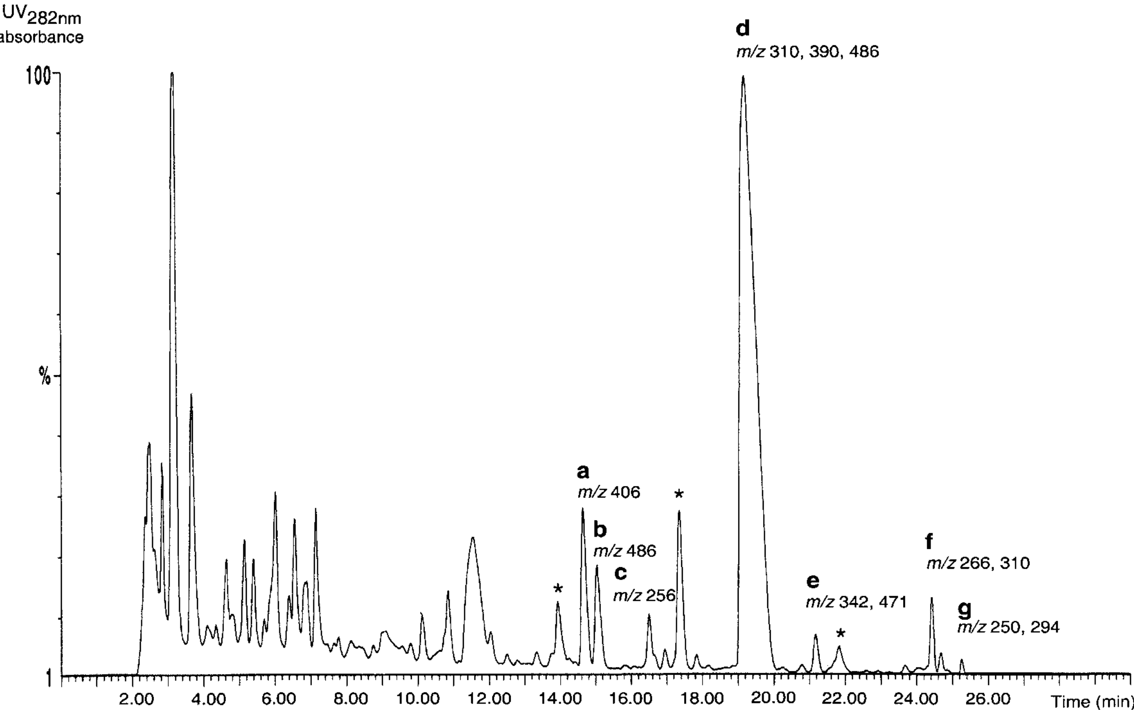

Figure 1. HPLC/UV$_{282nm}$ chromatogram of rat urine collected 0–8 h after a single oral dose of diclofenac 30 mg/kg. Peaks labelled **a** to **g** were identified as diclofenac metabolites by −ve electrospray oa-TOFMS. Peaks labelled * did not contain the chlorine isotopic pattern diagnostic of diclofenac, by oa-TOFMS.

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performing all LC/MS experiments, the LCT was calibrated in both positive and negative ion electrospray mode over a mass range of 100–1000 u using a 20 ng/μL solution of poly-DL-alanine (Sigma Aldrich, Dorset, UK) dissolved in methanol. A separate calibration line was used for each ion mode. To carry out exact mass measurement of the analytes as they eluted from the HPLC column, a single lock mass was infused via a Valco™ T-piece into the eluent stream prior to entering the electrospray probe. Leucine enkephalin (Sigma Aldrich, Dorset, UK) dissolved in 50:50 MeCN/H_2O was chosen as the lock mass as it produced both positive and negative ions ($M + H^+ = 556.2771$ and $M - H^- = 554.2615$). A solution of 0.5 ng/μL was infused using a Harvard 22 syringe pump at a flow rate of 10 μL/min to provide an ion current of approximately 300–500 counts per second to allow exact mass measurement. For all experiments, data were acquired over 100–1000 u at an acquisition rate of 0.95 s and an inter-acquisition delay of 0.05 s. Capillary voltages for sample ionisation were set to 3.2 kV for positive electrospray and 3.0 kV for negative electrospray. A sample cone voltage of 30 V was used for all experiments. The source temperature was set to 100 °C and the desolvation temperature was set to 250 °C. Nebuliser and desolvation gas flow rates were set to 1.5 L min^{-1} and 16.67 $Lmin^{-1}$, respectively. Again, MassLynx™ software was used for instrument control, data acquisition and data handling.

RESULTS

Some 40% of the diclofenac dosed to male rats (5 and 10 mg kg^{-1} oral and intravenous, respectively) is reported to be recovered in rat urine over 48 h, comprised of several phase I and II metabolites.[9] A typical HPLC/UV chromatogram obtained at the λ_{max} of diclofenac (282 nm) for the freeze-dried 0–8 h rat urine used here is shown in Fig. 1. A comparison of the chromatogram for predose urine indicated putative diclofenac metabolites labelled as peaks **a–g**, with the retention times given in Table 3. Apart from the fact that these new peaks absorbed UV at 282 nm and were, therefore, possibly diclofenac-related, no further structural information was available from the UV chromatogram. Whilst the UV λ_{max} of metabolites **a, d** and **e**, determined by the UV-DAD spectra shown in Fig. 2, were similar to that of diclofenac **g**, metabolites **b** and **f** gave a slightly shorter λ_{max} at *ca.* 273 nm. In constrast, the UV spectrum of the putative metabolite **c** gave a strong λ_{max} at 324nm. However, given the lack of detailed structural information provided by these spectra, the coelution of metabolites could not be discounted.

Further structural information on these metabolites was provided by the corresponding ^{35}Cl-monitored +ve electrospray ICPMS '*chloratogram*', depicting a number of chlorine-containing peaks **a–e** in the region 14–22 min, (Fig. 3(a)). The ability to generate such a chlorine-specific profile allows an estimate of the number and relative proportions of the diclofenac metabolites to be determined. By spiking a known amount of a chlorinated internal standard into the sample prior to separation, it would also be possible to quantify the diclofenac-related peaks directly. This is analogous to the use of ^{19}F NMR spectroscopy for the analysis of ^{19}F-containing compounds directly in biofluids.[1–3] It should also be noted that the use of ICPMS only allows the detection of chlorine-containing peaks but does not indicate whether such peaks are composed of single or multiple chlorine-containing compounds. Indeed, the major chlorine-containing peaks, **b** and **d**, were shown on investigation by oa-TOFMS to contain several co-eluting components (see below). In the case of diclofenac, the analyte contains two chlorine atoms. Although loss of a Cl atom via metabolism is not uncommon, ICPMS alone cannot reveal whether the metabolites contain both of the original Cl atoms. It should also be noted that not all of the diclofenac metabolites were seen in the '*chloratogram*', due to the marked detector response on elution with 100% methanol from the analytical column, resulting in the masking of chlorine peaks for metabolites **f** and **g**.

Because of the possibility of metabolic sulfation of hydroxylated metabolites, ICPMS was also used to monitor the separation for sulfur-containing materials. Unsurpris-

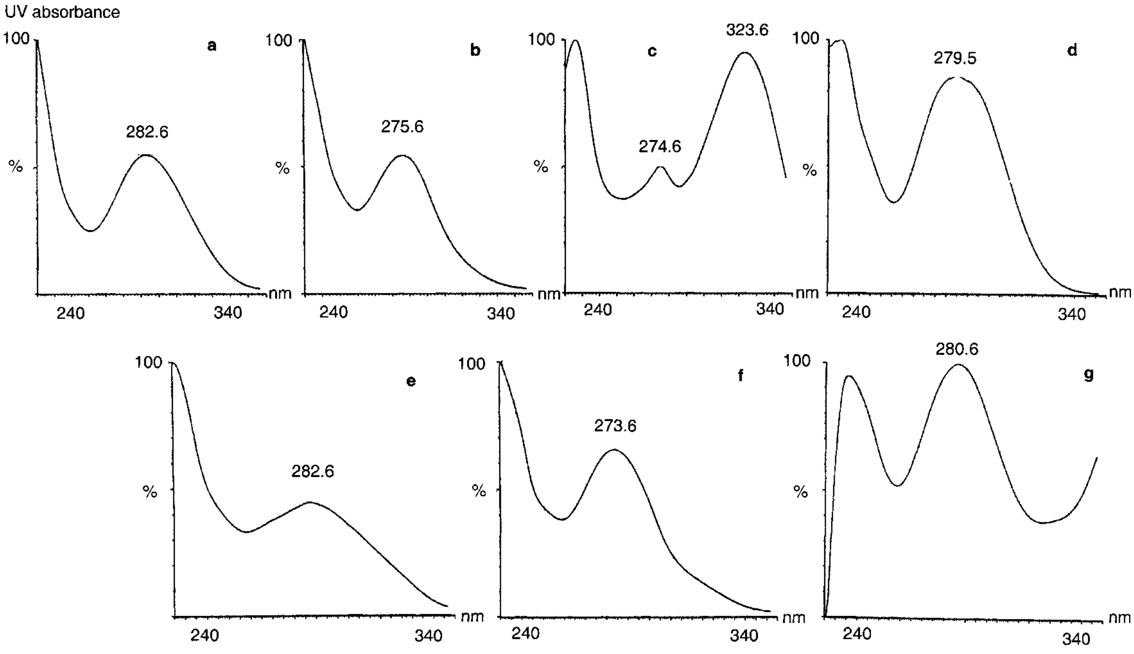

Figure 2. UV-DAD spectra acquired on-line for diclofenac metabolites **a–g**.

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Table 3. Assignment of diclofenac metabolites

Metabolite	UV r.t. (mins)	oa-TOF ($[M - H]^-$) (mDa/ppm error)	Elemental formula	Assignment
a	14.64	405.9552 (0.3/0.7)	$C_{14}H_{11}NO_7SCl_2$	Dihydroxylated sulfate
b	15.04 15.56	486.0364 (0.5/1.1)	$C_{20}H_{19}NO_9Cl_2$	Monohydroxylated ether glucuronide
c		255.9246 (0.8/3.1)	$C_6H_5NO_4SCl_2$	Monohydroxylated sulfate conjugate of cleavage product
d	19.19	486.0352 (0.7/1.5)	$C_{20}H_{19}NO_9Cl_2$	Monohydroxylated sulfate and monohydroxylated ether
		389.9598 (0.8/2)	$C_{14}H_{11}NO_6SCl_2$	Glucuronide
		310.0038 (0.2/0.7)	$C_{14}H_{11}NO_3Cl_2$ (fragment)	
e	21.17	471.0184 (0.4/0.9)	$C_{19}H_{18}N_2O_6SCl_2$	Mercapturic acid conjugate of diclofenac
f	24.42	310.0038 (0.2/0.7)	$C_{14}H_{11}NO_3Cl_2$	Monohydroxylated diclofenac
		266.0135 (0.5/1.7)	$C_{13}H_{11}NOCl_2$ (fragment)	
g	25.23	294.0098 (0.9/3.2)	$C_{14}H_{11}NO_2Cl_2$	Diclofenac
		250.0193 (0.2/1.2)	$C_{13}H_{11}NCl_2$ (fragment)	

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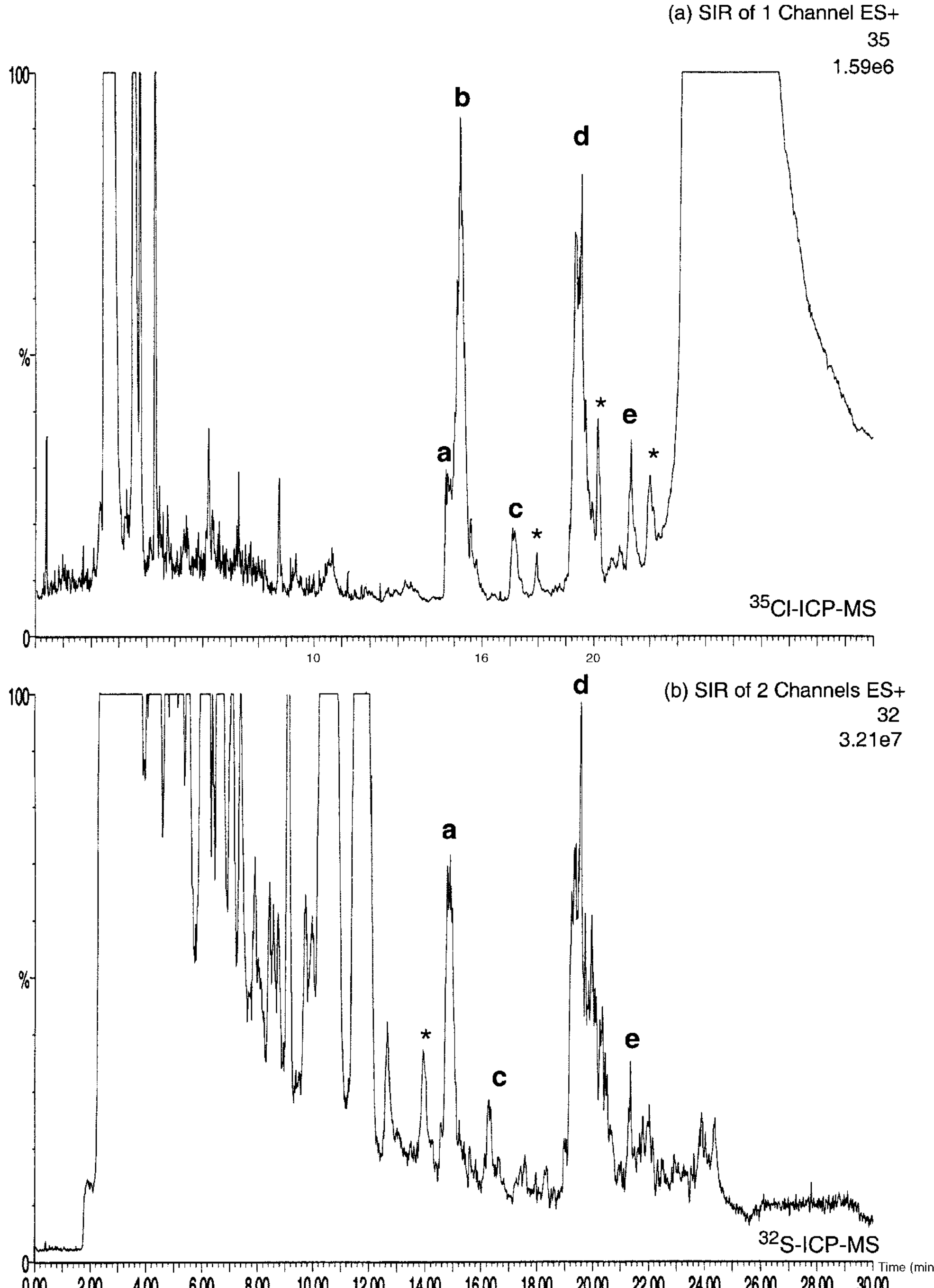

Figure 3. HPLC/ICPMS spectra (+ve electrospray) of 0–8 h rat urine (a) the ^{35}Cl-detected chloratogram shows several diclofenac-related peaks in the region 14–25 min. The baseline from 23 min was from 100% methanol in the mobile phase, (b) the ^{32}S-detected sulfatogram shows a complex background of sulfur-containing molecules endogenous to rat urine. Peaks labelled * did not contain the chlorine isotopic pattern diagnostic of diclofenac, by oa-TOFMS.

ingly, most of the major peaks in the +ve electrospray ^{32}S-ICPMS trace, or '*sulfatogram*', Fig. 3(b), were from endogenous metabolites naturally found in rat urine, none of which gave a corresponding chlorine-containing signal to uniquely identify the xenobiotic. Urine contains many sulfur-containing molecules, including cysteine, taurine, methionine and indoxylsulfate.[2] However, several sulfur-containing peaks with corresponding peaks in the ^{35}Cl-ICPMS trace were clearly observed for peaks **a, c, d** and **e** implying the presence of sulfur-containing conjugates of diclofenac.

Whilst the information from the '*chlorato-*' and '*sulfatograms*' was useful for locating peaks of interest in the chromatographic eluent, and would provide both a metabolite profile and a means of quantification, no detailed molecular structural data were forthcoming from the ICPMS measurement. Therefore, we used oa-TOFMS connected in parallel to ICPMS to obtain molecular mass spectra to aid in the characterisation of the diclofenac metabolites. A summary of the resulting data is given in Table 3. The oa-TOFMS total ion chromatogram (TIC) for the period 12 to 30 min is shown in Fig. 4. The largest UV-

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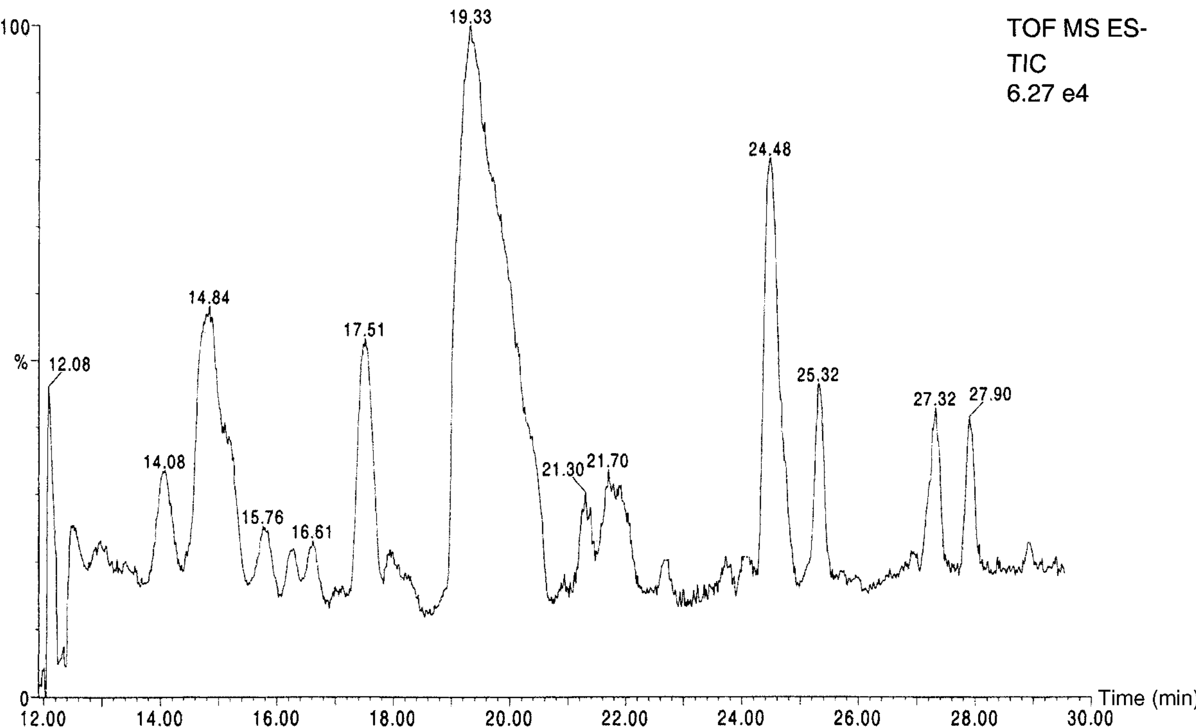

Figure 4. The total ion chromatogram (TIC) for 0–8 h rat urine, expanded for the region containing diclofenac metabolites.

absorbing peak (**d**) corresponded to the major TIC peak at 19.33 min. The −ve electrospray oa-TOFMS data on this peak (Fig. 5(d)) is consistent with the known sulfate conjugate of a monohydroxylated metabolite of diclofenac, having a molecular ion at *m/z* 390 ($M - H^- = 389.9598$, $C_{14}H_{11}NO_6SCl_2$) with loss of the sulfate group to give the fragment at *m/z* 310 ($M - H^- = 310.0038$, $C_{14}H_{11}NO_3Cl_2$). For the second largest UV-absorbing peak (**a**), corresponding to the TIC peak at 14.84 min, the oa-TOFMS data gave a molecular ion at *m/z* 406 ($M - H^- = 405.9552$, $C_{14}H_{11}NO_7SCl_2$), Fig. 5(a). This molecular ion and the early elution time are consistent with a dihydroxylated sulfate conjugate of diclofenac. Two remaining minor sulfur-containing peaks with corresponding chlorine peaks were observed for **c** and **e**, respectively. The molecular ion for **c**, the minor TIC peak at 15.76 min, was *m/z* 256 ($M - H^- = 255.9246$, $C_6H_5NO_4SCl_2$) and the diagnostic 9:6:1 pattern confirmed the presence of the dichloro-aromatic ring. Although this data would be consistent with monohydroxylation followed by sulfation with the cleavage product the absence of a corresponding sulfur peak in the 'sulfatogram' suggests that this is not the case. The identity of this minor unknown metabolite is still under investigation.

The oa-TOF mass spectrum shown in Fig. 5(e) for the minor sulfur-containing metabolite **e** at *m/z* 471 (TIC peak 21.30 min) is consistent with the presence of an *N*-acetylcysteinyl conjugate of diclofenac ($M - H^- = 471.0184$, $C_{19}H_{18}N_2O_6SCl_2$). The observed loss of 129 u to give the ion at *m/z* 342 is diagnostic of the presence of the sulfhydryl group.[10] This metabolite has not previously been reported as an *in vivo* metabolite of diclofenac. The site of conjugation was not clear from these data but may be at position 3′, with hydroxylation at position 4′, to give a structure analagous to the well-known *N*-acetylcysteine conjugate of paracetamol (acetaminophen).[10] Given the evidence that this route of metabolism for paracetamol is thought to be responsible for hepatotoxicity,[10] the presence of this putative *N*-acetylcysteine diclofenac conjugate may have implications for understanding the hepatotoxicity of this drug in humans. Further studies are in progress to fully characterise this metabolite.

The remaining diclofenac metabolites were also identified by oa-TOFMS (Table 3). Peaks **b** ($M - H^- = 486.0364$, $C_{20}H_{19}NO_9Cl_2$) and **d** ($M - H^- = 486.0352$, $C_{20}H_{19}NO_9Cl_2$) were isomeric ether glucuronides of hydroxylated diclofenac. Based on the literature these are likely to be conjugates of 4′-hydroxydiclofenac, 3′-hydroxydiclofenac or 5-hydroxydiclofenac.[9] Finally, diclofenac itself **g** ($M - H^- = 294.0098$, $C_{14}H_{11}NO_2Cl_2$) and a phase I monohydroxylated metabolite **f** ($M - H^- = 310.0038$, $C_{14}H_{11}NO_3Cl_2$) were also identified in the urine by oa-TOFMS. The spectroscopic data for the major urinary metabolites are summarised in Table 3. Additional HPLC/MS/NMR studies will be undertaken to determine the sites of biotransformation in these metabolites.

DISCUSSION

It is clear that the on-line combination of HPLC/ICPMS with oa-TOFMS provides a rapid and specific method for the detection and characterisation of chlorinated metabolites in biological samples. Quantification could also be performed based on the response of the detector to chlorine, which, unlike UV or molecular MS properties, will be independent of the structure of the metabolites. However, no detailed structural information, apart from the presence of chlorine, can be obtained on the metabolites from ICPMS alone. There is also no indication of the number of chlorine atoms present or whether co-elution of unresolved chlorine-containing metabolites occurs. Quantification of unknown chlorinated metabolites by HPLC/ICPMS alone will thus be subject to a number of caveats, similar to those based on the use of conventional radiolabels. However, as these results illustrate, the combination of HPLC/ICPMS with oa-

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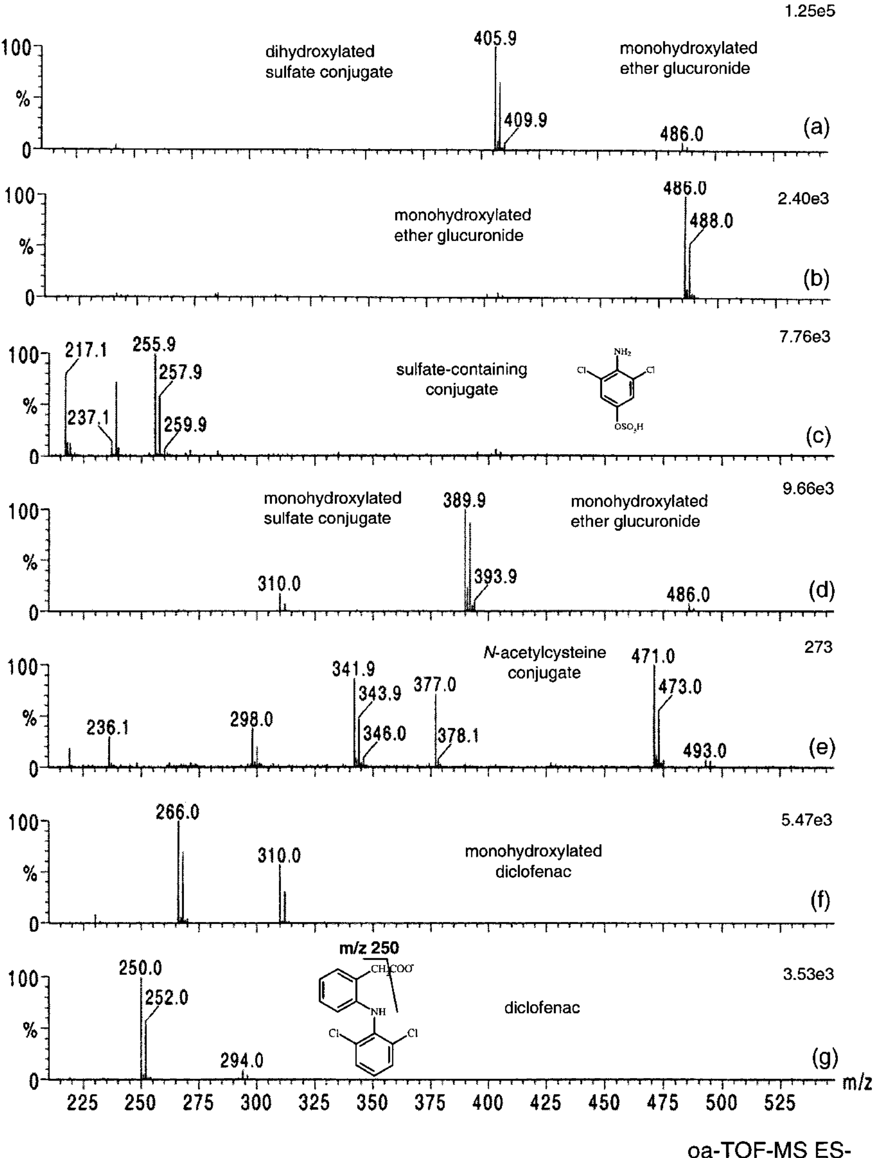

Figure 5. Extracted mass spectra for peaks **a** to **g** showing the distinctive chlorine isotopic substitution pattern, acquired by oa-TOFMS in −ve electrospray mode following injection of rat urine obtained 0–8 h post 30 mg/kg diclofenac.

TOFMS can enable both atomic and molecular mass spectra to be obtained simultaneously, thus providing a much more comprehensive description of the metabolite fate of a chlorine-containing drug (as well as an indication of the need to improve the separation in order to resolve co-eluting metabolites). Furthermore, the sensitivity of these mass spectrometers allowed the direct injection of untreated urine, which has potential benefits where unstable metabolites might be present.

The simultaneous analysis of phase I and II diclofenac metabolites directly in urine provided a realistic test of HPLC/ICPMS/oa-TOFMS for chlorinated drugs in intact biological samples. Based on the literature,[9] 40% of the administered diclofenac is recovered in urine in the period 0–48 h post dose. An estimated 10–15% of this is excreted in 0–8 h urine, of which 50% is accounted for by the sulfate conjugate of 4′-hydroxydiclofenac. Thus, the concentration of drug-related material injected on-column here was probably in the 200–1000 ng range, comprising of several metabolites. Unlike the ^{35}CI-ICPMS trace shown in Fig. 3(a), the ^{32}S-ICPMS trace shown in Fig. 3(b) was complicated by the presence of sulfur-containing molecules endogenous to urine. It was not possible to directly correlate drug-related molecules to observed peaks, although by using appropriate control samples the peaks due to endogenous substances could be eliminated. However, the information from the ^{35}Cl-ICPMS trace made it possible to identify the sulfur-containing diclofenac metabolites. Finally, the oa-TOFMS data on these peaks (Fig. 5) confirmed the presence of several sulfate conjugates and a previously unreported *in vivo* *N*-acetylcysteinyl conjugate of diclofenac.

CONCLUSIONS

These results have demonstrated that the combination of HPLC/UV-DAD/ICPMS/oa-TOFMS has great potential as a rapid, sensitive and highly selective tool for the qualitative detection and characterisation of chlorinated metabolites in biological fluids, without the need for authentic standards or

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radiolabelled drugs. Given the relatively widespread use of chlorine as a substituent in drugs and pesticides etc., HPLC/^{35}Cl-ICPMS/oa-TOFMS should be of widespread application in the study of the metabolic fate of these compounds.

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