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ORIGINAL ARTICLE

Geoffrey S Payne · Andrzej SK Dzik-Jurasz
Laura Mancini · Bernard Nutley · Florence Raynaud
Martin O Leach

Identification of biliary metabolites of ifosfamide using ^{31}P magnetic resonance spectroscopy and mass spectrometry

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Abstract Biliary excretion is a significant component in the metabolism of many drugs, but remains difficult to detect and characterise non-invasively. A previous publication recently described the detection of metabolites of ifosfamide in gall bladder in a guinea pig model using in vivo ^{1}H-decoupled ^{31}P 3-D magnetic resonance spectroscopic imaging and a clinical 1.5 T MR scanner.. Here high-resolution ^{31}P magnetic resonance spectroscopy (MRS) of extracted bile identifies peaks as parent ifosfamide (1.19 ± 1.47 mM; mean ± sd), carboxyifosfamide (2.04 ± 1.04 mM) and a major contribution from a previously unreported peak at 16.0 ppm (4.05 ± 2.38 mM). The unknown resonance was identified using liquid chromatography - mass spectrometry (LCMS) as the glutathione conjugate of ifosfamide (MW = 531). This was confirmed by analysing products from the reaction of glutathione with ifosfamide using LCMS and MRS. These results demonstrate how combined in vivo and analytical MRS, together with mass spectrometry, can help identify visceral routes of drug metabolism, thereby aiding understanding of ±drug disposition and mechanisms of action and toxicity. In particular, the distribution of ifosfamide and its metabolites into bile may be related to oxazophosphorine-related cholecystitis reported in patients.

G. S. Payne (✉) · A. S. Dzik-Jurasz · L. Mancini · M. O. Leach
Cancer Research UK Clinical Magnetic
Resonance Research Group,
Royal Marsden NHS Trust and
The Institute of Cancer Research,
Downs Road, Sutton, Surrey, SM25PT, UK
E-mail: gsp@icr.ac.uk

Present address: A. S. Dzik-Jurasz
Epix Pharmaceuticals, Cambridge, MA, USA

B. Nutley · F. Raynaud
Cancer Research UK Centre for Cancer Therapeutics,
The Institute of Cancer Research, Cotswold Road,
Sutton, Surrey, SM25NG, UK

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Introduction

Biliary excretion can have a significant influence on the pharmacokinetics and toxicology of many drugs and/or metabolites as many drugs undergo excretion via that route [1, 2]. Some drug classes such as the antibiotics may be chosen for their biliary accumulation especially in the treatment of biliary infection [3]. Drugs known to undergo biliary excretion [4] include docetaxel [5] and fluvastatin [6]. In addition, it is recognized that a wide spectrum of biliary excreted drugs may cause biliary dysfunction [7]. However, studies of biliary drug excretion in vivo using conventional methods remain highly invasive, requiring either direct ductal cannulation or the use of peroral endoscopic techniques [8]. Using in vivo ^{1}H-decoupled ^{31}P 3-D magnetic resonance spectroscopic imaging, and a clinical magnetic resonance (MR) scanner, we have recently observed in an animal model the biliary excretion of ^{31}P-containing metabolites of ifosfamide [9]. Ifosfamide is an alkylating agent used in the treatment of a wide range of diseases including cancer and autoimmune disease, and is metabolised to the active form in the liver [10].

Identification of the compounds detected in bile is critical for evaluating their clinical significance. High-resolution ^{31}P MR spectroscopy of extracted bile has shown the presence of several ifosfamide-related peaks not present in control bile. Several of the metabolites have been assigned on the basis of their chemical shift [9]. However, there are no references in the literature to a peak at the position of the dominant metabolite. Here, we describe the identification of this unknown biliary metabolite of ifosfamide, using analytical ^{31}P magnetic resonance spectroscopy (MRS) and mass spectrometry, as a glutathione conjugate of ifosfamide. We also

measure the concentrations of the different metabolites in the bile, and relate these quantities to the administered dose.

Materials and methods

Ten male Dunkin-Hartley guinea pigs (900 ± 20 g) were anaesthetised using intramuscular and intravenous fentanyl/fluanisone (0.5 mg/kg) and midazolam (0.5 mg/kg). The intramuscular route was used to induce anaesthesia with general anaesthesia being maintained via intravenous titration of the same anaesthetic. Venous access was secured via jugular vein cannulation. Ifosfamide (500 mg/kg) was infused through the cannula over approximately 15 min. All animal experiments were approved by the institution's animal ethics board. Animal care was prescribed by national and institutional standards. Approximately 2.5 h after ifosfamide administration (i.e. following in vivo MR measurements [9]), the animals were killed by CO_2 narcosis, the gall bladder was exposed and bile extracted using a sterile needle and syringe by direct puncture.

High-resolution^{31}P MRS ex vivo

High-resolution ^{31}P MR spectra were acquired of 600 µl bile samples, with 60 µl of D_2O added as a field lock, using 5-mm NMR tubes in an 11.74 T Bruker Avance spectrometer (Rheinstetten, Germany). Measurements were performed at room temperature (22°C). For each sample, 256 inverse-gated ^{1}H-decoupled acquisitions were obtained with a recovery delay of 20 s, giving a total measurement time of approximately 1.5 h—Measurements on one sample yielded approximate T_1s in the range 2–3.5 s for different metabolites, so a recovery time of 20 s should ensure the metabolites relax fully between acquisitions.

Mass spectrometer analysis

Bile samples were analysed by liquid chromatography mass spectrometry (LCMS), and metabolites further identified by liquid chromatography with liquid chromatography tandem mass spectrometry (LCMSMS).

A ThermoFinnigan (Hemel Hempstead, UK) "LCQ" ion trap mass spectrometer was used. Samples (25 µl) were injected into an Alltech Alltima C18 5u column (150 mm × 4.6 mm internal diameter). The mobile phase consisted of 0.1% aqueous formic acid (A) and methanol (B). The gradient (flow rate 1.0 ml/min.) started with 100% A, which was held isocratically for 0.5 min, followed by a linear increase to 10:90 (A:B) over 14 min and maintained at these conditions for a further 6.5 min. Eluant from the column entered into the mass spectrometer via a standard LCQ electrospray source operated in positive ion mode. Mass spectrometer conditions were: sheath gas 80, auxiliary gas 20 (both arbitrary units), capillary voltage 4.5 kV and heated capillary 250° C. The range of mass/charge investigated was 50–850, with the scan time set to an injection time of 200 ms or the time required to inject 2×10^8 ions if shorter.

Tandem mass spectrometry (MSMS) analysis spectra were also obtained by further fragmenting the ion selected with helium gas and energy in the ion trap.

Synthesis of glutathione conjugate of ifosfamide

Synthesis of the glutathione conjugate of ifosfamide was performed by mixing 50 mM ifosfamide and 50 mM glutathione in distilled water, titrated with sodium hydroxide to give a pH of 8.6. This pH was chosen to promote a reaction between glutathione and ifosfamide. In addition, the alkaline pH is characteristic of bile. The reaction was maintained at 27° C for 7 h and then analysed by LCMS as described above.

Results

High-Field^{31} P MRS studies of bile

An example high-resolution ^{31}P MR spectrum of bile is shown in Fig. 1, in which several ifosfamide-related peaks are observed. When a bile sample was spiked with ifosfamide, the amplitude of Peak 5 increased, which identifies it as ifosfamide. The frequency position was set to 15.85 ppm relative to H_3PO_4 [11]. Published data [11] then suggest from the chemical shift offsets that Peak 2 is carboxyifosfamide, while Peak 3 is either

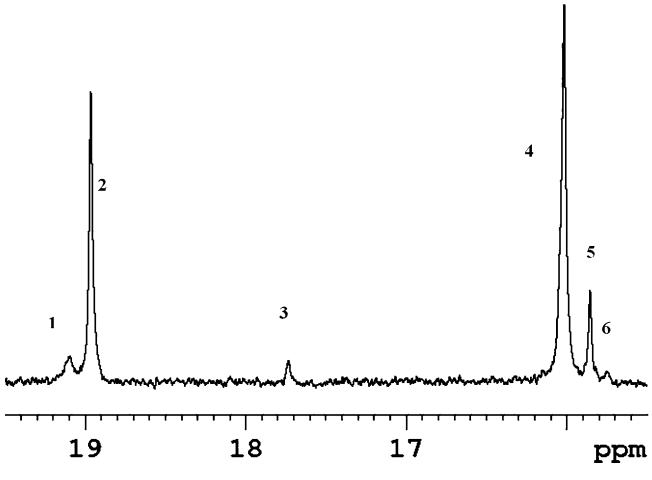

Fig. 1 ^{31}P MR spectrum from guinea pig bile taken approximately 2.5 h following administration of 500 mg/kg ifosfamide, measured at 11.74 T. Two hundred and fifty six inverse-gated acquistions were obtained with a recovery time of 20 s, giving a total acquisition time of approximately 1.5 h. Based on chemical shift and on spiking bile with known compounds, the peaks are identified as carboxyifosfamide (#2), 2- or 2,3-dechloroethyl ifosfamide (#3), and parent ifosfamide (#5). Based on the LCMS, work peak #4 is identified as a glutathione conjugate of ifosfamide. *Peaks (#1) and #6 have not been identified*

2-dechloroethyl ifosfamide or 2,3-dechloroethyl ifosfamide. However, the largest peak, at 16.02 ppm (Peak 4), has not been previously reported. Identification using Mass Spectrometry is described below. We have not attempted to identify Peak 1 or Peak 6 (which were only present in 5/10 samples).

Since data were acquired under fully relaxed conditions, it was possible to calculate concentrations from the signal amplitude relative to a 60 mM ifosfamide solution. Resonance frequencies are identical in all samples, but with some variation in concentraton (Table 1).

An estimate of the fraction of the administered ifosfamide present in the gall bladder at the time of measurement was made by assuming an average gallbladder volume of 4 cm^3. This indicates that a total of approximately 31.7 μ moles of ifosfamide-related compounds are measured in the bile, corresponding to approximately 1.8% of the injected ifosfamide dose. This estimate assumes that these compounds are fully MR-visible in the bile.

Results of LCMS analysis

Figure 2a shows the base peak trace from a sample of bile from an ifosfamide-treated guinea pig. The largest peak occurs at an elution time of 7.02 min, and is not present in control bile. In Fig. 2b is shown the selected ion trace at the mass/charge ratio = 261 as expected for ifosfamide, and corresponding to a retention time of 9.81 min. The full scan MS spectrum for the 9.81-minute peak is shown in Fig. 2d. This spectrum has both the correct $M+H^+$ ions ($m/z=261$ and 263) for a compound with the molecular formula of ifosfamide and the correct isotope ratio pattern for a compound containing two chlorine atoms. The ions seen at m/z 283 and 285 are consistent with the formation of sodium adducts ($M+Na^+$) of ifosfamide. Sodium adducts are often created in biological samples when analysed in electrospray-ionisation mode.

Figure 2c shows that the large peak at 7.02 min corresponds to $m/z=532$. Figure 2e shows the full scan spectrum for the peak eluting at 7.02 min. This spectrum is consistent with a compound having the molecular formula of the glutathione conjugate of ifosfamide where conjugation has occurred via reaction of one of the chlorine atoms of ifosfamide with the sulphur atom of glutathione. Such reactions of halogens with glutathione are well recognised in xenobiotic metabolism, often as a detoxification process [12].

The results suggest that the most intense ion (peak height 6.57×10^8) seen in the base peak trace and which had a retention time of 7.02 min. was derived from the glutathione conjugate of ifosfamide (m/z 532 trace) which had the same peak height and retention time. Although several other metabolites could be detected, they accounted for no more than 30% of the peak height of the glutathione ifosfamide conjugate. Unconjugated ifosfamide could still be detected in the bile as shown in the selected ion trace for ifosfamide (m/z 261). The peak height intensity for this compound (8.34×10^7), which had the same retention time and spectrum as pure ifosfamide and the compound spiked into bile (data not shown), is equivalent to about 13% of the peak height of the ifosfamide glutathione conjugate.

Table 1 Chemical shifts (mean and sd) and concentrations (mean and range) of ifosfamide-related peaks in bile ($n=10$; ND not detectable). The chemical shift is relative to H_3PO_4

Peak	ppm	Conc(mM)	Proportion of MR-visible Ifosfamide metabolites(%)
1	19.092 ± 0.001	0.49 (ND – 0.19)	6.1
2	18.964 ± 0.003	2.04 (0.2 – 3.8)	25.6
3	17.736 ± 0.004	0.16 (ND – 0.25)	2.0
4	16.017 ± 0.003	4.05 (1.05 – 8.1)	50.7
5	15.856 ± 0.002	1.19 (0.53 – 5.3)	15.0
6	15.749 0.004	0.055 (0 – 0.14)	0.7

Confirmation of identity of glutathione ifosfamide conjugate

Three approaches were used to confirm the identity of the major biliary metabolite as a glutathione conjugate of ifosfamide. The first of these was to compare the MSMS spectra of ifosfamide (Fig. 3a) and of glutathione (Fig. 3b) with that of the conjugate present in bile (Fig. 3c). The aim was to see if the pattern of fragmentation ions identified or mass differences between ions due to loss of neutral fragments could be related back to the structure of ifosfamide and glutathione within the proposed conjugate. The results show a similar pattern of neutral losses between glutathione and the conjugate. Examples include the loss of the gamma-glutamyl group (neutral loss of 129 Da) leading to formation of the m/z 179 ion in glutathione and m/z 403 ion in the conjugate. In addition, formation of the m/z 257 ion in the glutathione conjugate spectrum can only be derived from fragmentation of the ifosfamide moiety at the sulphur atom of glutathione.

The second approach to confirm the identity of this conjugate was to analyse the reaction products derived from mixing glutathione and ifosfamide under basic conditions as described above. The results showed that one of the major reaction products had the same retention time and full scan and MSMS spectra as the compound detected in bile (data not shown).

In addition, high-resolution ^{31}P MR spectra were acquired at 11.74 T of a glutathione-ifosfamide mixture after heating for 7 h (Fig. 4a), and of bile spiked with this glutathione-ifosfamide mixture (Fig. 4c). A spectrum of bile following administration of ifosfamide is shown for comparison (Fig. 4b). All experiments were performed with TR = 20 s and 32 acquisitions. In Fig. 4(a), Peak 3 is parent ifosfamide, while Peak 1 comprises the two possible glutathione conjugates in

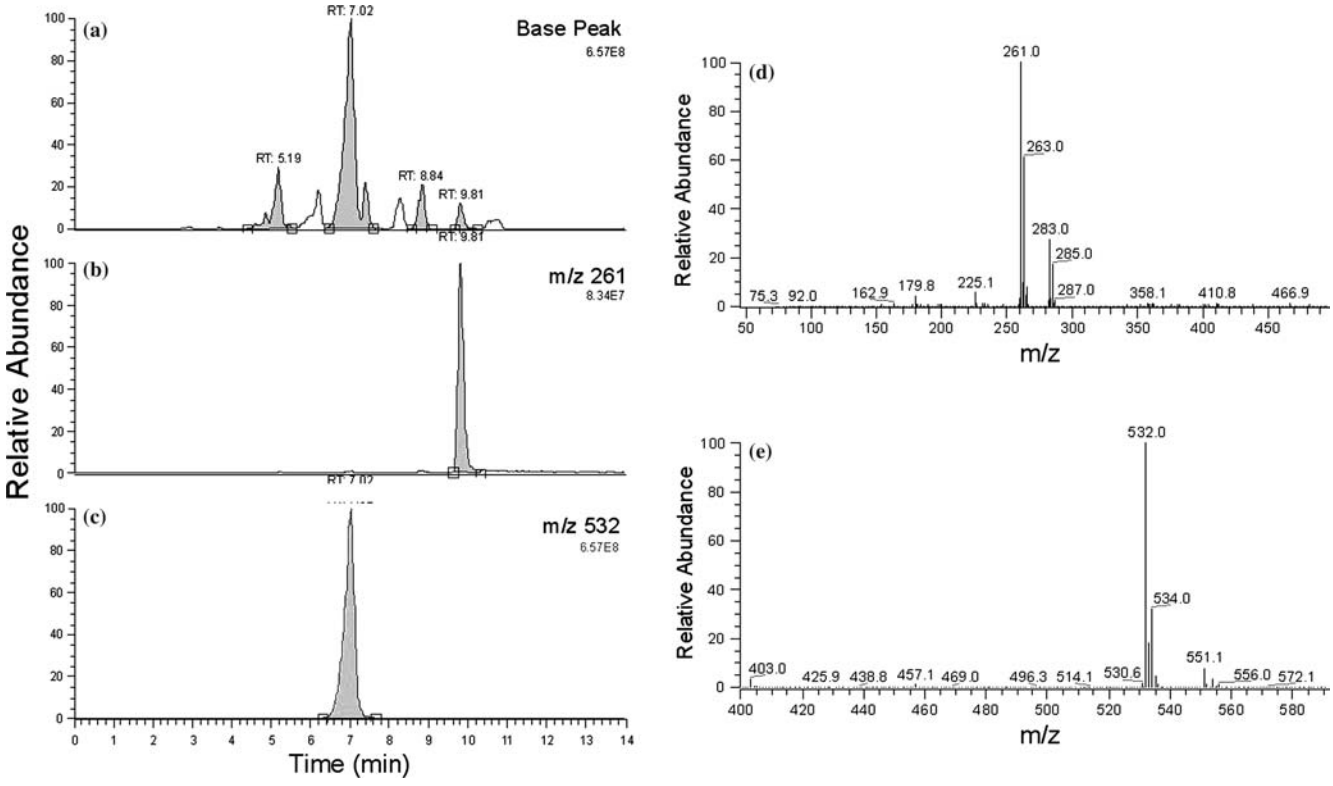

Fig. 2 LCMS data from bile of guinea pig following treatment with 500 mg/kg ifosfamide. **a** Relative signal as a function of time for the sum of all mass/charge ratios measured, **b** Selected ion trace for $m/z = 261$ **c** Selected ion trace for $m/z = 532$ **d** Full scan MS spectrum for peak eluting at 9.81 min **e** Full scan MS spectrum for peak eluting at 7.02 min

which one chlorine atom in ifosfamide is replaced by one glutathione. Peak 2 may be the double conjugate. In bile spiked by the glutathione-ifosfamide mixture, both peaks increase, supporting the assignment of the peak at 16.02 ppm as the glutathione conjugate.

Discussion

Some parent ifosfamide and several of its metabolites have been identified in the gall-bladder bile of a guinea pig model. The dominant metabolite has been shown to be a glutathione conjugate of ifosfamide; in general, such complexes represent end products in the detoxification pathways and therefore this complex is unlikely to have a pharmacological effect. Some parent ifosfamide has also been shown to be present, which is likely to be reabsorbed following excretion into the small bowel, and therefore contribute to a prolonged pharmacological effect. In the system studied, the amounts of all ifosfamide compounds present in bile are small compared with the administered dose. However, the presence of these ifosfamide metabolites has not previously been reported, and suggests that other metabolites not detectable with ^{31}P MRS (such as acrolein) may also be present, and which may have toxic consequences on the gall bladder, such as the reported cases of cholecystitis.

The presence and quantification of ifosfamide and its metabolites in human bile remain to be assessed. While it is not feasible to extract bile samples for high-resolution studies, in vivo MRS measurements have previously been performed in the gall bladder [13, 14], and give the opportunity for non-invasive measurements.

Gallbladder toxicity can be a significant event as acute cholecystitis carries a substantial morbidity and mortality. At particular risk are immunocompromised individuals and patients treated in the high-intensity setting. Gallbladder disease potentially leading to cholecystitis is prevalent in the population [15] though acalculous cholecystitis is becoming more widely recognized [16]. In addition, some drugs [17] including herbal remedies [18] are known to induce biliary disease. Many of these effects can lead to subsequent inflammation and infection though outright cholecystitis appears to be a rare event [19].

Conclusions

High-resolution ^{31}P MRS and mass spectrometry show that the main ifosfamide metabolite present in the gall bladder is a glutathione conjugate of ifosfamide. All visible ifosfamide metabolites together represent approximately 2% of the administered dose. Further work is required to assess implications of these metabolites for therapeutic effectiveness and gall-bladder toxicity in patients.

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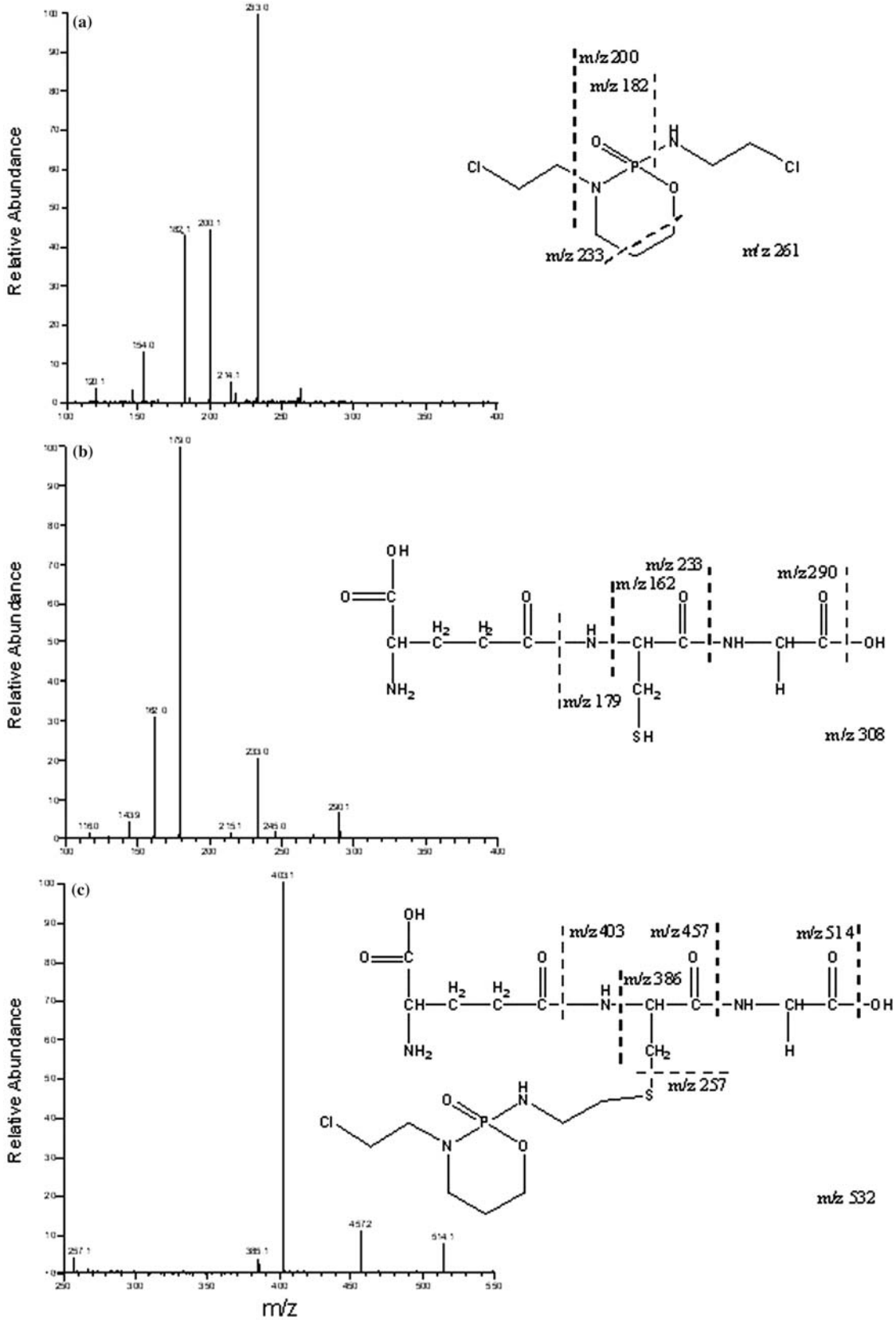

Fig. 3 Results of MSMS fragmentation measurements. **a** Parent ifosfamide **b** glutathione **c** peak eluting at 7.02 min from LC column of bile from ifosfamide-treated guinea pig

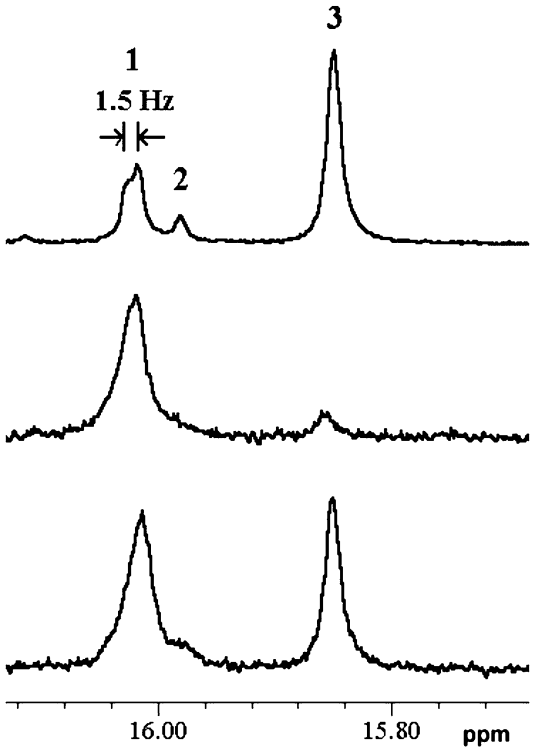

Fig. 4 ^{31}P MR spectra acquired at 11.74 T supporting the assignment of the peak at 16.02 ppm being a glutathione-ifosfamide conjugate. All experiments were performed with TR = 20 s and 32 acquisitions (total acquistion time approximately ten minutes). **a** The mixture of glutathione and ifosfamide after heating for 7 h. Peak 3 is parent ifosfamide, while Peak 1 shows the two possible glutathione conjugates in which one chlorine atom in ifosfamide is substituted by one glutathione. Peak 2 may be the double conjugate. **b** bile following administration of ifosfamide, **c** bile spiked with the glutathione-ifosfamide mixture. Both peaks increase, supporting the assignment of the peak at 16.02 ppm as the glutathione conjugate

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