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Application of inductively coupled plasma mass spectrometry and high-performance liquid chromatography—with parallel electrospray mass spectrometry to the investigation of the disposition and metabolic fate of 2-, 3- and 4-iodobenzoic acids in the rat

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Abstract

ICP-MS, HPLC-ICP-MS and HPLC-ICP-MS/ESI-MS have been applied to determine the disposition and metabolic fate of 2-, 3- and 4-iodobenzoic acids following intraperitoneal administration at 50 mg kg^{-1} to male bile duct cannulated rats. Quantitative excretion balance studies based on the determination of the total iodine content of urine and bile showed that all three iodobenzoic acids were rapidly excreted. Recoveries ranging from 95 to 105% of the administered doses were achieved within 24 h of administration. Metabolite profiles for urine and bile showed extensive metabolism with unchanged iodobenzoic acids forming a minor part of the total. A combination of alkaline hydrolysis and MS enabled the identification of the major metabolites of all three iodobenzoic acids as glycine and ester glucuronide conjugates with very little if any of the parent compounds excreted unchanged.
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1. Introduction

At all stages of drug discovery and development there is a need for information on the metabolism of candidate compounds. This requires the ability to identify and quantify unknown metabolites from in vitro or in vivo drug metabolism studies. Identification is often achieved using HPLC-MS, but for quantitative analysis of metabolites both MS and UV detection are inadequate as the response of these detectors is dependent on the molecular structure of the metabolites detected. Quantification thus requires authentic standards that are rarely available. To obtain quantitative metabolite data, radiochemical detection is usually applied but this requires the time-consuming and expensive synthesis of isotopically labelled compounds, which are not normally available in the early phases of drug discovery programs. There is hence a need for alternative methods of quantification.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is an element specific detection method and the sensitivity is in principle independent of the structure of the

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compound. This makes ICP-MS a promising technique for drug metabolism studies in that quantitative data can be obtained without the need for either standards or radiolabelling. The application of ICP-MS in combination with molecular mass spectrometry in the life sciences has recently been reviewed by Wind and Lehmann [1]. For substances containing heteroatoms such as phosphorous [2], sulphur [3,4], bromine [5], and chlorine [6], HPLC with ICP-MS detection can be applied to the analysis of complex mixtures, including biological fluids, for metabolism studies. Iodine, when present in a molecule, also provides an interesting opportunity for sensitive and specific detection and quantification and a limited number of applications exploiting this element have been reported. Thus, HPLC-ICP-MS, with iodine-specific detection, has been applied to pharmaceutical substances such as X-ray contrast media [2] or thyroid hormones, either in tablets [7], enzyme digests of bovine thyroid gland [8], in urine [9] or serum [10] and for analysis of iodine-containing compounds in human milk [11]. A recent HPLC-ICP-MS study has also demonstrated the use of this approach to obtain metabolite profiles following exposure of 2-fluoro-4-iodoaniline to earthworms [12]. We were therefore interested in examining the potential of ICP-MS, HPLC-ICP-MS and HPLC-ICP-MS/ESI-MS for investigating the metabolism and disposition of iodinated compounds in animals. Here, we describe a study of the fate of 2-, 3- and 4-iodobenzoic acids following i.p. dosing to bile-cannulated rats.

2. Experimental

2.1. Chemicals

2-, 3- and 4-iodobenzoic acids (98% purity) were purchased from Sigma–Aldrich Co. Ltd. (Dorset, UK). Ethanol, formic acid, sodium hydroxide and hydrochloric acid (37%) were of analytical grade and purchased from Fisher Scientific UK Ltd. (Loughborough, UK). Acetonitrile was of HPLC grade (Fisher Scientific UK Ltd., Loughborough, UK). Water was obtained from an Elga water purification system (Elgastat Maxima, Elga, High Wycombe, UK).

2.2. Animal dosing

Three male Wistar derived rats (250–300 g) were bile duct cannulated and acclimatised individually in glass metabolism cages 3 days prior to dosing. The animals were subjected to 12 h artificial light/dark cycles and were permitted free access to food and water throughout the study. Dosing solutions were made up by dissolving 2-, 3- and 4-iodobenzoic acid in 50% ethanol, adjusting the pH to 6.5–8 using diluted NaOH, to reach a final concentration of 50 mg ml^{-1} for 2- and 3-iodobenzoic acid and 40 mg ml^{-1} for 4-iodobenzoic acid. The rats were dosed i.p. with one compound per rat at 50 mg kg^{-1}. The remainder of the dosing solutions were stored at −20 °C until analysis. Urine and bile were collected prior to dosing and for the periods 0–6, 6–12, 12–24 and 24–48 h post-dose and stored at −20 °C until analysis. Cage wash was collected at 0–24 and 24–48 h postdose and stored at 4 °C until analysis.

2.3. Instrumental

Jasco PU-1580 HPLC pumps (Jasco Ltd., Great Dunmow, UK) and a CTC-HTC Pal autosampler (Presearch, Hitchin, UK) were used to introduce samples to a GVI Platform ICP-MS instrument (GV Instruments Ltd., Manchester, UK). The ICP-MS instrument was equipped with a PFA concentric nebuliser and a cooled double pass spray chamber and MassLynx software (GV Instruments Ltd.) was used for instrument control, data acquisition and analysis. The operating conditions of the instrument are shown in Table 1.

For determining the total iodine content of urine and bile for the excretion balance study chromatography was not performed and samples were introduced directly into the ICP-MS by flow injection into a carrier stream of water at a flow rate of 0.6 ml min^{-1}. For metabolite profiling, chromatographic separations were performed on a Polaris C18-A, 3 µm, 150 mm × 4.6 mm column with a matching guard column (Varian BV, Middelburg, the Netherlands) at a temperature of 40 °C obtained using an Eppendorph TC-50 column heater (Presearch). The mobile phase consisted of 30% acetonitrile and 0.1% formic acid in water for analysis of 3- and 4-iodobenzoic acid metabolites or 20% acetonitrile and 0.1% formic acid in water for analysis of 2-iodobenzoic acid metabolites. The flow rate of 1 ml min^{-1} was split using an accurate splitter (Presearch) allowing 50 µl min^{-1} into the ICP-MS and the remainder into a Jasco UV-1575 detector set at 254 nm. To avoid excess carbon build-up on the cones of the ICP-MS, the nebuliser gas was mixed with argon/oxygen (95/5%, v/v).

For the multiple hyphenation experiments, where both ICP-MS and ESI-MS techniques (HPLC-ICP-MS/ESI-MS) were used, the eluent (1 ml min^{-1}) was split with 50 µl min^{-1}

Table 1
Instrument operating conditions for ICP-MS

Parameter	Excretion balance	Metabolite profiling
Cooling gas flow (L min^{-1})	17.0	18.0
Plasma gas flow (L min^{-1})	0.8	0.8
Nebuliser gas flow (argon) (L min^{-1})	0.8	0.2
Nebuliser gas flow (argon/oxygen, 95/5%, v/v) (L min^{-1})	0	0.5
Plasma power (W)	1400	1650
Dwell time (ms)	300	300
Mass monitored	127	127
Spray chamber temperature (°C)	8	−7

directed into the ICP-MS and 250 μl min^{-1} into the UV detector and then to a API-365 triple quadrupole mass spectrometer (Applied Biosystems, Warrington, UK). A turbo ionspray inlet source was used in the positive mode and a Q1 full scan over the range *m/z* 100–600 was acquired. The source temperature was 450 °C, ionisation energy 4500 V, turbo ion spray gas 7 L min^{-1}, nebulising gas 14 (arbitrary figure), curtain gas 10 (arbitrary figure), declustering potential 20 mV, focusing potential 200 mV, entrance potential 10 mV and the mass set at unit resolution. Analyst software was used for instrument control, data acquisition and analysis.

2.4. Sample analysis

For the excretion balance study, a stock solution of 150 mM 2-iodobenzoic acid was prepared in 0.2 M NaOH. This was further diluted in control urine to prepare standard solutions of 0.1, 0.5, 1, 5, 10, 15 and 20 mM. A similar procedure was used to prepare quality control samples at final concentrations of 0.1, 5 and 20 mM. Standard solutions in bile were prepared by diluting the stock solution in control bile to concentrations of 0.1, 0.5, 1 and 5 mM with quality control samples at 0.15 and 5 mM. For analysis of cage wash and dosing solutions, aqueous standard solutions were prepared at concentrations of 0.1, 5, 10, 20 mM and quality control samples of 5 and 10 mM. The dosing solutions were diluted with water (1 + 49) and analysed in triplicate. The urine, bile and cage wash samples were analysed in duplicate. Samples were introduced by flow injection to the ICP with an injection volume of 10 μl.

For metabolite profiling, 20 μl of neat urine and bile samples were injected on column. Selected urine and bile samples were further analysed by HPLC-MS and subjected to base hydrolysis to obtain information on the identity of the metabolites. Base hydrolysis was performed by incubating samples with 1 M NaOH (2 + 1) at 37 °C for 24 h and then restoring the pH by adding an equivalent amount of 1 M HCl. Then 40 μl of the hydrolysed samples were injected on column.

3. Results and discussion

3.1. Excretion balance study

In order to determine the disposition of dosed compounds it is normal practice to perform an “excretion balance” study whereby the amount of compound-related material is determined in the excreta. This provides quantitative information on the rate and routes of elimination of the drug or xenobiotic. Bile cannulated animals were used here to avoid the problems often associated with poor extraction recoveries from faeces. For sample analysis, a method based on total iodine content in the samples was developed and partially validated. Linear standard curves ($r^2 > 0.993$) were obtained in urine, bile and water over the concentration range 0.1–20 mM iodine, which covered the concentration ranges found in the samples, with concentrations below 0.1 mM considered irrelevant. Based on the quality control samples, values for accuracy were found in the range 94–105% with relative standard deviations below 11% as seen in Table 2. Similar values were obtained regardless of the matrix used, indicating that the performance of the method was not matrix dependent. The ICP-MS method thus appeared to be well suited to determining iodine content in urine, bile and aqueous samples in order to obtain excretion balance data for iodobenzoic acid dosed to rats.

Table 2
Accuracy and precision data for ICP-MS analysis of iodine at *m/z* 127

Matrix	Iodine concentration (mM)	*n*	Accuracy (%)	R.S.D. (%)
Urine	0.1	6	101.5	8.2
	5	6	94.2	6.0
	20	6	97.9	6.7
Bile	0.15	6	104.4	10.2
	5	6	100.4	10.2
Water	5	3	98.5	9.9
	10	3	100.2	3.7

Table 3
Average iodine concentration obtained in dosing solutions ($n = 3$) and samples ($n = 2$) after i.p. dosing of 2-, 3- and 4-iodobenzoic acid to one rat per compound at 50 mg kg^{-1}*) iodine concentration <0.1 mM

Sample	Iodine concentration (mM)		
	2-Iodobenzoic acid	3-Iodobenzoic acid	4-Iodobenzoic acid
Dosing solution	3.52	3.08	2.62
Urine predose	–*	–	–
Urine 0–6 h	12.3	9.36	10.1
Urine 6–12 h	3.84	4.18	18.1
Urine 12–24 h	0.33	0.19	0.53
Urine 24–48 h	–	–	–
Bile predose	–	–	–
Bile 0–6 h	0.52	0.43	0.96
Bile 6–48 h	–	–	–
Cage wash	–	–	–

Table 4
Percent recovery in urine, bile and cage wash of 2, 3- and 4-iodobenzoic acids dosed i.p. to one rat per compound at 50 mg kg^{-1}

Sample	% Dose recovered		
	2-Iodobenzoic acid	3-Iodobenzoic acid	4-Iodobenzoic acid
Urine 0–6 h	70.1	76.4	22.5
Urine 6–12 h	16.0	6.1	32.4
Urine 12–24 h	4.8	10.9	26.4
Urine 24–48 h	0.0	0.0	0.0
Bile 0–6 h	11.1	8.2	12.1
Bile 6–48 h	0.0	0.0	0.0
Cage wash	0.0	0.0	0.0
Total	102.0	101.6	93.4

Following admimistration of 2-, 3- and 4-iodobenzoic acids at 50 mg kg^{-1} i.p. to bile duct cannulated rats urine, bile and aqueous cage wash were collected. The measured iodine concentrations in the dosing solutions, cage wash, urine and bile samples are shown in Table 3. These results show that practically no iodine was present in the predose samples or cage wash, and the highest iodine concentrations were found in the 0–12 h post-dose urine samples. As shown in Table 4, essentially a quantitative recovery of the test compounds was seen over the 24 h time course of the study via urine and bile (102, 101 and 93% for 2-, 3- and 4-iodobenzoic acids, respectively). The excretion of all three iodobenzoic acids was rapid with approximately 90% of the dose recovered in the 0–24 h urine and the remainder excreted in the bile within 6 h.

These results clearly demonstrate, for the first time, the potential of ICP-MS for quantitative excretion balance studies to be performed based on iodine-containing compounds without the need for radiolabelling.

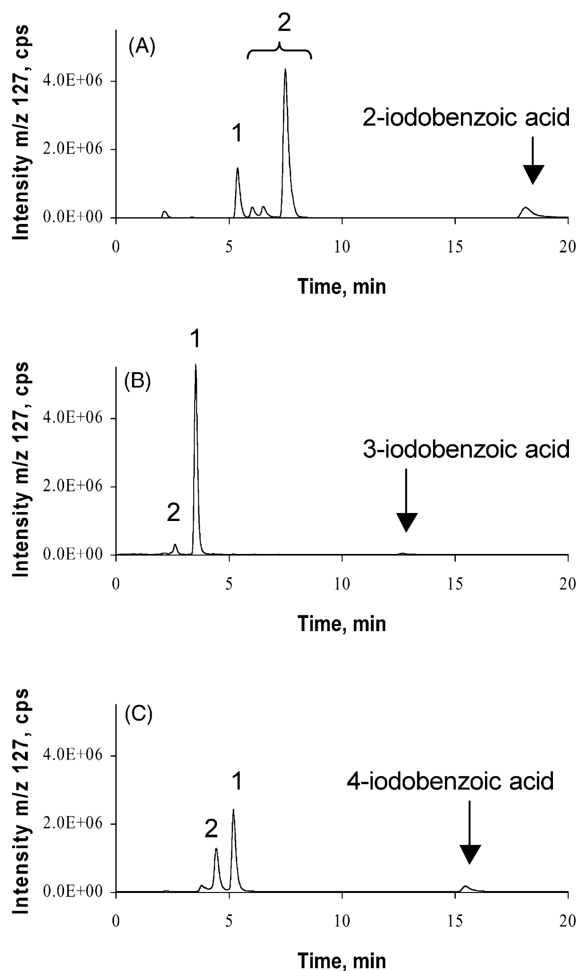

Fig. 1. 0–6 h urine profiles from (A) 2-iodobenzoic acid (B) 3-iodobenzoic acid and (C) 4-iodobenzoic acid. Peak identification: (1) glycine conjugates. (2) Ester glucuronide conjugates. 20 μl neat urine was injected on a Polaris C18-A, 150 mm × 4.6 mm column, 40 °C, eluted isocratically with 20% (in A) or 30% (in B and C) acetonitrile and 0.1% formic acid. Detection with ICP-MS at m/z 127.

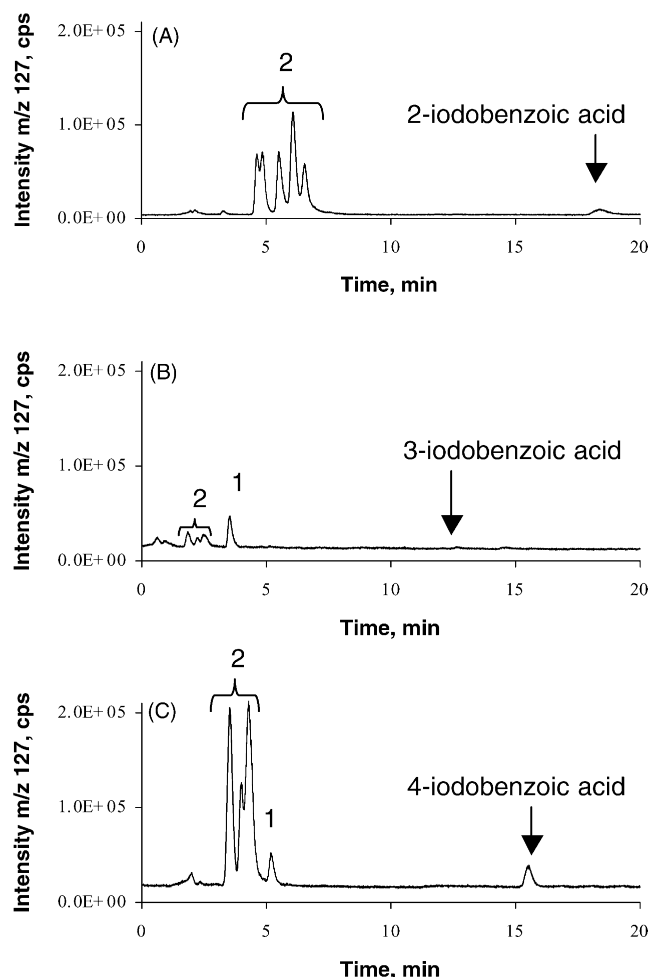

Fig. 2. 0–6 h bile profiles from (A) 2-iodobenzoic acid (B) 3-iodobenzoic acid and (C) 4-iodobenzoic acid. Peak identification: (1) glycine conjugates. (2) Ester glucuronide conjugates. 20 μl neat bile was injected on a Polaris C18-A, 150 mm × 4.6 mm column, 40 °C eluted isocratically with 20% (in A) or 30% (in B and C) acetonitrile and 0.1% formic acid. Detection with ICP-MS at m/z 127.

3.2. Metabolite profiling and identification

As well as excretion balance data, it is also very valuable to be able to obtain additional information regarding the metabolic fate of test compounds. The metabolism of 2-, 3- and 4-iodobenzoic acid was therefore investigated by profiling the urine and bile samples by HPLC-ICP-MS. Previous studies [7–12] have demonstrated the use of reversed-phase HPLC with ICP-MS as an iodine detector for drug substances and metabolites. However, for quantitative analysis the coupling of HPLC with ICP-MS has some limitations due to the influence of organic solvents on the plasma stability and analyte sensitivity [7–9,13]. For gradient elution HPLC, the changing amounts of organic solvents may result in varying responses and therefore an isocratic method for these samples was developed to avoid this issue. For HPLC-ICP-MS, methanol is generally preferred over acetonitrile as an organic modifier because of the lower carbon loading to

the plasma. However, acceptable resolution of the iodobenzoic acid metabolites was not achieved within a reasonable analysis time using isocratic methanol-based solvent systems whilst chromatographic methods based on 20–30% acetonitrile and 0.1% formic acid were able to achieve the required separation. To minimise the organic loading to the plasma the flow was split to direct only 50 $\mu l\,min^{-1}$ to the ICP-MS. Oxygen was also added to the nebuliser gas to avoid excess carbon build-up on the torch and cones of the ICP-MS.

The metabolite profiles of the 0–6 h urine and 0–6 h bile samples obtained for 2-, 3- and 4-iodobenzoic acids, based on iodine detection, are shown in Figs. 1 and 2. For the 6–48 h samples, the profiles were qualitatively similar and are hence not shown. The pre-dose samples showed only trivial amounts of iodine (data not shown) indicating that all the iodine-containing peaks were iodobenzoic acid-related compounds. These profiles reveal that 2-, 3- and 4-iodobenzoic acids were extensively metabolised with little unchanged parent compound excreted. For all three iodobenzoic acids, the urinary metabolite profile was dominated by one or two major peaks. For bile a more complex profile was generally obtained, with no particular metabolite predominating.

Since ICP-MS is an element-detection technique, these profiles do not reveal any structural information of the metabolites and other measures are required to elucidate the identity of the metabolites and purity of the peaks. For this purpose mass spectrometry is usually applied, and here a mass spectrometer was connected in parallel, as we have described elsewhere [5,6,14] to the HPLC-ICP-MS instruments along with a UV detector. As an example of the type of data aquired, typical total ion current full scan MS, UV and iodine detected ICP-MS chromatograms are shown in Fig. 3a–c, respectively for 4-iodobenzoic acid. Both the UV and the MS chromatograms show peaks from compound-related material as well as endogenous compounds further highlighting, if it were needed, the selectivity of the iodine-detected ICP-MS for quantitative metabolite profiling.

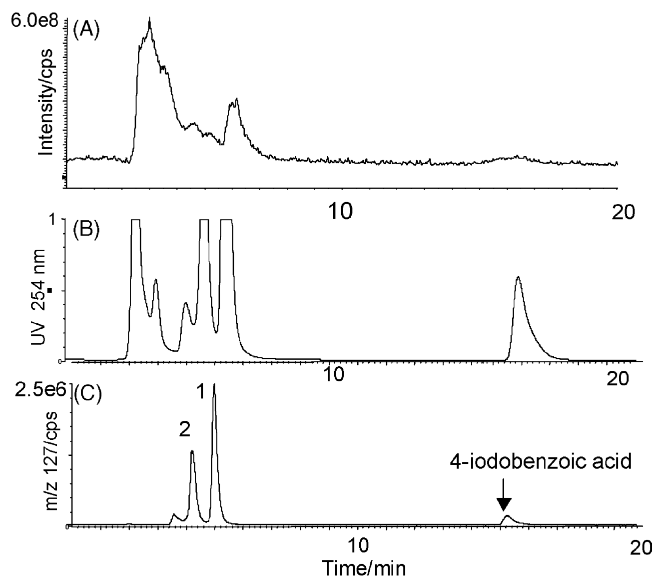

Fig. 3. 0–6 h urine profile following dosing of 4-iodobenzoic acid. Reversed phase isocratic HPLC with three modes of detection: (A) total ion current full scan MS m/z 100–600, (B) UV 254 nm and (C) iodine detection by ICP-MS at m/z 127. Peak identification: (1) glycine conjugates. (2) Ester glucuronide conjugates.

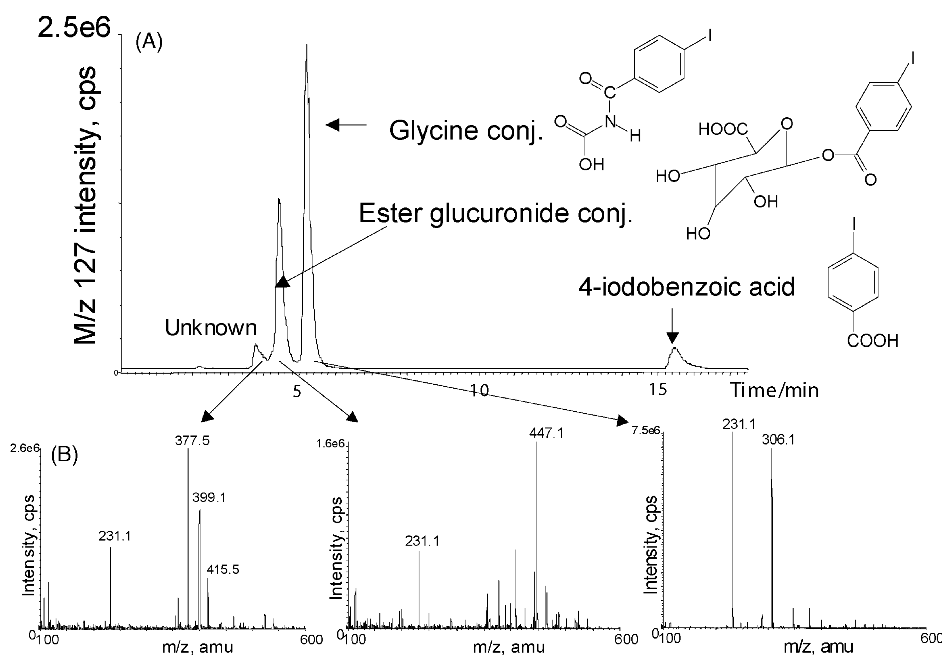

Fig. 4. 0–6 h urine profile following dosing of 4-iodobenzoic acid. Reversed phase isocratic HPLC with (A) iodine detection by ICP-MS at m/z 127 and (B) MS spectra obtained from metabolite peaks.

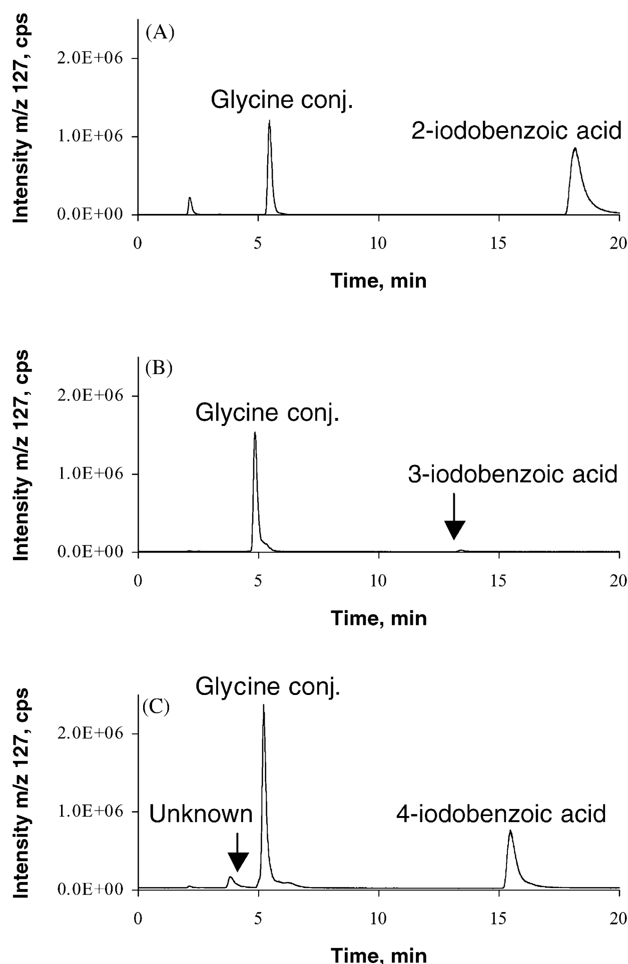

Fig. 5. Profiles after alkaline hydrolysis of urine samples 0–6 h from (A) 2-iodobenzoic acid (B) 3-iodobenzoic acid and (C) 4-iodobenzoic acid. 20 μl neat urine was treated with 1 M NaOH and injected on a Polaris C18-A, 150 mm × 4.6 mm column, 40 °C eluted isocratically with 20% (in A) or 30% (in B and C) and 0.1% formic acid. Detection with ICP-MS at *m/z* 127.

To identify the individual metabolites, iodine-detected peaks in the ICP chromatogram were used as guides to pinpoint relevant peaks in the total ion current mass chromatogram from which mass spectra were extracted. For the urine profile of 4-iodobenzoic acid, shown in Fig. 4, the major peak at 5.2 min had a *m/z* of 306 suggesting a glycine conjugate. An ion with a *m/z* of 447 was found in the other major peak (eluting at 4.4 min) suggesting the presence of an ester glucuronide (a typical metabolite of a small aromatic acid such as this), seen as a sodium adduct. A minor component with a *m/z* of 377 (and possibly sodium and potassium adducts at 399 and 415) at 3.8 min was also observed, but the identification of this metabolite was not pursued further as it accounted for a very small proportion of the dose. A common signal of *m/z* 231 was observed in all the mass spectra, most likely representing the intact iodobenzoyl group. For the bile profile of 4-iodobenzoic acid, the same metabolites were observed but in addition several further peaks with a *m/z* of 447 were present suggesting the presence of transacylated ester glucuronides. These findings are consistent with the literature, in that both glycine and ester glucuronide conjugates are widely found as benzoic acid metabolites [15]. In the urine profiles from 2- and 3-iodobenzoic acid, similar *m/z* values (306, 447) were found. However, in the case of 2-iodobenzoic acid the glycine conjugate was observed to elute before the ester glucuronides, reversing the order seen for both the 3- and 4-iodobenzoic acid metabolites. In the bile, several peaks at *m/z* 447 for putative transacylated ester glucuronides were observed in the profiles of both 2- and 3-benzoic acids. However, the ion at *m/z* 306 for the glycine conjugate was only detected in the 3-iodobenzoic acid samples.

To further investigate the identity of the metabolites, the urine and bile samples were treated with alkali to hydrolyse ester glucuronides (glycine and ether glucuronide conjugates are stable under these conditions). As seen in the urine HPLC-ICP-MS profiles in Fig. 5, the peaks postulated to be ester glucuronides all disappeared after alkaline hydrolysis with a related increase in peak area of the parent compound. In contrast, the glycine conjugates remained unaffected as did the minor unknown metabolite of 4-iodobenzoic acid. Similar effects were observed for the bile samples when alkaline hydrolysis was performed (data not shown). In summary 2-, 3- and 4-iodobenzoic acid were metabolised in the rat primarily to either glycine conjugates or ester glucuronides, but to varying degrees depending on the structure of the parent compound. These results are broadly similar to those obtained on rats and perfused rat kidneys using ^{125}I-labelled benzoic acids [16]. However, a direct comparison is not possible because of differences in the doses used (ca 0.5 mg kg^{-1}).

Quantification of the individual metabolites based on the HPLC-ICP-MS chromatograms was performed by relating the relative peak areas of the metabolites to the total iodine content found in the excretion balance study. By using this ap-

Table 5
Percent formation of ester glucuronides and glycine conjugates in urine and bile after dosing of 2-, 3- and 4-iodobenzoic acid i.p. to rats at 50 mg kg^{-1}

	% Formation								
	2-Iodobenzoic acid			3-Iodobenzoic acid			4-Iodobenzoic acid		
	Urine	Bile	Total	Urine	Bile	Total	Urine	Bile	Total
Glucuronides	60.3	10.7	71.0	5.4	4.6	10.0	18.6	9.7	28.3
Glycine conj.	19.5	0.0	19.5	87.1	3.6	90.7	58.1	0.7	58.8
Parent	11.0	0.4	11.4	0.8	0.0	0.8	3.7	1.8	5.5
Total	90.8	11.1	101.9	93.3	8.2	101.5	80.4	12.2	92.6

proach, the total degree of glycine and ester glucuronide formation of the 2-, 3- and 4-iodobenzoic acids was determined (see Table 5). The results show that 2-iodobenzoic acid was mainly glucuronidated whereas 3-iodobenzoic acid primarily formed glycine conjugates. For 4-iodobenzoic acid, the balance between glycine conjugation and ester glucuronidation was similar but the former still accounted for the majority of the dose. The unknown metabolite of 4-iodobenzoic acid accounted for less than 1% and is not presented in the table.

4. Conclusion

This limited study of the application of HPLC-ICP-MS to iodine-specific detection of the metabolites of 2-, 3- and 4-iodobenzoic acids in the urine and bile of rats clearly demonstrates the potential for the technique as an alternative to the use of radiolabelled compounds. Quantitative excretion balances could be performed as well as metabolite profiling. The study further shows the possibility of gaining additional information by having MS detection in parallel with ICP-MS detection.

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