A Study Utilising High Temperature UPLC with Mass Spectrometric Detection to Quantify the Metabolites of 2-, 3- and 4- Bromobenzoic Acids both *In Vitro* and *In Vivo*

The use of radiolabels (^{14}C and ^{3}H) during drug development programmes as a tracer for drug related materials during metabolic profiling is both extremely useful and expensive. An alternative method may be utilised dependent upon the presence of suitable elements within the drug and that is inductively coupled plasma mass spectrometry (ICP-MS) which has been successfully used in excretion pathway [1-3] and metabolic profiling studies [4-5]. Conventional mass spectrometers may also be coupled to provide structural information on compound(s) of interest [4-5]. There are downsides to the use of ICP-MS with the use of organic modifiers during HPLC studies which can lead to problems with the plasma which may result in reduced sensitivity and stability. Some of the problems may be addressed with the use of oxygen to the argon carrier gas but it is not a complete solution as plasma stress then comes into play.

Chromatographic theory predicts that as water increases in temperature it behaves in a manner analogous to a mobile phase containing organic modifiers in that its elutropic strength increases. Pure water at high enough temperatures has been shown [6-10] to be a useful mobile phase allowing elution of a wide series of differing polarity compounds than water at room temperature. This technique of HTLC has found use in metabolite profiling [6-10] where a wide range of polarities may be encountered with thermal gradients using pure water to elute the compounds as opposed to the standard practice of gradients based on increasing eluotropic strengths. Here we show how using HTLC with thermal gradients and ICP-MS and MS-MS in parallel as detection systems, isomers of bromobenzoic acid may be studied and analysed under both In Vito and In Vivo conditions thereby further demonstrating the possibilities of this new approach.

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EXPERIMENTAL

Chemicals

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

Sample Preparation

Standards solutions of 10 $mg.mL^{-1}$ of the individual bromobenzoic acids (2-BBA, 3-BBA and 4-BBA) were made up in water and then further dilutions prepared by taking 10 µL of the stock solution and diluting with 990 µL of water to make 1 $\mu g.mL^{-1}$ solutions.

In Vitro Studies

All of the bromobenzoic acid substrates were individually incubated with freshly prepared hepatocytes derived from male Wistar rats. Hepatocyte yield and viability was determined and from this the cell suspensions were diluted with the incubation buffer (made up of 100 mL of Hanks balance salt solution (895 mL of water and 5 mL of 7.5% $NaHCO_3$ + $MgCl_2$), to give cell counts of approximately 2 million cells / mL and the suspension (4 mL) transferred to incubation flasks (chosen to allow good air exchange). The flasks were placed in a shaking warm water bath maintained at approximately 37°C and allowed to equilibrate for a few minutes, at which point the individual benzoic acids were added to give a final concentration of 2.5 $\mu g.mL^{-1}$. Control incubations consisted of flasks containing each acid and buffer only, while another contained hepatocytes with 7-ethoxycoumarin (a model substrate used to demonstrate the qualitative metabolic viability of the cells). At intervals between zero and 4 hours, 0.5 mL aliquots were removed from the flasks and metabolism stopped by addition of an equal volume of ice-cold ethanol. Further aliquots of the incubations were also taken to assess cell viability during the experiment. The denatured samples were centrifuged for 3 minutes at 12000 rpm (d=15cm) and then stored at approximately -20°C until analysed. At all times the flasks and samples were protected from light (covered with aluminium foil) to prevent possible photolysis of substrates and products [11].

In Vivo Studies

Urine samples containing bromobenzoic acid metabolites were obtained from a previously described study, performed in bile cannulated rats following oral administration at 50 $mg.kg^{-1}$ [5].

INSTRUMENTAL

High Temperature Liquid Chromatography

In the hot water configuration an Acquity UPLC system (Waters, Milford, USA) was used to provide sample introduction (25 µL injection volume), solvent delivery and pressure control to the LC column contained in a GC-2014 oven (Shimadzu, Milton Keynes, UK). The GC oven was used to deliver the temperature gradient conditions found in *Table 2*. The separations were performed using a C18 bonded, 3.5 µm Gemini 50 x 2.1 mm column (Phenomenex, Macclesfield, UK) and the mobile phase was water with 0.1% formic acid. From the column the eluent was directed to a GVI Platform ICP-MS (GV Instruments Ltd, Manchester, UK) and an API 4000 Qtrap (Applied Biosystem, Warrington, UK) via a splitter valve set at 70%: 30% respectively. The ICP-MS was equipped with a Meinhard concentric nebuliser and a cooled double pass spray chamber with helium gas added to the collision cell to reduce argon-based interferences in the mass 80 region ($^{40}Ar^{40}Ar^{1}H^{+}$, $^{40}Ar^{38}Ar^{1}H^{+}$). MassLynx software (GV Instruments Ltd, Manchester, UK) was used for instrument control, data acquisition and analysis. The operating conditions of the instruments are shown in *Table 1*. The API 4000 Qtrap used a turbo ionspray inlet source in positive mode with a scan range of m/z 100-600. Analyst software was used for instrument control, data acquisition and analysis.

Table 1. Instrument operating conditions for ICP-MS.

Parameter	Setting
Cooling gas flow	18.0 L min^{-1}
Plasma gas flow	0.8 L min^{-1}
Nebuliser gas flow (argon)	0.8 L min^{-1}
Helium gas flow	1.0 mL min^{-1}
Plasma power	1600 W
Dwell time	300 ms
Mass monitored	79
Spray chamber temperature	-7°C

Conventional Chromatographic Conditions

The conventional HPLC separation, with methanol as the organic modifier, used the same HPLC system and column as above without the GC oven. The gradient began with 95% aqueous formic acid (0.1% v/v): 5% methanol held for one minute then rising though a linear gradient to 95% methanol: aqueous 5% formic acid over 5 minutes at a flow of 1 $mL.min^{-1}$. The data was collected on the ICP-MS as a bromatogram (m/z 79) and quantified using MassLynx software. The peaks were confirmed used the API 4000 Qtrap with the same instrument settings as employed for the HTLC methodology.

RESULTS AND DISCUSSION

Development of HTLC Conditions

For this work we required a single set of separation condition that would enable the metabolite profiling of all of three analytes following incubation with hepatocytes in ca. 10 min.

However, a general isothermal separation for all three bromobenzoic acids proved impossible to attain. Thus, at 100°C 2- and 3-bromobenzoic acids were eluted ca. 5 min or less but 4-bromobenzoic acid only eluted after 20 min. Raising the temperature to 110 °C reduced the retention time of 4 brombenzoic acid to 4 minutes, but eluted the 2- and 3-bromobenzoic acids in less than 2 minutes, which was too rapid for metabolite profiling.

As a result a temperature gradients were then investigated exploring the range 100 to 110 °C. As the viscosity of water decreases with temperature we exploited this to increase the flow rate, whilst maintaining a constant back pressure of 9000 psi through the separation (as described elsewhere [6]) to optimise the analysis and minimise run times. A number of temperature and flow gradients were tried to achieve the optimum analysis with the final temperature gradient used shown in *Table 1*, and the flow gradient illustrated in *Figure 1* which shows how the flow rate was increased with time and the increase in temperature to maintain a constant pressure in the system.

The metabolites and parent acids were eluted as the temperature was raised from 100 to 110 °C, after which the temperature was ramped to 135 °C to remove sample contaminants from column.

Table 2. Temperature gradient profile (with constant pressure at 9000 psi maintained by changing flow rate).

Time (min)	Temperature (°C)	Rate (°C min^{-1})
0	100	0
10	110	1
15	135	5
17	50	2
19	100	2

Between runs the temperature was reduced to 50°C and held for 5 minutes. This procedure was found to be essential to ensure that the column temperature could reliably be returned to the starting temperature of 100°C prior to the next analysis (simply reducing the oven temperature to 100°C meant that much longer equilibration times were needed).

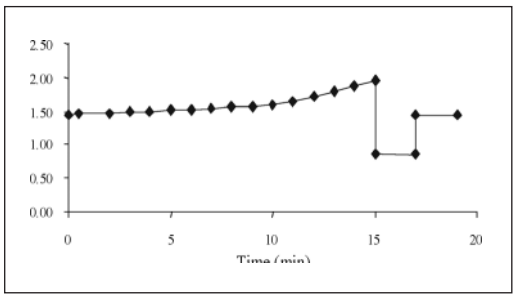

Figure 1. Graph indicating changing flow rate to maintain constant pressure in the column of 9000 psi.

In Vitro Sample Analysis

Having obtained HTLC conditions that resulted in good retention of the various parent compounds, metabolic profiles were obtained for each of the 2-, 3- and 4-bromobenzoic acid samples resulting from their in vitro incubation with rat hepatocytes. As well as profiling and quantification using ICP-MS detection via the Br element molecular information was obtained using the API 4000 Qtrap for structure determination. Representative 0 and 4hr profiles obtained following incubation of 4-bromobenzoic acid with hepatocytes are shown in *Figure 2* (obtained by monitoring m/z 79).

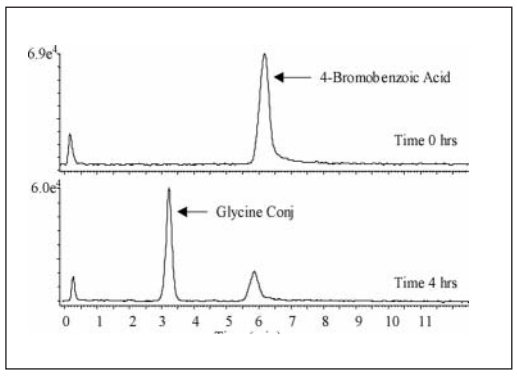

Figure 2. Profiles of 4-bromobenzoic acid after injection of hepatocyte incubate at T 0 hrs and T 4 hrs separated via a temperature gradient at constant pressure. The chromatograms were obtained by monitoring m/z 79. The amount of parent at T 0 hrs was measured at 2.2 µg.mL^{-1} reducing to 0.7 µg.mL^{-1} at T 4 hrs. The Glycine moiety at T 4 hrs was corresponded to 1.85 µg.mL^{-1}.

At time 0 hr a major bromine peak was seen for 4-bromobenzoic acid with a minor component eluting at the solvent front corresponding to inorganic bromide (present as a contaminant). The concentration of 4-BBA corresponded to 2.2 µg.mL^{-1}, or 55 ng on column (22 ng of bromine). After incubation with hepatocytes for 4 hours a new peak was detected in the bromatogram with a retention time of 3.2 minutes, corresponding to a concentration of ca 1.8 µg.mL^{-1} (45 ng eq. on column, 18 ng of bromine). Similarly the 2- and 3-substituted bromobenzoic acid profiles also showed differing amounts of metabolism to other peaks as seen in *Table 3*.

Table 3. Parent and glycine conjugate concentrations for the time period 0 to 4hrs for the hepatocytes incubation.

Time points (hr)	2 BBA (µg.mL^{-1})		Time points (hr)	3 BBA (µg.mL^{-1})		Time points (hr)	4 BBA (µg.mL^{-1})	
	A 1.4 (min)	B 2.4 (min)		A 3.4 (min)	B 6.0 (min)		A 3.3 (min)	B 6.3 (min)
0	-	n/a	0	-	2.3	0	-	2.2
0.5	0.03	2.1	0.5	0.2	1.1	0.5	0.2	1.4
1	0.4	1.5	1	0.5	1.0	1	0.5	1.4
2	0.1	1.5	2	1.0	0.7	2	0.9	0.8
4	0.2	1.3	4	2.0	0.5	4	1.8	0.7

Identification of the metabolite peaks was performed via MS using the API 4000 Qtrap. This analysis showed that the major peak resulting from the metabolism of all of the bromobenzoic acids by hepatocytes was the respective glycine conjugate, which gave observed m/z values 258/260 (sodium adducts m/z 280/282). Fragments m/z 183/185 were also observed, which were the result of in source fragmentation of the glycine conjugates [5]. Whilst all three compounds were metabolised by this route in the in vitro hepatocytes incubations 2-BBA showed reduced glycine conjugate formation compared to the 3- and 4- positional isomers.

Clearly, when using such high temperatures there may be concerns over stability, both of the analytes and the chromatographic system itself. With respect to the latter we noted no changes in retention or decreases in the efficiency of the chromatographic system during the analysis of the 30 in vitro samples run though the system here. To address the possibility that on-column degradation of the metabolites might be taking place we re-analysed then same samples using a conventional reversed-phase gradient chromatographic system. The results for 4-BBA are shown in *Figure 3*, with the conventional chromatography giving a very similar result to the HTLC separation (peak identity was reconfirmed by MS). The data from both chromatographic separations were compared and the peak ratios were found to be the same whichever technique was used, indicating that the high temperature had not adversely affected sample analysis (as shown in *Table 4*).

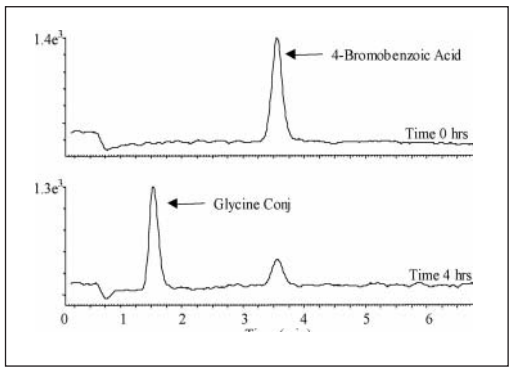

Figure 3. Profiles of 4-bromobenzoic acid after injection of hepatocyte incubate at T 0 hrs and T 4 hrs separated on a conventional gradient system consisting of methanol with 0.1% formic acid. The parent to glycine peaks ratio of 2.7 in the 4 hr sample for conventional LC was comparable to the HTLC result of 2.6.

Table 4. Comparison of the glycine and parent BBA area ratio on both conventional LC and HTLC for 3 BBA and 4 BBA.

3 BBA Time points	Hot water	conventional
0.5	0.2	0.2
1	0.5	0.6
2	1.5	1.4
4	3.4	3.4
4 BBA Time points	**Hot water**	**conventional**
0.5	0.1	0.2
1.0	0.4	0.4
2.0	1.0	1.0
4.0	2.6	2.7

A further benefit that derived from the use of HTLC with an entirely aqueous mobile phase was a marked increase in sensitivity for ICP-MS detection of bromine as indicated by an increase in the absolute signal (from ca. 1.4e^3, to 6.9e^4 in the case of 4-BBA when analysed by HPLC or HTLC respectively) and the obvious improvement in signal to noise for the HT separation. The reduced sensitivity of the conventional chromatographic method no doubt resulted from the need to add oxygen to the carrier gas to react with the carbon from the organic mobile phase in the plasma to produce carbon monoxide, which is then removed from the system.

The use of oxygen in this way minimises the problems associated with the use of organic modifiers in HPLC but has the unwanted effect of reducing the sensitivity of the detection of elements by placing the plasma under stress.

In Vivo Samples Analysis

Whilst in vitro studies can be extremely informative there is also a need for the analysis of samples such as urine and bile obtained from in vivo studies. Such samples are much more complex than those derived from in vitro incubations, and also can show a more complex metabolic profile. We therefore took the opportunity to reanalyse samples for these BBAs that had been obtained in an in vivo study undertaken on bile duct cannulated rats (previously reported by Jensen et al [5]) to see if these samples also gave comparable data on HTLC vs conventional HPLC.

In *Figure 4* a bromatogram showing the urine profile obtained after the direct injection of 10 µL of a urine sample obtained for the period 0-6 hrs following dosing with 3-bromobenzoic acid is shown.

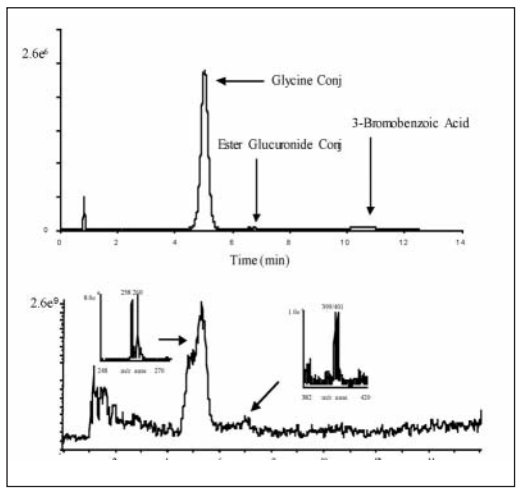

Figure 4. Urine profile (0-6 hrs) following dosage of 3- Bromobenzoic acid. Neat urine (10 µL) was analysed via HTLC as used for the in vitro incubations: A) bromatogram showing the metabolite profile, B) total ion chromatogram m/z 100-650 with inserts to show the molecular ions of the glycine (m/z 258/260) and ester glucuronide (m/z 399/401) conjugates.

This sample contained (as described in the original paper) the glycine conjugate as the major metabolite together with a small amount of the ester glucuronide conjugate (m/z 399/401). The profiles obtained for this sample (and indeed for all three compounds for the 0-6 hour post dose samples) was comparable to the original profile obtained using the conventional methodology. This is particularly noteworthy in the case of the ester glucuronides, which are relatively labile compared to the glycine conjugates and might have been anticipated to be subject to significant thermal degradation. `

CONCLUSIONS

It has been shown that the use of HTLC-ICP-MS, with parallel MS provides a useful alternative to conventional HPLC for the generation of metabolite profiles for bromine-containing compounds such as these BBAs. The results obtained for the metabolites of 2-, 3- and 4-bromobenzoic acids in vitro and in vivo were comparable to conventional LC indicating that the high temperatures used were not detrimental to the samples. The use of superheated water, with temperature programming to effect elution, instead of a more conventional water-organic mobile phase also had the added advantage of providing increased ICP-MS sensitivity compared to conventional HPLC.

References

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