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Short communication

Quantitative urinalysis of the mercapturic acid conjugates of allyl formate using high-resolution NMR spectroscopy

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Abstract

As end products of xenobiotic metabolism via glutathione conjugation, mercapturic acids (MCAs) can be used as markers to indicate exposure to allylic compounds as well as the rate and efficiency of their excretion. In addition, the formation of certain MCAs indicates metabolism via the known toxin acrolein, a strong electrophile. High-resolution ^{1}H NMR spectroscopy has been employed to quantitatively measure the presence of MCAs in the urine of Sprague–Dawley rats, collected in the 8 h following 25 and 50 mg kg^{-1} i.p. doses of allyl formate (AF), a model toxin. 3-Hydroxypropylmercapturic acid (HPMA) was found to be the only ^{1}H NMR-observable MCA excreted in the urine, exhibiting a percentage recovery of ∼20% at the 25 mg kg^{-1} dose level, and ∼30% at the 50 mg kg^{-1} dose level.

Keywords: Allyl formate; 3-Hydroxypropylmercapturic acid; Acrolein toxicity; NMR

1. Introduction

Allyl formate (AF) is a model hepatotoxin that has been reported to cause severe hepatic haemorrhagic periportal necrosis in rat following intraperitoneal dosing [1]. This toxicity results from conversion to allyl alcohol and subsequent formation of the toxic intermediate acrolein. A number of studies have focused on the metabolism and toxicity of allylic compounds, notably acrolein [2], allyl alcohol [3], allylamine [4], allyl halides [5], allyl cyanide [6], and allyl formate [1] (for a review see [7]), many of which have been found not only in industrial environments, but also in environmental pollutants, such as tobacco smoke. The biochemical analyses performed in these studies indicated that the major metabolic products are 3-hydroxypropylmercapturic acid (HPMA) and 2-carboxyethylmercapturic acid (CEMA), end products of the detoxification of acrolein via glutathione (GSH) conjugation (Fig. 1), or allyl mercapturic acid (ALMA), the result of direct GSH conjugation. Other studies showed that these were also the major metabolites for numerous other compounds including cyclophosphamide [8,9] and bromopropane [10]. In light of these studies, MCAs are now widely considered biomarkers for exposure to electrophilic chemicals [7,11,12].

From a health perspective, simple methods for quantitatively measuring the excretion of MCAs are useful as they can form part of a procedure to assess the degree of exposure to a number of electrophilic xenobiotics and help identify high-risk groups or safety issues in industrial environments [12].

MCAs have previously been identified and quantified by various methods, the most common being mass spectrometry (MS) and UV spectroscopy. Many of the methods described in the literature require derivitisation, radiolabelled compounds, or the use of HPLC for analytical separation [12].

High-resolution ^{1}H NMR spectroscopy has been successfully used for structural determination of urinary components [13,14], and lends itself to metabolite quantification due to the intrinsic property that while less sensitive than MS by many orders of magnitudes, NMR spectra can provide a

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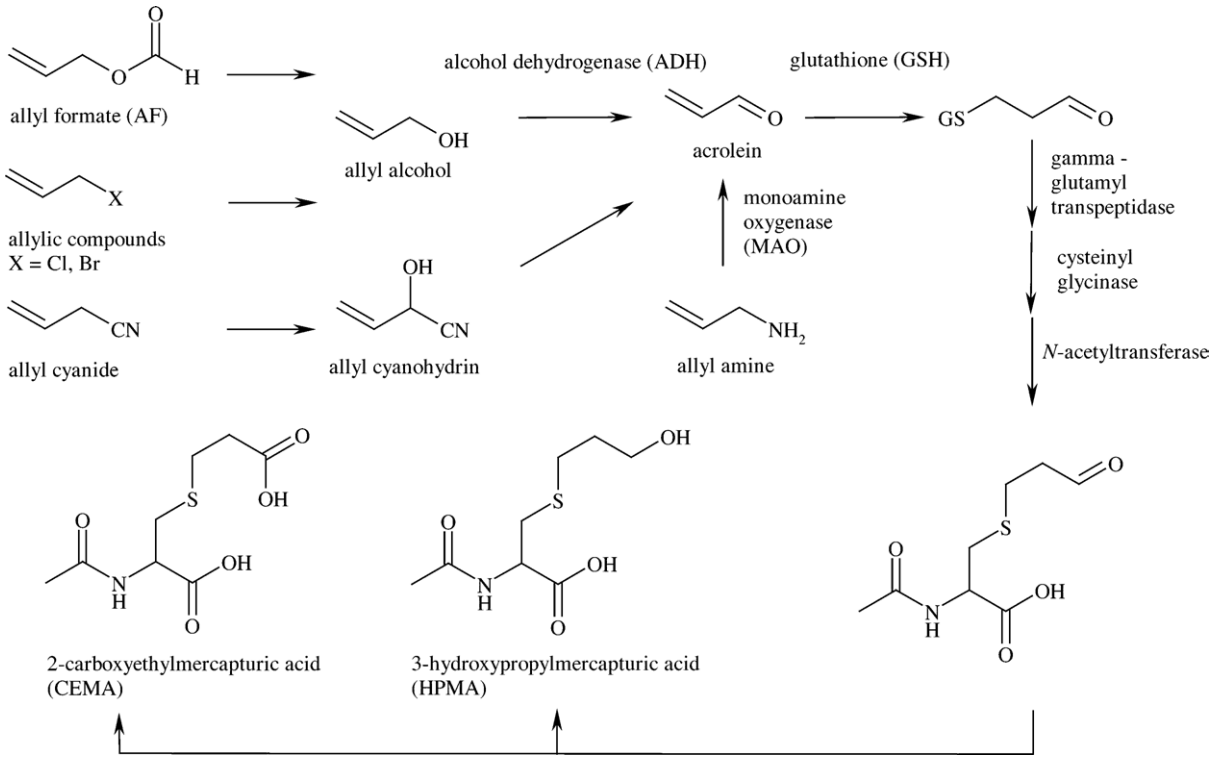

Fig. 1. The major route of detoxification of several allylic compounds via glutathione conjugation with acrolein.

huge range of information about biofluid components, their relative concentrations and their internal connectivity. It has the advantage that it is a simple, non-destructive technique requiring little sample preparation—ideal for certain applications in biological monitoring. High-resolution ^{1}H NMR has been used for metabolite structure elucidaton of MCAs, but received only a passing mention with respect to their quantification [15].

In this study we have quantified the MCA present in the urine of rats following administration of AF, using a calibration curve derived from *N*-acetylcysteine. We confirm that AF is partially metabolised via the GSH conjugation pathway, the major metabolite being HPMA. The percentage recovery of AF as HPMA appeared to alter with dose, but the significance of this could not be tested because of the large margin of error in sample volume measurements. The histopathological findings of this study confirm the known hepatotoxicity of AF, and highlighted the variability in response in the rat, which may relate to a large variation in HPMA recovery between individual animals.

2. Experimental

2.1. Chemicals and dosing

Allyl formate (ICN, Lot No. 23327) was administered to male, 5–6-week-old Sprague–Dawley rats, housed in individual metabolism cages ($21 \pm 3\,^{\circ}$C, relative humidity $55 \pm 15\%$). A standard diet (Purina chow 5002) and fresh water (acidified to pH 2.5 using HCl to prevent microbial growth) was available to each animal ad libitum. Twelve-hour light/dark cycles were maintained throughout the study, including a 6-day adaptation period. Animals were randomly separated into three dose groups (vehicle only (control), 25 and 50 mg kg^{-1}), receiving their dose in 10 ml saline i.p.

2.2. Sample collection

Urine samples were collected for 16 h prior to dosing, and then 0–8 and 8–24 h post-dose. The volume of each collection was recorded to the nearest 1 ml. Samples were stored at $-40\,^{\circ}$C until required for spectroscopic analysis. Prior to analysis, samples were thawed and centrifuged at 1200 *g* for 10 min to remove suspended solid debris. Two-hundred microliters of each sample was added to 200 μl phosphate buffer (pH 7.4, 0.2 M) containing D_2O (20%) and sodium 3-(trimethylsilyl)[2,2,3,3-$^{2}H_4$]propionate (TSP, 0.3 mM), and 200 μl HPLC-grade water, giving a final sample volume of 600 μl.

Rats were killed by exsanguination following anaesthesia using CO_2 after 48 h (five animals from each dose group) and 168 h (five animals from each dose group). Following death, animals were subjected to a thorough histopathological examination. Note: further collections of urine were made up until 48 and 168 h post-dose as part of another study, hence rats were not killed after the 24 h urine sample was taken. Collections made after 24 h were not analysed as part of this study.

2.3. ^{1}H NMR measurements

High-resolution ^{1}H NMR spectroscopy was performed on samples using a Bruker DRX600 spectrometer equipped with a 5 mm flow-probe, with samples introduced using a BEST flow-injection system (Bruker Biospin, Karlsruhe, Germany). Acquisitions were made using a proton resonance frequency of 600.13 MHz at 298 K.

A set of inversion recovery experiments were used to determine which resonance of interest had the longest t_1 relaxation time. One-dimensional NMR spectra were obtained using a NOESY [16] pulse sequence [90°–t_1–90°–t_m–AQ], with a t_1 time of 6 μs. A sufficient recycle delay (>5 ($T_{1(longest)}$)) was chosen so that full t_1 relaxation of the ^{1}H nuclei in the sample occurred. The water signal was suppressed by applying weak rf irradiation (power equivalent to 50 Hz) to the water resonance for 2 s at the beginning of each pulse sequence iteration and during the mixing time (t_m, 0.10 s). Sixty-four scans were used to obtain each spectrum following eight dummy scans. Each of the spectra were collected into 32 K data points, with a total spectral width of 20 ppm.

An exponential line-broadening factor of 0.3 Hz was applied to each FID, followed by Fourier transformation to give frequency domain spectra. Analysis of these spectra was performed on an Octane workstation (Silicon Graphics, California, USA) using XWIN NMR v3.1 (Bruker Biospin, Karlsruhe, Germany). Spectra were phase-corrected, referenced to TSP (δ 0.00), and baseline corrected manually. A spectrum of a pooled rat urine sample was taken to represent a typical background NMR signal. Difference spectra were obtained by subtraction of this background from each ^{1}H spectrum. The peak integral of the *N*-acetyl methyl protons in each difference spectrum was measured relative to that of the TSP reference (intensity of 1.00) in the original spectrum, and used for quantification as described below.

No repeat acquisitions were made to demonstrate reproducibilty of ^{1}H NMR analysis of urine, as this has previously been investigated by Keun et al. [17]. The use of biological replicates (n = 10 for each dose group) was intended to help fairly represent the biological variation between animals, but also subsumes variation due to the analysis procedure used.

A 2D COSY [18] spectrum was obtained using a sequence with pulsed field gradients for coherence selection and with water presaturation during the recycle delay of 2 s. Thirty-two collected scans of 2 K data points were obtained for each of 256 increments with a spectral width of 10.5 ppm in both transformed dimensions.

A sine-bell apodisation function and a line broadening of 0.3 Hz was applied prior to Fourier transformation in both dimensions. No linear prediction methods were employed.

2.4. Calibration

In this study, a calibration curve was constructed using *N*-acetylcysteine, the unconjugated equivalent of a MCA. The ^{1}H NMR spectrum of *N*-acetylcysteine shows a clear singlet at δ 2.06–2.08, a resonance that arises from the methyl group protons attached to the acetyl group. The equivalent region in the rat urine spectrum is uncluttered with other resonances, making this peak easily identifiable, and useful for quantification.

N-acetylcysteine was obtained from Sigma (St. Louis, Missouri, USA). Two-hundred-microliter aliquots of *N*-acetylcysteine solution were added to 200 μl control rat urine and 200 μl HPLC-grade water at concentrations of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mM.

The peak integral of the *N*-acetyl methyl protons in the difference spectra were plotted against the *N*-acetylcysteine concentration values and linear regression was used to derive a relation between the observed peak integral following subtraction of the control baseline, and the concentration of *N*-acetyl species present in each sample. The intercept of the line was set to zero—the value of a control spectrum subtracted from a control spectrum, giving the relation:

$$x = \frac{(y_{obs} - c)}{k}$$

where x is concentration of *N*-acetyl species; y_{obs} the *N*-acetyl peak integral relative to TSP; c the y-intercept of linear regression (set to zero); k the gradient of linear regression.

This relation was used to determine the urinary concentration of *N*-acetyl species in each sample. The percentage recovery was then calculated using the urinary volume, dose level and animal weight.

A calibration curve was also constructed for *N*-acetylcysteine in pure aqueous solution. Samples were prepared identically to the urine samples, except the 200 μl urine was substituted for an additional 200 μl HPLC-grade water. These samples were analysed in a similar way to the 'spiked' urine set.

3. Results

3.1. Clinical and histopathological findings

Rats in both dose groups had clear clinical signs such as piloerection and diarrhoea.

Individual reaction in the 50 mg kg^{-1} group showed considerable variation. Some animals exhibited no treatment-related changes other than the above clinical signs in contrast to others that displayed changes in clinical signs, clinical chemistry, macroscopic morphology and microscopic histopathology.

Of the rats killed after 48 h, three in the high dose group exhibited multifocal perilobular subacute parenchymal necrosis, including one that had several greyish foci on the main lobe of the liver approximately 10 mm in diameter.

Of the rats killed after 168 h, one in the high dose group displayed slight multifocal perilobular subacute

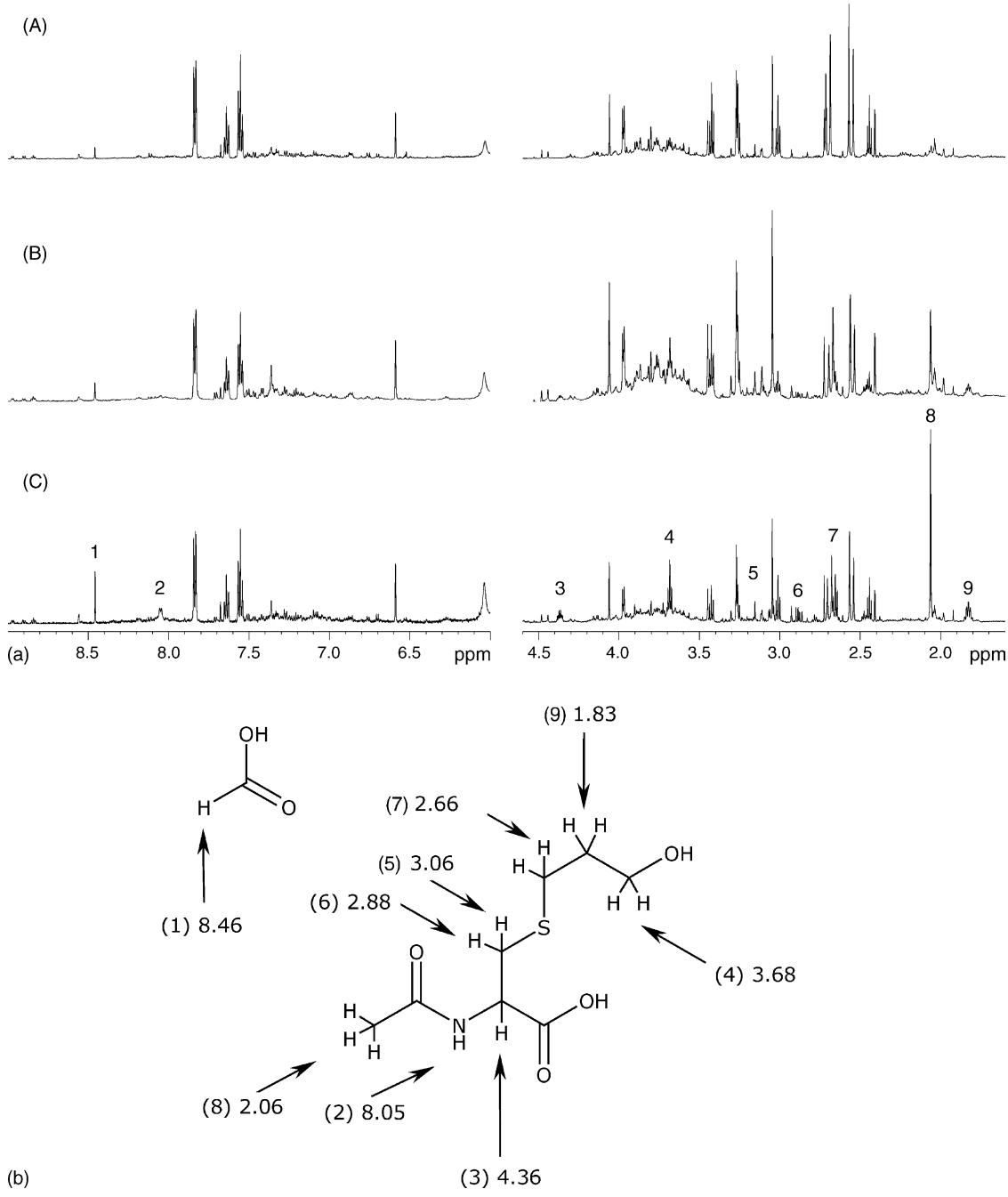

Fig. 2. (a) 1D NOESY 600 MHz ^{1}H NMR spectra of rat urine at three AF dose levels: Control (A), 25 mg kg^{-1} (B), 50 mg kg^{-1} (C). Numbered resonances correspond to those arising from HPMA, assigned in (b). (b) ^{1}H NMR assignments for HPMA and formic acid. Numbers correspond to peaks in (a). Chemical shifts are given in ppm.

parenchymal necrosis. Adjacent to the parenchymal necrosis this animal showed moderate focal subchronic granulomatous inflammation including slight proliferation of cholangial cells. This rat also exhibited yellow discoloration of the entire liver and an abscess 20 mm in diameter in the caudate lobe of the liver. This was considered to be treatment related. The variation in the observed histopathology may relate to variation in the recovery of AF as HPMA.

3.2. Metabolite identification

The spectra obtained for the samples collected in the 0–8 h time period showed a marked increase in intensity of the resonances arising from *N*-acetylcysteine conjugate protons. In fact the major observable increases in the ^{1}H NMR spectrum can be assigned to HPMA (Fig. 2). Chemical shifts for these resonances were in agreement with published literature; δ 1.83 m 2H ($HOCH_2C\mathit{H}_2CH_2S$–); δ 2.66 t

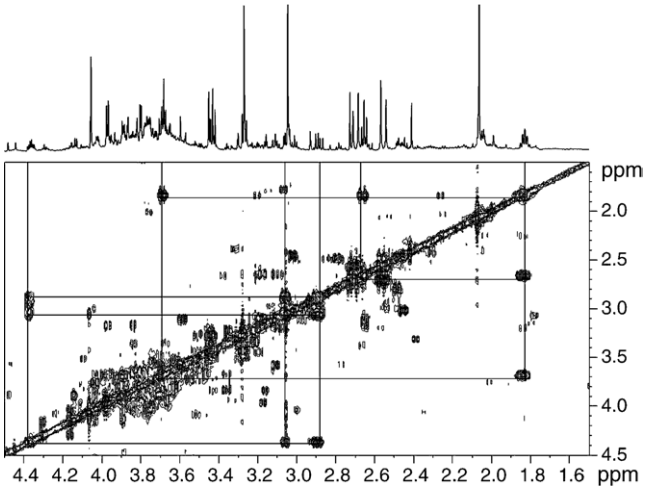

Fig. 3. 1D NOESY (top) and 2D COSY (bottom) 600 MHz ^{1}H NMR spectra of rat urine collected 0–8 h after dosing with 50 mg kg^{-1} AF i.p. The connectivity of HPMA protons is indicated.

2H ($HOCH_2CH_2CH_2S$–); δ 3.68 t 2H ($HOCH_2CH_2CH_2S$–); δ 2.88 m 1H and 3.08 m 1H ($-SCH_2CH$); δ 4.36 m 1H ($-SCH_2CH$); δ 2.06 s 3H (CH_3CO–); δ 8.05 s broad ($H_3CCONHCH$). The 2D COSY spectrum (Fig. 3) is consistent with the pattern of cross-peaks expected for HPMA, previously reported by Boor et al. [4].

The spectra of the urine samples collected from dosed animals in the 8–24 h period showed no indication of AF metabolites.

In addition to the assignments made for HPMA, one other peak intensity increase was observed—a singlet at δ 8.46. This can be assigned to the α-proton of formic acid moiety.

3.3. Metabolite quantification

Interactions between calibration compounds and their surrounding biofluid matrix, and effects caused by urinary components such as high salt concentrations, may distort the linearity of the signal/concentration relationship observed in NMR by reducing the rf power that is applied during the acquisition. In this instance, the peak intensities for the *N*-acetylcysteine calibration samples showed a strong linear correlation with the sample concentration over the observed concentration range, both in the presence ($R^2 = 0.997$) and absence ($R^2 = 0.997$) of urine, indicating that such distortions are of low magnitude. The relation between peak integral area and *N*-acetylcysteine concentration took the form $x = (y_{obs} - c)/1.02$ as explained in Section 2.4.

Increases in *N*-acetyl resonances, following dosing with AF, were assumed to have arisen from the excretion of MCAs alone, and not from other species with chemical shifts in the same region. Excretion of metabolites that give rise to background resonances in the *N*-acetyl region of the spectrum was assumed to remain constant in dosed and control animals, hence a representative control spectrum was used to subtract the baseline signals not arising from *N*-acetylcysteine species. The mean recovery of AF as HPMA for the low dose and high dose animals was 20 ± 3 and 30 ± 3%, respectively. The maximum possible error in the mean recovery introduced by imprecision in the measurement of urinary volume was estimated to be less than that apparently caused by biological variability, i.e. <±3%. Hence there would appear to be some differences in HPMA recovery according to dose, but the high variability across animals in recovery and urine volume mean that it is difficult to draw detailed conclusions. The mean urinary volume observed for the high dose group was nearly double that of the low dose group. This may well be a good indicator of the effects AF has on rat metabolism—either reducing the efficiency of water retention or stimulating release.

4. Discussion

Sprague–Dawley rats were given single intraperitoneal doses of AF at either 25 or 50 mg kg^{-1}. Urine was collected during the 0–8 and 8–24 h periods post-dose. Initial ^{1}H NMR analysis of the collections showed the only observable differences between dosed and control samples was the presence of HPMA, an MCA. This is the known major metabolite of AF, and was excreted during the first 8 h post-dose and not beyond, in addition to an increase in the concentration of formic acid.

The histopathological examination carried out as part of this study confirmed that AF evokes signs of acute, localised hepatotoxicity, which has been previously observed [1]. Much literature has been devoted to showing that allylic compounds are not generally the causative agent of observed toxicity, but that it is acrolein that is the toxic species. The conversion of allyl alcohol to acrolein is mediated by alcohol dehydrogenase, which is localised in the periportal region of the liver. This localisation, and thus the site of acrolein formation in the liver is now accepted as the reason AF causes region-specific necrosis.

A calibration curve was constructed using *N*-acetylcysteine, and used for quantification of HPMA using the *N*-acetyl methyl peaks seen in the ^{1}H NMR spectra. The calibration curves for *N*-acetylcysteine in both standard solution and 'spiked' into control urine were shown to be linear in the concentration range appropriate for the values observed in this study. The mean percentage recovery of AF as HPMA was found to be 20 ± 3% at the 25 mg kg^{-1} dose level and 30 ± 3% at 50 mg kg^{-1}, as summarised in Table 1. Two previous studies have attempted to quantify HPMA excretion in rat urine after treatment with AF. Clayton et al. administered AF at 75 mg kg^{-1} i.p. to Sprague–Dawley rats and estimated the recovery of the dose as HPMA to be ∼2% [15]. However quantities were estimated using NMR peak height and not integral intensities, possibly explaining this lower value (personal communication, A. Clayton). Kaye reported a recovery of HPMA ∼7% by weighing derivatives isolated by extraction,

Table 1
MCA recovery in the rat following administration of various model toxins, including allyl formate (this study)

Compound	Dose (mg kg^{-1})	Mean percentage recovery as HPMA* (%)	n
Allyl amine	150	48.7 ± 1.3	4
Acrolein	13	78.5 ± 2.2	4
Allyl alcohol	64	28.3 ± 0.9	4
Allyl chloride	76	21.5 ± 0.3	4
Allyl formate	50	30 ± 3	10
	25	20 ± 3	10
Allyl bromide	120	3.0 ± 0.7	4
Allyl cyanide	115	3.7 ± 0.9	4

Adapted from Sandjuda et al. [20].
* Quoted ± S.E.M.

but concludes that the true value could be substantially higher since the chief purpose of the isolation was to obtain the mercapturate in its pure form [19]. In agreement with our findings, Kaye did not observe the allylmercapturic acid. Literature values taken from Sanjuda et al. [20] for the excretion of MCAs, following gavage administration of allylic and related compounds to rat, are given in Table 1. It can be seen that the recovery of AF as HPMA determined in this study is comparable with that observed for allyl alcohol. As AF is an ester of allyl alcohol, it is unsurprising that these values are fairly similar, as AF simply requires hydrolysis to form allyl alcohol. A previous study deduced that the formation of HPMA is indicative of the hydrolysis step proceeding via acyl-oxygen fission [19].

It has been shown that the route of administration has a major influence on the recovery of other allylic compounds. In a study following oral administration of allyl chloride, 22% of the dose was recovered as HPMA, compared to <3% when dosed i.p. It has been suggested that the rate of oxidation of allyl chloride to allyl alcohol is dependant on the dose route and/or animal strain. The metabolism of allyl alcohol and allyl formate does not require this oxidation to occur, so differences in recovery of dose as HPMA, caused by dose may not be as pronounced. However, these findings should be taken into account when comparing the data in Table 1. The hydrolysis of AF is likely to be the reason for the observed increase of formic acid in dosed animal urine. Formic acid and formate salts are ubiquitous in biological systems, and it is likely that any excess would be partly utilised in a number of biochemical reactions, rather than excreted in the same way as HPMA as a mechanism for detoxification. Therefore we propose that an increased presence of formic acid in urine is indicative of AF metabolism when found alongside the presence of HPMA, but is in no way a quantitative measure of exposure. Because of this hydrolysis reaction, the toxicity exhibited by AF compound is therefore a combination of that caused by acrolein and that caused by formate metabolism. The LD_{50} for i.p. formate in mouse is 940 mg kg^{-1}, so the contribution to toxicity from formate is likely to be minimal.

A marked difference in the urinary volumes was observed between the low and high dose groups. This may be an indicator of the damage that AF metabolism inflicts or could be a consequence of increased excretion of HMPA.

One criticism of this study is that the accuracy of the results is most heavily affected by the initial urinary volume measurements. Some collections were small – as little as 2 ml – and measurements made to the nearest 1 ml give a huge margin of error that must be considered when drawing conclusions from data obtained in this study. In future work, collaborators performing animal studies will be encouraged to measure urinary volumes with greater precision so as to enhance the value of quantification data generated. This may require a change in approach to the way urine samples are collected, as volume measurements provide by far the greatest source of error in recovery calculations.

In this respect, the accuracy of urinary volume measurements and the difference in the volumes collected, may go someway to explaining the significant difference in recovery of AF as HPMA between the low and high dose groups. The literature values for HPMA recovery following dosing with allylic and related compounds are all quoted with comparatively low SEM. This suggests that either a more uniform response was exhibited by the test animals in these studies, or these values do not reflect all the errors involved in metabolite quantification.

Regardless of quantification issues, this study has confirmed that MCAs can be used as biomarkers for exposure to allylic compounds. ^{1}H NMR has been shown to provide a simple method for allylic metabolite identification and quantification—in this instance, HPMA. Whether the technique is applicable at much lower dose levels of allylic compounds, such as those encountered inhaling tobacco smoke, remains to be investigated. Previous studies using radiolablled acrolein have identified a number of other minor metabolites, which were not observable in this study [21,22].

This study has highlighted a benefit of incorporating NMR into procedures for identifying metabolites—it is useful in prospecting for interesting metabolites or changes that are not entirely expected or obvious, as it does not require the same degree of pre-selection of target as with MS or chemical derivitisation. In this instance, the changes in formic acid excretion following AF administration, that have not been previously reported, were not specifically targeted in this study, but formed part of the collected data.

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