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The use of mass spectrometry in the study of chemically-reactive drug metabolites. Application of MS/MS and LC/MS to the analysis of glutathione- and related *S*-linked conjugates of *N*-methylformamide*

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Abstract: The *S*-(*N*-methylcarbamoyl) derivatives of glutathione, cysteine and *N*-acetylcysteine, the *S*-linked conjugates derived from a reactive metabolite of *N*-methylformamide (NMF), were studied in mice dosed with an equimolar mixture of NMF and deuterium-labelled NMF. Following preparation of *N*-benzyloxycarbonyl derivatives in aqueous media, the title conjugates were isolated, purified as their methyl esters and subjected to analysis by fast atom bombardment mass spectrometry (FAB/MS), fast atom bombardment tandem mass spectrometry (FAB/MS/MS) or thermospray liquid chromatography/mass spectrometry (TSP LC/MS). Characteristic isotope clusters in the FAB or TSP mass spectra facilitated recognition of drug metabolites, while constant neutral loss (89 *u*) and daughter ion scanning tandem mass spectrometry (MS/MS) experiments provided unique structural information on the conjugates of interest. It is concluded that the combined use of stable isotopes, aqueous-phase derivatization and contemporary mass spectrometric techniques represents a powerful approach for the analysis of glutathione adducts and related *S*-linked conjugates of chemically-reactive drug metabolites.

Keywords: *Chemically-reactive drug metabolites; glutathione conjugates; cysteine conjugates; mercapturic acids; tandem mass spectrometry; liquid chromatography–mass spectrometry.*

Introduction

Chemically-reactive metabolites of drugs and other foreign compounds are of considerable importance from a toxicological standpoint, in that such short-lived species are believed to mediate the serious adverse effects of many therapeutic agents, industrial chemicals and environmental pollutants ([1–3] and references therein). The endogenous

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tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine, GSH) plays a particularly important role in the disposition of reactive metabolites through formation of the corresponding *S*-linked conjugates which may either be eliminated directly via the bile, or undergo metabolic degradation to cysteine or *N*-acetylcysteine adducts (mercapturic acids) which usually are excreted into the urine [4–6]. The molecular structures of these conjugates are of interest in that they provide a unique insight into the chemical nature of the reactive intermediates from which they are derived. In some cases, notably the GSH conjugates of simple haloalkanes [7, 8], the adducts themselves may be toxic and, therefore, research aimed at establishing the identities of these species is of fundamental importance in gaining an understanding of the underlying biochemical mechanisms of foreign compound-induced toxicity.

The characterization of GSH conjugates in biological matrices is attended by a variety of analytical difficulties which result from the high polarity, thermal instability, amphoteric nature and, in some instances, chemical reactivity of these adducts. Moreover, GSH conjugates often are present in biological fluids at low concentrations, thus necessitating the use of highly sensitive analytical techniques, principally mass spectrometry, for their identification. The advent of "soft" ionization methods for mass spectrometry, notably FAB or liquid secondary ion mass spectrometry ([9, 10] and references therein) and TSP LC/MS [11–13], have had a major impact in this field in that trace amounts of GSH conjugates may now be examined as the intact species. Moreover, when combined with the use of selective aqueous-phase derivatization procedures to facilitate the isolation of polar GSH conjugates from aqueous media [9, 14], these contemporary mass spectrometric techniques represent a powerful approach for the identification of products of the GSH pathway of xenobiotic metabolism.

In the work reported in this communication, we have extended our earlier studies on the formation of *S*-(*N*-methylcarbamoyl)glutathione (SMG; Fig. 1), the GSH conjugate derived from NMF (Fig. 1), an industrial solvent and experimental antineoplastic agent which undergoes oxidative metabolism to a chemically-reactive, hepatotoxic intermediate [9, 15]. The specific objectives of the present study were three-fold: (i) to assess the utility of the isotope cluster ("twin ion") technique [10, 16] as an aid to the recognition of SMG and related conjugates of NMF in biological fluids; (ii) to evaluate the potential of MS/MS for the class-specific detection of GSH conjugates in bile by monitoring for compounds which fragment with loss of the elements of methylglycine ($H_2N{-}CH_2{-}CO_2CH_3$; 89 *u*), a characteristic transition in the collisionally-activated dissociation (CAD) FAB mass spectra of GSH conjugate aryloxycarbonyl methyl ester derivatives [9]; and (iii) to conduct a preliminary comparison of FAB/MS/MS and TSP LC/MS for the analysis of the conjugates of interest in specimens of bile and urine from animals dosed with NMF.

Experimental

Materials

N-Methylformamide, benzylchloroformate and reagents for synthetic and analytical work were purchased from the Aldrich Chemical Co. (Milwaukee, WI, USA). [$^2H_3$-methyl]NMF (Fig. 1) with an isotopic purity of 97 atom % excess $^2H_3$ and [^{14}C-methyl]NMF, with a specific activity of 1 mCi $mmol^{-1}$ and a radiochemical purity of 97%, were prepared by treatment of [$^2H_3$]methylamine hydrochloride (MSD Isotopes, St. Louis, MO, USA), and [^{14}C]methylamine hydrochloride (ICN, Irvine, CA, USA),

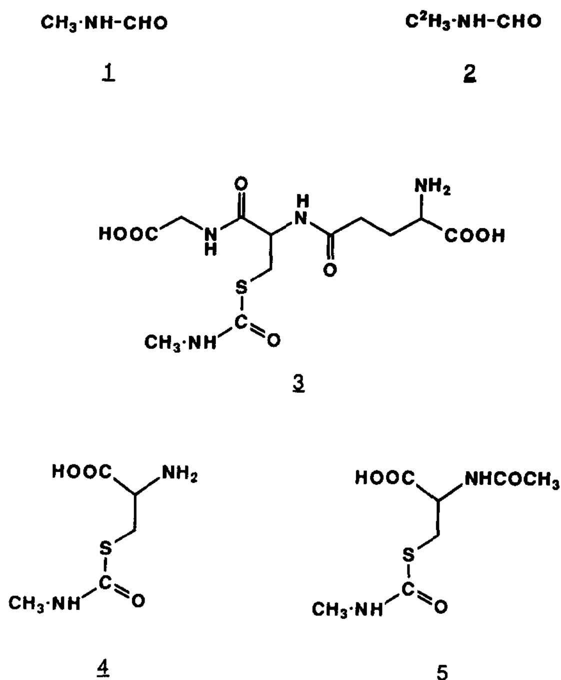

Figure 1
Structures of compounds referred to in the text. **1**, NMF; **2**, [$^2H_3$]NMF; **3**, the glutathione conjugate of NMF (SMG); **4**, the cysteine conjugate of NMF and **5**, the mercapturic acid derivative of NMF.

respectively, with excess ethyl formate [17]. Authentic samples of SMG and the corresponding cysteine and *N*-acetylcysteine conjugates of NMF (Fig. 1) were synthesized by reaction of methylisocyanate with GSH, cysteine and *N*-acetylcysteine, respectively, in aqueous acetonitrile solution. Details of these synthetic procedures will be reported elsewhere.

Animal experiments

Male BALB/c mice (25 g) were given an approx. equimolar mixture of unlabelled NMF and [$^2H_3$]NMF by intra-peritoneal injection at a total dose of 400 mg kg^{-1}. The administered drug also contained a small quantity of [^{14}C]NMF in order that recovery of the metabolites of interest could be monitored conveniently during extraction and chromatography steps. In experiments aimed at detecting SMG in bile, animals were killed by cervical dislocation 4 h after drug administration and their gall bladders were removed for collection of bile. Where the urinary conjugates were of interest, pooled urine specimens were collected for 24 h post-dose and aliquots were lyophylized. Both bile and dried urine samples were treated, as outlined previously [15], with excess benzylchloroformate to form *N*-benzyloxycarbonyl derivatives, which were then passed through C_{18} Sep–Pak solid-phase extraction cartridges and eluted with methanol. The dried extracts from this step were next treated with anhydrous methanolic HCl at room temperature for 2 h, and the resulting benzyloxycarbonyl methyl ester derivatives were either taken directly for analysis by TSP LC/MS or were purified by HPLC [9] prior to analysis off-line by FAB/MS and FAB/MS/MS.

Mass spectrometry

Derivatized extracts of bile were analysed by FAB/MS and FAB/MS/MS using a VG 70-SEQ hybrid tandem instrument of EBQQ geometry (VG Analytical Ltd, Manchester, UK), equipped with an Ion Tech fast atom gun and VG 11/250 data system. Conventional FAB mass spectra were obtained (from approx. 5–10 μg of sample) as described previously [9], while daughter ion spectra were recorded in the MS/MS mode employing CAD in the first (*rf*-only) quadrupole with argon (2×10^{-6} torr) as the collision gas and a collision energy of 25 eV. Constant neutral loss spectra were obtained under similar MS/MS conditions, and the magnet was scanned at 10 s decade^{-1} between *m/z* 100–700 with the quadrupole mass analyser offset by 89 *u*. Daughter ion spectra were recorded via the data system in the multi-channel analysis mode and were mass measured subsequently. Neutral loss spectra were recorded as single scans.

Derivatized conjugates of NMF in urine were analysed by TSP LC/MS, which was carried out using a Vestec 201 instrument (Vestec Corp., Houston, TX, USA) with a directly heated probe vaporizer. Thermospray probe control and tip temperatures were 115 and 174°C, respectively, and were adjusted to maintain a jet temperature of 204°C. The source block was held at 265°C. The HPLC system consisted of an LKB 2150 pump and LKB 2152 controller unit (LKB, Bromma, Sweden). Samples were injected via a Valco C6W manual injector (Valco Instruments, Houston, TX, USA) equipped with a 100-μl sample loop and separations were achieved using an Ultrasphere C_{18} column (15 cm × 4.6 mm, i.d.; 5-μm particle size) with acetonitrile–aqueous 0.1 M ammonium acetate (40:60, v/v) as mobile phase (flow rate 1.2 ml min^{-1}). Analyses were carried out in the "filament on" mode (filament current 350 μA) and mass spectra were acquired and processed by a Hewlett–Packard 59979C ChemStation data system (Hewlett–Packard, Palo Alto, CA, USA).

Results and Discussion

Biliary metabolites of NMF

An extract of bile from mice which had received a mixture of NMF, [$^2H_3$]NMF and [^{14}C]NMF was derivatized to yield benzyloxycarbonyl methyl esters, purified by HPLC (using radioactivity measurements to trace drug metabolites) and subjected to FAB/MS analysis. The resulting mass spectrum, which is reproduced in Fig. 2, revealed the presence of two prominent ions (at *m/z* 527 and 530) which were absent in the corresponding spectrum of control (drug-free) bile. Since the molecular weight of the benzyloxycarbonyl bis-methyl ester derivative of SMG is 526, this pair of signals was assigned tentatively to the MH^+ species of unlabelled SMG and [$^2H_3$]SMG present as biliary metabolites of NMF and [$^2H_3$]NMF, respectively. This conclusion was supported by the approx. 1:1 intensity ratio of the ions in question, as expected if bioactivation of NMF and subsequent conjugation with GSH were not subject to any appreciable deuterium isotope effect. However, no additional information on the identity of these biliary components could be derived from this FAB mass spectrum in view of the absence of any structurally-informative fragment ions.

In an attempt to confirm that the above biliary metabolites were indeed GSH conjugates, a constant neutral loss scanning MS/MS experiment was next conducted to reveal selectively those components of the sample which eliminated 89 *u* (methylglycine) as a neutral species upon collisional activation [9]. The spectrum recorded under these conditions is reproduced in Fig. 3, which indicates clearly that each of the ions at *m/z* 527

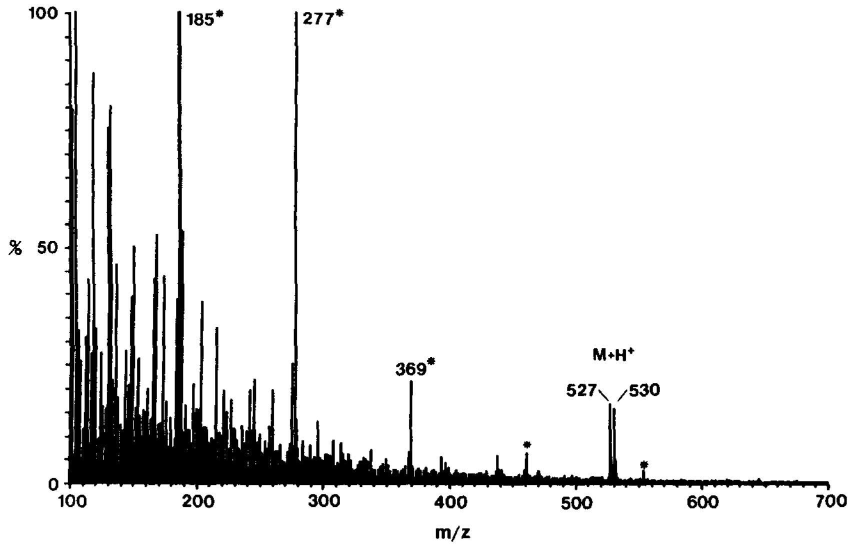

Figure 2
FAB Mass spectrum of a derivatized extract of bile collected from a mouse dosed intra-peritoneally with an approx. equimolar mixture of NMF and [$^2H_3$]NMF. The MH^+ ions from SMG and its deuterated counterpart are evident at *m/z* 527 and 530, while prominent ions derived from the glycerol matrix are denoted by asterisks.

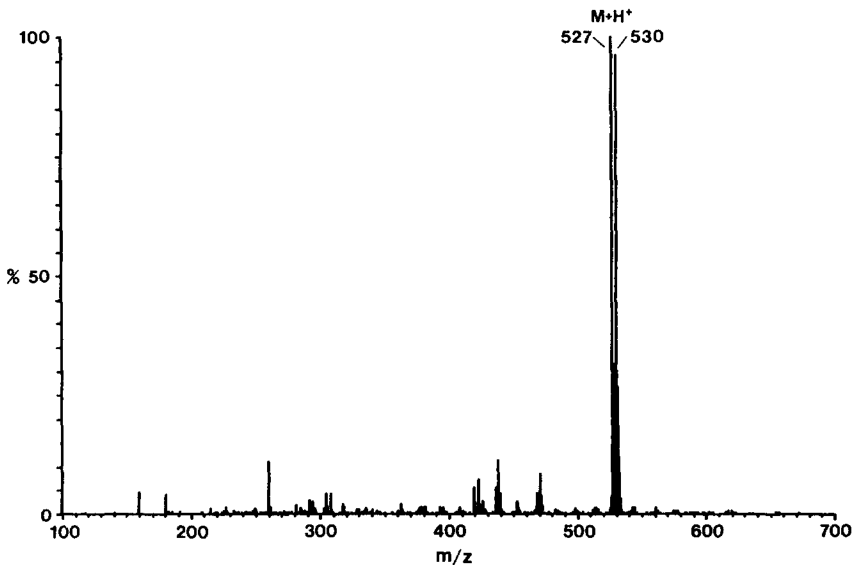

Figure 3
Constant neutral loss (89 *u*) spectrum of the same derivatized extract of bile employed for the conventional FAB mass spectrum shown in Fig. 2. Selective detection of SMG and [$^2H_3$]SMG in this scan mode was based on the fact that GSH conjugate methyl esters undergo a characteristic loss of the elements of methylglycine (89 *u*) under CAD conditions.

and 530 underwent the transition of interest, consistent with the interpretation that these signals were due to the protonated parents of SMG and its deuterated analogue. No additional isotopic clusters were evident in this neutral loss spectrum, which suggests that only one type of GSH adduct is formed during the metabolism of NMF. Thus, neutral loss scanning (89 *u*) appears to hold considerable promise for the detection of derivatized GSH conjugates as a structural class, while a similar approach (neutral loss scanning for 129 *u*) has been proposed for use with the corresponding underivatized adducts [10].

Positive identification of the above biliary metabolites of NMF/[$^2H_3$]NMF was obtained by recording their respective daughter ion spectra following CAD. When the ion at *m/z* 527 was selected by the first mass analyser, activated in the collision cell and the spectrum of daughter ions examined by scanning the second mass analyser, the spectrum depicted in Fig. 4 was obtained. This spectrum was closely similar to that recorded previously for the benzyloxycarbonyl bis-methyl ester derivative of SMG [9], and contained all of the fragment ions diagnostic for both the GSH moiety and the N-methylcarbamoyl substituent of this adduct. A similar CAD experiment on *m/z* 530 provided the daughter ion spectrum reproduced in Fig. 5, in which all of the characteristic fragments of SMG were again evident, some of which (e.g. the 'a' ion at *m/z* 441 formed by loss of the elements of methylglycine) were shifted 3 *u* to higher mass relative to the corresponding signals in Fig. 4 as a result of deuterium substitution on the *N*-methyl group. Thus, the two metabolites of NMF/[$^2H_3$]NMF present in this specimen of bile were identified unequivocally as SMG and its trideuteromethyl counterpart, the detection and characterization of which were facilitated greatly by the use of stable isotope labelling and MS/MS techniques.

In contrast to its behaviour under FAB conditions, the benzyloxycarbonyl bis-methyl ester derivative of SMG gave a rather unsatisfactory TSP mass spectrum when analysed under the reversed-phase LC/MS conditions employed here. Thus, the MH^+ ion, while present at *m/z* 527, was of very low relative abundance, and the spectrum was dominated by a prominent daughter ion at *m/z* 470 corresponding to ($MH^+ - CH_3NCO$) (data not shown). In view of the composition of the latter species, it did not shift in mass in the spectrum of the deuterated SMG derivative and no prominent "doublet" ions were noted in the spectrum of the biological conjugate derived from NMF/[$^2H_3$]NMF. Therefore, FAB/MS and FAB/MS/MS proved to be superior to TSP LC/MS for the detection and identification of derivatized SMG, despite the more lengthy sample work-up required for the FAB analyses. However, it may be noted that preliminary TSP LC/MS analyses of this conjugate under *normal phase* HPLC conditions (silica column, 100% isopropanol as mobile phase) gave spectra of improved quality, in that they exhibited prominent MH^+ and ($MH^+ - CH_3NCO$) ions.

Urinary metabolites of NMF

Preliminary experiments with the cysteine and *N*-acetylcysteine conjugates of NMF indicated that their respective derivatized products afforded abundant MH^+ ions under both FAB and TSP conditions. In light of the relatively rapid analysis times attainable with the (on-line) LC/MS approach, this technique was favoured in the present study for the search for conjugates of NMF in specimens of urine.

Derivatized urinary extracts from mice which had received NMF/[$^2H_3$]NMF contained a component which eluted from the HPLC column with the same retention time (1.4 min) as the methyl ester of the authentic *N*-acetylcysteine adduct of NMF. This compound afforded the TSP mass spectrum shown in Fig. 6, which contained an

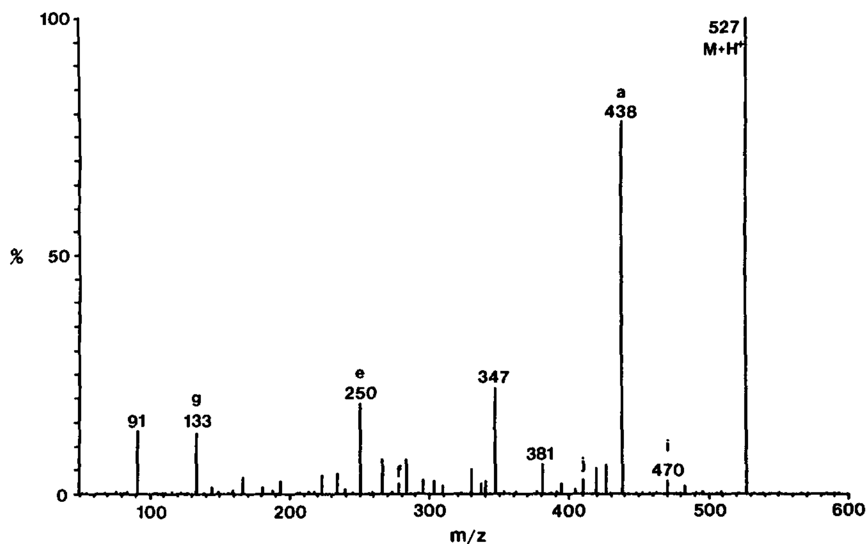

Figure 4
Daughter ion spectrum obtained by collisional activation of the MH^+ of derivatized SMG (at *m/z* 527) present in an extract of mouse bile. Structurally-informative fragment ions are denoted by lower case letters and arise from pathways discussed previously [9].

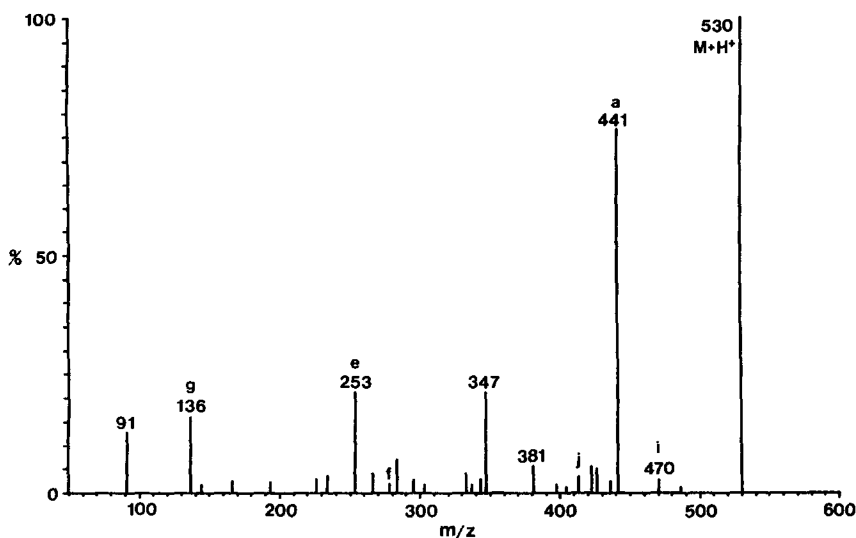

Figure 5
Daughter ion spectrum obtained by collisional activation of the MH^+ of derivatized [$^2H_3$]SMG (at *m/z* 530), recorded under identical conditions to that of the unlabelled conjugate depicted in Fig. 4.

abundant MH^+ ion (*m/z* 235) accompanied by a satellite at *m/z* 238 derived from the trideuterated conjugate. A corresponding pair of NH_4^+ adduct ions was evident at *m/z* 252 and 255, and an intense fragment produced by loss of the elements of methylisocyanate (57 *u*) from the protonated parent species was present at *m/z* 178. This latter daughter ion, having been generated by a process which involved removal of the

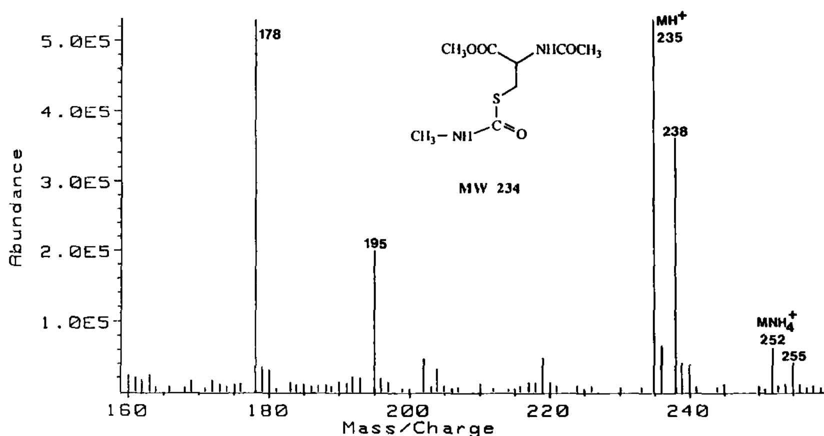

Figure 6
Mass spectrum of the methylated mercapturic acid derivative of NMF isolated from the urine of a mouse dosed with a mixture of NMF and [$^2H_3$]NMF. The spectrum was recorded under TSP LC/MS conditions, using acetonitrile–0.1 M ammonium acetate (40:60, v/v) as mobile phase for the HPLC separation.

deuterated methyl group, did not exhibit an isotope cluster. (The same was true for the corresponding NH_4^+ adduct ion at *m/z* 195.)

The cysteine conjugate of NMF, which yielded an *N*-benzyloxycarbonyl methyl ester derivative with $M_r = 326$, eluted somewhat later ($t_R = 5.6$ min) from the reversed-phase HPLC column than the above *N*-acetyl methyl ester on account of the lipophilic character of the benzyl moiety. Under TSP conditions, the derivatized cysteine adduct afforded prominent ions at *m/z* 327 (MH^+) and 270 ($MH^+ - CH_3NCO$), both of which were accompanied by NH_4^+ adduct ions (at *m/z* 344 and 287, respectively). When a search was made for this conjugate and its deuterated congener in urine samples from the mice given NMF/[$^2H_3$]NMF, none of the above ions was detected at the appropriate HPLC retention time with an intensity significantly above background. Therefore, it may be concluded that the cysteine conjugate of NMF is not excreted to any appreciable degree into mouse urine, but appears to undergo extensive *N*-acetylation to yield the corresponding mercapturate, *S*-(*N*-methylcarbamoyl)-*N*-acetylcysteine (Fig. 1) which is a known urinary metabolite of NMF in mice, rats and humans [18]. In light of this finding, an abbreviated analytical protocol, in which the derivatization step with benzylchloroformate is omitted, should prove adequate in mass spectrometric studies of urinary *S*-linked conjugates of NMF, since the mercapturic acid metabolite does not react with benzylchloroformate due to its blocked amino group.

Conclusions

The results of these investigations on the GSH, cysteine and *N*-acetylcysteine conjugates of NMF have further confirmed the analytical utility of the two-step derivatization sequence (formation of aryloxycarbonyl methyl esters) for the isolation of polar adducts from biological fluids, prior to examination by mass spectrometry. Use of the isotope cluster technique aided the recognition of drug-related products in the biological isolates on account of the appearance of conspicuous doublet ions in their mass

spectra, reflecting the presence of unlabelled and labelled molecules in a predetermined ratio. The advantages of tandem mass spectrometry were evident in the work with SMG, both in the conjugate class-specific search for GSH adducts (constant neutral loss scanning) and in the deduction of molecular structure of sputtered MH^+ parent species following collisional activation (daughter ion scanning). Although all of the above MS/MS experiments were performed in conjunction with FAB ionization, it seems probable that similarly favourable results would accrue from the application of TSP LC/MS/MS techniques [19]. Therefore, based on the findings of this study, it may be concluded that both FAB/MS and TSP LC/MS represent viable mass spectrometric approaches for the study of intact GSH conjugates, cysteine adducts and mercapturic acids, although one technique may prove superior to the other for specific applications (e.g. FAB/MS was preferable to TSP LC/MS for the analysis of derivatized SMG because of a higher yield of parent ions; TSP LC/MS, on the other hand, offered the advantage of speed of analysis in the detection of the urinary NMF mercapturate). The advent of the dynamic FAB interface [20] should enhance further the utility of FAB/MS/MS for investigations such as these, in that this mode of sample introduction potentially eliminates the requirement for time-consuming chromatography prior to MS/MS analysis.

Contemporary mass spectrometric methods and selective chemical derivatization procedures of the type described in this communication have facilitated greatly the detection and identification of products of the GSH conjugation pathway of foreign compound metabolism. Consequently, they have provided an indirect means by which the chemically-reactive precursors of these GSH adducts may be studied, and it now seems certain that such analytical approaches will play an integral role in the elucidation of biochemical mechanisms of metabolic activation and associated xenobiotic-induced toxicities.

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