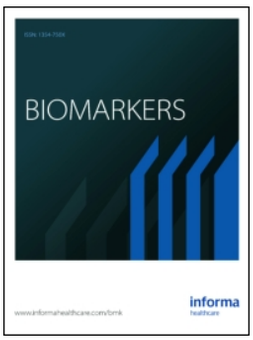

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REVIEW ARTICLE

Mercapturic acids: recent advances in their determination by liquid chromatography/mass spectrometry and their use in toxicant metabolism studies and in occupational and environmental exposure studies

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Abstract

This review describes recent selected HPLC/MS methods for the determination of urinary mercapturates that are useful as noninvasive biomarkers in characterizing human exposure to electrophilic industrial chemicals in occupational and environmental studies. High-performance liquid chromatography/mass spectrometry is a sensitive and specific method for analysis of small molecules found in biological fluids. In this review, recent selected mercapturate quantification methods are summarized and specific cases are presented. The biological formation of mercapturates is introduced and their use as indicators of metabolic processing of reactive toxicants is discussed, as well as future trends and limitations in this area of research.

Keywords

Internal exposure, liquid chromatography, mass spectrometry, mercapturic acid, toxicant metabolism, urinary biomarker

History

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Introduction

The measurement of urinary mercapturic acids (mercapturates) is important in characterizing human exposure to occupational and environmental toxicants. Toxicant concentrations found in the surrounding environment often do not correlate to an individual's internal dose. Estimates based on models may not be accurate due to variations in toxicant absorption and metabolism in exposed individuals (De Rooij et al., 1998). Determination of urinary mercapturates, which are the products of toxicant metabolism, provides useful biomarkers of individual toxicant absorption and internal dose (Vanwelie et al., 1992). The use of mercapturates as biomarkers of toxic occupational and environmental exposure has been extensively reviewed (De Rooij et al., 1998; Vanwelie et al., 1992) and a survey of HPLC separation and mass spectrometry techniques to quantitate these biomarkers has been undertaken in this review.

Mercapturate formation begins when glutathione (GSH), an endogenous tripeptide, reacts to inactivate an electrophilic toxicant or reactive toxicant metabolite either spontaneously or by catalysis with GSH transferase in the liver and in other organs (Figure 1). Next glutamyl and glycine moieties are enzymatically removed to form a cysteine conjugate. This conjugate is, in turn, *N*-acetylated to form what is generally a toxicant-specific mercapturate (Perbellini et al., 2002). The initial reaction between endogenous GSH and an electrophilic moiety on a compound is considered as a detoxification step, and prevents reaction of the electrophilic metabolite with cellular components such as proteins, lipids or DNA. The final *N*-acetylation reaction increases the polarity and hydrophilicity of the metabolite, making it more water soluble and allowing for urinary excretion and elimination (De Rooij et al., 1998; Vanwelie et al., 1992).

Mercapturate formation from a reactive toxicant is often complex and formation of multiple mercapturate products is possible. Urinary mercapturate determination provides a noninvasive tool to investigate up-stream toxicant activation. Generally, investigations of toxicant metabolism are more easily done in experimental animals. However, differences in toxicant metabolism between species, especially those between the rodents generally used and that of humans are often significant. Identification and quantification of the mercapturate products of reactive metabolites can demonstrate differences in toxicant activation and detoxification between species. An understanding of these differences in toxicant activation is necessary for accurate risk analysis, especially when data from human volunteer or occupational studies are limited.

In the interest of brevity, the mercapturate names shown in Table 1 and used throughout this review are truncated from

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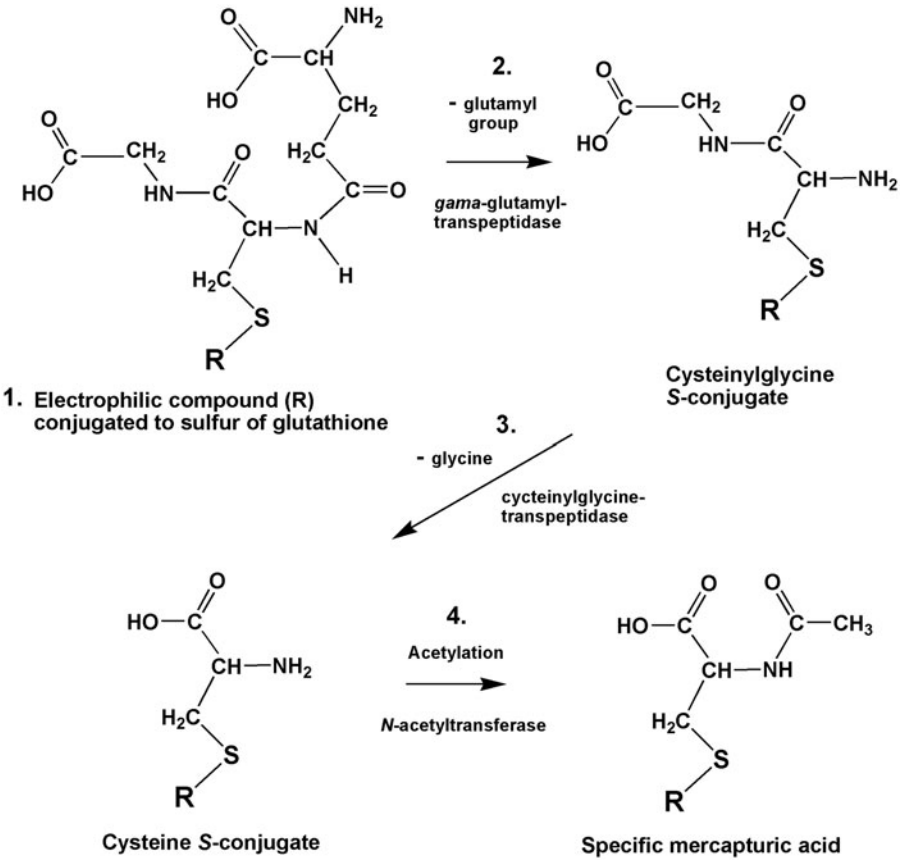

Figure 1. Mercapturate formation begins with the conjugation of an electrophilic group (R) of a toxicant or toxicant metabolite with the sulfur of glutathione to form a glutathione *S*-conjugate. Next glutamyl and glycine moieties are removed by transpeptidases to form a cysteine-*S*-conjugate that is *N*-acetylated to form a specific mercapturate. Adapted from Perbellini et al. (2002).

the systematic name of the *S*-conjugated electrophilic group except in the cases of acrylamide (AA) and the triazine and chloroacetamide herbicide derived mercapturates. Thus the systematic name for the mercapturate of benzene, *N*-acetyl-*S*-(phenyl)-L-cysteine is shortened to phenyl mercapturate. The names and abbreviations used for mercapturates vary throughout the current literature, and are generally derived from the *S*-conjugated electrophilic group. The abbreviations used in this review, as they appear in the cited methods, are listed in Table 1 by parent compound.

This review describes selected HPLC–MS methods for determination of mercapturates as biomarkers of human exposure to industrial chemicals for use in occupational and environmental studies. Mercapturates are the detoxification products of a wide range of heterogeneous electrophilic compounds (Table 1) and no single analytical approach for development of a method for a new specific mercapturate can be recommended. Alternatively, this work is a survey of the multiple analytical approaches described in recently published HPLC/MS mercapturate determinations.

The current review focuses on HPLC/MS determination of a broad range of mercapturates. In 2002, Perbellini reviewed methods for determining mercapturates in biological exposure monitoring. The scope of that review included gas chromatographic (GC), and high-performance liquid chromatographic (HPLC) methods, and it was limited to single mercapturates of benzene, toluene and xylene, and the detection of two of the mercapturate products of 1,3-butadiene and of trimethylbenzene (Perbellini et al., 2002). In this review, selected representative mercapturate methods are summarized in tabular format and highlights of the sample preparation and chromatography techniques used in these methods are briefly described. An overview of mercapturates as useful indicators of toxicant metabolism is presented. Mercapturates as specific indicators of toxicant exposure, metabolic activation and as tools to investigate toxicant metabolism and elimination are considered.

Tabular summaries of selected methods

Tables 2 and 3 summarize selected HPLC–MS methods reported for the detection and quantification of various mercapturates used in occupational and environmental studies. The terminology and abbreviations appearing in these tables indicate sample preparation techniques, chromatographic conditions and mass spectrometry detection modes reported for these methods, and are explained in more detail in the following sections of this review.

Sample preparation techniques

Successful determination of mercapturates by HPLC/MS requires separation of analyte mercapturates from interfering components found in urine. Proteins, numerous metabolites, salts and other components that make up the urinary sample matrix interfere with the sensitive and specific detection of the target mercapturates. Salts may alter the intensity of the analyte signal causing ion suppression or ion enhancement. Unrelated metabolites having a similar structure may co-elute from the chromatographic column with the target mercapturate. The necessary removal of these interferences makes

Table 1. Mercapturate abbreviation and common name by parent compound.

Parent compound	Abbreviation	*N*-acetyl-*S*-(R)-cysteine
Acrylamide	AAMA	Acetamidomethylmercapturate
	GAMA2	1-Carbamoyl-2-hydroxyethylmercapturate
	GAMA3	2-Carbamoyl-2-hydroxyethylmercapturate
	NASPC	*S*-Propionamidemercapturate
Acrylonitrile	CEMA	2-Cyanoethylmercapturate
	HEMA	2-Hydroxyethylmercapturate
	CHEMA	1-Cyano-2-hydroxyethylmercapturate
Acrolein	3-HPMA	3-Hydroxypropylmercapturate
Atrazine	AZMA	Atrazinemercapturate
Acetochlor	ACMA	Acetochlormercapturate
Alachlor	ALMA	Alachlormercapturate
Metolachlor	MEMA	Metolachlormercapturate
Benzene	PMA	Phenylmercapturate
1-bromopropane	NPMA	Propylmercapturate
2-bromopropane	iPMA	Isopropylmercapturate
1,3-butadiene	DHBMA	3,4-Dihydroxybutylmercapturate
	THBMA	2,3,4-Trihydroxybutylmercapturate
	bis-BDMA	2,3-Dihydroxybutylmercapturate
	MHBMA1	1-Hydroxymethyl-2-propenylmercapturate
	MHBMA2	2-Hydroxy-3-butenylmercapturate
Crotonaldehyde	CPMA	3-Carboxy-2-propylmercapturate
	HPMMA	3-Hydroxypropyl-1-methylmercapturate
Dimethylacetamide	AMMA	Acetamideomethylmercapturate
Dimethylformamide	AMCC	Methylcarbamoylmercapturate
Ethylene oxide	HEMA	2-Hydroxyethylmercapturate
Propylene oxide	HPMA	2-Hydroxypropylmercapturate
Glycidol	DHPMA	2,3-Hydroxypropylmercapturate
Styrene	PHEMA	1-Phenyl-2-hydroxyethylmercapturates
	4-VPMA	4-Vinylphenylmercapturate
Tetrachlorethylene	TCVMA	Trichlorovinylmercapturate
Trichlorethylene	1,2-DCVMA	1,2-Dichlorovinylmercapturate
	2,2-DCVMA	2,2-Dichlorovinylmercapturate
Toluene	BMA	Benzylmercapturate
Xylene	DPMA	2,4-Dimethylphenylmercapturate
	MBMA	*o*-Methylbenzylmercapturate
Polycyclic aromatic hydrocarbons		
Phenantherene	PheO-Nac	9,10-Dihydro-9-hydroxyl-10-phenanthrylmercapturate
Phenantherene diol epoxide	PheDE-Nac	2,3,4-Trihydroxy-1,2,3,4-tetrahydrophenanthrylmercapturate
Benzo(a)pyrene diol epoxide	rev-BPDE-7-Nac	8,9,10-Trihydroxy-7,8,9,10-tetrahydrobenzylmercapturate

sample preparation as critical to success as any other part of the analysis. A variety of sample preparation techniques have been applied in the methods reviewed. The simplest is dilution and filtration through 0.2 µm pore cellulose medium followed by direct injection (Yan et al., 2010). Initial acidification of urine is common to some sample preparation procedures, and in *S*-phenylmercapturic acid analysis samples were so treated (Maestri et al., 2005; Paci et al., 2007; Sterz et al., 2010). Other techniques use sample concentration with re-suspension in methanol or acetonitrile, or protein precipitation by acidification and centrifugation prior to analysis (Alwis et al., 2012; Sohn et al., 2005; Sterz et al., 2012; Wu et al., 2012). Most methods use a form of solid phase extraction (SPE) for sample preparation and clean-up. In SPE, urine is applied to chromatographic medium and is pulled through the medium under vacuum pressure. Target mercapturates are captured in the solid medium and several volumes of solvent are used to remove sample matrix components. Concentrated and purified analytes are then washed free from the medium with elution buffer or organic solvent for analysis. In simple manual SPE techniques, medium in syringes, disks or cartridges are used to extract 1–5 mL of urine.

Various SPE media are used to extract and concentrate target mercapturates from the urinary matrix: reversed phase (RP) (Li et al., 2005; Kellert et al., 2006; Kopp et al., 2008), RP-strong anion exchange (RP-SAX) (Melikian et al., 1999) or restricted access medium (RAM) phase online trap cartridges (Hou et al., 2012; Schettgen et al., 2008a). Restricted access media are specialized chromatographic phases that combine size-exclusion with other retention mechanisms. Internal surface-RP RAM combines silica gel particles having pores lined with RP C18, C8 or C4 alkane chains to retain small analyte molecules with an outer hydrophilic surface such as methyl cellulose.

Sample preparation is often the rate-limiting step in most bioassay methods. Automated sample preparation has become popular and two forms using SPE media have come into common use (Varma et al., 2010). Popular 96-multi-well sample plate format has been adapted to high-throughput SPE (Mallet et al., 2003). SAX medium in this format was used to increase extraction throughput in analyses of benzene and toluene mercapturates for studies of benzene exposure in smokers (Li et al., 2005) and gas station workers (Barbieri et al., 2004). Recently, Kuklenyik described a 96-well plate sample extraction and sample handling technique for analysis of four atrazine (ATZ) mercapturates for non-occupational exposure studies (Kuklenyik et al., 2012). The authors describe rapid optimization of sample extraction parameters,

Table 2. Targeted mercapturic acid biomarker analysis by LC/MS.

Parent compound	Sample preparation	Chromatography	Interface/detection	Target mercapturate	Limit of detection	Validation population	Reference
Acrylamide	SPE	RP, gradient CHOOH/ACN	ESI/SQ/SIM$^-$	AAMA d_4-AAMA	100 µg/L	Non-exposed volunteers	B'Hymer & Cheever (2007)
	RP-pretrap column switching	RP, gradient CHOOH/ACN	ESI/QQQ/MRM$^-$	NASPC $^{13}C_3$-NASPC	5 µg/L	Military officers	Li et al. (2005)
	RP-pretrap column switching	RP, isocratic CHOOH/ACN	ESI/QQQ/MRM	AAMA $^2H_3$-AAMA GAMA2 $^2H_3$-GAMA2 GAMA3 $^2H_3$-GAMA3	0.5 µg/L 1.0 µg/L	Smoking & non-smoking volunteers	Kellert et al. (2006)
	RP-pretrap column switching	HILIC, gradient NH_4CH_3COOH/ACN	ESI/QQQ/MRM$^-$	AAMA $^{13}C_3$-AAMA AAMA-sulfide $^{13}C_3$-AAMAsul GAMA $^{13}C_3$-GAMA	0.5 µg/L 0.1 µg/L 2.0 µg/L 0.1 µg/L 1.0 µg/L 0.5 µg/L	Non-exposed, nonsmoking volunteers	Kopp et al. (2008)
	SPE	RP, gradient CHOOH/ACN	ESI/QQQ/MRM$^-$	AAMA d_3-AAMA AAMA-sulfide d_3-AAMA-sul GAMA2 d_3-GAMA2 GAMA3 d_3-GAMA3	2.0 µg/L 3.0 µg/L 3.0 µg/L 3.0 µg/L	Non-exposed students	Zhang et al. (2015)
Acrylonitrile	RAM phase column switching	RP, gradient CHOOH/ACN	ESI/QQQ/MRM$^-$	CEMA $^2H_3$-CEMA HEMA $^2H_3$-HEMA	1 ηg/L 30 ηg/L	General population	Hou et al. (2012)
	Protein precipitation	RP, isocratic CHOOH/ACN	ESI/QQQ/SRM$^-$	CEMA d_3-CEMA HEMA d_4-HEMA	0.05 µg/L 0.05µg/L	Smokers & nonsmokers	Wu et al. (2012)
	RAM phase column switching	RP, isocratic CHOOH/ACN	ESI/QQQ/MRM$^-$	CEMA d_3-CEMA	0.05 µg/L	General population	Schettgen et al. (2012)
Acrolein	SPE	RP, isocratic CHCOOH/ACN	ESI/QQQ/MRM+	3-HPMA *N*-acetyl-cys	50 µg/L	Non-exposed volunteers	Mascher et al. (2001)
	SPE	RP, gradient NH_4 CH_3COOH /MeOH	APCI/QQQ/SRM$^-$	3-HPMA $^{13}C_3$-3-HPMA	0.9 ηg/L	Non-exposed volunteers	Carmella et al. (2007)
	Dilute, filter inject	HILIC, gradient $NH_4CHCOOH$/ACN	ESI/QQQ/MRM$^-$	3-HPMA d_3-3-HPMA	22 µg/L	Non-exposed volunteers	Yan et al. (2010)
Benzene	SPE	RP, gradient CH_3COOH/MeOH	ESI/SQ/SIM$^-$	PMA $^{13}C_6$-PMA	0.2 µg/L	Exposed workers	Maestri et al. (2005)
	SPE	RP, gradient CH_3COOH/MeOH	ESI/QQQ/MRM$^-$	PMA d_5-PMA	0.05 µg/L	Oil refinery workers	Paci et al. (2007)
	SPE-SAX	RP, gradient CH_3COOH/MeOH	ESI/QQQ/SRM$^-$	PMA $^{13}C_6$-PMA tt-MA $^{13}C_6$-ttMA	0.1 µg/L 1.0 µg/L	General populations	Melikian et al. (1999)

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Bromopropane	SPE	RP, gradient CH_3COOH/MeOH	ESI/SQ/SIM^-	NPMA d_7-NPMA	10 µg/L	Volunteers, exposed workers	Cheever et al. (2009)
1,3-Butadiene	SPE	RP, gradient CH_3COOH/MeOH	ESI/QQQ/MRM^-	DHBMA d_7-DHBMA	12.2 µg/L	Non-exposed volunteers	Carrieri et al. (2009)
	SPE	RP/WAX, gradient CH_3COOH/ACN	ESI/QQQ/SRM^-	THBMA d_3-THBMA	0.1 µg/L	Smoking cessation study	Kotapati et al. (2011)
	SPE	RP/WAX, gradient CH_3COOH/ACN	ESI/QQQ/SRM^-	*bis*-BDMA $^2H_6$-*bis*-BDMA	1.0 µg/L	Smokers, exposed workers	Kotapati et al. (2014)
	Concentrate/resuspend in MeOH	HILIC, gradient NH_4CH_3COOH/ACN	ESI/QQQ/MRM^-	1-MHBMA d_6-1-MHBMA 2-MHBMA d_6-2-MHBMA	0.05 µg/L 0.24 µg/L 	Non-exposed volunteers	Sterz et al. (2012)
	SPE	RP, gradient NH_4HCOOH/MeOH	ESI/QQQ/MRM^-	DHBMA d_7-DHBMA MHBMA d_6-MHBMA	0.14 µg/L 0.16 µg/L 	Non-exposed volunteers	Li et al. (2015a)
Dimethylacetamide	SPE	RP, gradient CH_3COOH/MeOH	ESI/SQ/IonTrap	AMMA AMCC	1500 µg/L	Fiber factory workers	Princivalle et al. (2010)
Dimethylformamide	SPE	RP, isocratic CH_3COOH/ACN	ESI/SQ/SIM^-	AMCC	2.0 µg/L	Workers, general populations	Perico et al. (2004)
	Protein precipitation	RP, isocratic CHCOOH/MeOH	ESI/QQQ/MRM^+	AMCC	4 µg/L	Exposed workers	Sohn et al. (2005)
Styrene	Filtration/acidification	RP, gradient CHCOOHNH4/MeOH	ESI/QQQ/SRM^-	R, R-1-PHBMA S, R-1-PHBMA R, R-2-PHBMA S, R-2-PHBMA	0.7 µg/L 1.0 µg/L 0.7 µg/L 0.7 µg/L	Exposed workers	Manini et al. (2000)
	RAM column Switching	RP/SAX, gradient CHCOOH/ACN	ESI/QQQ/MRM−	All PHEMAs	0.3 µg/L	General population	Reska et al. (2010)
	SPE	RP/SAX, gradient CHCOOH/ACN	ESI/QQQ/SRM−	4-VPMA	0.3 µg/L	Hand-lamination workers	Linhart et al. (2012)

ACN: acetonitrile; APCI: atmospheric pressure chemical ionization; ESI: electrospray ionization; HILIC: hydrophilic interaction chromatography; MeOH: methanol; MRM: multiple reaction monitoring; *N*-acetyl-cys; *N*-acetyl-cysteine; RAM: restricted access medium; RP: reversed phase; RP/WAX: mixed mode reversed phase-weak anionic interaction; RP/SAX: mixed mode reversed phase-strong anionic interaction; SIM: selected ion monitoring; SRM: selected reaction monitoring; SPE: solid phase extraction; SQ: single quadrupole; QQQ: triple quadrupole.

Table 3. Simultaneous determination of multiple mercapturic acids by LC/MS.

Parent compounds	Sample preparation	Chromatography	Interface/detection	Target mercapturate	Limit of detection	Validation population	Reference
Multiple determination	SPE	RP, gradient CHOOH/ACN	ESI/QQQ/SRM$^-$			General population smokers and nonsmokers	
PAH's							
Phenantherene				PheO-Nac	0.7 ηg/L		Upadhyaya et al. (2006)
				d_{10}-PheO-Nac			
Phenantherene diol epoxide				PheDE-Nac	1.0 ηg/L		Hecht et al. (2008)
				$^{13}C_6$-PheDE-Nac			
Benzo(a)pyrene				rev-BPDE-7-Nac	1.0 ηg/L		Upadhyaya et al. (2010)
				Phe-9OH-Nac			
Multiple determination	SPE	RP, gradient CHOOH/ACN	ESI/QQQ/MRM$^-$			General population	Schettgen et al. (2008b)
Acrolein				3-HPMA	5 µg/L		
Acrylamide				AAMA	2.5 µg/L		
				d_4-AAMA			
Dimethylformamide				AMCC	5 µg/L		
				d_4-AMCC			
Ethylene oxide				HEMA	0.5 µg/L		
				d_4-HEMA			
Propylene oxide				2-HPMA	5 µg/L		
Multiple determination	RAM phase column switching	RP, gradient CHOOH/ACN	ESI/QQQ/MRM$^-$			General population	Schettgen et al. (2009)
Acrylonitrile				CEMA	0.5 µg/L		
				d_3-CEMA			
1,3-Butadiene				DHBMA	5 µg/L		
				d_7-DHBMA			
				MHBMA	1 µg/L		
				d_6-MHBMA			
Multiple determination	LLE/SPE/ PFBBr derivitization	RP, gradient CHOOH/ACN	ESI/QQQ/MRM$^+$			Smoking cessation study	Scherer et al. (2010)
Acrylonitrile				CEMA	0.2 µg/L		
				d_3-CEMA			
Ethylene oxide				HEMA	0.3 µg/L		
				d_4-HEMA			
				MMA	0.3 µg/L		
				d_3-MMA			
Multiple determination	RAM phase column switching	RP, gradient HCOOH/ACN	ESI/QQQ/MRM$^-$			Diet controlled smokers & nonsmokers	Pluym et al. (2015)
Acrylonitrile				CEMA	0.08 µg/L		
				d_3-CEMA			
Crotonaldehyde				CPMA	1.91 µg/L		
				d_3-CPMA			
				HPMMA	0.49 µg/L		
				d_3-HPMMA			
Styrene				PHEMA1	0.03 µg/L		
				$^{13}C_6$-PHEMA1			
				PHEMA2	0.13 µg/L		
				$^{13}C_6$-PHEMA2			
Benzene				PMA	0.005 µg/L		
				d_5-PMA			

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Toluene				BMA d_7-PMA	0.027 µg/L		
Alkylating agents				HEMA d_4-HEMA	0.06 µg/L		
Ethylating agents				EMA d_5-EMA	0.008 µg/L		
Methylating agents				MMA d_3-MMA	0.88 µg/L		
Multiple determination	Concentrate/resuspend in MeOH	RP, gradient NH_4CH_3COOH/ACN	ESI/QQQ/MRM$^-$			Diet controlled smokers & nonsmokers	Pluym et al. (2015)
Acrolein				3-HPMA $^{15}N^{13}C_3$-3-HPMA	12.6 µg/L		
Acrylamide				AAMA d_3-AAMA	8.7 µg/L		
				GAMA d_3-GAMA	0.36 µg/L		
Dimethylformaide				AMCC d_3-AMCC	0.93 µg/L		
Propylene oxide				HPMA d_3-HPMA	1.3 µg/L		
1,3-Butadiene				MHBMA1 d_6-MHBMA1	0.09 µg/L		
				MHBMA2 d_6-MHBMA2	0.03 µg/L		
				DHBMA d_7-DHBMA	4.6 µg/L		
Multiple determination	RAM phase column switching	RP, gradient HCOOH/ACN	ESI/QQQ/MRM$^-$			Smokers & nonsmokers	Zhang et al. (2014)
Acrylonitrile				CEMA d_3-CEMA	0.015 µg/L		
Acrolein				3-HPMA d_3-3-HPMA	0.049 µg/L		
1,3-Butadiene				DHBMA d_7-DHBMA	0.053 µg/L		
Benzene				PMA d_5-PMA	0.013 µg/L		
Crotonaldehyde				HPMMA d_3-HPMMA	0.032 µg/L		
Multiple determination	Ionic liquid DLLME	HILIC, gradient HCOOH/ACN	ESI/QQQ/MRM$^-$			Smokers & nonsmokers	Li et al. (2015b)
Acrolein				CEMA $^2H_3$-CEMA	2.25 µg/L		
				3-HPMA $^2H_3$ -3-HPMA	0.40 µg/L		
Benzene				PMA $^2H_5$-PMA	0.11 µg/L		
1,3-Butadiene				DHBMA $^2H_7$-DHBMA	1.50 µg/L		
				MHBMA $2H_6$-MHBMA	1.15 µg/L		

(*continued*)

Table 3. Continued

Parent compounds	Sample preparation	Chromatography	Interface/detection	Target mercapturate	Limit of detection	Validation population	Reference
Crotonaldehyde				HPMMA $^{2}H_3$ –HPMMA	2.50 µg/L		
Styrene				PHEMA $^{13}C_6$ -PHEMA	0.05 µg/L		
Multiple determination	SPE	HILIC, gradient NH_4CH_3COOH/ACN	ESI/QQQ/MRM$^-$			Workers	Eckert et al. (2010)
Glycidol				DHPMA $^{13}C_2$-DHPMA	5.5 µg/L		
Ethylene oxide				HEMA d_4-HEMA	4.4 µg/L		
Propylene oxide				2-HPMA	7.0 µg/L		
Acrolein				3-HPMA d_3-3-HPMA	3.0 µg/L		
1,3-Butadiene				DHBMA d_7-DHBMA	4.5 µg/L		
				MHBMA d_6-MHBMA	5.0 µg/L		
Multiple determination	RAM phase column switching	RP, gradient CH_3COOH/ACN	ESI/QQQ/MRM$^-$			Smokers & nonsmokers	Eckert & Goen et al. (2014)
Ethylating agent				EthylMA d_5-EthylMA	2.1 µg/L		
Methylating agent				MethylMA d_3-MethylMA	5.1 µg/L		
1-Bromopropane				NPMA d_3-NPMA	2.5 µg/L		
2-Bromopropane				isoPMA d_3-isoPMA	2.0 µg/L		
Multiple determination	Protein precipitation	RP, gradient NH_4CH_3COOH/ACN	ESI/QQQ/MRM$^-$			General population	Alwis et al. (2012)
Crotonaldehyde				HPMMA $^{2}H_3$-HPMMA	1.3 µg/L		
Styrene				PHEMA $^{13}C_6$-PHEMA	0.7 µg/L		
Tetrachlorethylene				TCVMA $^{13}C_2$-TCVMA	3.0 µg/L		
Trichlorethylene				1,2-DCVMA ^{13}C-$^{2}H_3$ 1,2-DCVMA	12.6 µg/L		
				2,2-DCVMA ^{13}C-$^{2}H_3$ 2,2-DCVMA	6.5 µg/L		
Xylene				DPMA $^{2}H_3$-DPMA	5.0 µg/L		

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Multiple determination	SPE column switching	RP, gradient NH_4CH_3COOH/ACN	ESI/QQQ/MRM$^-$			Smokers & nonsmokers	Chiang et al. (2015)
Acrylamide				GAMA d_3-GAMA	0.014 µg/L		
1-Butadiene				DHBMA d_7-DHBMA	0.033 µg/L		
				MHBMA d_3-MHBMA	0.06 µg/L		
Acrylonitrile				CEMA d_3-CEMA	0.77 µg/L		
Benzene				PMA d_4-PMA	0.769 µg/L		
Xylene				2,4-DPMA d_3-2,4-DPMA	0.024 µg/L		
Multiple determination	SPE	RP, gradient CH_3COOH/MeOH	ESI/QQQ/MRM$^-$			Urban traffic wardens	Sabatini et al. (2008)
Benzene				PMA d_5-PMA	0.30 µg/L		
Toluene				BMA d_5-BMA	0.35 µg/L		
Xylene				MBMA 2-HPMA	0.40 µg/L		
Multiple determination	SPE	RP, gradient CH_3COOH/ACN	APCI/QQQ/MRM$^-$			Rural agrarian men	Norrgran et al. (2006)
Atrazine				AZMA $^{13}C_3$-AZMA	60 ηg/L		
Acetochlor				ACMA $^{13}C_6$-ACMA	48 ηg/L		
Alachlor				ALMA $^{13}C_6$-ALMA	39 ηg/L		
Metolachlor				MEMA $^{13}C_6$-MEMA	36 ηg/L		

ACN: acetonitrile; APCI: atmospheric pressure chemical ionization; DDLME: dispersive liquid-liquid extraction; ESI: electrospray ionization; HILIC: hydrophilic interaction chromatography; MeOH: methanol; MRM: multiple reaction monitoring; *N*-acetyl-cys: *N*-acetyl-cysteine; PFBBr: pentafluorobenzyl bromide; RP: reversed phase; RP-WAX: mixed mode reversed phase-weak anionic interaction; SPE: solid phase extraction; QQQ: triple quadrupole.

selecting between four extraction eluate compositions to increase analyte stability and maximize MS/MS signal intensity. Following extract elution into 2 µL square wells, further extract transfer and handling were eliminated by evaporation of extracts under nitrogen in the sample wells. Evaporated extracts were stored up to four days without sample degradation before reconstitution immediately before analysis. This format may be adapted to online analysis when high-throughput and the speed of fully automated analysis are necessary.

In a second form of high-throughput SPE, larger volumes of urine and high numbers of samples may be rapidly extracted using online sample extraction with column switching. Urine was passed through a trap linked to the chromatographic column using an online multiple valve system (Hou et al., 2012; Kellert et al., 2006; Kopp et al., 2008; Li et al., 2005; Schettgen et al., 2008a). In this technique, target mercapturate analytes are retained in the trap; when valve positions are switched, urinary proteins and salts are washed away to waste. A final switching of valves with a change to elution buffer carries analytes from the trap to the HPLC column for separation. Online SPE extraction using column switching has grown in popularity to create automated analyses that decrease overall analysis time by a substantial reduction in sample preparation steps. This trend may be expected to continue in the future as better automated HPLC–MS/MS systems become commercially available. Kuklenyik has described online SPE–LC–MS/MS method design and optimization by presenting three example applications including a determination of two mercapturates of ATZ (Kuklenyik et al., 2011). Online SPE–LC–MS/MS has been used extensively by Schettgen and collaborators (Reska et al., 2010; Schettgen et al., 2008a; Schettgen et al., 2009; Schettgen et al., 2012) in simultaneous determinations of biomarker mercapturates of aromatic compounds and volatile alkylating agents. Here, rapid automated sample extraction and µg/L sensitivity are combined in methods for occupational and environmental exposure assessment to tobacco smoke and urban air pollutants in the general populations.

Liquid chromatography

Reversed phase. Mercapturate metabolite analysis methods often use RP columns containing a non-polar stationary phase consisting of alkane chains (i.e. C18, C8 or C4). Commonly used mobile phases for tandem MS analysis contain volatile acids or buffers such as formic acid (HCOOH), acetic acid (CH_3COOH), ammonium formate (NH_4COOH) or ammonium acetate (NH_4CH_3COOH). Organic modifiers such as methanol (MeOH) and acetonitrile (ACN) are typically used with either isocratic or gradient conditions for analyte elution. RP chromatography is common with electrospray ionization MS (ESI-MS), but has the major limitation in the lack of retention of highly hydrophilic, ionic or polar molecules on the stationary phase. Mixed mode separation has also been reported in the literature. Kotapati combined RP and weak anionic exchange to determine THBMA, a highly polar mercapturate of 1,3-butadiene (Kotapati et al., 2011).

Ultra-high performance liquid chromatography (UHPLC)

Ongoing improvements to increase the speed and efficiency of separations are related to column technology and instrumentation. UHPLC utilizes shorter columns, 3–5 cm long and reduced particle sizes, smaller than 2 µm. Shorter columns result in faster analysis times and shorter equilibration time. Reduced particle size decreases analyte peak dispersion, thus enhancing peak resolution. Sub-2 µm particles produce sharper peaks while decreasing sample loading over conventional HPLC. These improvements in column efficiency and linear velocity can be expected to increase the number of theoretical plates (Varma et al., 2010). This in turn may increase sensitivity by increasing the signal to noise ratio of the detector. The mass spectrometer is a mass sensitive detector, not concentration dependent such as in ultraviolet detection; therefore, UHPLC may in some cases match or exceed the sensitivity of standard chromatographic systems.

The advantages of sub-2 µm particles working at higher pressures were reviewed (Nguyen & Schug 2008). However, very high pressure is required to push mobile phase through a column packed with smaller diameter particles. UHPLC has come into use for biomarker monitoring owing to the fact that most HPLC pump manufacturers are offering pumping systems capable of maintaining the high back pressure levels required for the technique. Standard HPLC pumping systems have traditionally maximum pressure levels of approximately 6000 psi (~420 atm) while UHPLC pumps are designed to handle pressures in excess of 15 000 psi (~1000 atm). These fundamental aspects and practical requirements of UHPLC have been reviewed (Wu & Clausen, 2007). Application of UHPLC specifically to bioanalysis has been reviewed (Varma et al., 2010). UHPLC analysis of structurally similar mercapturates found in the urine of smokers (Pluym et al., 2015; Wu et al., 2012) and multiple mercapturates in workers exposed to multiple volatile organic chemicals (Alwis et al., 2012) will be considered in later sections of this review.

Hydrophilic interaction chromatography. Biomonitoring of worker exposure to electrophilic alkylating agents that are carcinogenic, such as AA (International Agency for Research on Cancer, 1994), acrylonitrile (AN) (International Agency for Research on Cancer, 1999), styrene (International Agency for Research on Cancer, 1994) and 1,3-butadiene (International Agency for Research on Cancer, 2008), is important in industrial medicine (De Rooij et al., 1998; Vanwelie et al., 1992). The mercapturates of these compounds are polar, especially those containing hydroxylalkyl groups. These hydroxyalkyl mercapturates (HAMAs) are difficult to retain on RP columns without using highly aqueous conditions and nonvolatile mobile phase components that are not compatible with mass spectrometric detection. Ordinarily normal phase (NP) would be used to satisfy the separation conditions of highly polar analytes (Snyder et al., 2010), but NP is not easily made compatible with ESI-MS. Hydrophilic interaction chromatography (HILIC) has been used to overcome the mismatch between NP like chromatography and ESI-MS. Typically, HILIC mobile phases using high organic content (>80%) are ideal for ESI-MS analysis, and may

enhance ES-MS response (Nguyen & Schug, 2008). HILIC utilizes a polar stationary phase with an aqueous/polar organic solvent mobile phase, where water is introduced to play the role of a stronger eluting solvent. In HILIC separations, mercapturates are separated from urinary matrix by a partitioning mechanism between a water-enriched layer associated with a polar stationary phase and solvent containing ammonium formate or ammonium acetate and 5–15% water to maintain the water-enriched layer on the stationary phase. Since retention increases with hydrophilicity and polarity of the analyte, elution is driven by increasing water content in the mobile phase which is composed of high organic content, usually consisting of acetonitrile or alternatively, methanol. The HILIC mechanism has been discussed in great detail (Hemstrom & Irgum, 2006) and the advantages of HILIC when combined with ESI-MS detection have been reviewed (Nguyen & Schug, 2008). The application of HILIC in quantitative bioanalysis of compounds of pharmaceutical interest has been described (Jian et al., 2010).

HILIC columns have been reported for the determination of mercapturates having greater molecular polarity (Eckert et al., 2010; Kopp et al., 2008; Sterz et al., 2012; Yan et al., 2010), and this technique has become an important recent trend in mercapturate analysis. HILIC–ESI-MS appears to be a useful technique and has been used as a complement to RPLC–ESI-MS studies by Dekant and collaborators (Kellert et al., 2006; Kopp et al., 2008) in complementary studies of AA and glycidamide (GA) mercapturates and other polar AA metabolites (Table 2). Kopp used HILIC–ESI-MS to achieve baseline separation between two AA metabolites, AAMA-sulfoxide and GAMA. Failure to resolve these two metabolites could result in over estimation of urinary GAMA levels, and hence an over estimation of the potential risk of AA exposure in humans. A direct-injection method (Yan et al., 2010) was developed using HILIC to eliminate sample extraction used in earlier methods (Carmella et al., 2007; Mascher et al., 2001) to quantitate the acrolein mercapturate 3-HPMA. The method specificity, linearity, precision and accuracy met required FDA criteria (US Department of Health and Human Services, Food and Drug Administration, 2001). HILIC chromatography has also been used to simultaneously determine HAMAs of multiple alkylating agents in a single chromatographic run (Eckert et al., 2010; Sterz et al., 2012). These studies are considered later in this review describing simultaneous determination of mercapturates in a single chromatographic analysis.

Mass spectrometry modes of detection

In tandem HPLC/MS analysis, after target analytes are separated by chromatography, they are introduced into the mass spectrometer for analysis. This is done at the LC/MS interface where the chromatographic eluate is vaporized and the analyte molecules are ionized for mass selection and detection in the mass analyzer. The ion sources used in the methods reviewed here are ESI and atmospheric chemical ionization (APCI). These ionization techniques allow easy and robust interfacing of HPLC to tandem mass spectrometry (Zimmer, 2003).

In ESI, the liquid eluent from the HPLC system is passed through a small capillary needle held at a high electrical potential (2000–5000 V). This results in electrostatic nebulization of the liquid into droplets. The resultant droplets contain a net charge having the same polarity as the voltage placed on the needle. During desolvation of the droplets, the electric field increases in strength at the diminishing droplet surface and leads to the ejection of charged analyte ions upon final evaporation. The ESI source is a gentle and ''soft'' ionization technique and does not cause significant thermal degradation as that caused by other ion sources. ESI is able to ionize extremely polar/nonvolatile molecules, which may be more difficult for APCI; thus, it is generally considered more versatile than APCI. ESI also has a high level and efficiency of ionization, which leads to a higher level of detector sensitivity. ESI sources generally require water and acidic pH of the mobile phase to aid in ionization.

In APCI, the liquid eluent from the HPLC is heated and aerosolized by means of a nebulizer and a high flow of nitrogen gas. The aerosol is subjected to a corona discharge to form ions in a three step process. First, a corona discharge ionizes the nebulizing gas to form primary ions. These primary ions react immediately with the solvent molecules of the mobile phase forming reagent ions. Finally, the reagent ions react with the analyte molecules to form $[M+H]^+$ in positive-ion mode or $[M-H]^-$ in negative-ion mode. In APCI, this cascade of ionization reactions occurs in the gas phase, unlike ion formation from the liquid phase as in ESI. The APCI source allows for improved analysis of non-polar and medium polar volatile compounds. APCI is generally regarded as a more robust ionization method than ESI, and it is less susceptible to signal suppression resulting from co-eluting matrix components (Ackermann, et al., 2002; Korfmacher 2005; Matuszewski et al., 1998). APCI also requires little or no buffers in aqueous mobile phases to assist in ionization.

The basic function of a mass spectrometer is to measure the mass-to-charge ratios (*m/z*) of analyte ions. Mass spectrometers have various designs which have been reviewed elsewhere in the literature (Goddlett et al., 2001). Although mass spectrometers are used in qualitative identification of compounds, the monitoring of specific ions for quantitative determination is the focus of this discussion. For the mercapturate analyses surveyed for this review, single quadrupole (SQ) mass analyzers and tandem mass spectrometers using the triple quadrupole (QQQ) design dominate what is reported in the literature (Tables 2 and 3). The mass analyzer of the spectrometer separates the formed precursor ions. Analysis of analytes using SQ instruments is performed in Selected Ion Monitoring (SIM) mode in which only a selected *m/z* value is detected in the analysis. The majority of the methods found in this review use tandem transmission quadrupole instruments (MS/MS). In the case of multiple mercapturate metabolite analysis, all use the QQQ where precursor ions are selected in the first quadrupole, and allowed to pass into a collision chamber for collision-induced dissociation fragmentation into product ions. Transmitted from the collision chamber, fragmentation product ions will be separated by the third quadrupole for detection. Tandem mass spectrometry provides the greatest level of sensitivity

and specificity for the analysis method. This allows for detection of analytes in the presence of biological sample matrix components that would otherwise interfere with ultraviolet or fluorescence detection (B'Hymer & Cheever, 2010). For this reason, tandem MS detection is considered the method of choice for quantitation of metabolites in biological fluids (Matuszewski et al., 2003). The high sensitivity of the MS/MS detection is of particular importance in investigating low-level pollutant exposure in general populations in whom urinary mercapturates may be present in μg/L or ng/L levels.

Signal suppression is a well-known problem in HPLC–MS/MS analysis. The most common problem is that of ion suppression; the problem of ion enhancement, although rare, may also be encountered (Matuszewski et al., 1998). Both manifestations can be caused by sample matrix interferences from components within the sample. The mechanism of matrix induced ion suppression or enhancement is not fully understood (Kebarle & Tang, 1993; King et al., 2000). It is thought to originate from the competition between the target analyte and a co-eluting, undetected sample component reacting with the primary ions formed in the HPLC–MS/MS. This type of matrix effect may greatly change the reproducibility or the quantitation accuracy of a particular analyte.

Compensation for signal suppression by the use of an internal standard is a common strategy used in HPLC–MS/MS. The use of an internal standard will only be effective if the internal standard is subject to the same type of matrix effect as the analyte and must essentially chromatographically co-elute with the target analyte. Thus, ideally, the best internal standard is the stable isotopically labeled target analyte or a chemically similar homolog of the target analyte. For example, deuterated or carbon-13 analogs of the target analytes are typically used as internal standards. The internal standard is added to both the initial sample and spiked sample solutions before analysis. Calibration plots using analyte/internal standard peak-area ratios generated from the chromatograms, rather than the peak area of the target analyte alone, are then used to calculate accurate results. At the time of this writing, both analytical and isotopically labeled standards for the mercapturates reviewed in this work (Table 1) are commercially available or available through custom synthesis. Seven commercial sources of mercapturates are listed by Alwis et al., and many others are available (Alwis et al., 2012). This was not the case in several earlier published methods reviewed here when researchers used general methods of laboratory synthesis to produce the required mercapturate standards (Van Bladeren et al., 1980).

Mercapturates as indicators of toxicant metabolism

The metabolism of occupational and environmental toxicants in exposed individuals varies with toxicant dose, absorption and enzyme polymorphisms (De Rooij et al., 1998; Vanwelie et al., 1992). The excretion of mercapturates subsequently varies and their quantitation offers information about individual occupational and environmental exposure, internal dose and differences in metabolism. Metabolic activation of an electrophilic compound can produce multiple metabolites many of which may react with GSH to form a mercapturate. The determination of one or more mercapturate products of a parent compound may be required for informative biomonitoring (De Rooij et al., 1998; Vanwelie et al., 1992). The metabolism of several environmental toxicants and the formation of their mercapturates will be described briefly: these include AA, AN, 1,3-butadiene, benzene, toluene, styrene, 1-bromopropane (1-BP) and polycyclic aromatic hydrocarbons (PAHs). These examples demonstrate broadly how mercapturate determination is used in exposure biomonitoring and in the investigation of toxicant metabolism. Toxicant metabolism may include biological activation, detoxification and elimination of mutagenic or carcinogenic toxicants.

Acrylamide

AA is an extensively used industrial chemical intermediate with many applications such as a polymerizing agent in grouts or other AA polymers used in waste water treatment, soil stabilization and paper manufacture (Friedman, 2003). Low levels of AA are present in baked, fired and roasted foods, and mainstream and sidestream tobacco smoke are common sources of human exposure (Tornqvist, 2005). Both AA and its oxidative metabolite GA contain electrophilic groups capable of binding to cellular proteins, a property associated with AA neurotoxicity. Furthermore, GA, a reactive epoxide, binds to nucleophilic nucleic acids to form adducts with cellular DNA; this mechanism is regarded as the cause of AA carcinogenicity (International Agency for Research on Cancer, 1994). Thus, in human biomonitoring and health risk assessments of AA elimination routes, the metabolism and conversion to GA must be considered. AA may be detoxified by direct conjugation with GSH to form AAMA (Figure 2). GSH conjugation of GA, an epoxide, leads to formation of two isomeric mercapturates, GAMA2 and GAMA3. Quantification of AAMA is a measure of direct detoxification of AA, while determination of GAMA2 and GAMA3 measures AA bioactivation to a direct-acting mutagen. HPLC/MS analysis has been adapted to these purposes and five examples of this are listed in Table 2 (B'Hymer & Cheever, 2007; Kellert et al., 2006; Kopp et al. 2008; Li et al., 2005; Zhang et al., 2015). A method for simultaneous determination of urinary AA and the mercapturate, AAMA, was developed for occupational exposure monitoring (B'Hymer & Cheever, 2007). As indicated previously, AA exposure is not limited to the industrial environment. To examine the health risk posed by AA in food, methods for simultaneous quantitation of AAMA and of GA isoform mercapturates, GAMA2 and GAMA3, have been developed and reported in the literature during epidemiologic studies for dietary AA conversion to GA in non-occupationally exposed populations (Kellert et al., 2006; Kopp et al., 2008; Zhang et al., 2015).

Acrylonitrile

AN, a widely-used industrial chemical and component in tobacco smoke, is a thoroughly characterized chemical exposure hazard (International Agency for Research on Cancer, 1999). AN is not directly carcinogenic, but like AA, is potentially carcinogenic through an oxidative

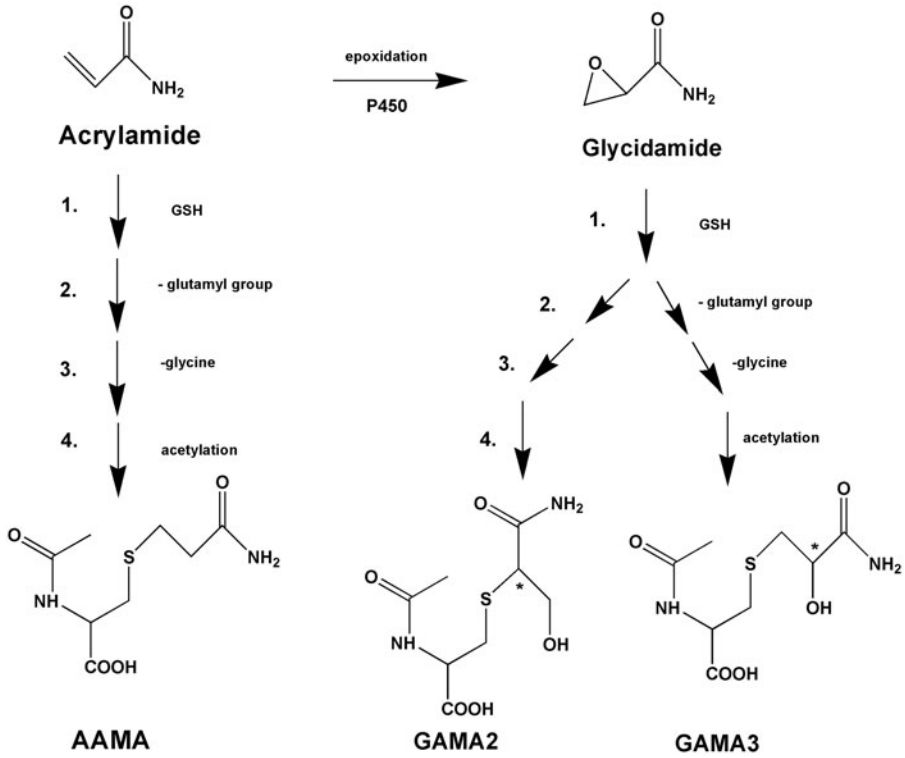

Figure 2. Simplified formation of acrylamide mercapturates beginning with direct glutathione (GSH) conjugation to form AAMA, or by GSH conjugation of glycidamide, the electrophilic epoxide metabolite of acrylamide to form isomeric mercapturates, GAMA2 and GAMA3. Asterisks (*) indicate the asymmetric carbons.

metabolite, cyanoethylene-epoxide (CEO) (Hou et al., 2012; Schettgen et al., 2012; Wu et al., 2012). If this epoxide is not detoxified by GSH conjugation or other mechanism, it can react with nucleophilic sites in DNA and function as direct-acting mutagens. AN is detoxified by direct GSH conjugation to form 2-cyanoethyl mercapturate (CEMA). CEO is detoxified and eliminated as 2-hydroxyethyl mercapturate (HEMA) or 1-cyano-2-hydroxyethylmercapturate (CHEMA) (Figure 3). Taken together CEMA, HEMA and CHEMA quantitation represents conjugative detoxification of AN, while HEMA and CHEMA quantitation is a measure of metabolic activation of AN to the reactive epoxide, CEO.

Aromatic solvents

Benzene and toluene are aromatic hydrocarbons used as solvents in industrial chemicals, as common additives in fuels and are components in cigaret smoke. Benzene is a human cancer hazard (International Agency for Research on Cancer, 1982) and the hematotoxicity of benzene has been elucidated during the past few decades (Arnold et al., 2013). A non-carcinogen toluene (International Agency for Research on Cancer, 1989) is often used as a less toxic substitute for benzene in inks, dyes, thinners, detergents and in chemical and drug preparation (American Conference of Governmental Industrial Hygienists (ACGIH), 2014). Historically, biological monitoring for both chemicals was done using their hydrolytic detoxification products. In the case of benzene, four metabolites, phenol, catechol, hydroquinone and *trans, trans*-muconic acid (*tt*MA), have been investigated and used as biomarkers (American Conference of Governmental Industrial Hygienists (ACGIH), 2014). However, their urinary levels are influenced by the metabolism of gut flora, diet and medication use, and smoking (Arnold et al., 2013). Similarly, the metabolism of toluene produces two hydrolytic metabolites, hippuric acid and *o*-cresol, that are influenced by diet and are not specific for occupational exposure (Cosnier et al., 2013). However, GSH detoxification of both these solvents produces corresponding mercapturates which may be used as biomarkers of exposure. Benzene metabolism forms phenylmercapturic acid (PMA) and toluene forms benzylmercapturic acid (BMA). Because a biomarker of exposure should be specific for chemical exposure, PMA and BMA that are free of dietary and endogenous interferences are preferred biomarkers for benzene and toluene exposure (Arnold et al., 2013; Cosnier et al., 2013). However, both are influenced by smoking. Therefore, in biomonitoring, study subjects are asked to refrain from smoking for 2 h before urine collection (Lovreglio et al., 2010).

In addition to toluene's side chain GSH metabolite BMA, three isomeric toluylmercapturates resulting from the arene oxidation of toluene's aromatic ring may be found in rat urine (Cosnier et al., 2012). One of these, S-*p*-toluylmercapturate, was demonstrated in urine from toluene exposed workers, and has been proposed as a biomarkers of exposure (Angerer et al., 1998). Similarly, arene oxidation of the aromatic ring in xylene and ethylbenzene leads to the formation of isomeric mercapturates (Gonzalez-Reche et al., 2003; Cossec et al., 2013).

1-Bromopropane

1-BP is an industrial solvent used as a substitute for chlorofluorocarbons or 1,1,1-trichloroethane in metal electronics degreasing, in adhesives, in aerosol solvents or in place of trichloroethylene or perchloroethylene in dry cleaning (National Institute for Occupational Safety and Health, 2013). Worker case studies report that 1-BP exposure

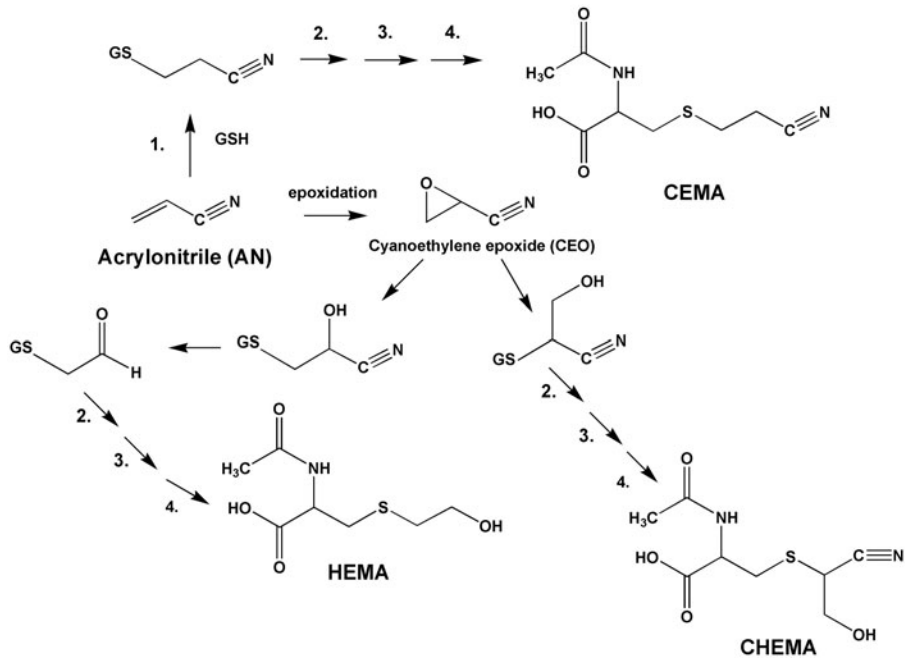

Figure 3. Simplified formation of acrylonitrile mercapturates by direct detoxification to CEMA, or by detoxification of bioactivated cyanoethylene-epoxide to form HEMA and CHEMA.

causes central and peripheral neurological disorders and changes in cellular blood components in workers including evidence of dose-dependent neurological and hematological changes in women working in 1-BP production plants (Ichihara, 2005; Li et al., 2010; Majersik et al., 2007; Morbidity Mortality Weekly Report, 2008; Raymond & Ford 2007; Sclar, 1999). Available epidemiologic studies have not reported cancer in humans exposed to 1-BP. However, based on recent animal studies, the National Toxicology Program concluded 1-BP is reasonably anticipated to be a human carcinogen. In these studies, the molecular alterations observed in experimental rodents that are associated with oxidative metabolism, genotoxicity and carcinogenesis are relevant to possible mechanisms of carcinogenicity in humans (US Department of Health and Human Services Public Health Service. National Toxicology Program, 2014). At the time of this writing, there are no standardized biological monitoring techniques for 1-BP. To compare and evaluate the suitability of several 1-BP metabolites as biomarkers of occupational exposure, recent animal exposure and human exposure studies are considered.

The metabolism of 1-BP is complex (Baines et al., 1977; Barnsley et al., 1966; Jones & Walsh, 1979; Sklan & Barnsley, 1968; Tachizawa et al., 1982). In rats, some absorbed 1-BP is metabolized rapidly through direct conjugation with GSH to form *n*-propylmercapturate with the release of free bromide (Br^-) ions (Jones & Walsh, 1979). Mercapturate formation, investigated most completely in rodents, produces four mercapturates; *n*-propylmercapturate, and three more derived from C2 or C3 oxidations of 1-BP by cytochrome P450 2E1 monooxygenase (CYP2E1). Oxidative metabolites, 1-bromo-2-propanol and bromoacetone (Barnsley et al., 1966) and 3-bromopropionic acid (3-BPA) (Jones & Walsh, 1979) are, in turn, conjugated with GSH to form 2-hydroxypropyl-mercapturate, 2-oxopropylmercapturate and 2-carboxyethylmercapturate, respectively (Figure 4).

Human exposure studies investigated urinary bromide level (Br^-) as a possible biomarker of 1-BP exposure using GC with electron capture detection (Kawai et al., 2001; Kawai et al., 2002; Zhang et al., 2001). The correlation between urinary Br^- and airborne 1-BP was significant, but background urinary Br^- was substantial ($\sim$8 mg/L). Further investigation found that intake of fruit, sea-food, some soft drinks and use of brominated vegetable oils influenced urinary Br^- levels (Horowitz, 1997; Kawai et al., 2002; Zhang et al., 2001). This lack of specificity limits the use of urinary Br^- levels for estimating human occupational exposure only when dietary or other bromide intake can be considered. Alternatively, other metabolites identified in rodents are specific products of 1-BP oxidation by CYP2E1; 1-bromo-2-propanol, bromoacetone and 3-BPA. Their mercapturates represent specific biomarkers that are free of dietary or other non-occupational interferences (Ichihara et al., 2001; Pombrio et al., 2001; Valentine et al., 2007). Urinary *n*-propylmercapturate levels were measured using GC/MS in post-shift urine samples of 47 workers in a 1-BP production plant. Urinary *n*-propylmercapturate levels increased with increasing 1-BP exposure in these workers (Valentine et al., 2007). Investigation of this mercapturate and one oxidative metabolite precursor, 3-BPA, as potential biomarkers of 1-BP exposure in highly exposed workers was performed in this laboratory (Mathias et al., 2012). HPLC/MS was used to quantify urinary mercapturates of 1-BP (Cheever et al., 2009) where the direct GSH conjugate, *n*-propylmercapturate was predominate in urine specimens taken from the most heavily exposed workers. The same urine samples when analyzed for 3-BPA using GC/MS (B'Hymer & Cheever, 2004) contained no 3-BPA. This result suggests that the predominate *n*-propylmercapturate, the product of direct conjugate of 1-BP with GSH, is a major detoxification route in highly exposed workers, and that oxidative metabolism of 1-BP is not a major metabolic pathway in humans. Studies of human metabolism of 1-BP are limited to analysis of *n*-propylmercapturate in

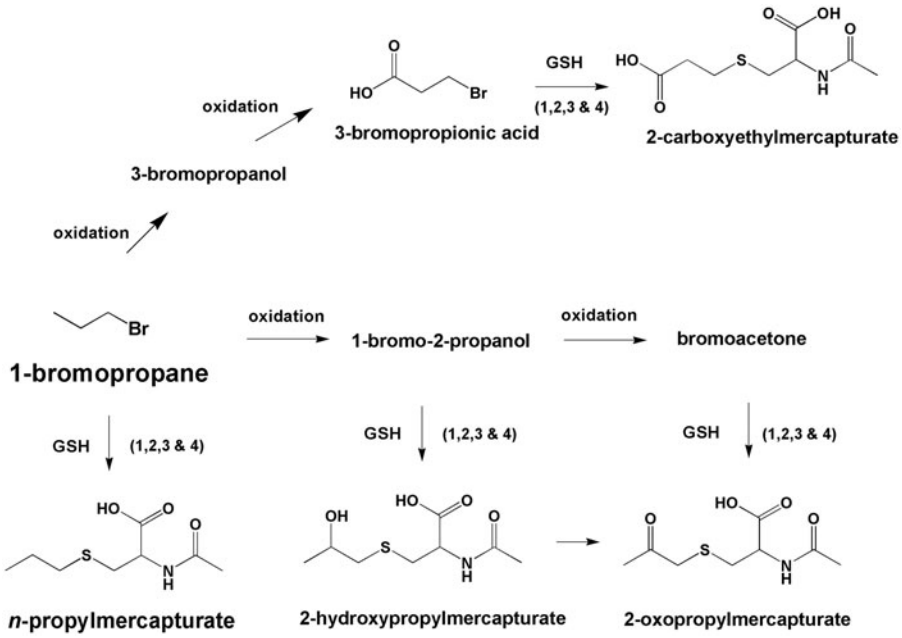

Figure 4. Metabolism of 1-bromopropane (1-BP) in the rat by multiple pathways. Direct conjugation of 1-BP forms *n*-propyl mercapturate or 1-BP may be oxidized to form metabolites which are conjugated with GSH to form other mercapturates.

worker urine (Ichihara et al., 2001; Valentine et al., 2007), and provide no explanation for these results. However, studies of oxidative metabolism of 1-BP by CYP2E1 in rodents by Garner demonstrated that 1-BP metabolism becomes saturated in highly exposed rats, but not in mice (Garner et al., 2006). In rats, 1-BP oxidative metabolism by CYP2E1 is dose-dependent and becomes blocked with increased toxicant dose. As a result *n*-propylmercapturate becomes a predominate urinary mercapturate. When 1-aminobenzotriazole, an inhibitor of oxidative metabolism was given to rats, all oxidative metabolites including 3-BPA, were eliminated from their urine leaving only *n*-propylmercapturate as the predominate urinary mercapturate, mirroring the effect of metabolite saturation of CYP2E1 activity that likely occurs in high 1-BP exposure in humans. These results suggest a difference between human and rat metabolism of 1-BP and that found in mice. In these studies, urinary mercapturate identification was used to investigate species differences in toxicant metabolism, and to deduce possible changes in the activity of up-stream metabolic pathways when the conditions of toxicant exposure in research animals or workers are changed. In summary, the mercapturates of 1-BP are specific for 1-BP exposure while bromide ion levels are not. Oxidative metabolites of 1-BP, 3-BPA or others are specific, but may not be produced during high exposures. Given these considerations, *n*-propylmercapturate, the product of direct GSH conjugation of 1-BP appears to be the best candidate biomarker of 1-BP exposure.

1,3-Butadiene

The genotoxicity of butadiene (BD) is attributed to its three epoxide metabolites, epoxybutene (EB), diepoxybutane (DEB) and epoxybutanediol (EBD), each of which is able to react with cellular protein such as hemoglobin, or with DNA as direct acting carcinogens (International Agency for Research on Cancer, 2008). Assay of their detoxification mercapturate products are useful to measure BD exposure, epoxide formation and detoxification, and may then be used to estimate genotoxic risk of exposure (Figure 5). Occupational exposure studies of BD monomer and rubber workers have used the EB mercapturate metabolite, 3,4-dihydroxybutylmercapturate (DHBMA) alone (Ammenheuser et al., 2001; Hallberg et al., 1997; Kelsey et al., 1995; Ward et al., 1996) or both EB mercapturates, DHBMA and 1-hydroxymethyl-2-propenylmercapturate (MHBMA) in rubber workers (Albertini et al., 2003; Fustinoni et al., 2004; Hayes et al., 2000), smokers (Urban et al., 2003), urban traffic workers (Sapkota et al., 2006) and in an unexposed population (Schettgen et al., 2009). The ratio of these mercapturates DHBMA/(DHBMA + MHBMA) serves as a relative comparison of hydrolytic detoxification versus direct GSH conjugative detoxification of EB (Kirman et al., 2010b; Urban et al., 2003). This ratio was used to examine species differences in BD metabolism (van Sittert et al., 2000). An understanding of these differences is necessary for accurate human risk analysis when toxicokinetic data based on more easily studied rodent metabolism is used when data from human volunteer or occupational exposure studies are limited (Kirman et al., 2010a; Richardson et al., 1999; van Sittert et al., 2000).

Alternative metabolism of EB leads to further epoxide formation to produce EDB and DEB. Of these, EDB is the most abundant BD epoxide metabolite in humans (Swenberg et al., 2007). Formation of both epoxides has been investigated in exposed workers by measurement of their hemoglobin adduct, N-(2,3,4-trihydroxybutyl)-valine (THB-Val) (Albertini et al., 2001,2003; Fustinoni et al., 2002; Hayes et al., 2000; Perez et al., 1997; Vacek et al., 2010). This measurement requires a blood specimen, and the analysis is technically demanding and expensive. Alternatively, determination of urinary 2,3,4-trihydroxybutylmercapturate (THBMA), the detoxification mercapturate of both EDB and DEB is an indirect measure of BD metabolism to EDB

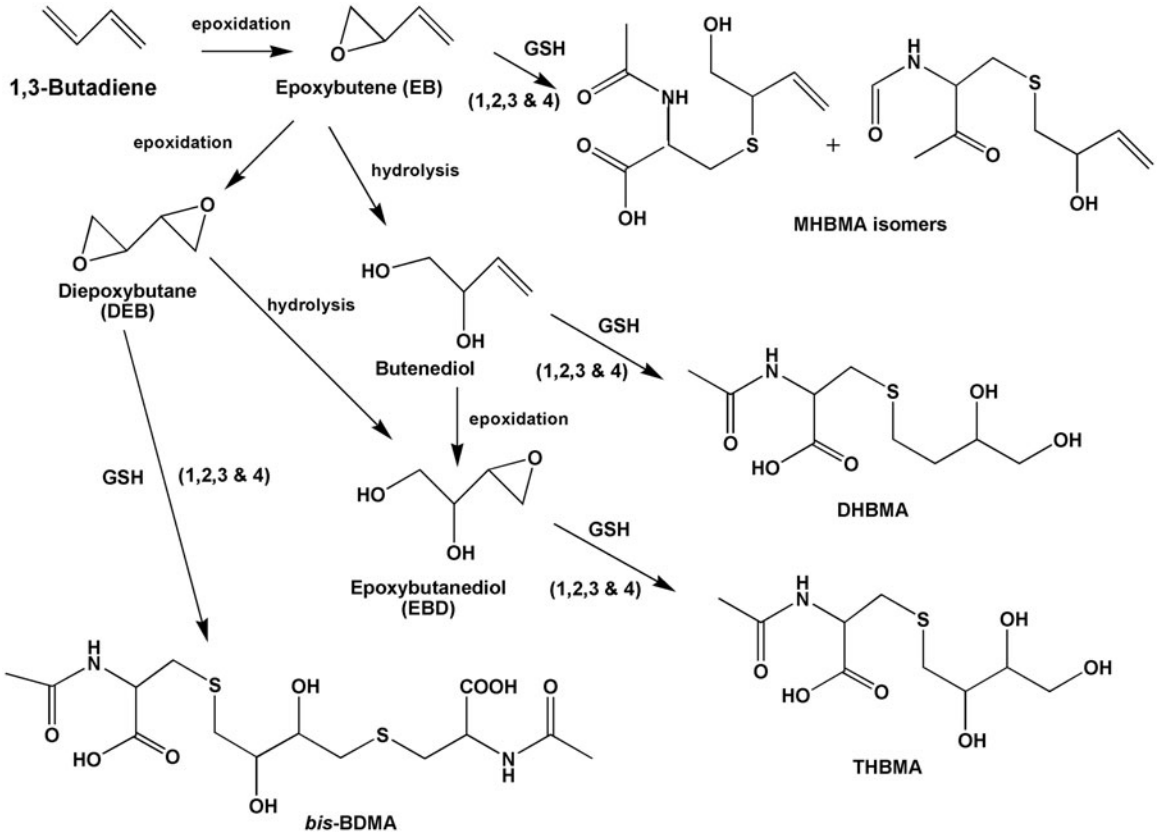

Figure 5. Alternate pathways of 1,3-butadiene metabolism and epoxide formation produce multiple mercapturates: isoforms MHBMA1 and MHBMA2; DHBMA; THBMA and *bis*-BDMA.

and DEB. THBMA is the most polar of the detoxification mercapturates of BD, and is poorly retained on RP media typically used in solid phase sample extraction and chromatography of less polar mercapturates. Kotapati adapted the method of Eckert using acidic sample extraction conditions to neutralize the carboxylate group of THBMA, improving analyte binding to the solid phase (Eckert et al., 2010; Kotapati et al., 2011). Chromatography was performed using mixed mode RP with weak anionic interaction to separate THBMA from other sample components. This method was expanded to include analysis of bis-BDMA, a mercapturate of DEB, as well as DHBMA and MHBMA for quantitation of all four mercapturates of BD (Kotapati et al., 2014). These recently developed HPLC/MS–MS methods for quantitation of urinary THBMA and bis-BDMA represent more cost effective biomarkers of EDB and DEB epoxide formation from BD than the currently used analysis of THB-Val adducts of hemoglobin.

Styrene

Styrene is an industrial monomer used worldwide in polymers, resins, latex paints and in manufactured building materials. The highest exposure potential occurs in open manufacturing of reinforced plastics and in hand-lamination workers (Miller et al., 1994). Oxidative metabolism of styrene occurs via two pathways both leading to products that contribute to its toxicity. Bioactivation may occur through oxidation of styrene's side chain to form styrene-7,8 epoxide (7,8-SO) a recognized animal carcinogen and a possible human carcinogen (International Agency for Research on Cancer, 1994). In humans the most abundant urinary metabolites, mandelic acid (MDA) and phenylglyoxylic acid (PGA), which represent the hydrolytic detoxification of SO, are used currently in occupational biomonitoring (American Conference of Governmental Industrial Hygienists (ACGIH), 2014). These metabolites are not specific for occupational exposure since trace amounts of styrene occur naturally in fruits, vegetables, nuts, beverages and in meats. In addition, styrene exposure may occur through tobacco smoke, consumer products and styrene vapor from building materials (American Conference of Governmental Industrial Hygienists (ACGIH), 2014). Because MDA and PGA are nonspecific, occupational exposure must be confirmed by measuring styrene in whole blood drawn at the end of the workday (American Conference of Governmental Industrial Hygienists (ACGIH), 2014). Alternative detoxification of 7,8-STO by GSH conjugation leads to formation of four isomeric phenylhydroxyethyl mercapturates (PHEMAs).

Investigations of styrene mercapturate formation have provided important information about the stereochemistry of styrene biotransformation (Figure 6). Oxidation of the side chain of styrene forms two stereoisomers of styrene epoxide, (S)7,8-STO and (R)7,8-STO. Each epoxide enantiomer when conjugated with GSH at either C7 or C8 of the side chain leads to two isomeric mercapturates designated PHEMA, MA1 and MA2. GSH conjugation of both S- and R-enantiomers yields four possible isomeric PHEMAs, the structures of which are shown in Figure 6 (Linhart et al., 1998). The relative proportions of the stereoisomers MA1 and MA2 was used to investigate the stereoselectivity of GSTM1

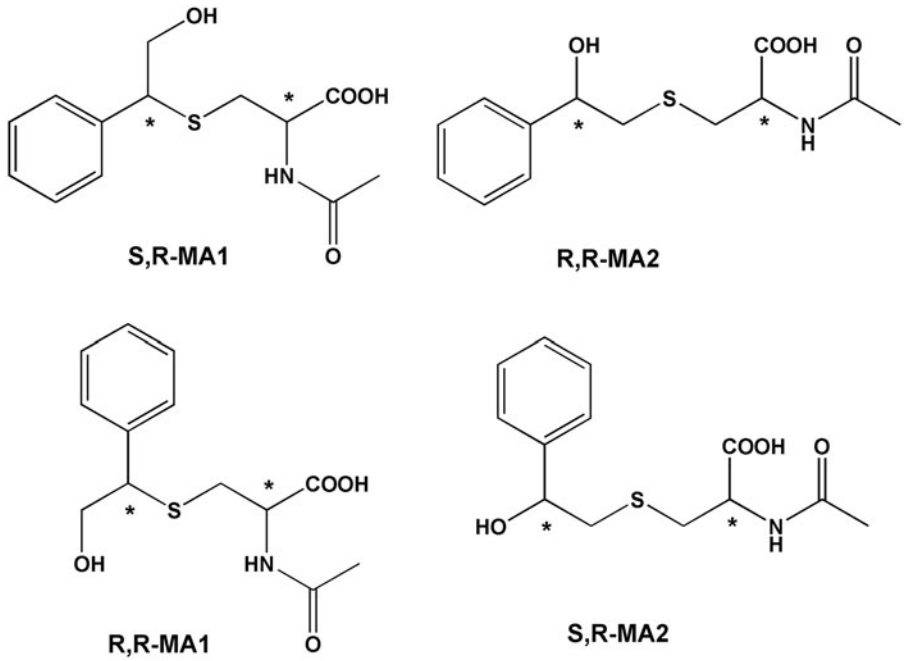

Figure 6. Stereoisomeric mercapturate products of styrene side-chain oxidation. Asterisks (*) indicate asymmetric carbons.

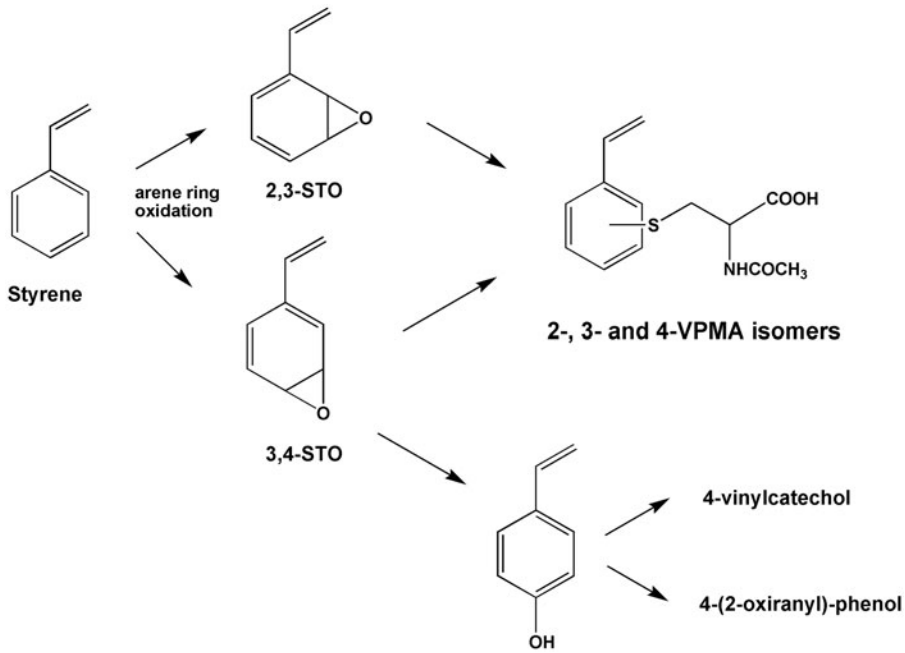

Figure 7. Ring oxidation of styrene produces 2,3- and 3,4-styrene oxides which may be further metabolized to 4-vinyl phenol and other reactive metabolites. Alternatively, the styrene oxides may be detoxified to produce three isomeric vinylphenol mercapturates 2-, 3- and 4-VPMA.

conjugation of 7,8-SO (De Palma et al., 2001; Fustinoni et al., 2008; Linhart et al., 1998). The most abundant stereoisomers R, R-MA1 and S, R-MA2 are derived from the less genotoxic (S)7,8-SO. This result suggests stereoselective conjugation of (S)7,8-SO at a less sterically hindered C8 over a more hindered C7 (Fustinoni et al., 2008). Formation of these mercapturates is a minor detoxification route, representing about 1% of inhaled styrene (Manini et al., 2003), but tandem HPLC/MS–MS analysis of these mercapturates offers a sensitive and specific tool for styrene biotransformation studies (Manini et al., 2000).

A second minor pathway of styrene bioactivation proceeds via oxidation on the aromatic ring to form arene oxides 2,3 and 3,4-styrene oxide (2,3 and 3,4-STO, respectively, Figure 7). 3,4-STO in turn may form 4-vinylphenol (4-VP), which has been found in the urine of workers exposed to styrene (Haufroid et al., 2002; Manini et al., 2003; Pfaffli et al., 1981; Watabe et al., 1982). 4-VP is more hepatotoxic and pneumotoxic than styrene and 7,8-STO at lower doses in rats and mice (Carlson, 2002; Carlson, 2004). Further studies indicate 4-VP metabolites including 4-vinyl catechol, -4-(2-oxiranyl)-phenol and electrophilic quinones could contribute to the hepatotoxicity of 4-VP (Carlson, 2004; Carlson, 2011; Zhang et al., 2011). Alternatively 2,3- and 3,4-STO may react with GSH to initiate formation of three isomeric vinylphenolmercapturates (VPMAs). Of these mercapturates, 4-VPMA, the product of 3,4-STO conjugation, is the most abundant VPMA isomer (Linhart et al., 2010; Linhart et al.,

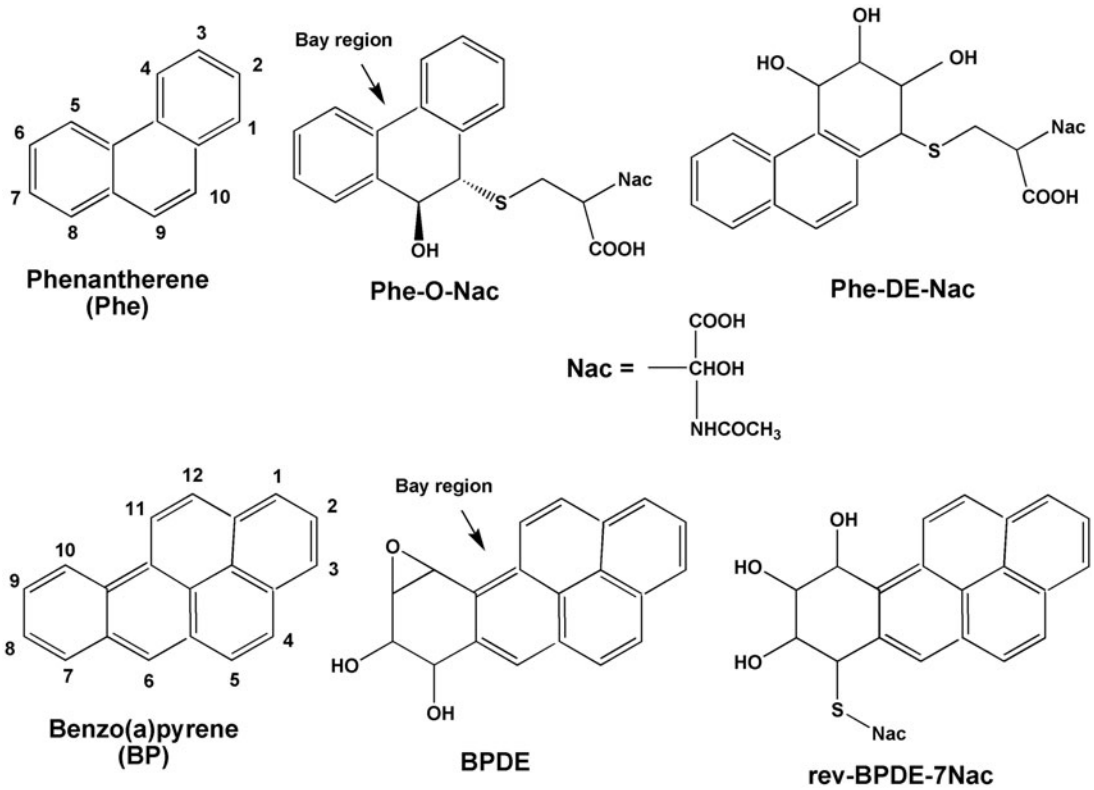

Figure 8. Mercapturate products of phenantherene (Phe) and benzo(a)pyrene (BP) indicate preferential GSH conjugation of the ''reverse'' diol epoxides over conjugation in the bay region of polycyclic aromatic hydrocarbons.

2012). The mercapturate products of 2,3-STO conjugation, 2- and 3-VPMA were detected only in trace amounts suggesting selective GSH conjugation at C4 of 3,4-STO over conjugation of 2,3-STO (Linhart et al., 2012). Formation of these mercapturates represents activity in a second toxicologically significant pathway of styrene activation and biotransformation (Linhart et al., 2012) (Table 2). Although determination of these styrene mercapturates provides toxicologically relevant information, they are minor metabolites and do not reflect absorbed dose as accurately as the currently used biomarkers MDA and PGA (De Palma et al., 2001).

The biotransformation of styrene is complex, but the sensitivity and specificity of tandem HPLC/MS analysis will make possible complete characterization of styrene metabolites in occupational settings (Manini et al., 2000; Manini et al., 2002) and in the low level exposures found in the general environment (Reska et al., 2010). Because the mercapturates of styrene are minor metabolites, they are not suited for quantitative estimates of internal absorbed dose. They are indicators of oxidative activation of styrene and are products of toxicologically relevant pathways of transformation. Their possible use as biomarkers of styrene activation and biotransformation awaits further study.

Simultaneous determinations mercapturates in occupational and environmental studies

In the past, one of the main technological limitations of mass spectrometers used in HPLC analysis has been the rate of data acquisition and the dwell time of monitoring the response at specific masses. Detector sampling rates must be rapid enough to obtain a sufficient number of data points across the analyte peak (Holland et al., 1983; Varma et al., 2010). Low data acquisition rates have been known for many years to lead to poor chromatographic peak integration and poor reproducibility of peak area determinations (Holland et al., 1983). Rapid data acquisition is necessary in order to minimize chromatographic peak distortion, which can be a problem with multiple analyte methods or spectral data collected from increasingly narrow chromatographic peaks such as with UHPLC. With improvements in data acquisition rate for MS systems, mostly from the advent of much more powerful computers, HPLC–MS methods have become more capable of determining multiple mercapturate analytes in a single chromatographic analysis.

The following sections consider simultaneous determinations of the mercapturates of multiple parent compounds in a single chromatographic analysis.

Polycyclic aromatic hydrocarbons

The carcinogenicity of benzo(a)pyrene (BP) is associated with diol epoxide formation by P450 oxidation in the bay region of this PAH. Inactivation and detoxification of BP were generally assumed to occur by GSH *S*-Transferase (GST) catalyzed GSH conjugation of bay region diol epoxides followed by further metabolism to urinary mercapturates (Figure 8). Molecular epidemiology and risk assessment studies based on this assumption had met only modest success (Upadhyaya et al., 2006). In investigating possible reasons for this, Hecht and collaborators recognized that the expected mercapturate products of GSH conjugation in the bay region

of PAHs had never been demonstrated in human urine. To reexamine the detoxification of PAHs, Upadhyaya developed a method to determine the mercapturates of phenantherene, the simplest of the PAHs having a bay region. The method was applied to the urine of 36 smokers. Detectable mercapturates were products of conjugation of the ''reverse diol epoxides'' of phenantherene, Phe-O-Nac and Phe-DE-Nac, and not products of GSH conjugation at the bay region (Hecht et al., 2008; Upadhyaya et al., 2006). The result was replicated in human hepatocyte culture where only trace amounts of mercapturates derived from the bay region of phenantherene were detected (Hecht et al., 2009). These results suggested that GST may have a substrate preference for GSH conjugation of reverse diol epoxides over those formed in the bay region of PAHs (Figure 8). Upadhyaya repeated hepatocyte incubations with culture medium containing the activated metabolite of BaP, 7,8-diol-9,10-epoxide (BPDE) or with the non-carcinogenic reverse diol epoxide 9,10-diol-7,8-epoxide (revBPDE). The majority of mercapturate products, again, were formed by preferential GSH conjugation of revBPDE by GST (revBPDE-7Nac). Mercapturate products of conjugation in the bay region of BPDE were detected in only one of 10 replicate incubations (Upadhyaya et al., 2010). These studies indicate that detoxification of BPDE by GSH conjugation is a minor pathway in humans, and that the gene polymorphisms of GST are not an important risk factor in the diol epoxide pathway of benzo(a)pyrene carcinogenesis.

Alkylating agents

Schettgen has developed RP simultaneous determinations for the mercapturates of high volume production alkylating chemicals AA, acrolein, dimethylformamide, ethylene and propylene oxides (Schettgen et al., 2008b) and for AN and 1,3-BD (Schettgen et al., 2009). These methods were applied to spot urine samples collected from smokers and nonsmokers having no occupational exposure. Both methods were sensitive enough to distinguish between smokers and nonsmokers and would be suitable for determination of background exposures of general populations to industrial emissions and polluted urban air.

The mercapturates of many alkylating agents contain highly polar hydroxyalkyl groups and are difficult to retain on RP media. To address this difficulty, Eckert et al. (2010) developed a HILIC method to determine six HAMAs, including the first reported determination of 2,3-dihydroxypropyl mercapturate of glycidol (Table 3). In agreement with earlier reports (Ding et al., 2009; Schettgen et al., 2009), Eckert reports only one MHBMA peak in urine of smokers. Most recently, Sterz combined UHPLC with HILIC to separate 1,3-butadienemercapturate isomers, MHBMA1 and MHBMA2 in human urine (Sterz et al., 2012, Table 3). Altogether, further studies are needed to better evaluate 1,3-butadiene metabolism. Perhaps, as in the complementary studies of AA by Dekant and collaborators (Kellert et al., 2006; Kopp et al., 2008), RPLC–ESI-MS/MS and HILIC–ESI-MS/MS may be used to investigate the isomers of MHBMA, and evaluate their utility as biomarkers in occupational exposure, in smokers and in urban populations.

Urban air pollutants

Simultaneous determination analysis is well suited to investigate complex exposures to volatile organic compounds (VOCs) in occupational settings, in exposure of urban populations to air pollutants and in cigaret smoke (Chiang et al., 2015; Ding et al., 2009; Eckert & Goen, 2014; Li et al., 2015b; Pluym et al., 2015; Scherer et al., 2010; Schettgen et al., 2008b; Zhang et al., 2014). Sabatini developed a simultaneous determination for the mercapturates of benzene, toluene and xylene (BTX) using FDA validation guidelines (US Department of Health and Human Services, Food and Drug Administration, May 2001) to measure BTX co-exposure in traffic warden exposures to automobile exhaust and urban air pollutants examining the urine of men and women, including smokers and nonsmokers (Sabatini et al., 2008, Table 3). To study urban populations exposed to cigaret smoke, Wu used an ultra-high performance small bore column to resolve structurally similar mercapturates of AN, CEMA and HEMA (Wu et al., 2012). UHPLC was used ambitiously by Alwis et al. to determine 24 mercapturate metabolites of 15 VOCs in the urine of multi-ethnic males and females including both smokers and nonsmokers (Alwis et al., 2012). Target mercapturates of this study included a third isomer of 1,3-butadienemercapturate, 4-hydroxy-2-buteneyl-mercapturate (MHBMA3). Although multiple analyte analysis is an obvious modern trend, the rate of data acquisition must be kept in mind when developing and validating new biomarker analytical methods (Holland et al, 1983). Low data acquisition rates can lead to distorted peak shape, poor peak area integration and ultimately inaccurate quantitation.

Scherer used pentafluorobenzyl-bromide (PFBBr) derivitization to determine exposure to alkylating agents in tobacco smoke for use in cigaret testing and smoking cessation studies (Scherer et al., 2010). Mercapturate analytes were enriched in urinary extracts by reaction with PFBBr and extracted twice with ethyl acetate for analysis. The method was developed using FDA guidelines for bioanalytical methods (US Department of Health and Human Services, Food and Drug Administration, May 2001). Method calibrations were performed in human urine to simulate ion suppression interferences from a urinary sample matrix. The validated method achieved the required specificity and sensitivity to distinguish between mercapturate levels in smokers of conventional cigarets, test cigarets with activated carbon filters, or an electronically heated smoking system. After smoking cessation, the method detected 74% and 90% reductions in the mercapturates of ethylene oxide and AN, respectively. Details of the study designs, study participant characteristics and cigaret characteristics are reported elsewhere (Frost-Pineda et al., 2008; Sarkar et al., 2008).

Herbicides

Triazine and chloroacetamide herbicides are extensively used in commercial, agricultural, urban and residential settings and have become ubiquitous environmental pollutants. Sensitive, automated high throughput methods for occupational and environmental biomonitoring of ATZ exposure were developed by Barr and collaborators (Kuklenyik et al., 2012;

Norrgran et al., 2006; Panuwet et al., 2008). By combining RP-hexyl phenyl chromatography with APCI ionization and multiple precursor-product ion monitoring (RP–APCI–MRM), these methods allow simultaneous determination of urinary ATZ, ATZ metabolites, their mercapturates and hydroxylated derivatives. The analysis was adapted to water and urine samples by adding online SPE extraction and concentration of analytes to determine ATZ, two ATZ mercapturates and four other ATZ metabolites (Panuwet et al., 2008). A manual mixed-polarity polymeric SPE preparation of a 2-mL urine sample was added to the RP–APCI–MRM analysis to determine multiple herbicides: phenoxyacetate ATZ; 3-chloroacetanilide herbicides acetochlor, alachlor, metochlor and their mercapturates (Norrgran et al., 2006). The method achieves LODs <1 μg/L, sufficiently sensitive to detect exposures in non-occupationally exposed general populations (Table 3). Most recently, Panuwet's online method was expanded to create a two-dimensional HPLC analysis that incorporates SAX and RP chromatographic separation modes using three multiple-port valves and three pumps (Kuklenyik et al., 2012). This system determines ATZ and 11 ATZ derivatives, including four mercapturates for toxicology and occupational exposure applications.

Conclusions

Early HPLC mercapturate determinations were limited by nonspecific ultraviolet or fluorometric detection techniques. GC techniques required cumbersome analyte derivitization for sensitive and specific determination of mercapturates. HPLC–MS now provides a powerful and useful tool for mercapturate quantification. HPLC–MS is a highly specific analysis method in which interfering or co-eluting substances found in urine are eliminated in the chromatographic column or are filtered from the analysis stream by ion selection in the mass spectrometer. Although mercapturates are often minor metabolites, tandem (MS/MS) offers the greatest level of analytical sensitivity for analysis, and its application has expanded the utility of urinary mercapturates from biomarkers of exposure to indicators of toxicant metabolism, biotransformation and elimination. Tandem MS analysis of detoxification products of minor but toxicologically significant pathways has identified differences in metabolism between species providing information useful in exposure risk assessment. Mercapturate determination has demonstrated unexpected substrate preferences in GSH conjugation of PAHs, and also has revealed species differences in the stereospecific biotransformation and detoxification of styrene. Advances in chromatographic techniques, such as HILIC or mixed mode have been applied to determination of HAMAs of AA and 1,3-butadiene to complement information provided by RP chromatography. The use of UHPLC has found application in the simultaneous mercapturate analysis, and is likely to expand as high-pressure pumping systems become more available commercially. These applications of HPLC–MS/MS to the simultaneous determination of multiple mercapturates as indicators of exposure and metabolic processing in individuals may be expected to provide useful information for estimating exposure risk in both occupational and environmental health studies.

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Declaration of interest

The authors hereby report that we have no conflict of interest with the material reported in this paper. The authors alone are responsible for the content and writing of this paper.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health (NIOSH) or the Centers for Disease Control and Prevention (CDC). Mention of company names and/or products does not constitute an endorsement by NIOSH.

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