Characterization of Thioether Compounds Formed from Alkaline Degradation Products of Enflurane

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Background: Renal toxicity has occasionally been observed after enflurane anesthesia. Although originally attributed to its oxidative metabolism to inorganic fluoride, serum levels of inorganic fluoride appear to be small to explain these renal effects. Formation of potentially nephrotoxic halogenated alkenes during alkaline degradation in carbon dioxide absorbers and subsequent bioactivation *via* the glutathione conjugation pathway may be considered as an alternative mechanism for renal toxicity. The aim of this study was to characterize the thioethers formed chemically and biosynthetically.

Methods: Alkaline degradation of enflurane was achieved by stirring with pulverized potassium hydroxide. Volatile degradation products were analyzed by ^{19}F nuclear magnetic resonance (NMR) analysis of head space gasses trapped in dimethyl sulfoxide (DMSO). Thioethers were generated chemically by trapping head space gasses in DMSO containing *N*-acetyl-L-cysteine or 2-mercaptoacetic acid as model thiol compounds. Glutathione conjugates were generated biosynthetically by passing head space through rat liver fractions in presence of glutathione. Products formed were analyzed by gas chromatography–mass spectroscopy and ^{19}F-NMR.

Results: Direct analysis of head space gasses showed formation of 1-chloro-1,2-difluorovinyl difluoromethyl ether and two unidentified fluorine-containing products as alkaline degradation products of enflurane. When trapped in DMSO–*N*-acetyl-L-cysteine–triethylamine, *N*-acetyl-S-(2-chloro-1,2-difluoro-1-(difluoromethoxy)ethyl)-L-cysteine was identified as the major product. Another *N*-acetyl-L-cysteine S-conjugate formed was *N*-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, a potent nephrotoxin in rats. ^{19}F-NMR analysis of glutathione conjugates formed after incubation with rat liver fractions resulted in formation of corresponding S-conjugates.

Conclusions: The current study demonstrates that alkaline degradation products of enflurane can be conjugated to thiol compounds, forming S-conjugates that could theoretically contribute to adverse renal effects observed occasionally with enflurane anesthesia. The *N*-acetyl-L-cysteine S-conjugates identified may be biomarkers to assess exposure of humans to alkaline degradation products of enflurane.

FLUORINATED volatile anesthetics, such as halothane, isoflurane, sevoflurane, desflurane, and enflurane, are widely used. Clinical studies and animal experiments have shown that several of these agents may induce various forms of adverse reactions. Enflurane is sus-

pected to cause liver damage, which may result from oxidative metabolism to a highly reactive trifluoroacyl halide that binds covalently to liver proteins, forming acylated neoantigens.[1,2] Rare cases of renal toxicity have also been reported with enflurane.[3–8] The increase in urinary alanine aminopeptidase excretion, which was observed in patients exposed to enflurane, suggests renal proximal tubular toxicity.[8] In this same study, it was shown that enflurane nephrotoxicity is increased by aminoglycosides. Studies on human volunteers receiving up to 8 h of enflurane anesthesia showed a reversible depression of renal concentrating capacity.[9,10] Enflurane-induced reduction of urinary concentrating ability and enzymuria have also been reported in animal studies.[11–13] Enzyme induction, obesity, and impaired kidney function appear to be risk factors for enflurane nephrotoxicity.[8,12–14]

The oxidative metabolism leading to the production of inorganic fluoride has been proposed as a possible mechanism of enflurane-induced nephrotoxicity.[5,11,15] However, serum concentrations of fluoride ions appear to be too low to explain enflurane nephrotoxicity.[16,17] In addition, sevoflurane exposure generates greater serum fluoride concentrations than enflurane but did not produce the same renal effects as enflurane.[9] Finally, the proximal tubular localization of enflurane nephrotoxicity is not consistent with the distal tubular localization of fluoride toxicity. Therefore, alternative mechanisms responsible for enflurane nephrotoxicity should be considered.

In recent years, numerous studies have been directed to the potential nephrotoxicity of halogenated alkenes formed on alkaline degradation of volatile anesthetics in carbon dioxide absorbers (*i.e.*, soda lime and Baralyme; Chemetron, St. Louis, MO). Various halogenated ethylenes are known to cause nephrotoxicity on administration to rats.[18] The mechanism of this nephrotoxicity consists of hepatic conjugation to glutathione, subsequent degradation by biliary, intestinal, and renal peptidases, and, ultimately, local bioactivation of cysteine S-conjugates by renal cysteine S-conjugate β-lyases.[18] Next to the β-lyase–mediated bioactivation, alternative bioactivation mechanisms of cysteine S-conjugates and mercapturates, such as sulfoxidation by cytochromes P450 and/or flavin-containing monooxygenase, have been proposed as well.[19–21] Compound A (2-[fluoromethoxy]-1,1,3,3,3-pentafluoro-1-propene), the halogenated alkene formed by alkaline degradation of sevoflurane, as well as its corresponding S-conjugates, produce nephrotoxicity in rats.[19,22–25] Although metabolites in-
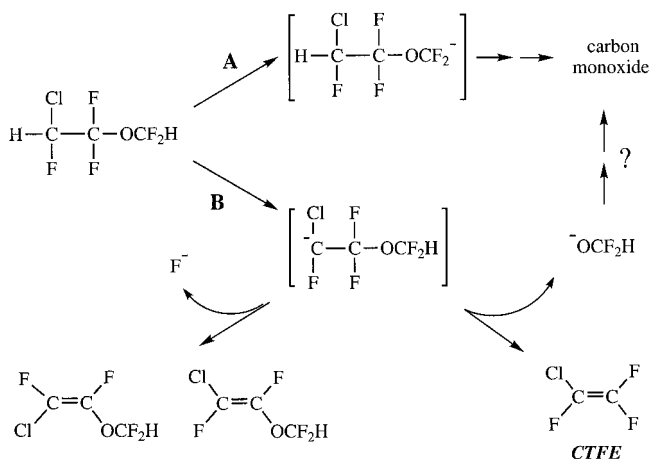

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Fig. 1. Formation of potentially toxic products on alkaline degradation of enflurane in carbon dioxide adsorbers. (*A*) Mechanism proposed for formation of carbon monoxide.[29] (*B*) Mechanism proposed for formation of halogenated alkenes.[30,31]

dicative for compound A exposure and subsequent glutathione conjugation have been identified in human urine,[26] its toxicologic significance to humans still is a matter of debate.[27] No case of nephrotoxicity attributable only to sevoflurane has been reported, and a large-scale retrospective analysis failed to show an increased incidence of renal toxicity in sevoflurane-treated patients.[28]

Enflurane is also known to be chemically degraded by the strong bases present in carbon dioxide absorbers, as indicated by the formation of carbon monoxide[29] and 1-chloro-1,2-difluorovinyl difluoromethyl ether[30,31]; the mechanisms explaining these products are shown in figure 1. The potential contribution of glutathione conjugation of the latter halogenated alkene in clinically observed cases of nephrotoxicity of enflurane, however, has not yet been the subject of investigations. The aim of the present investigation was to characterize the product(s) resulting from the reaction between enflurane degradation products and the thiol compounds *N*-acetyl-L-cysteine, 2-mercaptoacetic acid, and glutathione.

Materials and Methods

Materials

Enflurane was supplied by Abbott Laboratories (Abbott Park, IL). Chlorotrifluoroethylene, *N*-acetyl-L-cysteine, 2-mercaptoacetic acid, and glutathione were purchased from Sigma-Aldrich Co. (Steinheim, Germany). *N*-acetyl-S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine (CTFE-NAC) was synthesized and characterized as described previously.[32]

Methods

Isolation of Rat Liver Microsomes and Cytosols. Male Wistar rats (200–220 g) were obtained from Harlan (Zeist, The Netherlands) and were acclimated for 1 week after delivery to the laboratory. The animals were fed a standard laboratory diet from Hope Farms (Woerden,

The Netherlands) and had access to food and water *ad libitum*. Rats were killed by decapitation, and livers were excised and homogenized at 4°C in 2 volumes of 50 mM potassium phosphate (pH 7.4) with 0.9% sodium chloride using a Potter-Elvehjem homogenizer. The homogenates were centrifuged for 20 min at 12,000*g*, and the obtained supernatant was centrifuged for 60 min at 100,000*g*. The obtained supernatant was used for cytosolic incubations. The microsomal fraction was washed twice by resuspension in 50 mM potassium phosphate buffer (pH 7.4 with 0.9% sodium chloride) and centrifuged for 60 min at 100,000*g*. The washed pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.4), 0.9% sodium chloride, and 25% glycerol and stored at −80°C until use. Protein concentrations determined with Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munich, Germany) using bovine serum albumin as a standard were 38.59 and 13.10 mg/ml for cytosols and microsomes, respectively. Animal experiments were approved by the Animal Welfare Committee of the Vrije Universiteit (Amsterdam, the Netherlands).

Alkaline Degradation of Enflurane. Enflurane (73.6 g, 0.4 mol) was added to a 250-ml round bottom flask equipped with a magnetic stirrer, a septum, and a reflux cooler. After cooling on ice, 22.4 g (0.4 mol) potassium hydroxide, which was pulverized by mortar under nitrogen in advance, was added. Potassium hydroxide was selected because it is shown to be the most active hydroxide in enflurane degradation.[31] During equilibration to room temperature and while stirring, heat formation was noticed with the formation of gasses, consistent with previous observations.[33] Thirty minutes after the initiation of the spontaneous reaction, the mixture was refluxed at 80°C while being stirred vigorously for an additional 30 min. Gasses formed were guided to the thiol-containing reaction mixtures by tubing at the outlet of the reflux cooler.

Analysis of Head Space Gasses. Volatile degradation products were analyzed by transferring the gas phase–head space of the enflurane–potassium hydroxide mixture through a test tube containing 2 ml hexadeuterated dimethyl sulfoxide (DMSO-d_6) with the aid of a slow nitrogen stream. After 60 s, the DMSO-d_6 was transferred to a 5-mm nuclear magnetic resonance (NMR) tube and analyzed by ^{19}F-NMR.

Chemical Synthesis of Thioethers of Enflurane Degradation Products. Thiol compounds used to trap reactive enflurane degradation products were *N*-acetyl-L-cysteine and 2-mercaptoacetic acid. *N*-acetyl-L-cysteine was selected as trapping agent because the mercapturic acids formed may be potential biomarkers to evaluate human exposure to enflurane-derived alkenes and their conjugation to glutathione.[26,34] Analysis of mercapturic acids by gas chromatography–mass spectroscopy (GC-MS) is also facilitated by their characteristic fragmentation patterns.[35] Regioselectivity and mechanism of S-

conjugation (addition *vs.* substitution reaction) was determined by ^{19}F-NMR analysis. 2-Mercaptoacetic acid was used as trapping agent because the S-conjugates formed also are potential urinary metabolites derived from glutathione conjugation[18] and because the lack of a chiral center was helpful in interpretation of the complicated ^{19}F-NMR spectra.

Thioethers of alkaline degradation products of enflurane were chemically synthesized by transferring the gas phase–head space of the enflurane–potassium hydroxide mixture to a mixture of 1 g of thiol compound and 0.5 ml triethyl amine in 5 ml DMSO. The reaction was proceeded for 4 h. Products formed were analyzed by ^{19}F-NMR and GC-MS. For GC-MS analysis of thioether compounds formed, a 1-ml sample of the reaction mixture was added to 5 ml 2N hydrochloric acid and extracted twice by 5 ml ethyl acetate. The combined ethyl acetate layers were evaporated to dryness by a nitrogen stream while warming at 45°C. The residue was redissolved in 0.5 ml ethyl acetate and subsequently treated with an excess of freshly prepared ethereal diazomethane. After reacting for 20 min, the excess diazomethane was removed by a nitrogen stream. One microliter of the resulting solution was analyzed by GC-MS.

Direct ^{19}F-NMR analysis of the reaction mixture demonstrated a high concentration of unchanged enflurane, which obscured the ^{19}F-NMR signals of other products. Therefore, to remove enflurane, the DMSO solution was added to 50 ml of 2N hydrochloric acid and extracted twice by 50 ml ethyl acetate. The combined ethyl acetate layers were evaporated to dryness by rotaevaporation. The residue was redissolved in 3 ml deuterium oxide containing 5 mg sodium carbonate. The deuterium oxide solution was transferred to a 5-mm NMR tube and analyzed by ^{19}F-NMR.

Glutathione Conjugation of Enflurane Degradation Products by Rat Liver Cytosol and Microsomes. Glutathione conjugates of alkaline degradation products of enflurane were generated biosynthetically by slowly passing the gas phase–head space of a enflurane–potassium hydroxide mixture through a 25-ml incubation mixture containing 10 mM glutathione and rat liver microsomal protein (2.5 mg/ml) or rat liver cytosolic protein (7.5 mg/ml) in 50 mM potassium phosphate buffer (pH 7.4). After 2 h at 37°C, the incubations were terminated by the addition of 10 ml 2N HCl. Denaturated proteins were precipitated by centrifugation at 4,000 rpm for 10 min, after which the supernatants were isolated, frozen, and subsequently lyophilized to dryness. The residues were redissolved in 1 ml deuterium oxide, transferred to a 5-mm NMR tube, and analyzed by ^{19}F-NMR.

Glutathione S-conjugate of chlorotrifluoroethylene (CTFE) was generated biosynthetically by passing chlorotrifluoroethylene gas through a 25-ml incubation mixture containing 10 mm glutathione and rat liver cytosolic protein (7.5 mg/ml) in 50 mM potassium phosphate buffer (pH 7.4).[36] After 30 min of incubation at 37°C, the reaction was terminated by addition of 10 ml 2N HCl. The reaction mixture was prepared for ^{19}F-NMR as described above.

Analytical Equipment. [19]F nuclear magnetic resonance spectra were measured on a Bruker Avance 250 System (Rheinstellen/Karlsruhe, Germany) operating in the ^{19}F-NMR mode at 235.3574 MHz. Chemical shifts were relative to trifluoroacetic acid (in case of deuterium oxide as solvent) or $CFCl_3$ (in case of DMSO as solvent).

Gas chromatography–mass spectroscopy analyses were conducted in an HP 5890/MSD system (Hewlett-Packard Co., Palo Alto, CA). An ID-BPX5 capillary column (25 m) was obtained from SGE Ltd. (Ringwood, Victoria, Australia). The operation temperatures were 270°C (split injector and transfer line) and 280°C (ion source, electron impact ionization, electron energy of 70 eV). Column head pressure was 29 psi (195 kPa). For analyses of methylated products trapped by *N*-acetyl-L-cysteine and 2-mercaptoacetic acid, the column temperature was programmed from 45°C (2.5 min) to 270°C at 20°C/min.

Results

Analysis of Volatile Products of Alkaline Degradation of Enflurane

On ^{19}F-NMR analyses of head space gasses trapped in DMSO, various signals could be observed (fig. 2). Enflurane, without base treatment, was shown to contain low amounts of unidentified fluorine-containing contaminants with signals at −83.75 and −83.92 ppm, respectively (fig. 2B). On base treatment, seven new signals were observed (fig. 2A). The coupling patterns of five of the signals observed were fully consistent with that expected for the fluorine atoms present in the cis- and trans-isomers of 1-chloro-1,2-difluorovinyl difluoromethyl ether. One isomer (I) was formed in slight excess over the other isomer, which, next to its different coupling constants, allowed assigning the signals to the correct structures (table 1). Next to the signals from 1-chloro-1,2-difluorovinyl difluoromethyl ether, two singlets were observed at −78.78 and −82.54 ppm when measured in proton-decoupled mode (fig. 2A). In the presence of proton coupling, these signals transferred to doublets with coupling constants of 79 and 77 Hz, respectively, indicative for geminal fluorine–hydrogen coupling. However, the identity of these fluorine-containing products remains to be established. Chlorotrifluoroethylene, which was anticipated as an alternative degradation product (fig. 1), could not be identified directly as enflurane degradation product in the DMSO fraction.
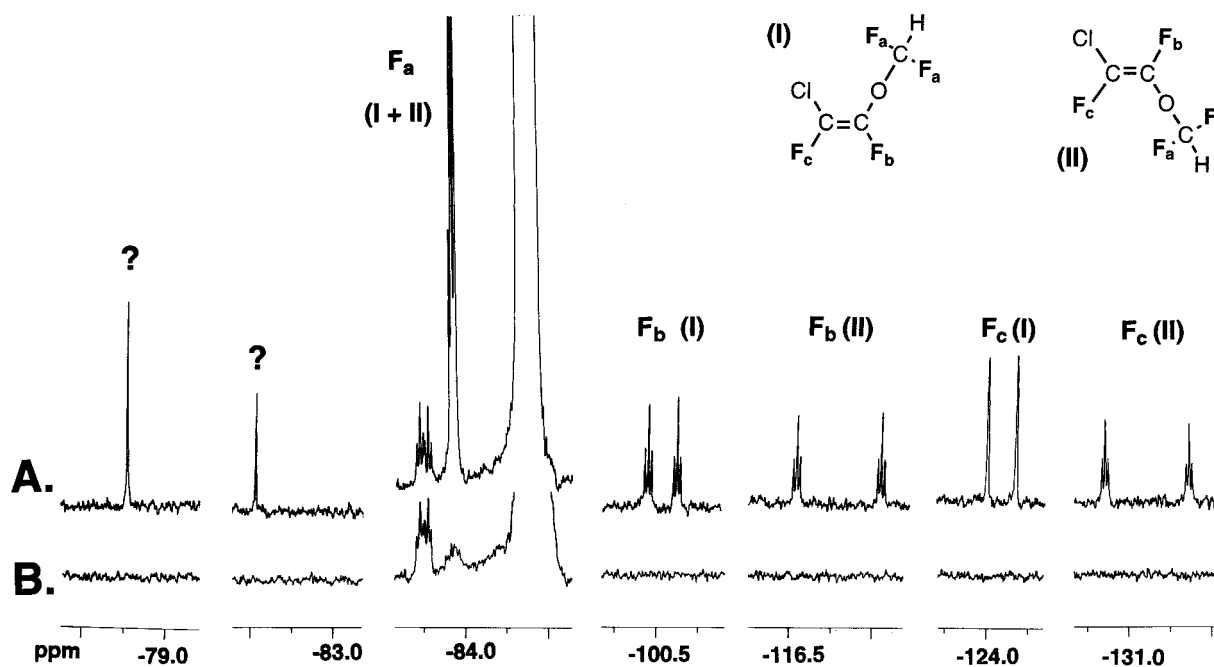
Fig. 2. Details of ^{19}F nuclear magnetic resonance spectra of head space samples trapped in dimethyl sulfoxide-d$_6$. (*A*) Head space sample of enflurane treated with pulverized potassium hydroxide; (*B*) head space sample of untreated enflurane. Spectra shown were recorded in proton-decoupled mode; for details of coupling constants, see table 1. ppm = parts per million.

Analysis of Chemically Synthesized Thioethers by Gas Chromatography–Mass Spectroscopy

Thioether compounds formed from reaction between enflurane degradation products and the thiol compounds, *N*-acetyl-L-cysteine and 2-mercaptoacetic acid, were initially analyzed by GC-MS. GC-MS analysis of methylated extracts of reaction mixtures containing *N*-acetyl-L-cysteine revealed the formation of three products with fragments ("key ions") characteristic for methyl esters of mercapturic acids (m/z 88, 117, 144, and 176[30]); retention times were 9.85, 10.30, and 10.45 min, respectively. Figure 3A shows the total ion chromatogram of one of five independent trapping experiments. The peak at retention time 9.85 min was identified unequivocally as the methyl ester of CTFE-NAC, based on the identical mass spectrum and reten-

tion time with that of synthetic CTFE-NAC methyl ester. A major peak at 10.30 min (peak I) and a minor peak at 10.45 min (peak II) both had mass spectra that could be attributed to the addition reaction of *N*-acetyl-L-cysteine to 1-chloro-1,2-difluorovinyl difluoromethyl ether. In an attempt to clean up one of the reaction mixtures by repetitive basic and acidic extractions, it was noticed that the intensity of peak I significantly decreased, whereas peak II strongly increased simultaneously, suggesting a rearrangement reaction (fig. 3B). Both peaks showed (1) a relatively weak molecular ion at m/z 341, (2) the fragmentations of [$M^{+\bullet}$-59] and [$M^{+\bullet}$-101] typical for methyl esters of *N*-acetyl-L-cysteine S-conjugates,[32,37] and (3) the so-called "key ions" m/z 176, 144, 117, and 88[35] derived from the mercapturic acid methyl ester moiety. Figure 4A shows the mass spectrum of peak I.

Table 1. ^{19}F-NMR Spectra of Alkaline Degradation Products of Enflurane

		^{19}F Nuclear Magnetic Resonance Spectrum	
Compound	F Atom*	Chemical Shift (ppm)†	Coupling Constants (Hz)
Trans-1,2-difluoro-2-chloro-1-(difluoromethoxy)-ethylene	F_a	−83.90	$^3J_{FF}$ = 4 (d); $^1J_{FH}$ = 70 (d)
	F_b	−116.76	$^2J_{FF}$ = 120 (d); $^3J_{FF}$ = 5 (t)
	F_c	−131.11	$^2J_{FF}$ = 120 (d); $^4J_{FF}$ = 5 (t)
Cis-1,2-difluoro-2-chloro-1-(difluoromethoxy)-ethylene	F_a	−83.90	$^3J_{FF}$ = 4 (d); $^1J_{FH}$ = 70 (d)
	F_b	−116.76	$^2J_{FF}$ = 41 (d); $^3J_{FF}$ = 5 (t)
	F_c	−131.11	$^2J_{FF}$ = 41 (d); $^4J_{FF}$ = 0 (t)

* Labels refer to fluorine atoms as indicated in figure 2. † Solvent: hexadeuterated dimethyl sulfoxide (DMSO-d$_6$); parts per million (ppm) relative to fluorotrichloromethane (CFCl$_3$).
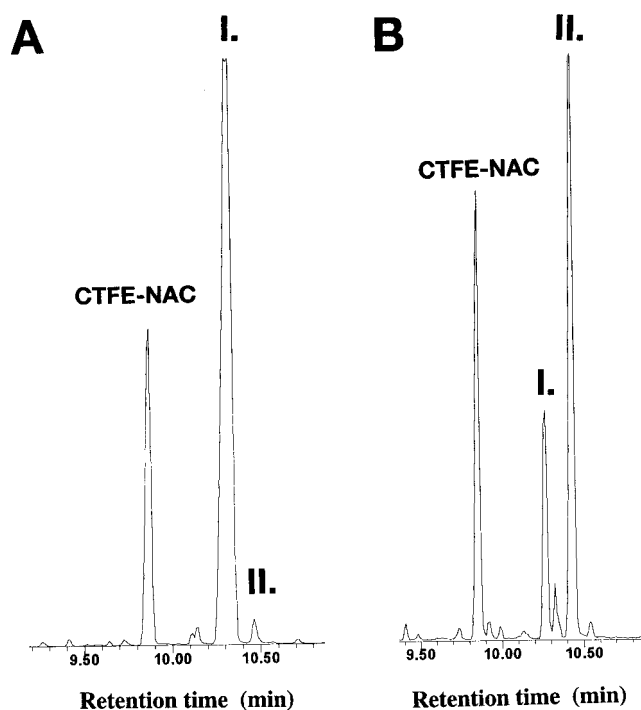
d = doublet; t = triplet.

Fig. 3. Total ion chromatograms of gas chromatography–mass spectroscopy analyses of methylated extracts of reaction mixtures of enflurane degradation products with *N*-acetyl-L-cysteine in dimethyl sulfoxide–triethylamine. (*A*) Initial products extracted for the reaction mixture; (*B*) after repetitive basic and acidic extractions.

Analyzing enriched fractions of both mercapturic acids by ^{19}F-NMR enabled us to elucidate the structures of both fluorine-containing moieties (see below).

Gas chromatography–mass spectroscopy analysis of extracts of reactions with 2-mercaptoacetic acid as thiol compound indicated formation of corresponding thioethers, as was observed with *N*-acetyl-L-cysteine. 2-Mercaptoacetic acid also reacted to 1-chloro-1,2-difluorovinyl difluoromethyl ether by an addition reaction: the major product as 6.93 min showed a relatively strong molecular ion at m/z 270 (fig. 4B). As was observed in extracts of *N*-acetyl-L-cysteine conjugates, a second but smaller peak with the same molecular ion (m/z 270) and with slightly longer retention time (7.14 min) was observed (data not shown). At a retention time of 6.0 min, a small chlorine-containing peak was observed with a molecular ion of m/z 222. This minor product was identified as the methyl ester of S-(2-chloro-1,1,2-trifluoroethyl)-2-mercaptoacetic acid by comparison with the reference compound that was synthesized by reaction of commercial chlorotrifluoroethylene with 2-mercaptoacetic acid in DMSO–triethylamine (data not shown).

No thioethers were formed when enflurane was reacted directly with the thiol compounds in DMSO–triethylamine, indicating that only alkaline degradation products are involved in thioether formation (data not shown).

Analysis of Chemically Synthesized Thioethers by [19]F Nuclear Magnetic Resonance Analysis

Figure 5A represents the ^{19}F-NMR spectrum of the initial product isolated from the reaction mixture containing *N*-acetyl-L-cysteine in DMSO–triethylamine. Consistent with the GC-MS analysis, a number of the signals (indicated by asterisks) were indicative for the formation of CTFE-NAC, as was proved by standard addition of the reference compound CTFE-NAC to the NMR tube. Based on the chemical shifts, multiplicity, and FF- and FH-coupling patterns of the remaining ^{19}F-NMR signals in figure 5A, the major product was identified as *N*-acetyl-S -(2-chloro-1, 2-difluoro-1-(difluoromethoxy)ethyl)-L-cysteine. Because of the presence of three chiral carbon atoms in this structure, of which the carbon of the *N*-acetyl-L-cysteine group only is in the L-configuration, four different diastereomeric forms (RR-L, RL-L, LR-L, and LL-L) of this compound exist. Because of the high sensitivity of fluorine NMR chemical shifts to subtle environmental changes, the signals from each individual diastereomeric form could be distinguished by ^{19}F-NMR (table 2). When measured in the proton-decoupled mode, fluorine atom F_d shows four doublets between −71.1 and −71.6 ppm resulting from $^2J_{FF}$ coupling by fluorine atom F_c. Fluorine atom F_c shows four doublets of triplets, resulting from coupling by F_d (doublet) and the CF_2HO group (triplet). The two fluorine atoms of the CF_2HO group are not identical because of the proximity of chiral centra and therefore produce AB systems. The left part of the AB systems of the four diastereomeric forms coincide at −7.36 ppm, whereas the right part of the AB systems was different for each diastereomer.

When trapping of the halogenated ethylene in DMSO–triethylamine containing 2-mercaptoacetic acid, only two doublets of triplets was observed for fluorine atom F_c (fig. 5B). Because the mercaptoacetic acid moiety in contrast to *N*-acetyl-L-cysteine lacks a chiral carbon atom, the RR isomer of the fluorine-containing moiety cannot be distinguished from its LL isomer. In addition, the RL isomer cannot be distinguished from the LR isomer. For the same reason, the CF_2HO group demonstrates only two AB systems, in contrast to the four AB systems observed in the *N*-acetyl-L-cysteine-adduct. For 2-mercaptoacetic acid adduct, the chemical shift of fluorine atom F_d appeared to be almost identical for all four different diastereomers.

[19]F nuclear magnetic resonance analysis of the fraction enriched with the second *N*-acetyl-L-cysteine conjugate (peak II in fig. 3) showed, next to the presence of CTFE-NAC, dramatic changes of the other fluorine signals. Signals of fluorine atoms F_c and F_d almost disappeared completely, whereas major changes were also observed between −6 and −11 ppm. After careful analysis of the chemical shifts and FH- and FF-coupling pattern (in the presence and absence of proton coupling),
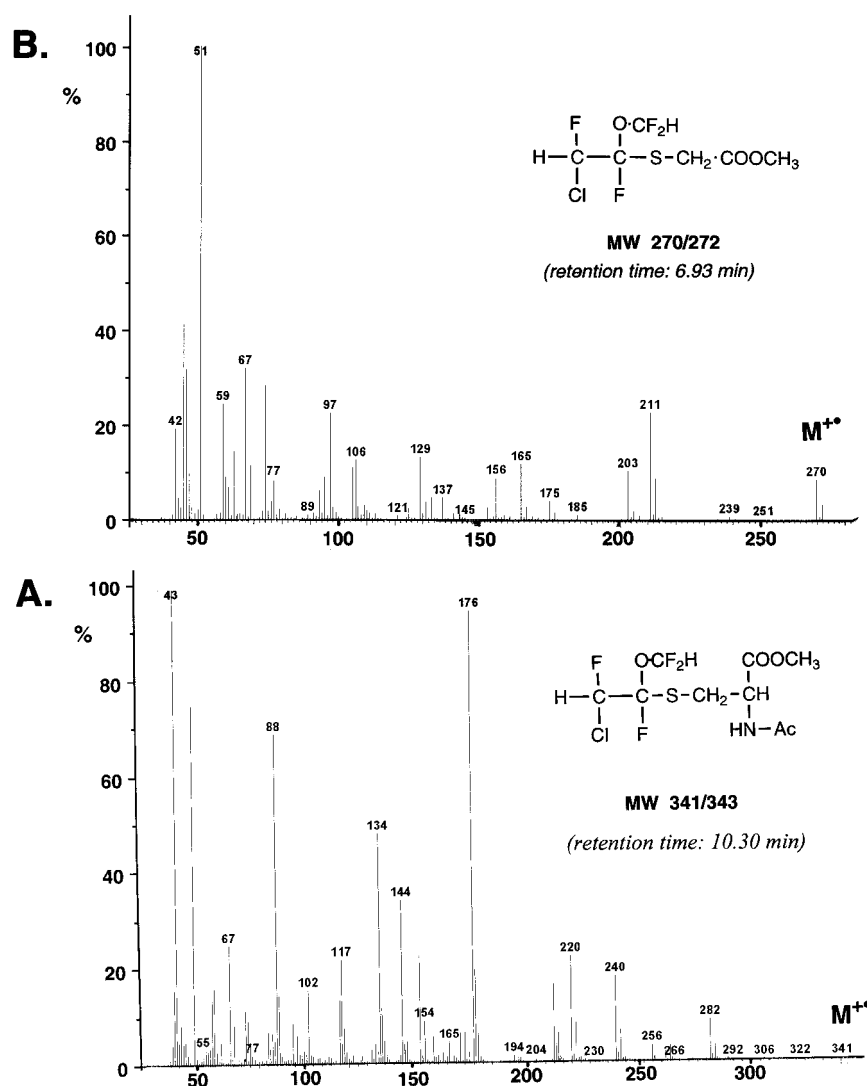
Fig. 4. Electron impact mass spectra of methylated extracts of reaction mixtures of enflurane degradation products with thiol compounds in dimethyl sulfoxide–triethylamine. (*A*) Extract of reaction with *N*-acetyl-L-cysteine; (*B*) extract of reaction with 2-mercaptoacetic acid.

this product is identified as *N*-acetyl-S-(2-chloro-2-(difluoromethoxy)-1, 1-difluoroethyl)-L-cysteine. Apparently, during basic and/or acidic conditions during the extractions, an exchange of fluorine atom F_d and the CF_2HO group of the original product takes place.

Analysis of Biosynthetical Glutathione S-Conjugates by ^{19}F Nuclear Magnetic Resonance Analysis

Figures 6A and 6B show the ^{19}F-NMR spectra of the products formed on incubation of enflurane degradation products with 10 mM glutathione in the presence of rat liver fractions. To obtain ^{19}F-NMR signals with good signal-to-noise ratios, 50,000 scans had to be collected over a 51-h period. As presented in figure 6B, the ^{19}F-NMR spectrum of the cytosolic incubation showed the presence of signals closely resembling those observed in that of the synthetic mixture of *N*-acetylcysteine S-conjugates (fig. 5B). Again, four doublets of triplets were observed at −21.95, −22.34, −25.02, and −25.41 ppm, which correspond to fluorine F_c of *N*-acetyl-S-(2-chloro-1,2-difluoro)-1-(difluoromethoxy)ethyl)-L-cysteine (fig.

5B). Furthermore, again four partially overlapping AB systems are observed between −7.45 and −9.45 ppm, corresponding to fluorine atoms F_A and F_B, as well as the four doublets between −71.81 and −72.30 ppm, corresponding to F_d (fig. 5B). Next to the similar chemical shifts, the FF-coupling constants of the signals of the four fluorine atoms are almost identical to those observed in the corresponding *N*-acetyl-L-cysteine conjugate. Therefore, the structure of one of the glutathione conjugates appears to be S-(2-chloro-1,2-difluoro-1-(difluoromethoxy)ethyl)-glutathione. Interestingly, the four diastereomers of S-(2-chloro-1,2-difluoro-1-(difluoromethoxy)ethyl)-glutathione are formed in a ratio of 3:1:1:2, according to the intensity of the signals corresponding to the four fluorine atoms. Apparently, glutathione conjugation of 1-chloro-1,2-difluorovinyl difluoromethyl ether by cytosolic glutathione S-transferases is stereoselective to some extent.

Next to signals attributed to S-(2-chloro-1,2-difluoro-1-(difluoromethoxy)ethyl)-glutathione, an AB system between −9.25 and −12.35 ppm and two triplets at
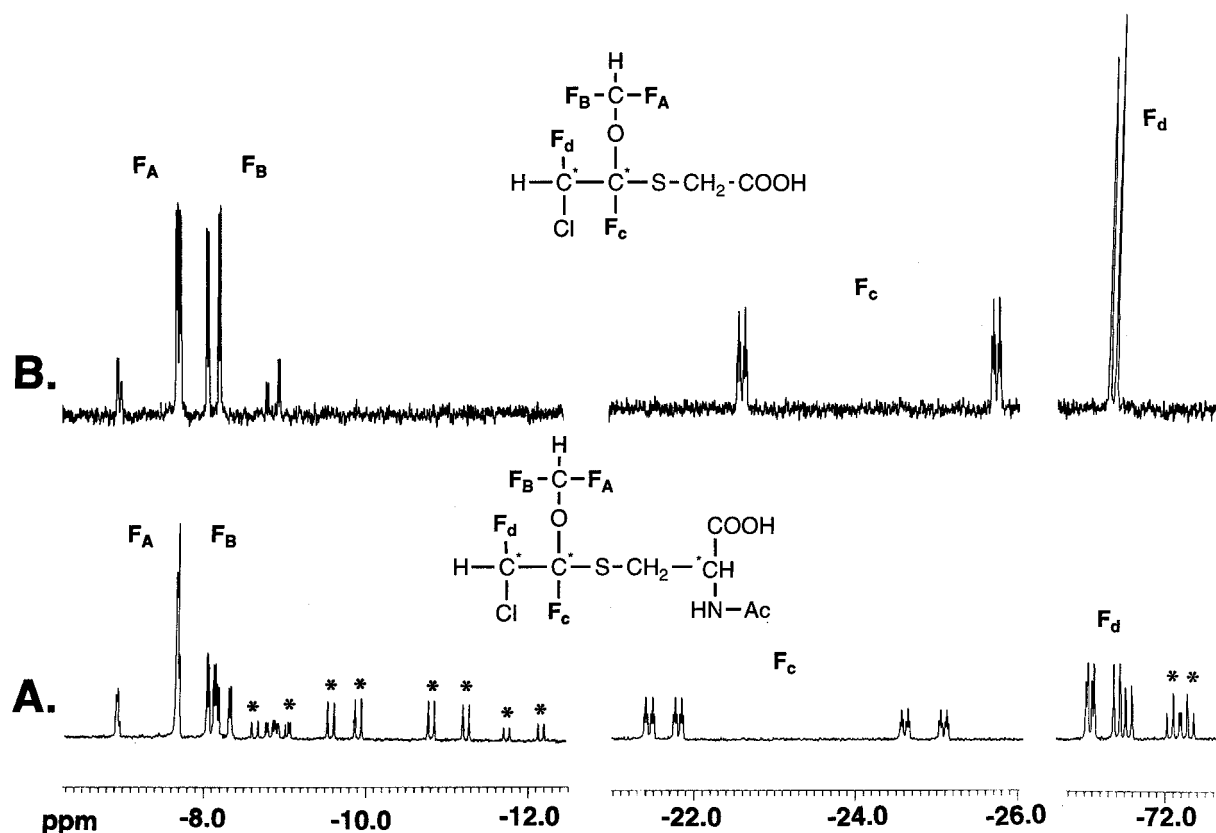
Fig. 5. ^{19}F nuclear magnetic resonance spectra of extracts of reaction mixtures of enflurane degradation products with thiol compounds in dimethyl sulfoxide–triethylamine. (*A*) Extract of reaction with *N*-acetyl-L-cysteine (single extraction). (*B*) Extract of reaction with 2-mercaptoacetic acid. The spectra shown were recorded in proton-decoupled mode; for details of coupling constants, see table 2. Signals derived from CTFE-NAC are indicated by asterisks. ppm = parts per million.

−73.01 and −73.13 ppm ($^{2}J_{FF}$ = 18 Hz) were observed (fig. 6B). These signals, indicated by asterisks, are identical to those present in the ^{19}F-NMR spectrum of biosynthetic chlorotrifluoroethylene–glutathione (CTFE-GSH; fig. 6C). The two triplets at −73 ppm are derived from the CFClH group of the two diastereomeric forms of CTFE-GSH, as described previously.[36] Therefore, these results confirm the formation of chlorotrifluoroethylene-derived S-conjugates from alkaline degradation products of enflurane.

On ^{19}F-NMR analysis of microsomal incubations with enflurane degradation products (fig. 6A), relatively weak signals were observed when compared with those obtained from the cytosolic incubations. The AB system between −9.2 and −12.3 ppm and the triplet at −73.1 appear to be derived from CTFE-GSH. The signals, however, are broadened to some extent, probably because of the high concentration of glycerol in the microsomal concentrate. Consistent with the observation of Dohn *et al.*,[36] only one diastereomer of S-(2-chloro-1,1,2-trifluoroethyl)-glutathione is formed by microsomal glutathione S-transferase, as indicated by the presence of only one triplet at −73.1 ppm. According to the extremely weak signals, at −8.4, −22, and −72 ppm, only very small amounts of S-(2-chloro-1,2-difluoro-1-(difluorometh-

oxy)ethyl)-glutathione appear to be formed in the microsomal incubation.

Discussion

The current study aimed to characterize the thioether compounds formed by the reaction of enflurane degradation products with thiol compounds. Head space analysis of our enflurane–potassium hydroxide mixtures by ^{19}F-NMR confirmed the previously described formation of 1-chloro-1,2-difluorovinyl difluoromethyl ether as an alkaline decomposition product of enflurane.[30,31] In addition, two as yet unidentified minor fluorine-containing products were observed by ^{19}F-NMR analysis.

Gas chromatography–mass spectroscopy and ^{19}F-NMR analysis of the thioethers formed after reaction of enflurane degradation products with *N*-acetylcysteine revealed formation of two products, which were identified as *N*-acetyl-S-(2-chloro-1,2-difluoro-1-(difluoromethoxy)ethyl)- L -cysteine and CTFE-NAC. The first product is indicative for an addition reaction to 1-chloro-1,2-difluorovinyl difluoromethyl ether at the carbon bearing the difluoromethoxy moiety. GC-MS and ^{19}F-NMR analysis did not show any products derived from an addition–elimination reaction, in contrast

Table 2. ^{19}F-NMR Spectra of Identified S-conjugates of Degradation Products of Enflurane

Compound	F Atom*	^{19}F-NMR Spectrum	
		Chemical Shift (ppm)†	Coupling Constants (Hz)
A: S-(1,2-difluoro-1-(difluoromethoxy)-2-chloroethyl)-2-mercaptoacetic acid (fig. 5A)			
Diastereomer 1	$F_{A,B}$	AB system: −7.41 and −8.43	J_{AB} = 173; $^3J_{FF}$ = 5 (d); $^1J_{FH}$ = 71 (d)
	F_c	−22.62	$^2J_{FF}$ = 19 (d); $^3J_{FF}$ = 5 (d); $^2J_{FH}$ = 4 (d)
	F_d	−71.39	$^2J_{FF}$ = 18 (d); $^1J_{FH}$ = 48 (d)
Diastereomer 2	$F_{A,B}$	AB system: −7.48 and −8.26	J_{AB} = 173; $^3J_{FF}$ = 5 (d); $^1J_{FH}$ = 71 (d)
	F_c	−25.76	$^2J_{FF}$ = 19 (d); $^3J_{FF}$ = 5 (d); $^2J_{FH}$ = 4 (d)
	F_d	−71.39	$^2J_{FF}$ = 18 (d); $^1J_{FH}$ = 48 (d)
B: *N*-acetyl-S-(1,2-difluoro-1-(difluoromethoxy)-2-chloroethyl)-L-cysteine (fig. 5B)			
Diastereomer 1	$F_{A,B}$	AB system: −7.38 and −8.22	J_{AB} = 173; $^3J_{FF}$ = 5 (d); $^1J_{FH}$ = 71 (d)
	F_c	−21.52	$^2J_{FF}$ = 19 (d); $^3J_{FF}$ = 5 (t); $^2J_{FH}$ = 5 (d)
	F_d	−71.15	$^2J_{FF}$ = 19 (d); $^1J_{FH}$ = 48 (d)
Diastereomer 2	$F_{A,B}$	AB system: −7.37 and −8.31	J_{AB} = 173; $^3J_{FF}$ = 5 (d); $^1J_{FH}$ = 71 (d)
	F_c	−22.49	$^2J_{FF}$ = 19 (d); $^3J_{FF}$ = 5 (t); $^2J_{FH}$ = 5 (d)
	F_d	−71.47	$^2J_{FF}$ = 19 (d); $^1J_{FH}$ = 48 (d)
Diastereomer 3	$F_{A,B}$	AB system: −7.37 and −8.36	J_{AB} = 173; $^3J_{FF}$ = 5 (d); $^1J_{FH}$ = 71 (d)
	F_c	−24.71	$^2J_{FF}$ = 18 (d); $^3J_{FF}$ = 5 (t); $^2J_{FH}$ = 5 (d)
	F_d	−71.13	$^2J_{FF}$ = 18 (d); $^1J_{FH}$ = 48 (d)
Diastereomer 4	$F_{A,B}$	AB system: −7.36 and −8.52	J_{AB} = 173; $^3J_{FF}$ = 5 (d); $^1J_{FH}$ = 71 (d)
	F_c	−24.95	$^2J_{FF}$ = 18 (d); $^3J_{FF}$ = 5 (t); $^2J_{FH}$ = 5 (d)
	F_d	−71.61	$^2J_{FF}$ = 18 (d); $^1J_{FH}$ = 48 (d)

* Labels refer to fluorine atoms as indicated in figure 5.   † Solvent: deuterium oxide; parts per million (ppm) relative to trifluoroacetic acid.

^{19}F-NMR = ^{19}F nuclear magnetic resonance; d = doublet; t = triplet.

to previous studies with compound A that showed both addition and addition–elimination reaction products after conjugation to thiols.[19] The second product identified, CTFE-NAC, amounted to approximately 30% of the *N*-acetylcysteine conjugates formed and may be explained by the addition reaction of *N*-acetylcysteine to chlorotrifluoroethylene, which is a known nephrotoxin in rodents.[38] A mechanism that may explain formation of chlorotrifluoroethylene is presented in figure 1. However, we were not able to detect chlorotrifluoroethylene directly, which may be explained by its high volatility.

^{19}F nuclear magnetic resonance analyses of the glutathione S-conjugates formed after incubation of enflurane degradation products in presence of rat renal fractions indicated formation of S-conjugates with structures consistent to that of the chemically synthesized *N*-acetylcysteine S-conjugates (figs. 5 and 6). All fluorine signals present in the ^{19}F-NMR spectrum of the cytosolic incubation could be attributed to the addition product of glutathione to 1-chloro-1,2-difluorovinyl difluoromethyl ether and the glutathione S-conjugate of chlorotrifluoroethylene, CTFE-GSH. Apparently addition–elimination reactions to 1-chloro-1,2-difluorovinyl difluoromethyl ether also did not occur during more physiologic conditions. Interestingly, conjugation of glutathione to 1-chloro-1,2-difluorovinyl difluoromethyl ether by cytosolic glutathione S-transferases showed some stereoselectivity, as indicated by the different abundance of the four diastereomers formed. Consistent with previous

studies,[36] conjugation of glutathione to chlorotrifluoroethylene by cytosolic glutathione S-transferases did not show stereoselectivity. Incubation of enflurane degradation products with microsomal fraction resulted in lower concentrations of fluorine-containing glutathione S-conjugates, the major product being CTFE-GSH. Consistent with the study of Dohn *et al.*,[36] only one diastereomer was formed by microsomal glutathione S-transferases.

As discussed in the Introduction, few cases of renal toxicity have been associated previously to enflurane exposure.[3–8] Enzymuria was indicative for proximal tubular toxicity and has also been reported in animal studies.[8,11–13] It might be hypothesized that formation of potentially nephrotoxic S-conjugates, on exposure to alkaline degradation products of enflurane, may be responsible for or contribute to previously reported renal effects of enflurane. At least one of thioethers identified in the current study, CTFE-GSH, is known as a very potent nephrotoxin in rats.[39] The high nephrotoxicity of this compound is explained by its enzymatic degradation by renal γ-glutamyltranspeptidase and dipeptidases to form S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine (CTFE-Cys) and by subsequent local activation by renal β-lyases forming highly reactive products.[39] Reactive intermediates identified on trapping experiments with model nucleophiles are chlorofluorothionoacyl fluoride and 1,1,2-trifluorothiirane.[40] Whether the other thioether identified, S-(2-chloro-1,2-difluoro-1-(difluoromethoxy)ethyl)-glutathione, is also neph-

Fig. 6. ^{19}F nuclear magnetic resonance spectra of biosynthetic glutathione conjugates of enflurane degradation products. (*A*) Rat liver microsomal incubation; (*B*) rat liver cytosolic incubation; (*C*) biosynthetic chlorotrifluoroethylene–glutathione conjugate formed using rat liver cytosolic incubation. The spectra shown were recorded in proton-decoupled mode. Signals derived from chlorotrifluoroethylene–glutathione are indicated by asterisks. ppm = parts per million.
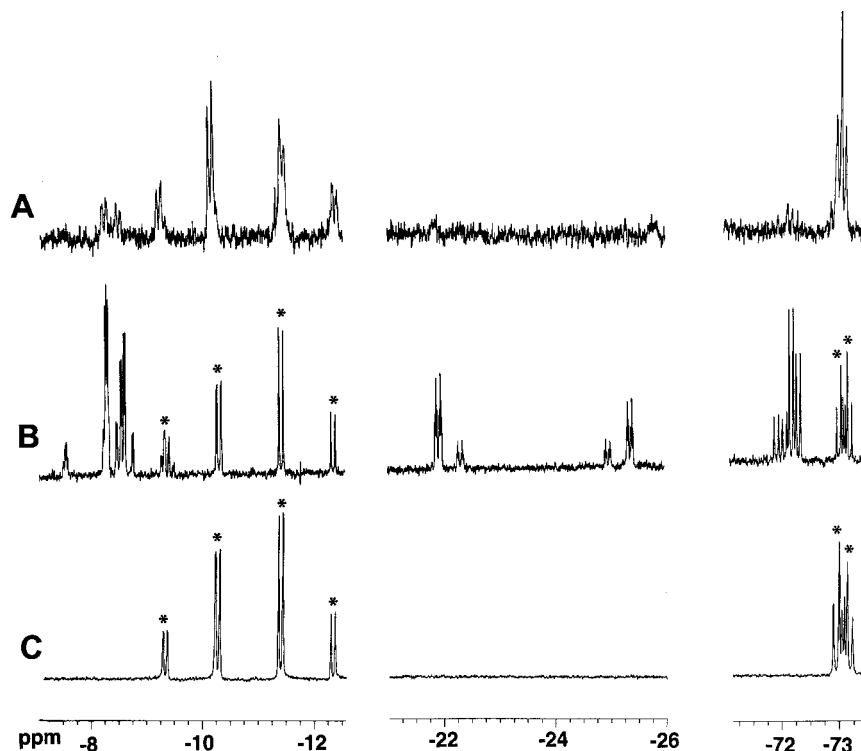
rotoxic, and whether this is mediated by β-lyase–dependent bioactivation, remains to be established.

An approach to test the hypothesis of whether enflurane-anesthetized patients are exposed to its alkaline degradation products and whether these products are metabolized *via* the glutathione conjugation pathway is the quantification of urinary thioethers as biomarkers of exposure. Glutathione S-conjugates are known to be excreted partially as their corresponding *N*-acetylcysteine conjugates (also known as mercapturic acids), which often are applied as biomarkers of exposure to electrophilic chemicals.[34] Because S-bound substituents of glutathione conjugates of halogenated compounds typically are not modified during processing of glutathione conjugates to mercapturic acids,[34] the *N*-acetylcysteine conjugates characterized in the current study may be applied to determine exposure to alkaline degradation products of enflurane. However, because *N*-acetylation of cysteine S-conjugates represents a detoxification pathway competing with β-lyase reaction, biomarkers reflecting the latter bioactivation pathway are required as well.[41] It has been proposed that urinary excretion of haloacids, which are hydrolysis products of β-lyase–derived reactive intermediates, may be applied as biomarkers for the β-lyase pathway. In case of compound A and perchloroethylene, for example, urinary excretion of 3,3,3-trifluoro-2-fluoromethoxypropanoic acid and dichloroacetic acid, respectively, were considered to reflect the β-elimination reactions of their corresponding cysteine S-conjugates.[26,41,42] Based on the levels of excretion of both *N*-acetylcysteine conjugates and haloacids in rat and human studies, it was concluded that

Fig. 7. Reactive intermediates and hydrolysis products that may be anticipated from cysteine conjugate β-lyase–mediated activation of S-(2-chloro-1,2- difluoro-1-(difluoromethoxy)ethyl)-L-cysteine.
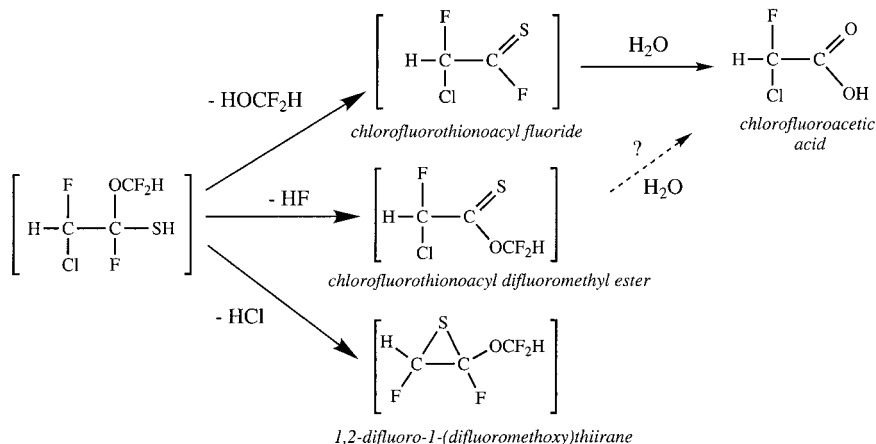
humans are likely to be less sensitive to nephrotoxicity of compound A and perchloroethylene than rats.[26,41,42]

After the current characterization of the thioethers of enflurane degradation products, haloacids formed *via* β-lyase catalyzed bioactivation of their corresponding cysteine S-conjugates can be anticipated. In case of S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, chlorofluoroacetic acid has previously been identified as the hydrolysis product of chlorofluorothionoacyl fluoride, which is one of the two reactive intermediates formed by β-lyase–catalyzed bioactivation.[38–40] In case of S-(2-chloro-1,2-difluoro-1-(difluoromethoxy)ethyl)-L-cysteine, the thiol compound formed after β elimination theoretically can rearrange to three different reactive intermediates (fig. 7) when applying the known rearrangement reactions of structurally related thiol compounds.[40] Interestingly, one of the anticipated products, chlorofluorothionoacyl fluoride, is identical to that formed from S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine. Therefore, chlorofluoroacetic acid theoretically may result from both thioethers of enflurane degradation products. However, the proposed hydrolysis products derived from the β-lyase–dependent bioactivation of S-(2-chloro-1,2-difluoro-1-(difluoromethoxy)ethyl)-L-cysteine remain to be established.

Previously, ^{19}F-NMR analysis of urine of rats and humans exposed to pure enflurane revealed two fluorine-containing haloacids next to inorganic fluoride.[43] The major haloacid identified was difluoromethoxy difluoroacetic acid, which results from hydroxylation of the ethyl moiety of enflurane. Unexpectedly, small amounts of trifluoroacetic acid were identified as well in human urine. The haloacids derived from oxidative metabolism of enflurane, therefore, are not likely to be confounders for the anticipated biomarkers of the β-lyase pathways.

In conclusion, alkaline degradation of enflurane produces halogenated alkenes that are conjugated to glutathione by addition reactions. The glutathione S-conjugates formed may be processed to potentially nephrotoxic thiol compounds. Analysis of the presently described mercapturic acids and anticipated hydrolysis products of β-lyase metabolites in urine of patients exposed to enflurane may help to elucidate the exposure to alkaline degradation products of enflurane and to investigate the involvement of these compounds in cases of clinically observed renal effects of enflurane.

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