The influence of cytochrome P450 enzyme activity on the composition and quantity of volatile organics in expired breath

James M. Mathews, James H. Raymer, George R. Velez, C. Edwin Garner and John R. Bucher

We have previously described a method to capture, identify and quantify volatile components in expired breath. The purpose of this research is to provide a non-invasive means to measure biomarkers of metabolism *in vivo*. In the present studies, the effect of 1-aminobenzotriazole (ABT), an inhibitor of diverse cytochrome P450 (P450) enzymes, on the composition of volatile organic chemicals (VOCs) expired in the breath of male F-344 rats was determined in parallel with the catalytic activities and total content of hepatic P450. Intraperitoneal administration of ABT (100 mg kg^{-1}) to rats resulted in markedly diminished hepatic microsomal P450 content and activities. The extent of inhibition was near maximal at 4 h, at which time approximately 50% of the total P450 content, about 65% of the CYP1A2 activity, 55% of the CYP2E1 activity, and about 80% of CYP2B activity were lost. Inhibition was maintained to 48 h post-dosing, but P450 content and activities had largely been restored by day 7. Concomitant with the inhibition of P450 were corresponding increases (up to several hundred-fold) in the molar amount of volatiles appearing in the breath of ABT-treated animals, and the rebound of P450 levels was attended by corresponding decreases in the appearance of breath volatiles. These studies indicate that P450 plays a major role in the metabolism of VOCs appearing in breath, and that these chemicals can serve as markers on P450 activity *in vivo*.

Keywords: *cytochrome P450, breath, volatile organic compounds.*

Abbreviations: *ABT, 1-Aminobenzotriazole; ANH, Acetanilide hydroxylase; BND, Benzphetamine N-demethylase; BSA, Bovine serum albumin; HEPES, 4-(2-Hydroxyethyl)-1-8 piperazineethanesulphonic acid; PNP, p-Nitrophenol; P450 or CYP, Cytochrome P450; TCDD, 2,3,7,8-Tetrachlorodibenzo-p-dioxin; VOCs, Volatile organic chemicals.*

Introduction

A number of non-invasive methods have been developed that allow estimation of cytochrome P450 activities *in vivo* using breath analyses. Most involve administration of an isotopically labelled substrate, followed by measurement of labelled CO_2 appearing in breath. The activities measured have included the N-demethylation of caffeine, O-demethylation of methacetin, O-deethylation of phenacetin (Kruger *et al.* 1991) and the N-demethylation of erythromycin (Watkins *et al.* 1989). However, the breath also contains a number of naturally occurring, low molecular weight volatile components. These include a variety of saturated and unsaturated straight- and branched-chain hydrocarbons, aldehydes, alcohols and ketones, among others. Some of these compounds have been shown to be produced during the autooxidation of lipids (Frankel 1982), and breath levels of ethane and pentane have been used as markers of lipid peroxidation *in vivo* (Wade and van Rij 1985).

We have developed a method to capture, identify and quantify volatile components in expired breath (Raymer *et al.* 1994a). The purpose of this research is to provide a non-invasive means to monitor changes in sensitive markers of metabolism, and to relate changes in breath components with xenobiotic-induced pathology or altered metabolism. We have found that most of the volatile components in the breath of rats are consistent in composition and quantity from day to day, show little evidence of circadian variation and are not affected by differences in the stage of the oestral cycle. There are, however, minor but clear sex-related differences in some breath components (Raymer *et al.* 1994b).

1-Aminobenzotriazole (ABT) is a mechanism-based inhibitor of cytochrome P450 enzymes in rats (Ortiz de Montellano and Mathews 1981). ABT does not stimulate lipid peroxidation or cause increases in the levels of serum transaminases *in vivo* (Ortiz de Montellano *et al.* 1984), and is effective against a broad spectrum of P450 isozymes (Ortiz de Montellano *et al.* 1981, Mathews and Bend 1986, Meschter *et al.* 1994). A recent publication of Meschter *et al.* (1994) described changes of rather minor toxicological significance associated with 13 weeks of treatment of Sprague–Dawley rats with ABT, during which total hepatic cytochrome P450 levels and all of the isozyme-selective alkoxyresorufin dealkylase activities were decreased to less than 30% of control levels throughout the study. These results demonstrate that ABT is well tolerated in rat and, apart from inhibition of cytochrome P450, causes no derangement of biochemical processes.

The goal of the current study was to examine the influence of cytochrome P450 activities on the pattern of breath components. Based on the work of Allerheiligen *et al.* (1987) and others, we anticipated that many of the components of breath, particularly the short-chain alkanes, alkenes, aldehydes, ketones and alcohols, might be substrates for cytochrome P4502E1 (CYP2E1), and that it might be possible to monitor functional changes resulting from induction or inhibition of specific P450 isozymes by monitoring changes in a particular, or a set of, breath components. This report describes the results of such experiments using ABT as a general cytochrome P450 inhibitor. We have found that inhibition of the cytochrome produces a dramatic increase in breath components, suggesting that the cytochromes P450 have a major 'housecleaning' function under normal physiological conditions.

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METHODS

Male F344 rats, 12–16 weeks old, were obtained from Charles River Laboratories, Inc. (Raleigh, NC) and, upon arrival, were examined by a veterinarian for evidence of disease, quarantined for 1 week, and examined again before release to the study. Prior to experiments, rats were housed (maximum of four per cage) in polycarbonate cages and provided with tap water and NIH07 diet (Ziegler Brothers, Inc., Gardners, PA) *ad libitum*. Contact bedding for rats was Ab-Sorb-Dri hardwood chips (Lab Products, Maywood, NJ). Three animals were used for each test group. During collection of exhaled breath, animals were housed in specially designed, Battelle-type, polycarbonate restrainers purchased from CH Technologies (Westwood, NJ). These restrainers function to keep the rat from backing out of the nose-port during the collection period. Animals were acclimated to these restrainers for a minimum of 2 h on 2 days immediately prior to being used in any definitive study.

The volatile organic compounds (VOCs) exhaled by Fischer 344 rats were collected using a nose-only, non-rebreathing breath collection system described previously (Raymer *et al.* 1994a). The system was open in design and relied on mass flow controllers to regulate both the introduction of clean, humidified air and the withdrawal of exhaled breath in a balanced manner. The system accommodated the simultaneous collection of breath from three rats held in Battelle-type restrainers. The restrainers were modified to include an auxiliary nitrogen purge to minimize the infiltration of VOCs from the room and from excreta into the collected breath. The VOCs from 90 l of pooled breath were collected onto two Tenax-GC sorbent traps in series and analysed by thermal desorption/gas chromatography with flame ionization or mass spectrometric detection (Raymer *et al.* 1994a). Gas chromatography in conjunction with electron impact (70 eV) high resolution mass spectrometry (VG ZAB-E, Fisons Instruments, Danvers, MA) and Fourier transform infrared spectroscopy (Mattson Cryolect with Sirius 100 spectrophotometer, Analytical Technologies Inc, Madison, WI) provided additional chemical characterization to support proposed structural assignments based on unit resolution mass spectrometry. The molar yields of the volatile components were calculated from GC analytical data as previously described (Raymer *et al.* 1994a). The chromatographic peak areas were used to calculate first grams of compound (assuming a response factor), then moles. This value was used to calculate a compound production rate (PR) with units of fmol of compound per 100 g rat per min. Production of volatiles was also normalized against CO_2 production in several experiments, and yielded similar findings.

ABT was purchased from Aldrich Chemical Co. (Milwaukee, WI) and administered i.p. to rats at a dose of 100 mg kg^{-1}. Deionized/distilled water was used as a vehicle for the ABT-treated animals. In previous work (Raymer *et al.* 1994b) it was demonstrated that administration of aqueous vehicles had no significant effect on the composition and quantities of breath volatiles. Post-dose breath samples (90 l) were collected from a group of three rats during the periods 3–5 h, 24–26 h, 47–49 h, and 1 week (169–171 h). This experiment was repeated twice with groups of three naive rats per experiment (for a total of three replicates of $N = 3$), with an extra group added to allow a fourth replicate of the pre-dose (0 h) total volatiles determination.

Separate groups of rats (three per timepoint) were dosed for determination of hepatic microsomal P450 activities. These rats were sacrificed by asphyxiation with carbon dioxide at the designated timepoints, and hepatic microsomes were prepared from the excised livers as previously described (Ortiz de Montellano *et al.* 1981). The microsomal pellets were resuspended to a protein concentration of ca. 10 mg ml^{-1} in 2.5 mM HEPES buffer (pH 7.4) containing 0.15 M KCl, flash frozen in liquid nitrogen, and stored at –70 °C. Protein concentration was determined by the method of Bradford (Bradford 1976), using a BioRad Protein Assay kit (BioRad Laboratories, Hercules, CA). A standard curve was prepared using bovine serum albumin (BSA). P450 content of the microsomal preparations was determined from the carbon monoxide difference spectrum using the procedure of Omura and Sato (1964).

Benzphetamine N-demethylation (BND) activity, a marker for cytochrome P4502B catalytic activity (Soucek and Gut 1992), was determined by quantitating the amount of formaldehyde liberated from the N-demethylation of benzphetamine according to a modification of the method of Nash (Nash 1953). The reaction mixtures contained 100 mM potassium phosphate buffer (pH 7.3), 1–2 mg of microsomal protein, 2 mM benzphetamine hydrochloride and 2 mM NADPH in a final volume of 1.0 ml. The reaction was initiated with NADPH and incubated for 10 min at 37 °C. Incubations were terminated by the addition of 0.6 ml of a 10% trichloroacetic acid solution with mixing, then centrifuged at 1500 *g* for 20 min. An aliquot of supernatant (1 ml) was added to 0.5 ml of the freshly prepared Nash reagent, and allowed to stand at 50 °C for 15 min then at room temperature for 5 min prior to measurement of absorbance at 405 nm with a BioTek EL340 Biokinetics Reader (Biotek Instruments, Inc., Winooski, VT). A standard curve was prepared using formaldehyde.

Acetanilide hydroxylase (ANH) activity, a marker for the activity of CYP1A2, was measured using a modification of the HPLC assay method of Liu *et al.* (1991). Briefly, microsomes were incubated with acetanilide for 20 min at 37 °C, after which the mixtures were chilled in ice and the product extracted with ice-cold ethyl acetate. Extracts were analysed by reversed phase HPLC using a 4.6 mm × 25 cm Supelco C_{18} DB column and a linear gradient mobile phase eluting initially with water/methanol/acetonitrile, 78:17:5, v/v/v. The percentage of acetonitrile was held at 5%, and the solvent composition changed linearly to 76.5:18.5:5 over a period of 10 min, then to 57:38:5 from 10 to 13 min, to 9.5:85.5:5 from 13 to 20 min, then back to the initial conditions from 20 to 25 min. The flow rate was 1 ml min^{-1}, and the eluting hydroxyacetanilide (retention time 3.5 min) was resolved from acetanilide (retention time 8.5 min), detected and quantitated by measuring absorbance at 254 nm.

p-Nitrophenol (PNP) hydroxylase activity, a marker for the activity of CYP2E1, was measured using the method of Koop (1986) in which the conversion of PNP to 4-nitrocatechol is measured. The reaction mixture was maintained at 37 °C and contained 0.1 M potassium phosphate buffer (pH 7.4), 0.2 mg microsomal protein, 0.1 mM PNP, and 1 mM ascorbate in a volume of 1 ml. Reactions were initiated by addition of 10 µl of 100 mM NADPH and stopped after 10 min by addition of 200 µl of ice-cold 1.5 N $HClO_4$. The mixture was centrifuged, 1 ml of supernate was removed and the colour was developed by addition of 100 µl of 10 N NaOH. A standard curve was prepared using authentic standards of 4-nitrocatechol. The absorbance at 490 nm was determined for each resulting solution using a Biotek EL 340 Microplate Bio Kinetics Reader.

Statistical treatment of data

The values for the total content and enzyme activities of cytochrome P450 were compared by ANOVA followed by Dunnett's test. The values for exhalation of total volatiles were compared using a paired *t* test. Statistically significant differences were determined at the $\alpha = 0.05$ level.

Results

System performance

Several compounds were routinely detected and measured in the system blank (collection of VOCs and 90 l of humidified air passed through the collection system), including pentane and low concentrations of other aliphatic, aromatic, and some oxygenated compounds. Pentane, benzaldehyde, and acetophenone were the major background contaminants and arose from the Tenax-GC itself. A system background was collected on each experiment day, and the concentrations of

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the compounds were consistent (see below). The large (90 l) sample volume required to concentrate sufficient masses of breath-related VOCs, however, began to saturate the sorbent and some VOCs broke through (Krost *et al.* 1982, Raymer *et al.* 1994b). Therefore, compounds such as acetone could not be quantified on a mass basis. Calculated amounts do, however, accurately reflect changes in concentration in the exhaled breath as long as the chromatographic detector is not saturated. Recoveries for alcohols, aliphatic and aromatic hydrocarbons, aldehydes, esters, sulphides, and thiazole at low ng l^{-1} concentrations were in excess of 98%, and thus, can be reliably collected and determined using this system (Raymer *et al.* 1994a, b). Such compounds represent the vast majority of volatile chemicals reported to be found in human breath (Conkle *et al.* 1975, Krotoszynski *et al.* 1977, Phillips and Greenberg 1992). Low concentrations of carboxylic acids, thiols, and primary amines were shown not to be reliably collected and analysed, but it is speculated that the majority of chemical classes are accurately represented.

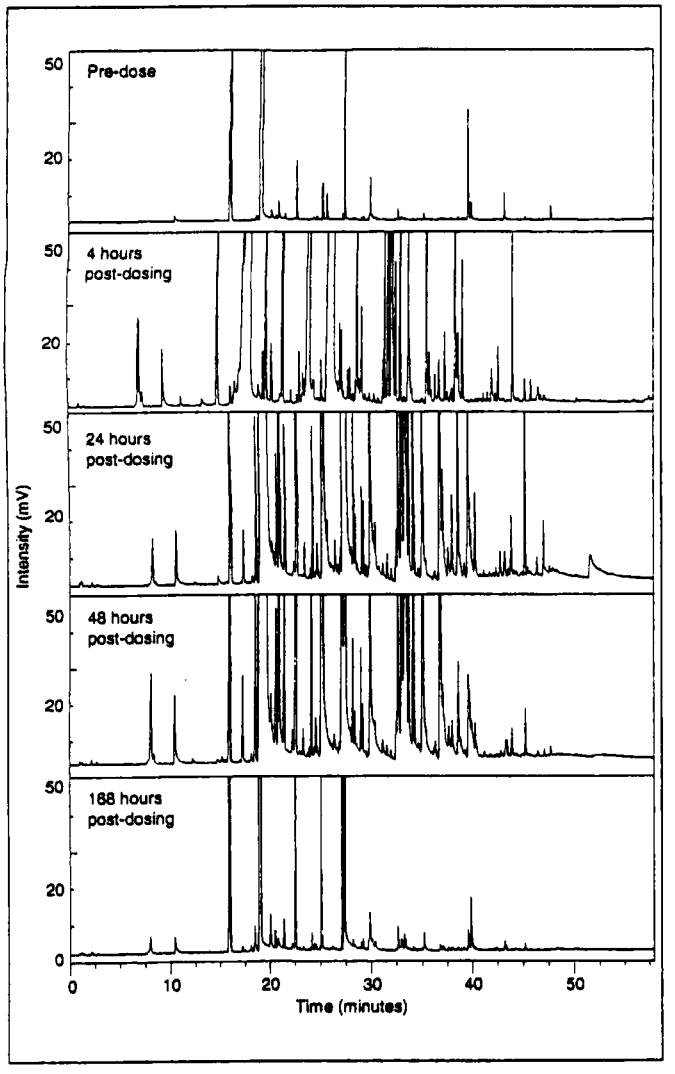

Figure 1. Gas chromatograms of breath components captured from rats pre-dose and at various times following treatment with ABT (100 mg kg^{-1} i.p.).

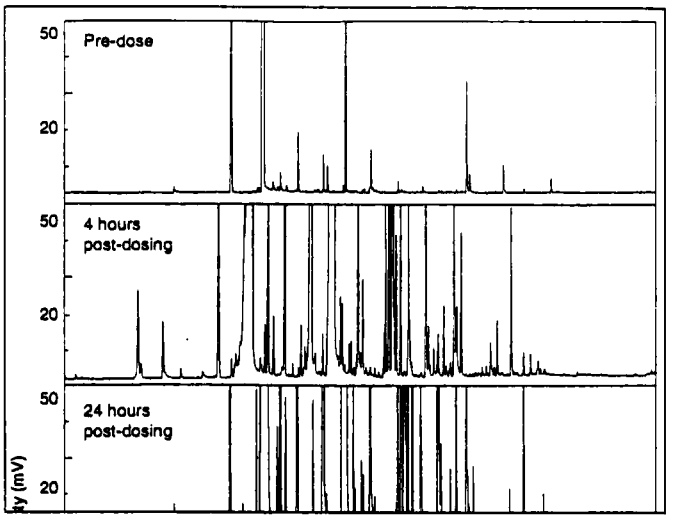

Figure 2. Time courses of the total mass of VOCs exhaled (●) and the content of hepatic microsomal P450 (■) from rats following treatment with ABT (100 mg kg^{-1} i.p.). Values are means ± standard deviation for $N = 3$ rats, except for the pre-dose (0 h) VOCs, for which $N = 4$ rats. *Significantly different from control (0 h), $p < 0.05$.

Cytochrome P450 and breath volatiles

Chromatograms showing the VOCs collected from breath pre-dose and 4, 24, 48, and 168 h after treatment with ABT are shown in Figure 1. The changes in the exhaled VOCs were pronounced, reached a maximum at 24 h post-dosing and returned to the approximate pre-dose levels at 168 h post-dosing (Figure 2). Compounds presumed to be of endogenous origin were ranked in order of the total molar amounts exhaled in the 24 h post-dose sample (Table 1). These chemicals include many low molecular weight alcohols, aldehydes, ketones, alkenes and alkanes associated with the products of lipid peroxidation. The relative change in the amounts of these selected volatiles at 24 h post-dosing is also shown in Table 1. *n*-Octane was increased in breath about 200-fold above its pre-dose level, and 10 other chemicals were increased from 20- to about 75-fold by 24 h following ABT treatment. A number of chemicals presumed to be of exogenous origin also showed marked increases (Table 2). These included the carcinogens benzene and 1,2-dichloroethane (US DHHS, 1991).

Using separate groups of rats, the effect of ABT on total hepatic microsomal content and activities of P450 enzymes were determined in parallel to the determination of the profile of volatiles produced. The content and activities of cytochrome P450 enzymes were markedly decreased by intraperitoneal administration of ABT (Table 3). The extent of inhibition was near maximal at the 4 h timepoint, at which time approximately 50% of the total P450 content, about 65% of the ANH activity (associated with CYP1A2) and 55% of the PNP-hydroxylase activity (associated with CYP2E1) were lost. Additionally in preliminary experiments, about 80% of BND activity (associated with the phenobarbital-inducible form 2B1) was lost at the 4 h timepoint (data not shown). The overall degree of inhibition was maintained to 48 h post-dosing. P450 content returned to levels that were equal to control levels 1 week post-dosing. Similarly,

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Compound[a]	Production rate (fmol per 100 g rat per min)		
	Pre-dose (CV)[b]	24 h post-dose (CV)[c]	% Change[d]
Acetone	190000 (39)	1000000 (13)	426
2-Butanone	1200 (68)	40000 (68)	3233
n-Hexane	700 (18)	16000 (75)	2186
3-Methylfuran	nd[e] (—[f])	10000 (59)	—
2-Propanol	880 (74)	9100 (86)	934
n-Octane	14 (121)	2700 (62)	19186
2-Pentanone	400 (50)	4500 (40)	1025
n-Hexanal	190 (16)	4300 (46)	2163
n-Octene/C_8H_{16} isomer	54 (43)	4200 (76)	7678
Dimethyl sulphide	360 (36)	2200 (92)	511
2-Heptanone	680 (116)	2000 (59)	194
Acetaldehyde	970 (19)	2000 (40)	106
4-Heptanone	38 (79)	1800 (16)	4637
2-Octene	nd (—)	1500 (33)	—
3-Methylpentane	200 (31)	1000 (111)	400
$C_7H_{14}O$ ketone	29 (90)	880 (40)	2934
n-Heptane	33 (29)	790 (34)	7294
4-Methyl-3-heptanol/saturated hydrocarbon	45 (33)	680 (49)	1411
$C_5H_{12}O$ alcohol/C_8H_{16} isomer	66 (24)	530 (46)	703
n-Nonane	21 (67)	450 (51)	2043
Dimethyloctadiene isomer	18 (19)	380 (36)	2011
Methylvinylketone/3-methylfuran	94 (13)	360 (56)	283
C_7H_{12} isomer	69 (10)	330 (47)	378
3-Methyl-2-butanone/2-methyl-2-butenal	41 (36)	280 (75)	583
4-Methyl-2-pentanone	22 (13)	260 (65)	1082
n-Heptanal	550 (15)	160 (74)	−71
3-Heptanone	32 (3)	160 (51)	400
$C_{10}H_{18}$ isomer	nd (—)	140 (86)	—
1-Hexene/C_6H_{12}/2-methyl-1-pentane	22 (73)	130 (62)	491
2-Methyl-3-butene-2-ol	13 ($n = 1$)	120 (65)	823
2-Methylfuran	20 ($n = 1$)	120 (44)	500
2-Pentylfuran	20 ($n = 1$)	120 (44)	500

Table 1. Breath components presumed to be of endogenous origin sorted by decreasing production rate in 24-h post-ABT treatment.

[a] Based on GC/MS identification; multiple entries indicate co-elution. [b] $n = 4$; CV = coefficient of variation. [c] $n = 3$.

[d] (24 h–(Pre-dose)/(Pre-dose)) ×100. [e] nd = not detected. [f] CV and % change could not be calculated ($n = 1$ or not detected).

there was no statistically significant difference in the PNP-hydroxylase activity 1 week post-dosing compared with pre-dose levels, but ANH activity recovered to only about 60% of control. Concomitant with the inhibition of P450 was a corresponding increase in the molar amount of volatiles appearing in the breath of ABT-treated animals, and the resynthesis of P450 was attended by corresponding decreases in the appearance of breath volatiles (Figure 2).

Discussion

In the present work, the effect of inhibition of cytochrome P450 on the composition and concentration of volatile components in the breath of rats was investigated. Rats were treated with ABT, a mechanism-based ('suicide') inhibitor of cytochrome P450 enzymes (Oritz de Montellano and Mathews 1981, Meschter *et al.* 1994). The natural, ongoing process of lipid peroxidation probably provides the source of most of the volatile organic compounds that were monitored in the present experiments (Frankel 1982), and it is concluded that the increases reported here result from inhibition of P450 rather than stimulation of the production of VOCs.

While most of the VOCs detected in the present work may be attributed to either xenobiotic or endogenous origins, there remains some uncertainty in these assignments. The possible contribution of metabolic products of ABT to the breath components is not certain because there has been no reported investigation of potential volatile metabolites of ABT to date. It may be speculated that the marked increase in benzene was due in part to metabolism of the benzyne precursor, ABT, and subsequent conversion to benzene. It is not possible from these data to determine whether the increase in breath benzene was

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Compound[a]	Production rate (fmol per 100 g rat per min) Pre-dose (CV)[b]	24h Post-dose (CV)[c]	% Change[d]
1,2-Dichloroethane	9400 (28)	230000 (58)	2347
Benzene	76 (61)	45000 (12)	59111
Chlorobenzene	56 (12)	7300 (19)	12936
Tetrachloroethylene	25 (48)	4300 (37)	17100
Methylene chloride/methyl acetate/acetonitrile	200 (97)	3400 (77)	1600
Toluene	120 (49)	2000 (10)	1567
Methylcyclopentane	57 (56)	900 (95)	1479
Cyclopentane	53 (58)	780 (25)	1372
Methylmethacrylate	47 (20)	490 (39)	943
Trichlorofluormethane	40 (36)	180 (19)	350

Table 2. Breath components presumed to be of exogenous origin sorted by decreasing production rate in 24 h post-ABT treatment.

[a] Based on GC/MS identification; multiple entries indicate co-elution.

[b] CV = coefficient of variation; $n = 4$. [c] $n = 3$.

[d] ((24 h–(Pre-dose))/Pre-dose) × 100.

due to the metabolism of ABT, or resulted from outgassing from a basal body burden, or from natural processes not previously described. However, in subsequent investigations using a non-benzenoid P4502E1 inhibitor, only trace amounts of benzene were detected in expired breath (Mathews *et al.* 1995). Similarly, the other 'industrial' volatile chemicals detected in these studies may presumably reflect the normal body burden of volatiles from exposures to commercial products, plastics, laboratory chemicals, etc. The rats typically showed high breath concentrations of 1,2-dichloroethane, and this was markedly increased by ABT treatment.

Consistent with the known cross-reactivity of ABT with catalytically diverse P450 isozymes (Ortiz de Montellano *et al.* 1981, Meschter *et al.* 1994) ABT inactivated the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-inducible form 1A2, ethanol-inducible 2E1, and the phenobarbital-inducible form 2B1. The timecourse of inhibition was monitored throughout the onset of inhibition, maximal effect, and eventual resynthesis of the enzymes, and the production of VOCs in breath was quantitated during those same intervals. Changes in the production of VOCs closely paralleled those of total P450 content and CYP2E1 activity, but not the activity of CYP1A2. No conclusion could be drawn concerning CYP2B1.

These findings demonstrate that cytochrome P450 plays a major role in the metabolism of volatile components that appear in breath. As such, measurement of the production of total breath volatiles, or perhaps selected components, holds promise as a means of monitoring the activities of cytochrome P450 isozymes *in vivo*. P450 serves not only to detoxify compounds, but also to make them more water-soluble to facilitate their removal and preserve the homeostasis of biological membranes. The structure and crucial functions of these membranes are particularly sensitive to their fluidity and microcrystalline states. As most of the volatile organics detected in the present studies are effective solubilizers of lipid membranes, prevention of their accumulation may be of particular importance. The toxicity data available on individual breath components range from sparse to relatively complete. Based on LD_{50} data, many of the compounds considered to be of endogenous origin appear to be relatively non-toxic, but some, such as *n*-hexane, are neurotoxic (Altenkirch *et al.* 1982), and others may have unappreciated toxic properties (Benedetti *et al.* 1979). It is often assumed that challenges to biological systems are principally of xenobiotic origin, but it is clear from these studies that the total mass of endogenous compound production may be equal to or greater than that encountered from external sources. Also, conditions which cause a decreased metabolic capacity may result in toxicity through accumulation of certain chemicals of endogenous origin.

While the lack of isozyme specificity of ABT precludes its use in a precise elucidation of which P450 isoform(s) are involved in the metabolism of these endogenously-generated volatiles, more specific inhibitors are available which are being employed in determining the contribution of particular isozymes to the metabolism of selected volatile components in breath. P450 isozymes involved in the metabolism of endogenous compounds commonly display much greater substrate specificity than do those that metabolize xenobiotics, and it is possible that a limited number of isozymes have high catalytic activity in metabolizing volatile organics of endogenous origin. Experiments with specific inhibitors are in progress to establish potential biomarkers of activity of selected P450 isoforms through component analysis of the breath of mammals.

Time post-dosing (Ch)	Cytochrome P-450[a] (nmol mg^{-1} protein)	Acetanilide hydroxylase (nmol mg^{-1} protein min^{-1})	*p*-Nitrophenol hydroxylase (nmol mg^{-1} protein min^{-1})
Control	0.94 ± 0.03	0.42 ± 0.07	1.0 ± 0.1
4	0.49 ± 0.09* [47.9][b]	0.15 ± 0.04* [64.3]	0.45 ± 0.16* [55.0]
24	0.48 ± 0.04* [48.9]	0.13 ± 0.07* [69.0]	0.32 ± 0.03* [68.0]
48	0.53 ± 0.10* [43.6]	0.15 ± 0.08* [64.3]	0.33 ± 0.13* [67.0]
168	0.94 ± 0.05 [0.0]	0.24 ± 0.04* [42.9]	0.85 ± 0.10 [15.0]

Table 3. Microsomal cytochrome P-450 content and catalytic activities following treatment with ABT (100 mg kg^{-1} i.p.).

[a] Mean ± standard deviation for $N = 3$ rats. [b] Percent loss versus control. * Significantly different from control ($p < 0.05$).

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Acknowledgements

This work was performed under National Institute of Environmental Health Sciences contract No. NO1-ES-05290. The authors are grateful to Drs Mike Cunningham and Dan Morgan for their review of the manuscript and to Ms Sherry A. Ennis for her assistance in the preparation of this manuscript.

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Received 16 January 1996, revised form accepted 26 February 1996

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