High-Performance Liquid Chromatographic–Fluorescent Method to Determine Chloroacetaldehyde, a Neurotoxic Metabolite of the Anticancer Drug Ifosfamide, in Plasma and in Liver Microsomal Incubations

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Chloroacetaldehyde (CA) is a nephrotoxic and neurotoxic metabolite of the anticancer drug ifosfamide (IFA) and is a dose-limiting factor in IFA-based chemotherapy. Plasma levels of CA in IFA-treated cancer patients are often difficult to determine due to the lack of a sufficiently sensitive and specific analytical method. We have developed a simple and sensitive HPLC method with fluorescence detection to measure CA formation catalyzed by liver cytochrome P450 enzymes, either *in vivo* in IFA-injected rats or *in vitro* in liver microsomal incubations. This method is based on the formation of the highly fluorescent adduct 1-N^6-ethenoadenosine from the reaction of CA with adenosine (10 mM) at pH 4.5 upon heating at 80°C for 2 h. The derivatization mixture is directly injected onto a C18 HPLC column and is monitored with a fluorescence detector. Calibration curves are linear ($r > 0.999$) over a wide range of CA concentrations (5–400 pmol). The limit of detection of CA in plasma using this method is < 0.1 μM and only 50 μl of plasma is required for the assay. By coupling this method with a recently described HPLC–fluorescent method to determine acrolein, a cytochrome P450 metabolite of IFA formed during the activation of the drug by 4-hydroxylation, the two major, alternative P450-catalyzed pathways of IFA metabolism can be monitored from the same plasma samples or liver microsomal incubations and the partitioning of drug between these two pathways thereby quantitated. This assay may prove to be useful for studies of IFA metabolism aimed at identifying factors that contribute to individual differences in CA formation and in developing approaches to minimize CA formation while maximizing IFA cytotoxicity. © 1999 Academic Press

Ifosfamide (IFA)[2] and its structural isomer cyclophosphamide (CPA) are alkylating agents commonly used in the treatment of malignant tumors (1). IFA is more active than CPA in the treatment of certain cancers, such as soft tissue sarcomas and ovarian cancer (2). IFA also exhibits lower myelotoxicity than CPA, which makes it possible to administer higher doses of IFA to cancer patients. IFA is a prodrug that must be metabolized to the active alkylating species isophosphoramide mustard via a 4-hydroxylation reaction catalyzed by a cytochrome P450 enzyme (3). IFA can also undergo an alternative P450-catalyzed N-dechloroethylation reaction that generates the therapeutically inactive metabolites 2- and 3-dechloroethylifosfamide and a neurotoxic and nephrotoxic byproduct, chloroacetaldehyde (CA) (Fig. 1). Consequently, factors that decrease the extent of N-dechloroethylation to yield CA may increase both the effective dose of active drug and its therapeutic effect, while at the same time decreasing IFA-induced host toxicity.

In cancer patients, about 45% of IFA is typically metabolized to CA via the N-dechloroethylation pathway, while in the case of CPA only ~10% of the parental drug is N-dechloroethylated to yield CA (4). Consequently, neurotoxicity occurs in about 20% of patients treated with IFA, but is rare in CPA-treated patients

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[2] Abbreviations used: AUC, area under the curve; C_{max}, peak plasma concentration; $t_{1/2}$, half-life; P450, cytochrome P450; CA, chloroacetaldehyde; CPA, cyclophosphamide; IFA, ifosfamide; UT, untreated; BNF, β-naphthoflavone; PB, phenobarbital; DEX, dexamethasone.

(5–8). Depletion of glutathione and lipid peroxidation are two possible mechanisms for CA-induced neurotoxicity and nephrotoxicity (9–11). CA primarily causes tubule toxicity rather than hemorrhagic cystitis, which is caused by acrolein, an IFA metabolite formed during the decomposition of 4-OH-IFA to yield isophosphoramide mustard, the therapeutically active metabolite (12). CA can, however, also bind to DNA (particularly adenine bases) and form etheno adducts (13–15).

Despite the importance of CA in IFA-induced toxicities, measurements of CA levels in IFA-treated cancer patients are reported infrequently because of the lack of a sufficiently specific and sensitive method to monitor CA. Several HPLC and GC methods for the determination of CA have been described (4). Most of the early methods were developed to detect acetaldehyde or other aliphatic aldehydes and are thus not specific for CA (4). Since aldehydes are commonly found in human plasma, these nonspecific methods are not suitable for accurate analysis of CA in plasma of IFA-treated patients. A more specific HPLC method was developed by Ruzicka and Ruenitz (16) and modified by Kaijser *et al.* (17). This method is based on the derivatization of CA with thiourea to form 2-aminothiazole, which can be separated by HPLC and monitored at 254 nm. This method has been successfully used in our laboratory to measure CA formed from IFA both in rat plasma (18) and in *in vitro* microsomal incubations (19). However, this method has low sensitivity and a high background due to interfering substances, despite the fact that an ion-exchange extraction step is performed to partially purify the samples prior to HPLC analysis. Drawbacks of the ion-exchange extraction step include the fact that it is a labor-intensive, time-consuming process that introduces sample-to-sample variability. A GC method has also been developed to detect CA (20). Access to and familiarity with GC instrumentation are required to apply this method.

In the present study, a simple and sensitive assay method was developed for CA based on the formation of the fluorescent adduct $1-N^6$-ethenoadenosine upon reaction of CA with adenosine (Fig. 1). This method was adapted from the HPLC–fluorescent assay for adenosine reported by Jacobson *et al.* (21) and Zhang *et al.* (22). This method is shown to be very useful for the determination of CA as a metabolite of IFA present in plasma samples and formed in *in vitro* liver microsomal incubations.

MATERIALS AND METHODS

Chemicals. Adenosine and $1-N^6$-ethenoadenosine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). CA [50% (w/v) solution in water] was purchased from Aldrich Chemical Co. (Milwaukee, WI). HPLC-grade methanol was purchased from J. T.

Baker (Phillipsburgh, NJ). Racemic IFA was provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). 4-Hydroperoxy-IFA and 4-hydroperoxy-CPA were obtained from Dr. J. Pohl (ASTA Pharma, Bielefeld, Germany).

Pharmacokinetics. Male Fischer 344 rats (8–10 weeks old, 175–200 g) were purchased from Taconic Inc. (Germantown, NY) and maintained in individual cages in the Boston University Laboratory for Animal Care Facility with food and water *ad libitum.* A catheter was inserted into the right atrium via the right jugular vein in rats to be used for pharmacokinetic analyses (18, 23). After 3 days recovery from surgery, an intravenous injection of IFA (100 mg/kg) was given to the rats. Blood (~250 μl at each time point) was withdrawn from the catheter at each of 10 time points (0, 4, 10, 25, 40, 60, 90, 120, 180, and 270 min) after IFA injection. At each time point, the volume of blood withdrawn was replaced with an equal volume of 0.9% saline. Blood samples were mixed with 10 U heparin and centrifuged at 14,000 rpm in an Eppendorf centrifuge (16,000g) at room temperature for 4 min. An aliquot of plasma (50 μl) was then taken and immediately stabilized with 10 μl of 10 mM formaldehyde to facilitate measurement of CA. Plasma samples were deproteinized using 20% trichloroacetic acid (40 μl) and then centrifuged at 16,000g for 4 min. A portion of the supernatant (72.5 μl) was removed, transferred to a clean screw-cap tube (to prevent sample loss during derivatization), and then neutralized with 7.5 μl of 5 N NaOH. A second aliquot of plasma (50 μl) was used to measure acrolein after derivatization by a published method (18, 23) followed by HPLC analysis using a fluorescence detector (24) as described below. For time point 0, a separate aliquot of plasma was taken and spiked with 1 nmol of CA for calibration.

In vitro microsomal assays. Rat liver microsomes were prepared as described (19, 25). Incubations were carried out in a shaking water bath in open tubes containing 100 μl, total volume, of 100 mM potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, 1 mM NADPH, 100 μg rat liver microsomal protein, and 0.25 mM IFA or CPA. Reactions were incubated at 37°C for 20 min and stopped by the sequential addition of 40 μl of ice-cold 5.5% ZnSO$_4$, 40 μl of saturated barium hydroxide, and 20 μl of 0.01 M HCl. After spinning at 14,000 rpm for 20 min, the supernatant was split into two 80-μl aliquots for the determination of CA and acrolein, respectively. CA was derivatized with adenosine as described below. Acrolein was derivatized with 3-aminophenol as described elsewhere (19, 24). Briefly, 45 μl containing 6 mg/ml 3-aminophenol and 6 mg/ml hydroxylamine hydrochloride (both dissolved in 1 N HCl) was added to each 80-μl sample, followed by heating at 90°C for 30 min in the dark. The samples

were allowed to cool to room temperature and then a portion of each (typically 50 μl) was injected onto a C18 HPLC column as described below. Calibration curves for acrolein were generated using 4-hydroperoxy-IFA or 4-hydroperoxy-CPA incubated with bovine serum albumin (100 μg) under the same assay conditions.

Derivatization of CA with adenosine. Typical conditions for derivatization were as follow: to an 80-μl sample containing CA (see above), 10 μl of 2 M sodium acetate (pH 4.5) was added, followed by 10 μl of 100 mM adenosine (dissolved in 0.25 N HCl) to give a final concentration of 10 mM adenosine. Samples were then heated at 80°C for 2 h to derivatize the CA. Variations of these conditions were tested to optimize the derivatization, as cited in the figure legends. During and after heating, the samples were protected from light by covering with aluminum foil. After cooling to room temperature, the samples were briefly centrifuged and a portion (typically 25 μl) was directly injected onto a C18 HPLC column for analysis (see below). Samples could be stored at 4°C prior to HPLC analysis for at least 48 h without noticeable degradation.

HPLC analysis. The HPLC system used in this study was equipped with two Rainin pumps (DYNAMAX solvent delivery system, Model SD-200), a Waters autosampler (WISP 710B), and a Waters 474 scanning fluorescence detector. The system was controlled using Waters Millenium[32] software. Samples were chromatographed on a Luna C18 (2) column (5 μm, 150 × 4.60 mm, Cat. No. 00F-4252-E0, Phenomenex, Torrance, CA). This column is stable at the low pH required for the detection of acrolein in the form of 7-hydroxyquinoline. Using a C18 guard column, a single C18 column can be used for the analysis of ~5000 microsomal assay samples under these assay conditions without any significant loss in resolution. Moreover, the same HPLC system and column can be used for determination of both CA and acrolein. For determination of CA, samples were eluted with 20% methanol in water in isocratic mode with a flow rate of 1 ml/min. The fluorescent product 1-N^6-ethenoadenosine eluted at 5.7 min and was detected with a Waters 474 fluorescence detector set at an excitation wavelength of 270 nm and an emission wavelength of 411 nm (gain 10 and slit width 18). For determination of acrolein, the fluorescent product 7-hydroxyquinoline was eluted with 20% methanol in 0.5% phosphoric acid (pH $\sim$ 1.5) at a flow rate of 1 ml/min and monitored at an excitation wavelength of 350 nm and an emission wavelength of 515 nm (retention time of metabolite $\sim$3.2 min). Quantitation was based on integrated peak areas, which were calculated by Millennium software.

Data analysis. Data were managed using Microsoft Excel software. Linear regression analyses were performed using the graphic software package GraphPad Prism (GraphPad Software, San Diego, CA). Pharmacokinetic parameters (AUC, C_{max}, $t_{1/2}$) were calculated using WinNonlin software (Pharsight Corp., Mountain View, CA). The limit of detection was defined as the sample concentration that gives a peak height three times the level of baseline noise. The limit of quantitation was defined as the lowest level of compound that could be precisely and accurately measured. In this study, we defined limit of quantitation as the concentration at which triplicate analysis of samples would give a relative standard deviation within 10%.

RESULTS

Assay for CA based on derivatization with adenosine. The formation of 1-N^6-ethenoadenosine by reaction of adenosine with a large excess of CA (Fig. 1) was previously used to measure low concentrations of adenosine present in plasma (22, 26), heart extract (21), rat brain (27), fetal perfusates (28), and other extracellular fluids (29). 1-N^6-Ethenoadenosine is highly fluorescent and can be analyzed with high sensitivity by HPLC with fluorescence detection. A linear relationship between the amount of 1-N^6-ethenoadenosine and the integrated peak area was observed over a wide range of 1-N^6-ethenoadenosine concentrations (from 0.02 to 200 pmol) (Fig. 2). The limit of detection for 1-N^6-ethenoadenosine is 0.02 pmol and the limit of quantitation is 0.05 pmol, in accordance with a previous report (22). In view of this high sensitivity for 1-N^6-ethenoadenosine detection, we sought to determine whether this assay could be adapted to quantitate CA after derivatization with a large excess of adenosine.

The formation of 1-N^6-ethenoadenosine from CA + adenosine is a nonenzymatic reaction and can proceed at room temperature (24°C), but at a very low rate (22). It requires 6 days to reach saturation in the presence of 440 mM CA and 1 μM adenosine. An increase of temperature dramatically accelerates the reaction. Samples heated at 37, 55, 80, and 100°C required 48 h, 10 h, 1 h, and 15 min, respectively, to reach a plateau of product formation (22). However, 1-N^6-ethenoadenosine is unstable at 100°C and quickly degrades after 30 min incubation. Heating at 80°C for 1–2 h was suggested as a preferred incubation condition as a compromise to achieve derivatization within a relatively short time and while minimizing sample degradation (22). We therefore examined the time course for 1-N^6-ethenoadenosine formation at 80°C in the presence of 10 mM adenosine and 1 μM CA in a total reaction volume of 100 μl. The reaction reached a plateau after 90 min incubation and the product did not degrade after 150 min (Fig. 3A). Therefore, heating at 80°C for 2 h was used in all subsequent incubations.

Adenosine was used at a concentration of 10 mM in all experiments, which, although not quite saturating
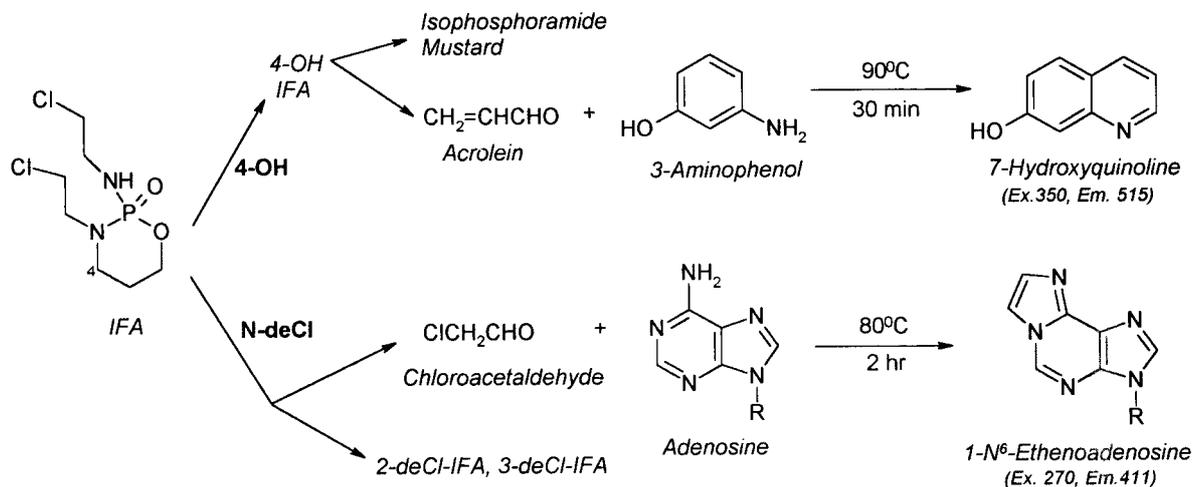
FIG. 1. Scheme of IFA metabolism showing the two alternative, P450-catalyzed pathways of IFA metabolism (4-hydroxylation, top pathway; N-dechloroethylation, bottom pathway). Also shown are the derivatization reactions for the IFA metabolites CA and acrolein. 2-deCl-IFA, 2-dechloroethyl-IFA; 3-deCl-IFA, 3-dechloroethyl-IFA; R, ribose ring.

with respect to CA derivatization (Fig. 3B), avoided the precipitation of adenosine that was observed at pH 4.5 when higher concentrations (>20 mM) were used. In pharmacokinetic studies in rats given a single intravenous injection of IFA at 100 mg/kg body wt, the plasma levels of CA typically range from 1 to 200 μM (18). Plasma CA concentrations are typically no more than 10 μM in patients receiving standard IFA treatment (17, 20). Accordingly, the concentration of adenosine (10 mM) used in the derivatization reaction is still at a 50- to 1000-fold molar excess over CA concentrations likely to be present in plasma samples. Under these conditions, the efficacy of CA derivatization is only 38–40%, lower than the value of 65–74% obtained
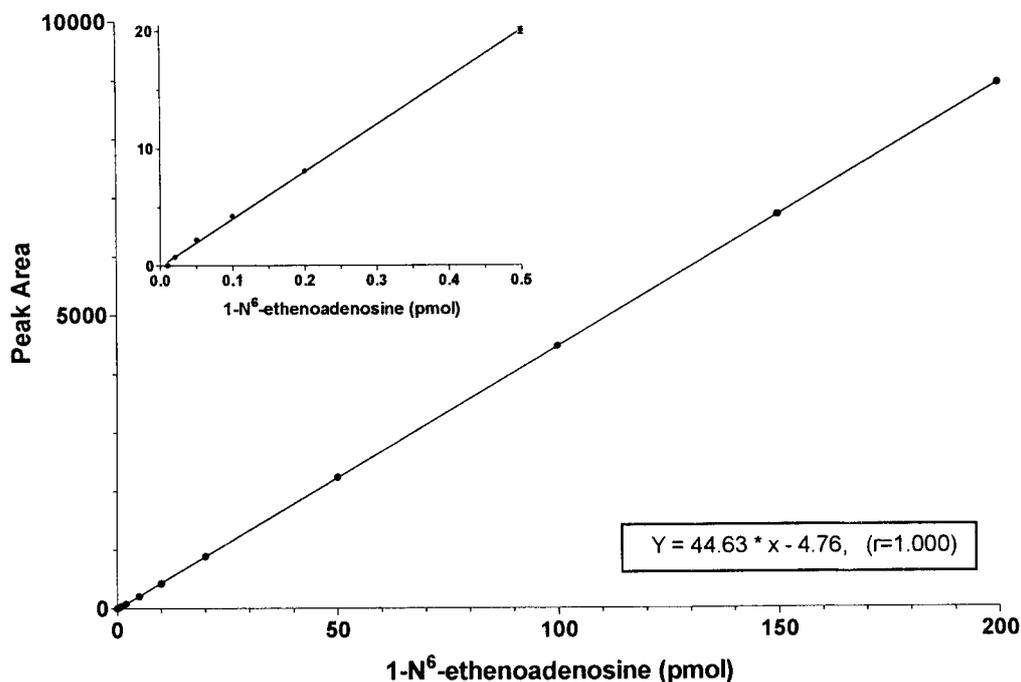
FIG. 2. Standard curve of 1-N^6-ethenoadenosine. 1-N^6-Ethenoadenosine was dissolved in distilled water and serial dilutions (from 0.01 to 200 pmol) were directly injected onto a C18 HPLC column and then eluted as described under Materials and Methods. Integrated peak areas of fluorescent activity were calculated using Millennium software. (Inset) Standard curve from 0 to 0.5 pmol 1-N^6-ethenoadenosine. Each point represents mean ± SD values based on triplicate HPLC determinations.
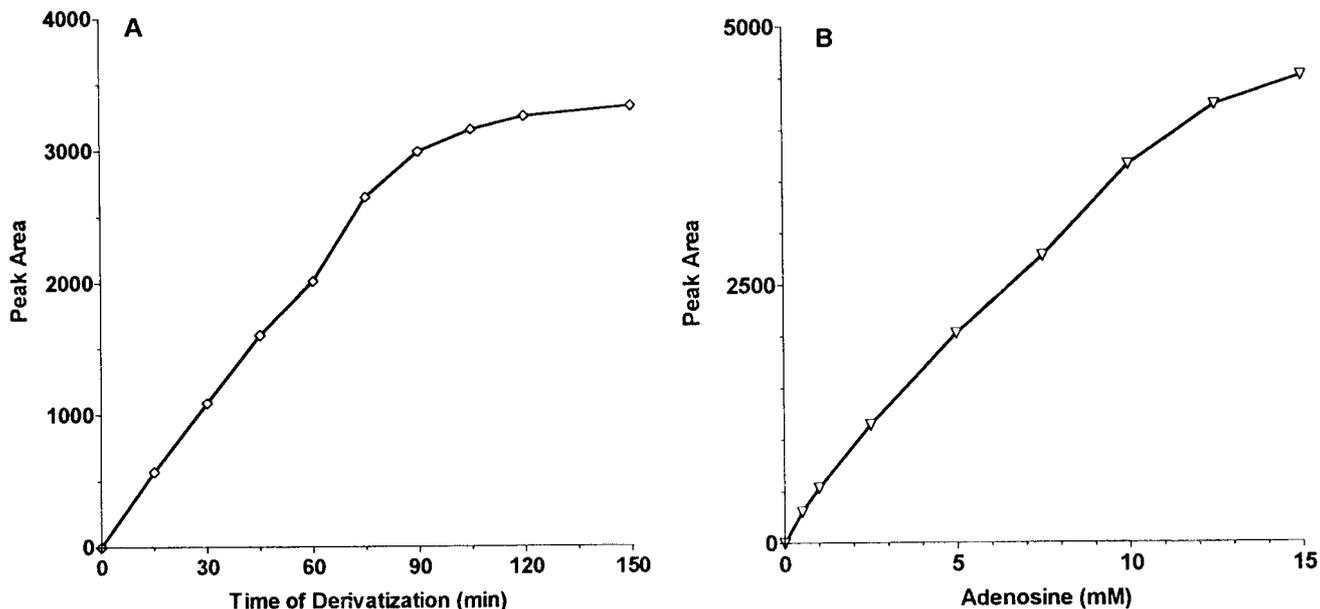
FIG. 3. Optimization of the conditions for derivatization of CA by adenosine. (A) Optimal time for derivatization. CA (100 pmol) was derivatized under the conditions described. Samples were removed from the heating block at different time points for analysis of 1-N^6-ethenoadenosine. (B) Optimal adenosine concentration. CA (100 pmol) was derivatized with different concentrations of adenosine under the conditions described. Each point represents mean ± SD values based on triplicate determinations.

when a large excess of CA (440 mM) is used to derivatize and thus assay for low levels of adenosine (22). A high sensitivity of CA detection and quantitation can still be achieved, however, despite the relatively low conversion efficacy, because of the highly fluorescent nature of 1-N^6-ethenoadenosine.

The conversion of CA to 1-N^6-ethenoadenosine under optimal conditions was linear from 5 to 400 pmol CA (Fig. 4), with a limit of detection and limit of quantitation for CA of 2 and 5 pmol, respectively. These detection and quantitation limits are almost 50–100 times higher than the corresponding values for 1-N^6-etheno-

$$Y = 15.27 * X + 95.40 \ (r^2 = 0.9992)$$
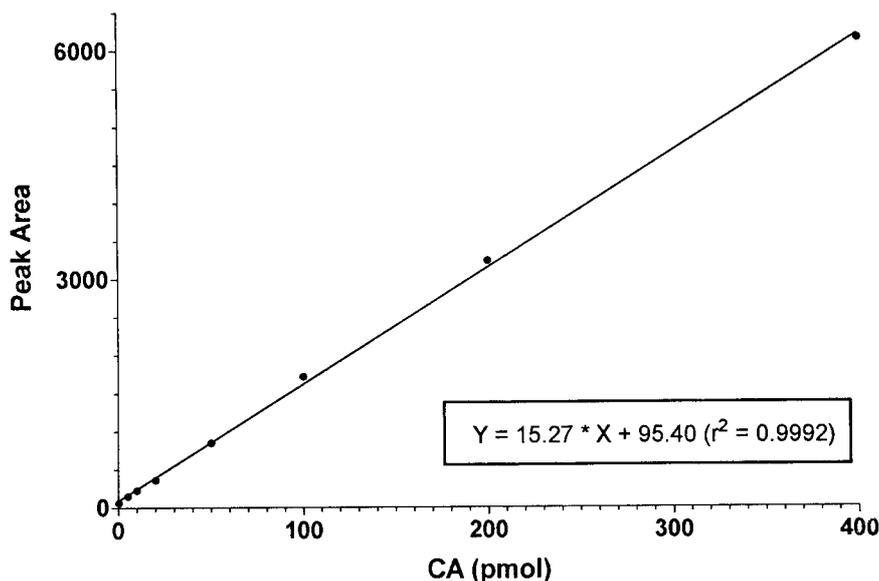
FIG. 4. Standard calibration curve for quantitation of CA. Different amounts of CA were reacted with 10 mM adenosine under the standardized conditions described under Materials and Methods. The fluorescent product 1-N^6-ethenoadenosine was quantitated by HPLC. Each point represents mean ± SD values based on triplicate determinations.
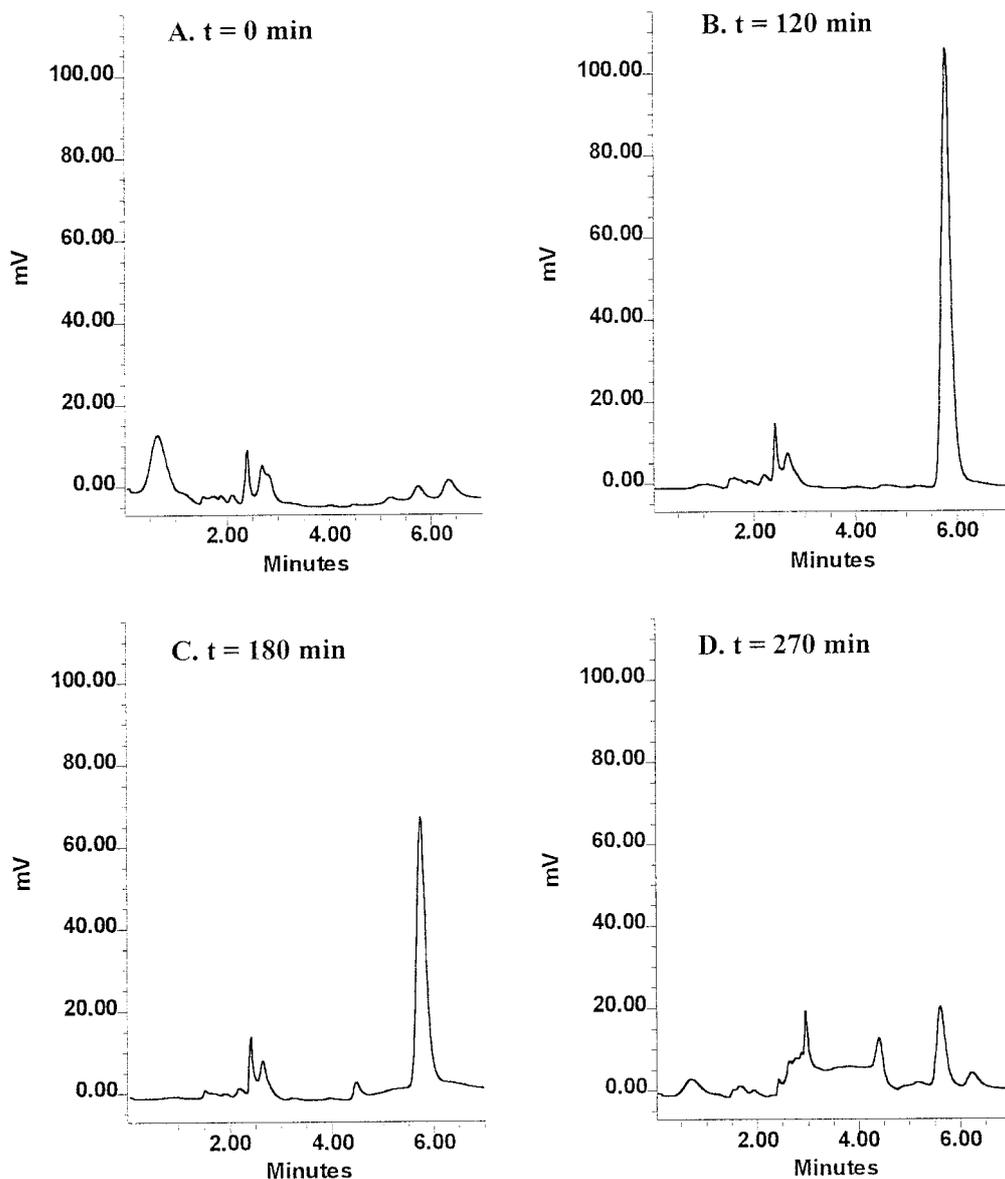
FIG. 5. Representative HPLC chromatograms from plasma samples taken from rats administered IFA. IFA injection and processing of plasma samples, followed by derivatization and HPLC analysis of CA, were carried out as described under Materials and Methods. An aliquot (25 μl) of each final 100-μl sample after derivatization was injected onto a C18 HPLC column to give the chromatographs shown. (A) CA analysis in plasma sample at time (t) = 0 min (time before IFA administration). (B) Sample at t = 120 min after IFA administration. (C) Sample at t = 180 min. (D) Sample at t = 270 min.

adenosine noted above (0.02 and 0.05 pmol, respectively). This discrepancy is primarily due to the presence in CA-free control plasma samples of a small peak, corresponding in size to ~1 pmol 1-N^6-ethenoadenosine. However, this small peak did not preclude the detection of low plasma CA levels, since even with a limit of detection for CA of 2 pmol, as little as 0.1 μM CA could be detected in a 50-μl plasma sample. This value (0.1 μM CA) compares favorably to plasma CA levels of up to 10 μM found in cancer patients administered IFA (17, 20).

Application to pharmacokinetic analysis of IFA metabolism. We next utilized this method to measure plasma levels of CA and acrolein in rats injected with IFA intravenously at a standard single dose of 100 mg/kg. Background plasma levels of CA were very low (Fig. 5A) compared to the fluorescence intensity obtained in plasma samples from IFA-treated rats, even after an extended period of decay of the initial plasma CA signal (Fig. 5B at 120 min, Fig. 5C at 180 min, and Fig. 5D at 270 min; cf. Fig. 6). By coupling this method with the measurement of acrolein formed via the 4-hy-
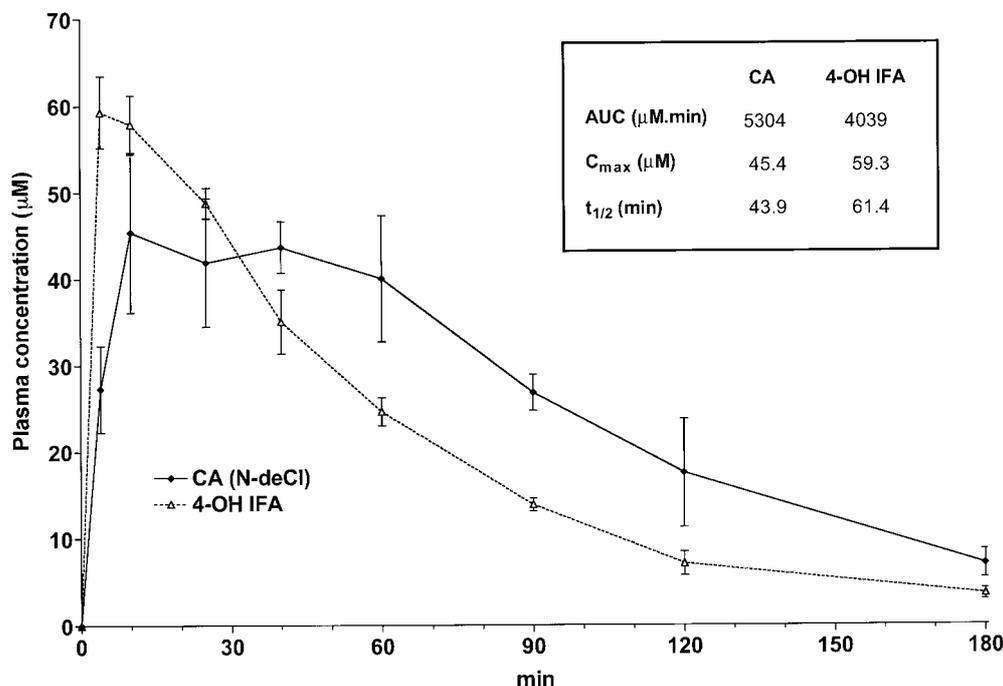
FIG. 6. Plasma CA concentrations after IFA administration. A single intravenous injection of IFA (100 mg/kg) was given to three individual adult male Fischer 344 rats. Blood was withdrawn at each of the times indicated. Plasma CA levels were determined as described under Materials and Methods. Quantitation was based on integrated peak areas. Each point represents mean ± SD plasma concentrations for three individual IFA-treated rats. The table presents pharmacokinetic parameters calculated from the data using WinNonlin software.

droxylation pathway, complete rat pharmacokinetic profiles for both major pathways of cytochrome P450-catalyzed IFA metabolism could be determined from a single set of plasma samples (Fig. 6). These are CA formation via the N-dechloroethylation pathway, measured as the 1-N^6-ethenoodenoisine adduct, and acrolein formation from the initial 4-hydroxy-IFA metabolite, determined as the 7-hydroxyquinoline adduct (Fig. 1). The results obtained are in general accordance with plasma CA concentrations in IFA-treated rats reported previously after derivatization of CA with thiourea to form 2-aminothiazole (18). AUC values for both CA and 4-OH IFA determined by this method were ~10% higher but were much more consistent between individual rats compared to the values reported previously (Fig. 6, legend). Moreover, the percentage of IFA metabolized by N-dechloroethylation, 56.8% based on the present method, compares favorably to the value of 63% reported previously (18). C_{max} values shown in Fig. 6 were ~20% higher than in that earlier study.

Trichloroacetic acid (20%) was used to precipitate plasma proteins when carrying out the pharmacokinetic analyses. Perchloric acid was found to be unsuitable due to the presence of UV-interfering substances (cf. excitation wavelength of 270 nm for 1-N^6-ethenoadenosine). Perchloric acid may be used provided that an additional extraction step is introduced to remove the acid (26). Zinc sulfate and barium hydroxide may

be used to precipitate microsomal proteins during *in vitro* assays (see below), but these reagents were inefficient with respect to the complete removal of plasma proteins, as indicated by the abundance of precipitate that formed during the derivatization step.

Application to liver microsomal activity assays. The present method could also be adapted for monitoring the two alternative pathways of P450-catalyzed IFA metabolism (Fig. 1) in *in vitro* metabolism studies using rat liver microsomes (Table 1), human liver microsomes, and cDNA-expressed cytochrome P450 enzymes (data not shown). Table 1 summarizes the results obtained with this method in a comparison of IFA metabolism catalyzed by uninduced rat liver microsomes (UT) with liver microsomes prepared from rats treated with one of three P450-inducing agents: β-naphthoflavone (BNF), phenobarbital (PB), and dexamethasone (DEX), each of which induces a distinct subset of liver P450 enzymes with a distinct capacity to metabolize IFA and CPA (19). BNF decreased the rate of microsomal metabolism by both pathways for both drug substrates. Overall, the percentage of IFA subject to N-dechloroethylation was decreased from ~36 to 22% following BNF treatment. PB-induced rat liver microsomes showed a dramatic four- to sixfold increase in the metabolism of IFA and CPA by both metabolic pathways (Table 1, values in parentheses). Thus, while

TABLE 1

IFA and CPA 4-Hydroxylase Activities and N-dechloro-ethylation Activities Catalyzed by Uninduced and Drug-Induced Rat Liver Microsomes

	4-OH (nmol/min/mg protein)	N-deCl (nmol/min/mg protein)	% N-deCl
		IFA	
UT	0.32 ± 0.01 (1.0)	0.18 ± 0.01 (1.0)	36.1
BNF	0.23 ± 0.01 (0.7)	0.07 ± 0.00 (0.4)	22.1
PB	1.72 ± 0.22 (5.4)	1.13 ± 0.13 (6.2)	39.6
DEX	1.11 ± 0.06 (3.5)	0.19 ± 0.01 (1.0)	14.6
		CPA	
UT	0.36 ± 0.03 (1.0)	0.09 ± 0.01 (1.0)	19.8
BNF	0.20 ± 0.02 (0.6)	0.05 ± 0.00 (0.5)	18.8
PB	1.72 ± 0.02 (4.7)	0.36 ± 0.10 (4.0)	17.4
DEX	0.42 ± 0.01 (1.2)	1.01 ± 0.05 (11.2)	70.7

Note. Activities were determined at 0.25 mM IFA or CPA as described under Materials and Methods. The values in parentheses represent fold induction compared to untreated rats (UT group). Data shown are mean ± SD values based on triplicate microsomal enzyme assays.

the overall rate of metabolism was increased, the percentage of drug undergoing N-dechloroethylation to yield CA did not change compared to uninduced liver microsomes (Table 1, last column, UT vs PB). Liver microsomes from DEX-induced rats displayed selective increases in two of the four reactions measured, IFA 4-hydroxylation and CPA N-dechloroethylation. Accordingly, the percentage of drug undergoing N-dechloroethylation decreased from 36.1 to 14.6% for IFA, while it increased from 19.8 to 70.7% for CPA in DEX-induced rat liver microsomes. The fold induction of each activity in response to P450 induction determined using the present method is strikingly similar to corresponding sets of values obtained using the more time-consuming and less sensitive previous assays (19). One difference is that the absolute N-dechloroethylation activities determined using the present methods are lower, giving a somewhat lower overall percentage of N-dechloroethylation. However, the percentage N-dechloroethylation values obtained in the present study are closer to the values calculated from rat pharmacokinetic data (23).

DISCUSSION

The HPLC–fluorometric method for CA quantitation described here has several advantages over previous HPLC/UV methods for CA determination (17). First, the new fluorometric method is more sensitive and can detect as low as 0.1 μM CA using a small volume (50 μl) of plasma. Previous HPLC/UV methods could not achieve this sensitivity and required several-fold higher amounts of plasma (18), a factor that limits the number of time points that can safely be taken from a single animal during a pharmacokinetic study. GC methods provide a similar sensitivity for the determination of 2- and 3-dechloroethyl-IFA formed during the N-dechloroethylation of IFA in the same reaction that yields CA (Fig. 1) (30). However, although 2- and 3-dechloroethyl-IFA are produced in amounts equimolar with CA, the dechloroethylated IFA metabolites can be further metabolized via 4-hydroxylation *in vivo* (4). Therefore, plasma 2- and 3-dechloroethylifosfamide levels may not accurately represent the overall extent of N-dechloroethylation and thus the total exposure to CA *in vivo.*

Second, this new method is very specific for CA. Potential interfering substances, such as other aldehydes, do not affect CA determination, as evidenced by the low background fluorescent intensity observed in time 0 plasma samples. Of note, CA can also be generated from several common industrial chemicals, including carcinogenic haloaliphatics, such as vinyl chloride and 1,2-dichloroethane (13, 14). Other mono-haloacetaldehydes, including bromoacetaldehyde, may react with adenosine and form 1-N^6-ethenoadenosine (14). Populations with high exposure to these chemicals may therefore have higher background levels of CA in their plasma, suggesting that the present assay may also be useful for monitoring chemical exposure leading to CA formation in these individuals.

Third, the method described in this report is simple. Samples can be directly derivatized and then injected onto an HPLC without the need for the labor-intensive and time-consuming solid-phase extraction step using Bond Elut SCX cation-exchange columns required with previous HPLC methods for CA determination. Elimination of these tedious extraction steps also eliminates a significant source of tube-to-tube variation and error. Elimination of the extraction step makes it possible to process many more samples in parallel with a substantial reduction in effort.

Coupled with the HPLC–fluorometric method for measuring acrolein, a metabolite of IFA formed during the activation of this drug via the 4-hydroxylation pathway; one can readily determine both CA and acrolein from the same set of samples, both in the context of pharmacokinetic studies and when carrying out *in vitro* microsomal enzyme studies. Using the present methods, both IFA metabolic pathways (Fig. 1) can also be determined in a single incubation step during *in vitro* microsomal assays. This provides for more accurate results and, in particular, enables one to calculate the percentage of IFA undergoing N-dechloroethylation with improved reliability and with a savings of time and materials.

In conclusion, we have developed a simple, sensitive, and specific method to measure plasma CA concentra-

tions as well as CA formed during liver microsomal N-dechloroethylation of the cancer chemotherapeutic prodrugs IFA and CPA. This method is also being used in this laboratory to determine acrolein and CA formation by cultured tumor cells transduced with specific human cytochrome P450 genes (31) as part of a gene therapy strategy for cancer treatment (32). This assay method may also be used to identify human P450 enzymes that catalyze N-dechloroethylation of CPA or IFA and to explore pharmacological methods to minimize the formation of CA by modulating the flux of drugs through the N-dechloroethylation pathway.

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