Determination of Acrolein in Urine by Liquid Chromatography and Fluorescence Detection of Its Quinoline Derivative

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We describe an assay for acrolein in urine, employing derivatization with *m*-aminophenol in the presence of ferrous sulfate solution in sulfuric acid. The derivative (7-OH quinoline; DER) and the internal standard (quinine-bisulfate; IS) were separated on a 10-μm particle, 8 mm × 10-cm C_{18} cartridge in conjunction with a radial compression system using a mixture of 0.05 *M* dibasic ammonium phosphate solution (pH 2.5):acetonitrile:methanol (92:6:2) at a flow rate of 3 mL/min as a mobile phase. The effluent was monitored fluorometrically at excitation and emission wavelengths of 360 and 495 nm, respectively. The retention times of DER and IS under these conditions were 4.3 and 26 min, respectively, and no interference in the assay from any endogenous substance or other concomitantly used drug was observed. The assay was highly linear ($r > 0.994$) in the range 1–20 μg/mL of acrolein in urine (CV at different concentrations, ≤7.9%). This method can serve to monitor acrolein pharmacokinetics in patients.

KEY WORDS: liquid chromatography; derivatization; cyclophosphamide metabolites; therapeutic monitoring; bone marrow transplant; cancer.

INTRODUCTION

Cyclophosphamide is effective in the treatment of lymphoma, leukemia, breast carcinoma, and a host of other neoplasms (1); and it is often used at high doses as part of the immunosuppressive conditioning regimens in bone marrow transplant (BMT) recipients. In spite of the routine use of mesna (sodium mercaptoethane-sulfonate) to prevent urinary toxicity of the reactive metabolites of cyclophosphamide such as acrolein, hemorrhagic cystitis, a well-documented toxicity of acrolein (2), occurs in some patients with alarming severity. A high concentration of acrolein may result from accentuated metabolism of cyclophosphamide and/or delayed renal excretion. Cyclophosphamide is converted by liver microsomal P-450 mixed-function oxidase to 4-hydroxy cyclophosphamide which is in equilibrium with its acyclic tautomeric form, aldophosphamide. While 4-hydroxyphosphamide is dehydrogenated to 4-ketocyclophosphamide, another inactive metabolite, aldophosphamide, is further oxidized by the liver aldehyde oxidase to the inactive metabolite, carboxyphosphamide. Finally, aldophosphamide undergoes nonenzymatic cleavage presumably in cells susceptible to cytolysis yielding phosphoramide mustard and acrolein, both of which are highly cytotoxic (3).

The kinetics of formation and elimination of acrolein in patients has not been addressed, presumably because of a lack of an analytical assay for this compound in urine or any other biological fluid. In this report, we describe an expedient and accurate assay for acrolein in urine. This method is currently being used to monitor acrolein in urine of bone marrow recipients following cyclophosphamide administration and to investigate its pharmacokinetics in these patients.

MATERIALS AND METHODS

Chemicals and Reagents

We used an analytical sample of acrolein stabilized with approximately 3% water and 200 ppm hydroquinone (Sigma Chemical Company, St. Louis, MO) for this assay. Quinine bisulfate (Internal Standard), hydroxylamine hydrochloride (both from Fisher Scientific Co., Fair Lawn, NJ), *m*-aminophenol (Aldrich Chemical Company, Inc., Milwaukee, WI), ferrous sulfate (Sigma Chemical Company, St. Louis, MO), and 7-OH quinoline (Pfaltz & Bauer, Inc., Waterbury, CT) were employed as received. Dibasic ammonium phosphate, phosphoric acid, sulfuric acid, acetonitrile, and methanol were all high-performance liquid chromatography (HPLC) grade from Fisher Scientific Company. Water for HPLC was prepared by passing deionized water through a Norganic Cartridge and a 0.45-μm (pore size) membrane filter (Millipore Company, Milford, MA) to remove trace amounts of organic compounds.

Fresh solutions of acrolein (100 mg/L) and quinine bisulfate (60 mg/L) in water were prepared immediately before use. The derivatizing reagent (500 mg of *m*-aminophenol and 300 mg of hydroxylamine hydrochloride in 5 ml of 2.5 *M* sulfuric acid) and ferrous sulfate (10%, w/v, in 2.5 *M* sulfuric acid) were prepared every 3–4 days and stored at 4°C in light-protected bottles.

Chromatography

We used a liquid chromatograph consisting of a System Gold Programmable Solvent Module 126, an Analog Interface Module 406 (both from Beckman Instruments, Inc., Altex Division, San Ramon, CA), a Waters 715 Ultra Wisp Sample Processor, a Radial Compression Separation Module (Z-Module) equipped with a 10-μm-particle packed, an 8-mm × 10-cm MB C_{18} Radial Pak Cartridge and a Guard-Pak Precolumn Module with a C_{18} insert, and a fluorescence detector Module 420 AC (Waters Associates) set at excitation and emission wavelengths of 360 and 495 nm, respectively. A Tondon PCA-40 computer for the System Gold controller and processor connected with a Citizen 180E printer was employed for collecting the data and recording the chromatograms. A photodiode array detector (Model 990) (Waters Associates) was used for generating the ultraviolet/

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visible spectra of the compounds as they were eluted from the chromatograph.

Mobile Phase

To prepare the mobile phase, 920 mL of 0.05 *M* dibasic ammonium phosphate solution adjusted to pH 2.5 with phosphoric acid, 60 mL of acetonitrile, and 20 mL of methanol were thoroughly mixed and passed through a 0.45-μm (pore size) membrane filter. After degassing, the mobile phase was pumped at a flow rate of 3 mL/min with a pressure of ≤1000 psi.

Derivatization of Acrolein

The sample was centrifuged at 2800 rpm for 10 min and a 1-mL aliquot was transferred to a clean tube containing 0.5 mL of the *m*-aminophenol solution and 0.5 mL of ferrous sulfate solution. After the internal standard (250 μL of 60 mg/L) was added, the mixture was vigorously stirred for 30 sec and heated in a boiling water bath for 15 min. The tube was then chilled in an ice bath, and the clear liquid was transferred to a microvial of the autosampler which was programmed to inject 25 μL into the cartridge. A blank urine sample containing no acrolein was prepared and chromatographed similarly.

Standard Curve and Linearity

The linearity of the assay was examined by preparing standard curves in the range of 1–20 μg/mL on 10 different days. To construct such a curve, appropriate amounts of acrolein were added to 1-mL aliquots of blank human urine to yield the following concentrations: 1, 2, 4, 6, 8, 10, and 20 μg/mL. The samples were derivatized and analyzed as described above. The standard curve was established by plotting the peak height ratio (acrolein DER/internal standard) against the concentration of acrolein added to blank urine.

Precision and Accuracy

The accuracy and intrarun (within-day) precision of the described assay were examined by supplementing pooled blank urine samples with three different amounts of acrolein to yield the concentrations 1, 8, and 20 μg/mL, which represent low, medium, and high concentrations (Table I). Each sample was divided into 10 replicates, which were analyzed individually according to the described procedure, and the coefficient of variation (CV) of the concentration, which reflects the intrarun precision at each level, was calculated. The deviation from perfect (theoretical) accuracy was calculated as 100 [(amount found − amount added)/amount added]. We determined the interrun (between-day) precision from the coefficient of variation (CV) of the peak height ratio (drug/internal standard) generated on 5 days for concentrations ranging between 1 and 10 μg/mL.

Table I. Accuracy and Intrarun Precision of Acrolein Assay in Urine

Amount added (μg/mL)	Amount found (μg/mL)	Coefficient of variation (%)	Deviation from perfect accuracy (%)[a]	Number of experiments
1.0	0.99	7.9	−1.0	10
8.0	7.93	5.3	−0.9	7
20.0	20.81	5.0	+4.1	10

[a] Estimated as 100 [(amount found − amount added)/amount added].

Specificity of the Assay

The specificity of the assay was examined by preparing concentrated solutions (≥100 μg/mL) of drugs that may be concomitantly used with cyclophosphamide in water, methanol, or acetonitrile and subjecting a small aliquot of each to chromatography under the same conditions described above. A list of these drugs and retention times is provided in Table II.

Applicability of the Assay

We investigated the applicability of this assay by measuring the concentration of acrolein in urine samples collected from a bone marrow recipient during the first 12-hr and the subsequent 24-hr periods after the administration of a high dose of cyclophosphamide. The analysis was performed as described above, and the concentration of acrolein was calculated by the use of a standard curve constructed on the same day.

RESULTS AND DISCUSSION

In an attempt to identify the compound which gives rise to the peak observed after derivatization of acrolein with *m*-aminophenol, we injected an aqueous solution of 7-OH quinoline which was conceived to be the product of the above reaction (4) into the chromatograph under the afore-

Table II. Retention Times of Drugs that May Be Concomitantly Administered with Cyclophosphamide

Drug	Retention time (min)
Aspirin	ND[a]
Teniposide	ND
Etoposide	ND
Lomustine	7.14
Dacarbazine	ND
Cytarabine	7.93
Cyclosporine A	ND
Carmustine	ND
Azathioprine	ND
Ifosfamide	ND
Carboplatin	ND
Fluorambucil	ND
Methotrexate	14.28
Thiotepa	ND
Sodium Salicylate	ND
Indomethacin	ND
Thioguanine	ND
Chlorambucil	ND
Betamethasone	ND
Diclofenac	ND
Mesna	ND

[a] Not detected.

mentioned conditions and subjected the eluate to photodiode array detection. As demonstrated in Fig. 1, the retention time and the ultraviolet spectrum observed were identical to those generated for the derivative. Further, when we supplemented the derivatization mixture with 7-OH quinoline, the derivative peak and spectrum were accentuated, with no other peak showing, indicating that the derivative is indeed 7-OH quinoline.

Figure 2 depicts representative chromatograms of a blank urine sample, a urine sample supplemented with acrolein and quinine bisulfate, and a urine sample collected from a patient who received 1740 mg of cyclophosphamide. As demonstrated in this figure, the peaks are sharp and symmetrical, with median retention times of acrolein derivative, 7-OH quinoline, and quinine bisulfate of 4.3 min (range, 3.3–4.5 min) and 26.3 min (range, 25.2–27.1 min), respectively.

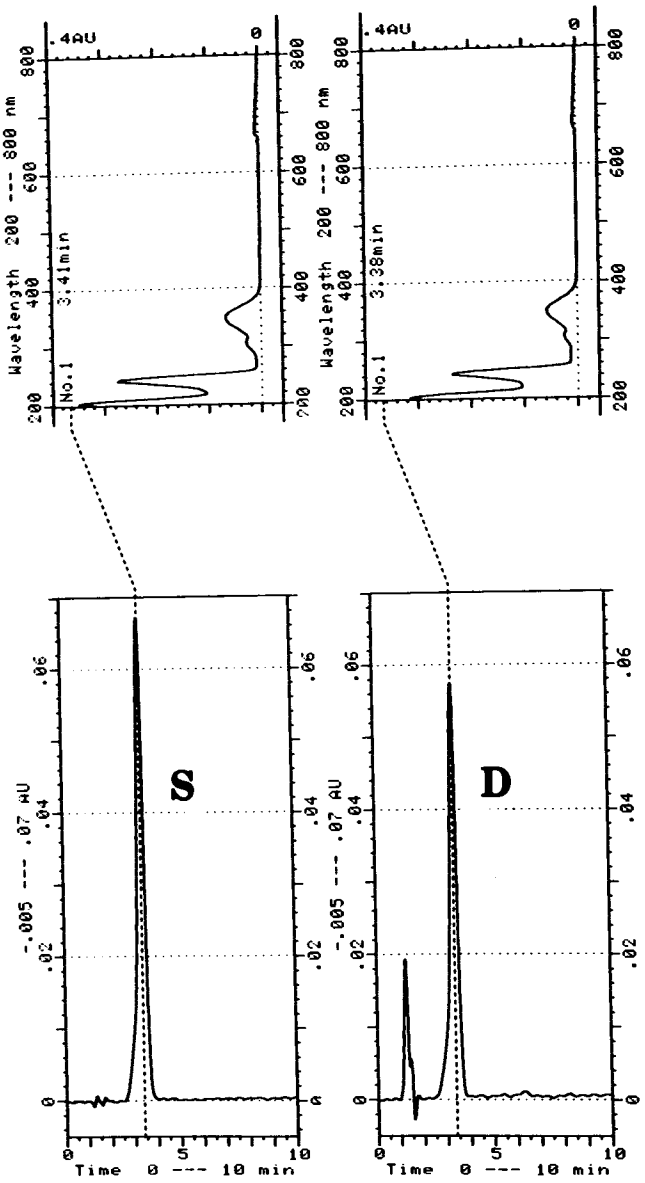

Fig. 1. Typical chromatograms and ultraviolet spectra observed by a photodiode array detector for an aqueous solution of 7-OH quinoline (S) and for the acrolein derivative with *m*-aminophenol (D).

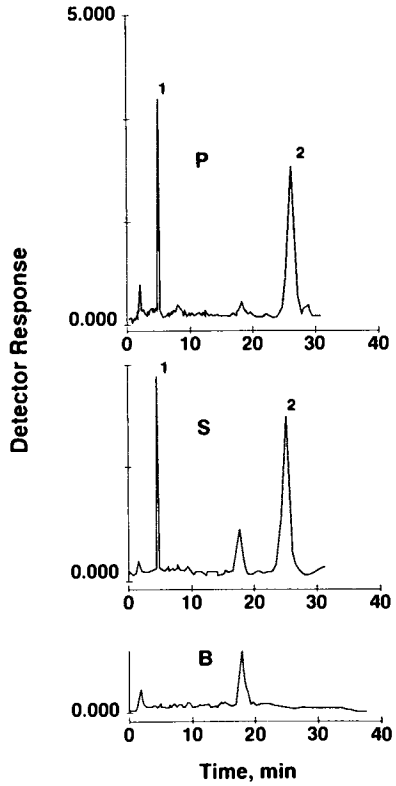

Fig. 2. Representative chromatograms of (B) a blank urine sample; (S) a blank urine sample supplemented with 10 μg/mL of acrolein and 15 μg/mL of quinine-bisulfate, IS; and (P) a patient's urine sample collected during the first 12 hr after intravenous administration of 1740 mg of cyclophosphamide and supplemented with 15 μg/mL of IS. The concentration of acrolein measured was 12.25 μg/mL. Peak 1 is that of acrolein derivative (7-OH quinoline), and Peak 2 is that of the internal standard (IS).

The assay was highly linear in the range examined (1–20 μg/mL) and the correlation coefficient (*r*) of the peak height ratio (acrolein DER/internal standard) versus acrolein concentration curve was consistently >0.994 (mean, 0.9964; range, 0.9941 to 0.999; $n = 10$). The specificity of the method was equally good where no interference from any drug commonly used with cyclophosphamide in a combined cancer chemotherapy was observed (Table II). Most of these drugs are nonfluoroscent under the conditions (excitation and emission wavelength, mobile phase) used and hence were nondetectable. Also, as exemplified in Fig. 2, we noted no interference from any endogenous substance originating from any urine sample collected from more than 10 different individuals for use as blank in this work.

The conditions employed for the derivatization of acrolein in urine were optimal, being the result of extensive experimentation where the impact of heating, acid medium, and reagent concentration were studiously investigated.

Effect of Concentration of Derivatizing Reagents upon Reaction

Initially, derivatization of acrolein in urine was performed by the use of *m*-aminophenol in the presence of hydroxylamine hydrochloride. However, the reaction yield

was prohibitively small. The addition of ferrous sulfate (100 mg/ml) as a catalyst produced a sharp increase in the yield. Similarly, increasing the concentration of *m*-aminophenol from 25 to 50 mg/mL amplified the relative yield (urine/water) by almost twofold. However, a further increase in the concentrations failed to exert any effect. Also, changing the concentration of hydroxylamine hydrochloride while maintaining constant concentrations of *m*-aminophenol and ferrous sulfate had no impact on the yield. We found that adding this substance to the derivatizing reagent solution merely prolongs its stability for a longer period of time.

Effect of Heating Time

The impact of heating time on the derivatization reaction in urine was examined over the range 10–90 min. While the relative yield (urine/water) observed after 10 min of boiling was 70%, it reached a maximum when the mixture was allowed to boil for 15 min. However, increasing the time of heating to 45 or 90 min caused a sharp drop in the yield, plausibly due to a possible reaction of the derivative with urine constituents.

Effect of Acid Medium

In an attempt to select the most appropriate medium for the reaction, various acids, i.e., sulfuric, acetic, phosphoric, and hydrochloric, were employed under the same heating and reagent concentration conditions. The highest yield was observed with sulfuric acid, presumably due to its catalytic effect. We also examined the effect of the concentration of sulfuric acid over the range 0.05 to 5 *M*. The highest yield was obtained at a concentration ranging between 0.05 and 0.5 *M*, however, the chromatograms of blank urine samples in this low molarity range included some interfering peaks which were not present at $M = 2.5$. It should be noted that the 7-OH quinoline derivative was stable in the autosampler microvial for at least 72 hr after derivatization at room temperature (i.e., no change in the peak height was observed after repeated injections over this period). Also, repeated analyses on different days of urine samples collected from a bone marrow recipient after cyclophosphamide administration and stored at −20°C yielded similar results, indicating that acrolein is stable at this temperature.

Accuracy and Precision

We determined the accuracy and precision of the assay at various concentrations of acrolein as described above. As shown in Table I, the deviation from perfect accuracy ranged between −1 and +4.1%, which signifies a good assay accuracy. The intrarun (within-day) precision was also good where the CV value of the concentration obtained at low, medium, and high concentration was ≤7.9% (Table I). Similarly, the CV values of the peak height ratios generated on 5 days for the concentrations 1, 6, and 10 μg/mL were 6.9, 6.6, and 4.4%, respectively, indicating an equally good interrun (within-day) precision.

Patient's Sample Analysis

The concentrations of acrolein in the urine samples collected from a bone marrow recipient during the first 12-hr and the subsequent 24-hr periods after intravenous administration of 1740 mg cyclophosphamide were 12.25 and 22.6 μg/mL, respectively, which correspond to 12.2 and 44 mg (total amounts) of acrolein excreted in the urine, respectively.

In conclusion, the analysis presented here for acrolein is suitable for monitoring its pharmacokinetics.

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