GC Analysis of Imidazopyrazole in Plasma Using Nitrogen-Specific Detection

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Abstract □ A sensitive, specific GC assay for imidazopyrazole in plasma was developed using nitrogen-specific detection. The samples are extracted with methylene chloride containing 7-bromo-imidazopyrazole as the internal standard and the extract derivatized with pentaflourobenzoyl chloride prior to isothermal chromatography on an OV-17 column. Peak-height ratio measurements produced linear standard curves over the concentration range of 0.045–40 μg/ml. The practical limit of sensitivity was 50 ng/ml and typical between-run variability for replicate analysis of a control specimen produced a coefficient of variation of 5.1%. This method is applicable to the study of the pharmacokinetics of imidazopyrazole following therapeutic doses and was used to support such studies in parallel with Phase I clinical studies in children.

Keyphrases □ Imidazopyrazole—GC analysis, plasma, nitrogen-specific detection □ Plasma—GC analysis of imidazopyrazole, nitrogen-specific detection □ Nitrogen-specific detection—GC analysis of imidazopyrazole, plasma □ GC analysis—imidazopyrazole in plasma

Imidazopyrazole[1] (2,3-dihydro-1-H-imidazo[1,2,-6]-pyrazole[2], I) is an investigational antineoplastic agent which selectively inhibits DNA synthesis (1). Preclinical studies have shown I to have significant antitumor activity especially against L-1210 leukemia cells, including those variants resistant to similar chemotherapeutic agents (2). The suggested mechanism of action is inhibition of ribonucleotide reductase, and in mice the drug showed the capacity to synchronize tumor, bone marrow, and duodenal crypt cells in the S-phase of the cell cycle (3). Phase I clinical trials of this novel agent have been initiated in children and adults with initial doses of 150 mg/m^3 body surface area (4), and an obvious need to collect early pharmacologic disposition data exists.

A limited number of methods for determining I in biological media have been preliminarily reported including liquid scintillation of radiolabeled drug (5), radioimmunoassay (6), and electron-capture GC (7). The former two methods require reagents not readily available and lack evidence of specificity, while the latter suffers from the lack of ruggedness generally associated with electron-capture detection when applied to analysis of biological specimens. The present method was developed to allow nitrogen-specific detection and still maintain adequate sensitivity to support Phase I clinical and pharmacokinetic studies in children being treated for cancer.

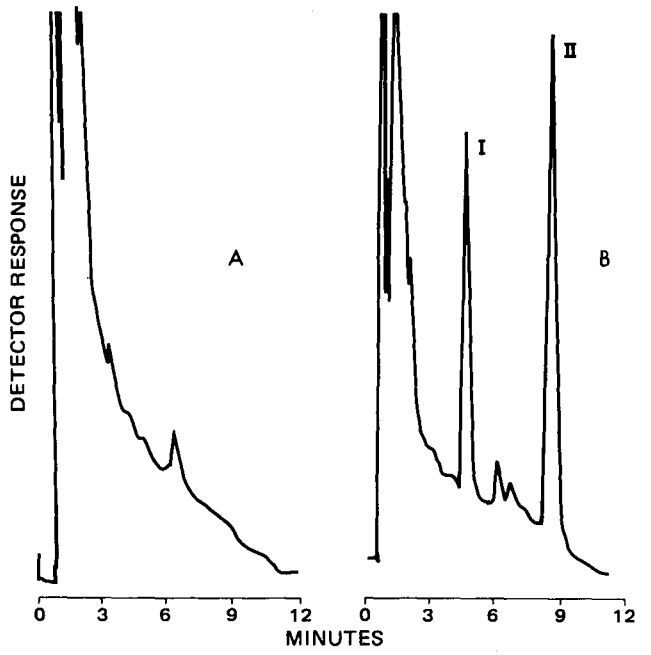
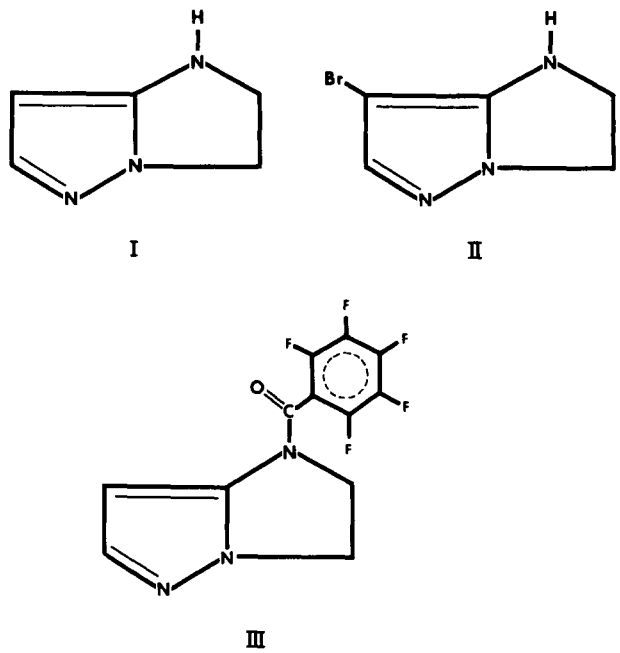


Figure 1—*Gas-liquid chromatographic tracing of (A) a blank serum sample and (B) a serum sample to which was added 2.37 μg/ml of I and internal standard at an attenuation of 16 × 10^{-11}.*

EXPERIMENTAL

Chemicals and Reagents—Compound I and 7-bromoimidazopyrazole[3] (7-bromo,2,3-dihydro-1-H-imidazo[1,2-6]pyrazole, II) were used directly. Pentafluorobenzoyl chloride[4] was used as received and stored under dry nitrogen at −20° in 1-ml ampules to protect from moisture. A 1.0 *M*, pH 10.5 carbonate buffer was prepared using reagent grade sodium carbonate[5] and sodium bicarbonate[5]. Methanol and methylene chloride were both HPLC grade[6]; all other chemicals and solvents were reagent grade. All glassware was acid washed then treated with 5% dichloromethylsilane in toluene[5] and rinsed successively with toluene and



[1] National Cancer Institute, Bethesda, Md.
[2] NSC 51143.
[3] Dr. Louis Malspeis, Ohio State University, Columbus, Ohio.
[4] Aldrich Chemical Co., Milwaukee, Wis.
[5] Matheson, Coleman and Bell, Norwood, Ohio.
[6] Fisher Scientific, Fairlawn, N.J.

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Table I—Recovery of I Added to Human Serum

I Added, μg/ml	n	I Found, μg/ml (mean ± SE)
0.048	10	0.054 ± 0.003
0.48	9	0.47 ± 0.011
1.91	10	2.03 ± 0.069
4.93	4	4.71 ± 0.32
9.85	4	10.27 ± 0.30
19.70	4	18.59 ± 0.56
39.40	4	40.37 ± 0.92

methanol and then oven dried. Deionized water was prepared using a filter reverse osmosis system[7].

Instrumentation—A gas chromatograph[8] equipped with a nitrogen phosphorous thermionic detector and 1-mV recorder was used. Chromatography was performed on a U-shaped glass column (1.8 m × 2 mm i.d.) packed with 3% OV-17 on 100/120 mesh[9], which was conditioned overnight at 250° and pretreated with several injections (10–25 μl)[10]. Nitrogen at a flow rate of 30 ml/min was used as the carrier gas, and air and hydrogen flows were optimized for maximum detector response. Operating temperatures were: oven, 195°; injector, 235°; and detector, 250°. Mass spectral data were obtained using a GC–MS–computer system[11]. Chromatography was performed using a coiled glass column (1.2 m × 2-mm i.d.) packed with 3% OV-101 on 100/120 mesh[9] operated isothermally at 170° with an injector temperature of 250°. The instrument was equipped with a jet separator and used 70 V as the ionization energy.

Standard Curves—Stock standards were prepared by dissolving 1 mg of I in 10 ml of water and 1 mg of II in 10 ml of methylene chloride. These were diluted to produce working standards of 10 μg/ml and stored at 4° where they were stable for at least 2 weeks. Appropriate aliquots were added to human serum[12] to produce either a low-concentration series of 0.05, 0.1, 0.5, 1.0, and 2.0 μg/ml of I (1 μg of II), or a high-concentration series of 1, 5, 10, 20, 30, and 40 μg/ml of I (28 μg of II). Calibration curves were constructed from peak-height ratio measurement of I/II.

Analytical Procedure—Seven milliliters of methylene chloride, 50 μl (5 μg) of internal standard (II) solution, 2 g of sodium chloride, and 1 ml of carbonate buffer (1.0 *M*, pH 10.5) were added to 1.0 ml of a patient

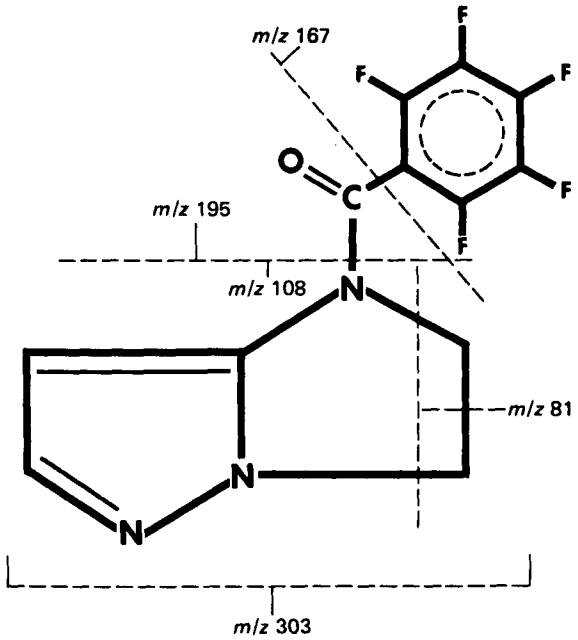

Figure 2—*Proposed mass fragmentation of III. See text for conditions.*

[7] Millipore Corp., Bedford, Mass.
[8] Varian Aerograph Model 2100, Varian Assoc., Palo Alto, Calif.
[9] Applied Science Laboratories, State College, Pa.
[10] Aquasil Pierce Chemical Co., Rockford, Ill.
[11] Hewlett-Packard Model 5992B/9825A, Hewlett-Packard, Santa Clara, Calif.
[12] Irvine Scientific, Santa Ana, Calif.

Table II—*In Vitro* Stability of I [a]

Hours	Concentration of I, μg/ml: pH 7.4 Phosphate Buffer, 0.1 *M*	Human Serum	5% Serum Albumin
0	1.04	0.95	1.01
1	1.03	0.77	0.88
2	1.10	0.79	0.92
4	1.06	0.69	0.79
8	1.03	0.67	0.81
24	1.01	0.62	0.73
48	—	0.62	—

[a] Solutions originally spiked with 1.0 μg/ml of I and stored under refrigeration.

plasma or spiked serum sample in a 15-ml centrifuge tube. The tube was then mechanically shaken for 30 min, centrifuged at 2000 rpm for 10 min, and the lower (organic) layer filtered into a clean tube containing 1 g of sodium sulfate. This was vortexed for 1 min and allowed to stand for 10 min. The organic phase was transferred to a clean tube to which was added 5 μl of pentafluorobenzoyl chloride. This was incubated for 30 min in a 50° water bath, and 0.5 ml of methanol was added. The tube was reincubated for 15 min and then evaporated to dryness under a stream of dry air at room temperature. The residue was redissolved in 2 ml of methylene chloride, shaken with 5 ml of carbonate buffer for 20 min, centrifuged for 10 min, and the lower (organic) layer transferred to a 5 ml conical centrifuge tube. This was evaporated to dryness under air and redissolved in 50 μl of methanol prior to injection of 1–2 μl into the gas chromatograph.

Clinical Study—Human plasma samples were obtained from pediatric oncology patients receiving I under an approved Phase I study protocol of the Childrens Cancer Study Group. Doses were administered by rapid intravenous infusion over 30–60 min, and blood samples of 1–3 ml were collected prior to dosing and at 0.25, 0.5, 1.0, 2.0, 6.0, 12.0, and 24.0 hr postdose. Blood samples were immediately centrifuged and the plasma separated and stored at −80° until analyzed.

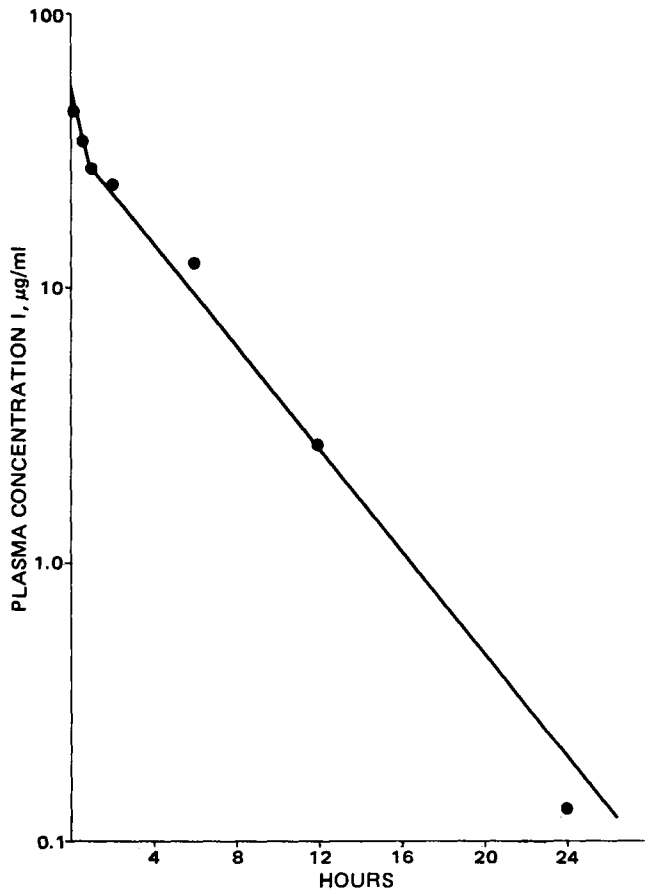

Figure 3—*Patient plasma–time profile of I following an intravenous bolus infusion of 450 mg.*

RESULTS AND DISCUSSION

Typical chromatograms from the analysis of a human serum blank and a spiked serum standard are shown in Fig. 1. Under the analytical conditions described, the retention times of derivatized I and II were 4.0 and 8.7 min, respectively. Standard curves prepared from a series of duplicate serum standards were linear over two concentration ranges typically encountered in pharmacokinetic monitoring. Linear regression for a series of low concentration standard (0.05–2.0 μg/ml) curves produced a slope of 1.642 ± 0.068, an intercept of −0.043 ± 0.063 with a mean correlation coefficient of 0.992. Corresponding results for the high-concentration range (1–40 μg/ml) produced a slope of 0.106 ± 0.002, an intercept of −0.0119 ± 0.031, and a mean (r) of 0.998. This reflects excellent between-run reproducibility, which is further supported by the recovery data over a wide concentration range shown in Table I. Replicate analysis of two spiked serum control specimens resulted in between-run coefficients of variation of 6.3% ($\bar{x}$ = 0.107 μg/ml) and 3.8% ($\bar{x}$ = 4.54 μg/ml). The practical lower limit of sensitivity for this procedure, for which a signal–baseline noise ratio of 3:1 can be seen, was 50 ng/ml. This is the same as that reported using a GC–electron-capture detection procedure (7). The entire procedure requires ~4 hr and allows analysis of ~20 specimens/analyst/working day.

The identity of the derivatized I GC peak was confirmed by comparing its mass spectrum to that of authentic, synthesized pentafluorobenzoyl imidazopyrazole (III). These were identical and demonstrated a parent peak at m/z 303 and a base peak at m/z 195, which corresponded to the pentafluorobenzoyl fragment. Other characteristic peaks occurred at m/z 167, 108, and 81 as seen in the proposed fragmentation pattern in Fig. 2.

The pharmacokinetic behavior of I was followed in four pediatric patients receiving intravenous bolus doses in a Phase I clinical trial. The plasma time course for one patient who received 450 mg is shown in Fig. 3. The mean terminal phase half-life and volume of distribution for all patients was 4.4 hr and 1.5 liters/kg, respectively. These values were in reasonable agreement with those reported for adult patients (6) ($t_{1/2}$ = 3–10 hr, Vd = 5–15 liters), although other widely divergent values using less specific radioactivity detection methods have also been reported (8). No other data in pediatric patients have been reported to date.

In the course of studying the reproducibility of the assay, it was observed that significant decreases in measured concentrations of I occurred if the spiked serum sample was allowed to sit unfrozen over a few hours. This had not been reported in the previous literature and appears to have serious implications for I measurements in biological media. A limited stability study was performed of I in pH 7.4 buffer, human serum, and 5% purified serum albumin. The results are summarized in Table II and indicate a relatively rapid decline in I in the presence of protein, which appears to stabilize at a level of 65–70% of the initial level over time.

A preliminary experiment examining the possibility of rapid irreversible protein binding suggested that this did not explain the relatively rapid decline observed. Although this problem can be circumvented by either immediate analysis of samples or quick freezing in methanol–dry ice, the mechanism of this apparent instability merits further study. Lack of recognition of this problem could lead to altered serum concentrations and errors in pharmacokinetic analysis *in vivo*.

The method reported appears sufficiently sensitive, reproducible, and rapid to support extended pharmacokinetic studies of I in humans or animals provided caution is used in rapidly handling samples to avoid an apparent instability in biological specimens.

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Stability of Concentrated Aqueous Solutions of Pralidoxime Chloride

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Abstract □ Concentrated aqueous solutions of pralidoxime chloride degrade more rapidly than dilute solutions. The rate and degree of degradation is dependent on the initial and final pH as well as the container in which the solution is stored. The effects of glass, metal, plastic, and rubber stoppers on the stability of concentrated and dilute solutions are discussed. The stability and shelf lives of 50% aqueous concentrates at different temperatures were determined.

Keyphrases □ Pralidoxime chloride—stability of concentrated aqueous solutions, kinetics □ Stability—concentrated aqueous solutions of pralidoxime chloride, kinetics □ Kinetics—comparison of stability in concentrated aqueous solutions of pralidoxime chloride

Pyridinium oximes in combination with atropine provide effective therapy in poisoning by many organophosphorus cholinesterase inhibitors (1). More clinical data are available for pralidoxime salts than other pyridinium oximes, as this oxime is in wider use. Pralidoxime also reportedly produces few undesirable side effects (2, 3). It is generally accepted that, for therapeutic efficacy, pralidoxime plasma concentrations should be at least 4 μg/ml (4). A 600-mg im dose is required to produce this level. A dilute solution would require an injection of an impractical volume. Since pralidoxime chloride is very water soluble, 30–50% concentrates can be used to provide a 4-μg/ml plasma level (5, 6). The purpose of this study was to determine stabilities and the effect of containers on the shelf life of aqueous concentrates of pralidoxime chloride[1].

[1] The opinions or assertions contained herein are the private views of the author and not to be construed as reflecting the views of the Department of the Army or the Department of Defense.

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