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## Note

# Determination of pyrazoloimidazole in plasma and urine by gas–liquid chromatography using nitrogen–phosphorus detection

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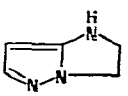
IMPY (2,3-dihydro-1*H*-pyrazolo[2,3-*a*]imidazole or pyrazoloimidazole, Fig. 1) is an anticancer agent currently undergoing clinical trials based on its antitumor activity in several tumor test systems[1]. Biological studies have shown it to be an effective reversible inhibitor of DNA synthesis[1,2]. Subsequent studies showed that IMPY inhibits ribonucleotide reductase[1,3]. Interest in IMPY was stimulated by its unique characteristics compared to other reductase inhibitors such as guanazole and hydroxyurea. IMPY is most effective against mouse L1210 leukemia when administered once every four or eight days, whereas guanazole, hydroxyurea or other ribonucleotide reductase inhibitors demonstrate optimal activity when administered daily[1]. The schedule-independent nature of IMPY has been partially explained by the fact that *in vivo* inhibition of DNA synthesis is observed for up to 16 h following intraperitoneal administration, as compared to only 5 h for guanazole or hydroxyurea[1]. Although these observations suggest that the disposition and metabolism of IMPY may play an important role in its biological activity, there is no information n the literature concerning its pharmacologic behavior.



IMPY

Fig. 1. Structure of IMPY.

To carry out studies of the disposition and metabolism of IMPY, we have developed a sensitive and specific gas chromatographic assay for the parent drug in biological samples employing a specific nitrogen–phosphorus detector (NPD). Quantitative analysis is accomplished with the use of 2,2-dipyridyl as internal standard. We have applied this assay to the determination of IMPY in plasma and urine of rabbits following intravenous administration of the drug at several dose levels.

## EXPERIMENTAL

### *Materials and methods*

All solvents were glass distilled and obtained from Burdick & Jackson (Muskegon, Mich., U.S.A.). IMPY was provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Chromatographic analyses were performed on a microprocessor-controlled Hewlett-Packard 5840A gas chromatograph equipped with an NPD. Silanized glass columns (6 ft. × 2 mm I.D.) were packed with 3% SP-2100 DB on Supelcoport (100–120 mesh) and conditioned overnight (245°, gas flow-rate 300 ml/min) prior to analysis. Injector, oven and detector temperatures were 250, 175 and 300°, respectively. Hydrogen gas flow-rate was 3 ml/min, air 90 ml/min and nitrogen 30 ml/min (Air Products, Allentown, Pa., U.S.A.).

### *Plasma and urine analysis*

Heparinized plasma or urine (1 ml) was added to a 16 × 125 mm screw-capped centrifuge tube*, and 10 or 20 μg 2,2-dipyridyl (1 or 2 μg/μl in methanol) added as internal standard. The pH was adjusted to 10 with 0.05 *M* sodium hydroxide (plasma) or 0.10 *M* sodium hydroxide (urine), chloroform (6 ml) added, and the samples shaken for 20 min on a mechanical shaker. After centrifugation (1000 *g*, 15 min), the chloroform layer was transferred to another tube containing a small portion of anhydrous sodium sulfate and briefly vortexed. The dried chloroform extract (5 ml) was transferred to a 12-ml conical centrifuge tube, butanol (100 μl) added, and the chloroform removed under a stream of nitrogen. Aliquots (1 μl) of the remaining butanol were directly injected for gas chromatographic analysis. The concentration of IMPY was determined by peak area ratios of drug to internal standard compared to values obtained from plasma and urine standard curves, obtained by analysis of control plasma or urine (1 ml) to which IMPY (0.25–20 μg) and 2,2-dipyridyl (10 or 20 μg) had been added.

## RESULTS AND DISCUSSION

Gas chromatograms obtained after analysis of rabbit plasma samples are shown in Fig. 2. The NPD provides not only excellent sensitivity, but also eliminates the appearance of many plasma contaminants due to its selectivity. Tailing, a frequent occurrence with underivatized amines[4], was a problem with several silicon column packings, but was avoided with a phase prepared commercially for the analysis of basic molecules (3% SP-2100 DB, Supelco, Bellefonte, Pa., U.S.A.).

Extraction of IMPY was optimal with chloroform, but sample concentration by evaporation of solvent to dryness resulted in significant losses due to the volatility of the drug. These losses were completely prevented by the addition of butanol, removal of the chloroform and analysis of the remaining butanol mixture. Owing to the volatility, chloroform extraction recoveries at low levels could not be precisely determined, but were at least 75%. Minor variations in the day-to-day response of the NPD, and application of the butanol sample concentration technique made the

* All glassware was silanized prior to use.

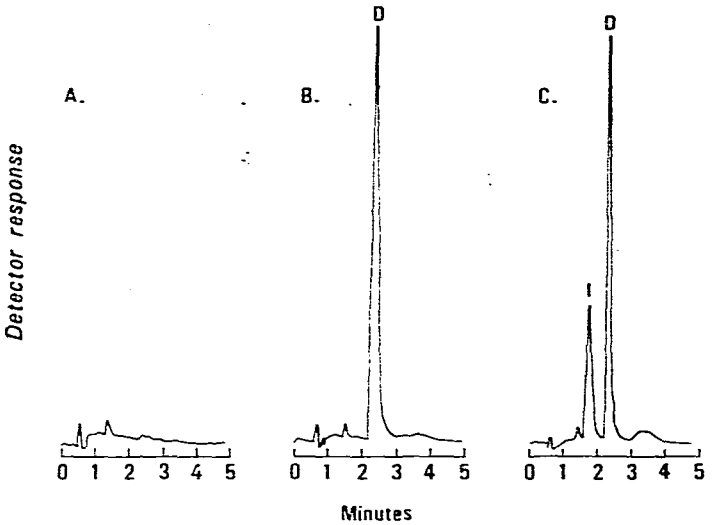
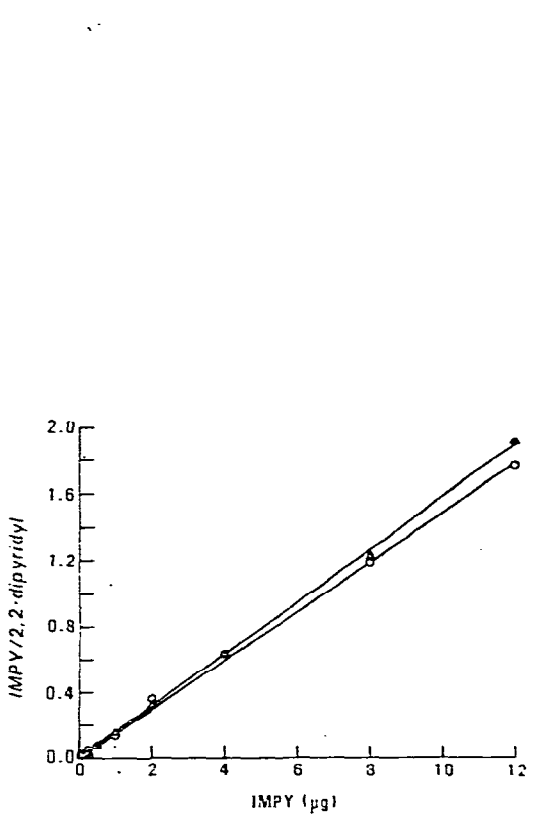



Fig. 2. Chromatograms of (A) blank rabbit plasma sample, (B) rabbit plasma sample to which 10 $\mu$g 2,2-dipyridyl was added as internal standard and (C) rabbit sample containing 2 $\mu$g/ml IMPY and 10 $\mu$g 2,2-dipyridyl. Samples were analyzed as described under Materials and methods.



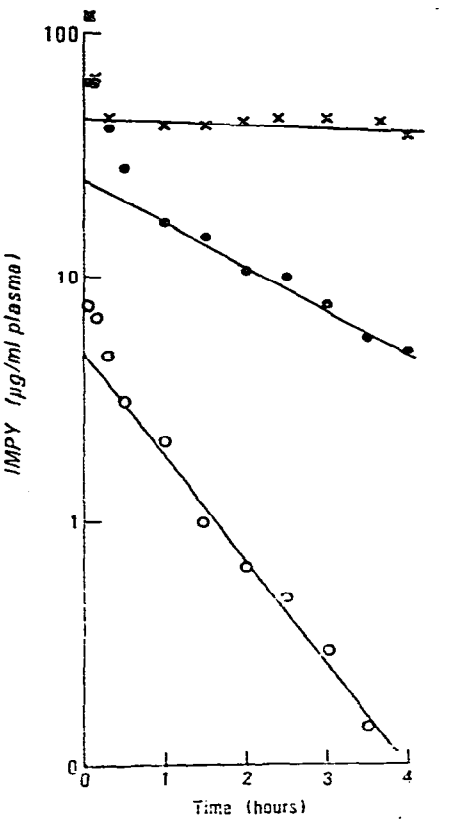




Fig. 3. Standard curves for IMPY in plasma (△) and urine (○) with 10 $\mu$g 2,2-dipyridyl as internal standard.

Fig. 4. Plasma levels of IMPY in rabbits following rapid intravenous administration of 5 (○), 25 (◑) or 150 (X) mg/kg.

use of an internal standard important for the assay. We selected 2,2-dipyridyl, a nitrogen heterocycle which is responsive to the NPD. The 2,2-dipyridyl/IMPY ratios were unchanged by extraction and sample concentration, and chromatographic separation from IMPY was adequate.

Standard curves for plasma and urine are shown in Fig. 3. By adding greater amounts of internal standard, linear standard curves were obtained over wider concentration ranges. It was noted that the retention time of IMPY varied slightly with concentration, (less than 10 sec over a 1000-fold concentration range), with larger amounts injected having longer retention times. This was not observed with similar nitrogen heterocycles (including 2,2-dipyridyl) and was not prevented by changes in column conditioning, flow-rates or column loading with IMPY. This was not a problem, however, as the IMPY peak never overlapped the internal standard or plasma contaminants.

The assay was applied to the analysis of IMPY in plasma and urine following intravenous administration of approximately 5, 25 or 150 mg/kg to three rabbits. The plasma time–log concentration curves at all three dose levels are shown in Fig. 4. It is interesting to note that the second phase of the removal of the drug becomes progressively slower as the dose of the drug is increased, the half-life being 45, 99 and 640 min at a dose of 5, 25 and 150 mg/kg, respectively. The distributive phase is similar at all three dose levels, being approximately 10 min. Urinary recoveries at all dose levels were very low, less than 2% 4 after administration of the drug. Recoveries at longer time periods (24 to 96 h) were less than 3%. Current single dose levels in clinical trials vary, but are in the range of 10 to 20 mg/kg, and will be increased as trials progress. The assay could therefore be readily applied to the determination of IMPY in patients undergoing clinical trials.

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