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Note

# High-performance liquid chromatographic analysis of imidazopyrazole (NSC 51143) in serum

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Imidazopyrazole [2,3-dihydro-1H-imidazo(1,2-6)pyrazole, NSC 51143, IMPY] is an investigational antineoplastic agent which selectively inhibits DNA synthesis [1]. Pre-clinical studies have shown IMPY to have significant anti-tumor activity especially against LI210 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/$m^2$ body surface area [4], and an obvious need to collect early pharmacologic disposition data exists.

A limited number of methods for determining IMPY in biological media have been preliminarily reported, including liquid scintillation of radiolabelled drug [5], radioimmunoassay [6] and electron-capture gas chromatography (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. We have recently reported a GC method employing nitrogen-specific detection [8] which has been used in support of phase I clinical and pharmacokinetic studies of IMPY in children. While this has

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proved sensitive enough to support phase I clinical and pharmacokinetic studies of IMPY in children, the overall reliability and ruggedness of high-performance liquid chromatography (HPLC) has prompted development of the present method which has comparable sensitivity and improved reproducibility.

## EXPERIMENTAL

### *Chemicals and reagents*

IMPY was obtained from the National Cancer Institute (Bethesda, MD, U.S.A.) and used directly. The internal standard, 2,3-dihydro-1-benzoylimidazo(1,2-6)pyrazole (ϕ-IMPY), was synthesized using a modification of a known reaction [9] in which a two-fold molar excess of benzoyl chloride (Gold Label; Aldrich, Milwaukee, WI, U.S.A.) was added slowly to 10 ml of a solution of 500 mg of IMPY in 2.5 *N* sodium hydroxide solution in an ice bath. After standing for 10 min, this was heated at 50°C for 10 min then cooled, and the resulting precipitate was separated and recrystallized three times from methanol. The structure was confirmed by ultraviolet and infrared spectroscopy, nuclear magnetic resonance and GC—mass spectrometry. The m.p. was 165—166°C. The structures of IMPY, ϕ-IMPY and the pentafluorobenzoyl (PFB) derivative of IMPY are shown in Fig. 1. Acetonitrile was HPLC grade (Omnisolve; MCB, Cincinnati, OH, U.S.A.), all other chemicals and solvents were reagent grade. Distilled water was purified by passing it through a reverse-osmosis four-filter system (Millipore, Bedford, MA, U.S.A.). Stock standard solutions of IMPY were prepared in purified water at concentrations of 10—100 μg/ml. Internal standard solutions were prepared in methanol at a 100 μg/ml concentration. These were refrigerated at 4°C and found to be stable for several weeks.

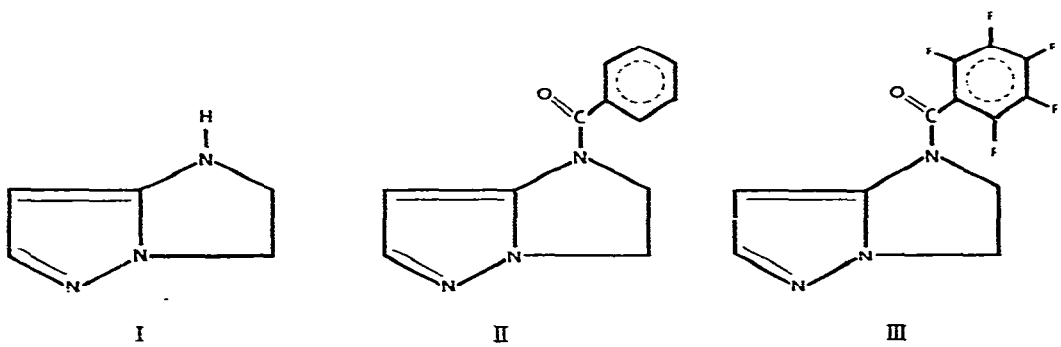


Fig. 1. Chemical structures of imidazole-pyrazole (IMPY, I), 2,3-dihydro-1-benzoylimidazo-(1,2-6)pyrazole (ϕ-IMPY, II), and the derivatized drug (PFB-IMPY, III).

### *Chromatographic conditions and instrumentation*

A Waters Assoc. (Milford, MA, U.S.A.) Model 202 liquid chromatograph equipped with a Model U6K injector and a Model 440 UV detector was used

for the analyses. Chromatography was performed on a 25 cm × 4.6 mm I.D. stainless-steel RP-8, 5 μm, Ultrasphere (Altex, Berkeley, CA, U.S.A.) column with an MPLC 3-cm, 10 μm precolumn (Brownlee, Santa Clara, CA, U.S.A.). The mobile phase consisted of acetonitrile—water (45:55) at a flow-rate of 1.5 ml/min and a pressure of 71.4 bar. The separation was run at ambient temperature at a wavelength of 254 nm.

*Extraction procedure*

To 1.0 ml of a serum sample or standard in a 15-ml centrifuge tube were added 7 ml of dichloromethane, 50 μl of internal standard solution (5 μg of ϕ-IMPY), 2 g of sodium chloride and 1 ml of 1.0 *M* carbonate buffer, pH 10.5. The tube was mechanically shaken for 30 min, centrifuged at 850 *g* for 10 min and the organic layer was filtered through Whatman No. 1 paper into a clean tube containing 1.7 g of sodium sulphate. This was vortexed for 1 min, allowed to stand for 10 min and the organic phase transferred to a tube containing 5 μl of pentafluorobenzoyl chloride. This was heated at 50°C for 30 min and then 0.5 ml of methanol was added. The tube was again heated at 50°C for 15 min to react the excess acylating reagent and then evaporated to dryness under dry air at room temperature. The residue was redissolved in 2 ml of dichloromethane and shaken with 5 ml of 1.0 *M* carbonate buffer, pH 10.5. The organic layer was transferred to a 5-ml conical centrifuge tube, evaporated to dryness under dry air and reconstituted in 50 μl of methanol; 5—20 μl were injected into the liquid chromatograph.

*Quantitation*

Standard curves were generated over the range 0.1—20 μg/ml in serum and IMPY concentrations were determined by calculating peak-height ratios of drug to internal standard.

*Mass spectrometric analysis*

The structure of the derivatized IMPY was assessed using a gas chromatograph—mass spectrometer—computer system (Hewlett-Packard Model 5992B/9825A, Santa Clara, CA, U.S.A.). GC was performed using a coiled glass column (1.2 m × 2 mm I.D.) packed with 3% OV-101 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.) operated isothermally at 170°C with an injector temperature of 250°C. The instrument was equipped with a jet separator and used a 70-V electron-impact ionization source.

*Rabbit pharmacokinetic studies*

The procedure was used to analyze the in vivo disposition of IMPY administered intra-arterially at doses of 250—1000 mg/m$^2$ to New Zealand white rabbits. Serial blood samples were drawn from an indwelling ear artery cannula up to 12 h after drug administration. The plasma was immediately separated from the cells and quick-frozen in a methanol—dry-ice bath (—68°C) until analyzed for IMPY.

## RESULTS AND DISCUSSION

Typical chromatograms from a human serum blank and spiked human serum are shown in Fig. 2. Under the analytical conditions described, retention times for $\phi$-IMPY and derivatized IMPY were 3.5 and 6.9 min, respectively. No significant interferences from extracted blank rabbit and human serum were observed. Peak shape was generally symmetrical and allowed calculation of IMPY concentrations from peak-height ratio measurement. Standard curves prepared from spiked human and/or rabbit serum were linear over the range 0.1—20 $\mu$g/ml ($r$ = 0.997). Between-run reproducibility and recovery were examined over this working concentration range with typical coefficients of variation in the 3—7% range (see Table I). The practical limit of sensitivity from a 1.0-ml serum sample which produced a 3:1 signal-to-baseline-noise ratio was 80 ng/ml. This is within the same magnitude as that reported in a gas chromatographic electron-capture procedure [7] and in our own GC—nitrogen-

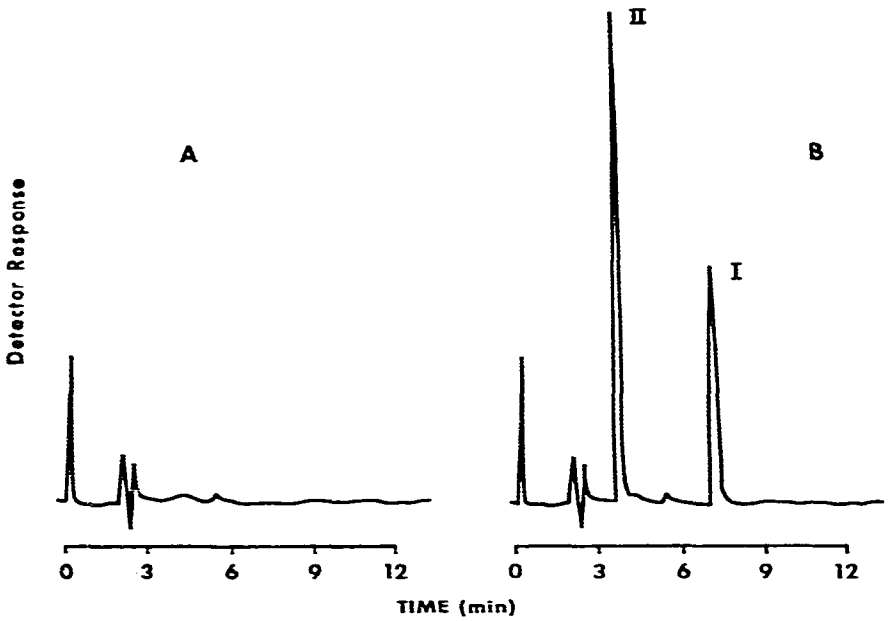


Fig. 2. High-performance liquid chromatograms of (A) extracted serum blank and (B) human serum standard spiked with 2.52 $\mu$g/ml IMPY and internal standard at an attenuation of 0.02 a.u.f.s.

TABLE I

RECOVERY AND BETWEEN-RUN VARIABILITY OF IMPY-SPIKED SERUM SAMPLES

| IMPY added ($\mu$g/ml) | IMPY found ($\mu$g/ml) | $n$ | S.D. | C.V. |
|---|---|---|---|---|
| 0.10 | 0.113 | 4 | 0.0044 | 3.9 |
| 2.48 | 2.42 | 4 | 0.065 | 2.7 |
| 7.23 | 7.13 | 4 | 0.374 | 5.3 |
| 12.25 | 11.96 | 4 | 0.1026 | 0.9 |

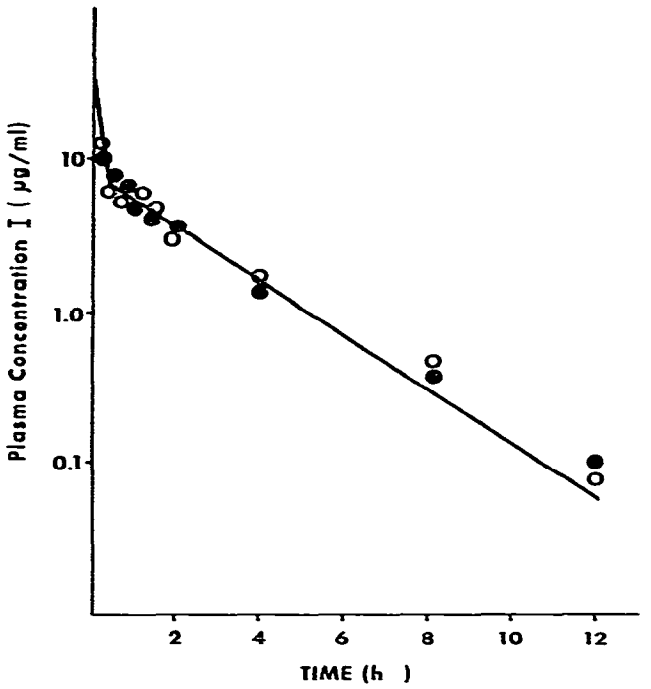


Fig. 3. Serum time course of IMPY in a rabbit receiving a 250 mg/m$^2$ intra-arterial bolus determined by GC analysis (○) and by HPLC (●) analysis.

detection procedure [8]. The entire procedure reported here required about 4 h and allows analysis of 20—25 specimens per analyst per working day.

The present procedure and the nitrogen-detection GC procedure [8] were compared by drawing and analyzing duplicate serum samples from a rabbit receiving 250 mg/m$^2$ IMPY as an intra-arterial bolus. The comparative results are seen in Fig. 3. The overall reliability of the HPLC procedure was better in our laboratory, probably due to the relative instability of the derivatized bromo-IMPY, which is used as the internal standard in the GC procedure. Between-run reproducibility was also substantially better in the present procedure.

The identity of the derivatized IMPY chromatography peak was confirmed by collecting the appropriate mobile phase fraction and, following solvent removal, subjecting the material reconstituted in methanol to GC—mass spectrometric analysis for comparison with authentic PFB-IMPY, which had been prepared in our laboratory. These were identical and demonstrated a parent peak at *m/e* 303 and a base peak at *m/e* 195, which corresponds to the pentafluorobenzoyl fragment. Other characteristic peaks occurred at *m/e* 167, 108 and 81, as seen in the proposed fragmentation pattern in Fig. 4.

The pharmacokinetic behavior of IMPY was determined in rabbits receiving intra-arterial doses ranging from 250 to 1000 mg/m$^2$. The serum time course was followed up to 12 h and was fitted to a two-compartment open model using the non-linear regression program AUTOAN/NONLIN [10]. The parameters derived for three animals each, at the 250 mg/m$^2$ and 1000 mg/m$^2$ dose,

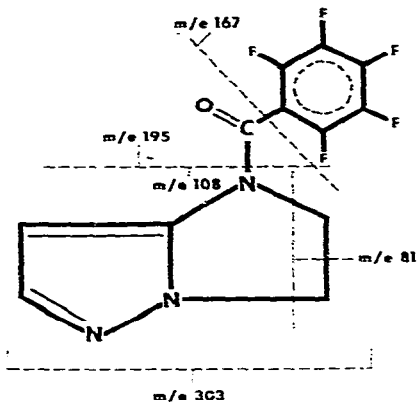


Fig. 4. Proposed mass fragmentation of 2,3-dihydro-1-pentafluorobenzoyl-imidazo(1,2-6)-pyrazole (III). See text for conditions.

TABLE II

PHARMACOKINETIC PARAMETERS FOR IMPY IN RABBITS FOLLOWING INTRA-ARTERIAL BOLUS DOSES

Three animals were used at each dose level.

| Parameter* | 1000 mg/m² dose (mean ± S.D.) | 250 mg/m² dose (mean ± S.D.) |
|---|---|---|
| $\alpha$ (min$^{-1}$) | 0.105 ± 0.074 | 0.21 ± 0.14 |
| $\beta$ (min$^{-1}$) | 0.007 ± 0.002 | 0.008 ± 0.003 |
| $t_{1/2\beta}$ (min) | 109.8 ± 24.9 | 87.9 ± 23.0 |
| $k_{10}$ (min$^{-1}$) | 0.040 ± 0.05 | 0.134 ± 0.18 |
| $k_{12}$ (min$^{-1}$) | 0.043 ± 0.03 | 0.048 ± 0.052 |
| $k_{21}$ (min$^{-1}$) | 0.028 ± 0.011 | 0.026 ± 0.013 |
| $V_d$ (l/kg) | 0.92 ± 0.98 | 0.233 ± 0.33 |
| $V_{d_{ext}}$ (l/kg) | 3.31 ± 1.92 | 2.27 ± 0.37 |
| Dose (mg) | 237 ± 26 | 50.2 ± 2.0 |

*Symbolism for pharmacokinetic parameters is that of AUTOAN [10].

are illustrated in Table II. There are at present no data from rabbit studies in the literature to allow comparison. There has been a report by Malspeis et al. [7] that IMPY, administered as an intravenous dose of 250 mg/m² to a single dog, demonstrated saturability in its kinetics and that typical log—linear disappearance was not observed until 2 h following the dose. The wide dose range selected here was designed, in part, to examine whether this could be demonstrated in rabbits. The data indicate widely varying parameter estimates between animals but do not suggest any non-linear kinetic behavior. More studies are needed in animals and humans to examine this issue.

In our previous paper describing the GC—nitrogen-detection analysis of IMPY, it was observed that the measured IMPY from a spiked serum sample declined significantly if allowed to stand unfrozen for a few hours [8]. This phenomenon was observed in the present procedure as well and all specimens should be rapidly frozen after collection to avoid the introduction of error caused by some unknown in vitro degradation of IMPY in serum.

## REFERENCES


1 H.L. Ennis, L.H. Moller, J.J. Wang and O.S. Selawry, Biochem. Pharmacol., 20 (1971) 2639.
2 R.W. Brockman, S.C. Shaddix, J.W. Carpenter, N.F. BuBois and R.F. Struck, Proc. Amer. Assoc. Cancer Res., Abstract 81 (1978) 19.
3 A. Krishran and R. Ganapathi, Cancer Res., 40 (1980) 1103.
4 B. Yap, W. Murphy, M.A. Burgess, M. Valdivieso and G.P. Bodey, Cancer Treatm. Rep., 63 (1979) 1849.
5 L.M. Allen, Proc. Amer. Assoc. Cancer Res., Abstract 433 (1979) 107.
6 K.L.L. Fong, D.H.W. Ho, G.P. Bodey, B.S. Yap, R.S. Benjamin, N.S. Brown and E.J. Freireich, Proc. Amer. Assoc. Cancer Res., Abstract 826 (1979) 204.
7 L. Malspeis, J.J.V. DeSousa, A.E. Staubus and H.B. Bhat, Proc. Amer. Assoc. Cancer Res., Abstract 617 (1979) 153.
8 G.W. Boswell and J.L. Cohen, J. Pharm. Sci., (1982) in press.
9 S.B. Matin and M. Rowland, J. Pharm. Sci., 61 (1972) 1235.
10 A.J. Sedman and J.G. Wagner, AUTOAN — A Decision Making Pharmacokinetic Computer Program, Publication Distribution Service, Ann Arbor, MI, 1974.