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## Note

# Gas chromatographic determination of pyrazoloimidazole

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Pyrazoloimidazole (Fig. 1) is a patented [1] pyrazolo[2,3-*a*]imidazolidine that is active against leukaemia. This paper describes a gas chromatographic procedure for the determination of pyrazoloimidazole as its trifluoroacetyl derivative in spiked human plasma and urine samples.

In an evaluation of direct gas chromatographic methods, the analysis was complicated by excessive peak tailing, so the chromatography of derivatives was investigated. The best peak shape was obtained with the trifluoroacetyl derivative. 7-Chloropyrazoloimidazole, prepared in the laboratory, was used as an internal standard. Both compounds were derivatized with trifluoroacetic anhydride after extraction from plasma or urine samples. The derivatives were then determined by gas chromatography.

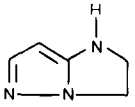


Fig. 1. Chemical structure of pyrazoloimidazole.

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## EXPERIMENTAL

### *Instrument and operating conditions*

A Hewlett-Packard Model 5840A gas chromatograph equipped with a nitrogen- phosphorus flame-ionization detector was used. The chromatograms, the peak areas and the retention times were recorded with a Hewlett-Packard Model 5840 electronic integrator. The stationary phase was 3% OV-17 on Gas-Chrom Q (100—120 mesh) packed into a 1.8 m × 2 mm I.D. coiled glass column. The following operating conditions were found to be optimal: column temperature, 170°C; injection temperature, 200°C; detector temperature, 275°C; carrier gas (helium) flow-rate, 30 ml/min; air flow-rate, 50 ml/min; and hydrogen flow-rate, 30 ml/min.

### *Chemicals and reagents*

Pyrazoloimidazole was supplied by the National Cancer Institute (Bethesda, MD, U.S.A.). 7-Chloropyrazoloimidazole was synthesized in the laboratory. Trifluoroacetic anhydride was obtained from Aldrich (Milwaukee, WI, U.S.A). All solvents were of analytical-reagent grade: *n*-hexane, methylene chloride, and methyl isobutyl ketone (Omni MCB, Deerfield, IL, U.S.A.), ethyl acetate and benzene (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). A buffer solution of pH 10.5 was prepared by mixing 80 ml of 0.1 *M* sodium carbonate with 20 ml of 0.1 *M* sodium bicarbonate solution.

### *Glassware*

All glassware was cleaned and siliconized prior to use. All reactions and extractions were carried out in 15 × 125 mm test-tubes (Pyrex 9826) with PTFE-lined screw-caps.

### *Synthesis and identification of the internal standard, 7-chloropyrazoloimidazole*

To about 500 mg of pyrazoloimidazole, add 5 ml of chlorinated glacial acetic acid (prepared by passing chlorine gas for 10 min into 50 ml of glacial acetic acid). After 15 min, add 5% sodium hydroxide solution dropwise until alkaline (pH 8) and then filter through double filter-paper. Extract the filtrate with benzene (3 × 25 ml). Dry the benzene extract over anhydrous sodium sulphate, filter and concentrate to about 1 ml. Chromatograph the final extract on TLC 1000 GF-type plates (Analabs, North Haven, CT, U.S.A.) using ethyl acetate—benzene (2:1) as the solvent system. Scrape off the TLC zone of the product and extract with ethyl acetate (3 × 30 ml). Evaporate the ethyl acetate extract to dryness and crystallize from benzene—*n*-hexane, then dry the product under reduced pressure (1 mmHg) at 56°C for 4 h.

Using this procedure, 200 mg of white crystals of 7-chloropyrazoloimidazole were obtained.

The NMR spectrum of the compound in $C^2HCl_3$ shows a multiplet at 4.04 and a singlet at 7.27. The mass spectrum shows the parent peak (molecular ion peak) at mass 143.

*Calibration graph and derivatization procedure*

Two calibration graphs were constructed from a series of methylene chloride solutions of pyrazoloimidazole and the internal standard. The first was prepared using 2—13 μl of a methylene chloride solution of pyrazoloimidazole (8.1 ng/ml) and 5 μl of the internal standard in methylene chloride (35 ng/ml). The second was prepared using 2—14 μl of a methylene chloride solution (100 ng/ml) and 10 μl of the internal standard in methylene chloride (200 ng/ml).

Evaporate these series of concentrations to dryness under nitrogen, then derivatize by adding 50 μl of trifluoroacetic anhydride and 0.5 ml of methylene chloride, cap the tubes tightly, sonicate for 15 min and evaporate to dryness under nitrogen. Dissolve the residue in 100 μl of methyl isobutyl ketone, sonicate for 5 min, centrifuge for 5 min to remove traces of turbidity and inject 3 μl of the clear solution into the gas chromatograph. Calculate the ratio of the peak area of pyrazoloimidazole to that of the internal standard and plot against weight to obtain the calibration graph.

*Procedure for assay of human plasma and urine*

*Samples spiked with pyrazoloimidazole.* To 1 ml of plasma or urine samples spiked with pyrazoloimidazole in a clean, dry, siliconized screw-capped test-tube with a PTFE-lined cap, add an appropriate amount of the internal standard. Acidify by addition of 0.5 ml of 0.5 *M* hydrochloric acid and extract with 6 ml of *n*-hexane by vortexing for 2 min. Centrifuge for 10 min, aspirate the *n*-hexane layer off and render the aqueous layer alkaline by addition of 5 ml of carbonate buffer solution (pH 10.5). Add 1 g of sodium chloride and extract with 6 ml of methylene chloride by shaking for 15 min in a mechanical shaker. Centrifuge as before, aspirate off the aqueous layer and discard. Dry the methylene chloride extract by addition of 0.5 g of anhydrous sodium sulphate. Vortex the mixture for 1 min, set aside for 10 min and transfer 4 ml of the dried methylene chloride extract into another clean, siliconized test-tube. Evaporate to dryness under nitrogen. Derivatize the residue as before and inject into the gas chromatograph.

## RESULTS AND DISCUSSION

Trifluoroacetic anhydride reacts with the imino group of pyrazoloimidazole or 7-chloropyrazoloimidazole to give the compounds shown in Fig. 2. This reaction is quantitative under the conditions of the method. Derivative preparation is simple and rapid. Complete elution of the volatile derivatives requires less than 5 min (Figs. 3 and 4).

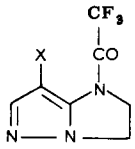


Fig. 2. Reaction products after reaction of pyrazoloimidazole or 7-chloropyrazoloimidazole with trifluoroacetic anhydride. X = H for pyrazoloimidazole; X = Cl for 7-chloropyrazoloimidazole.

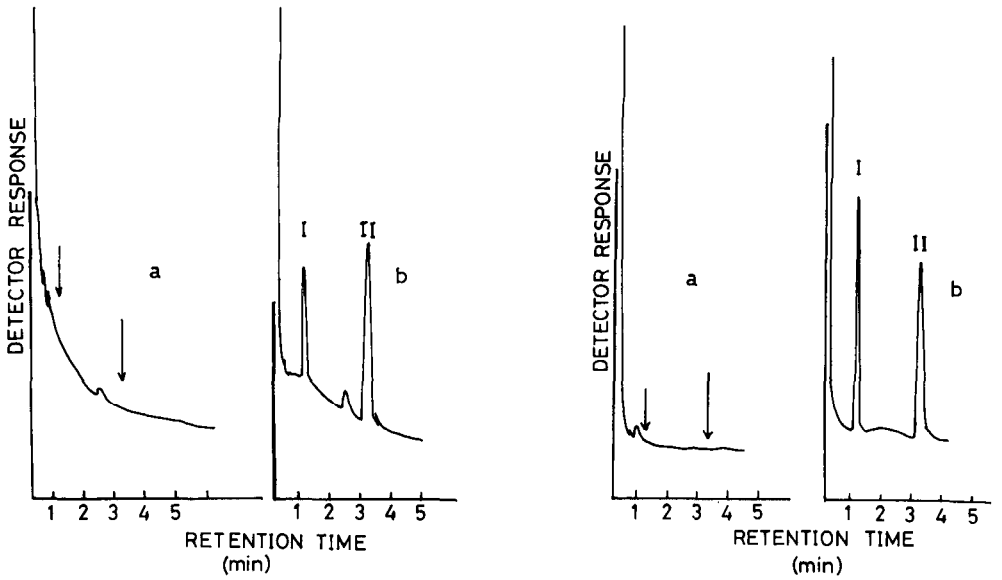


Fig. 3. Chromatograms of human plasma after extraction and derivatization with trifluoroacetic anhydride. (a) Blank plasma (1 ml); the arrows show the absence of signals at the retention times of pyrazoloimidazole and the internal standard. (b) Plasma containing pyrazoloimidazole (I) and the internal standard (II).

Fig. 4. Chromatograms of human urine after extraction and derivatization with trifluoroacetic anhydride. (a) Blank urine (1 ml); the arrows show the absence of signals at the retention times of pyrazoloimidazole and the internal standard. (b) Urine containing pyrazoloimidazole (I) and the internal standard (II).

The calibration graphs were constructed from five points for pyrazoloimidazole concentrations using two ranges, the low range (16.2—105.3 ng) and the high range (200—1400 ng). Regression analysis indicated a linear relationship between peak area ratio and concentration using the two ranges. A summary of the results of the regression analysis is presented in Table I. In addition, the value of the intercepts was shown to be not significantly different from zero according to the *t*-test (Table I).

The recoveries of pyrazoloimidazole from spiked plasma and urine samples were determined at different concentrations of pyrazoloimidazole by comparing the amount recovered in each extracted spiked plasma or urine sample with the linear regression obtained from the claibration graph. The mean recoveries were 97.22—103.85% (Tables II and III).

The precision of the method for the determination of pyrazoloimidazole in spiked plasma and urine samples is indicated by the coefficient of variation (C.V.) of the mean amount recovered from extracted plasma and urine samples repeated on five occasions. The C.V. values were found to be less than 2% (except at the 16-ng level) (Tables II and III). This concentration, 16.2 ng/ml, may be taken as the limit of determination of the method, although lower concentrations could still be detected.

Analytical studies indicated that extracts from blank human plasma and urine do not show peaks that interfere with the determination of pyrazolo-

TABLE I

REGRESSION ANALYSIS OF CALIBRATION GRAPHS

| Parameter | Range (ng) | |
|---|---|---|
| | 200—1400 | 16.2—105.3 |
| Slope ± S.D. | $5.1 \cdot 10^{-4} \pm 8.8 \cdot 10^{-6}$ | $8.6 \cdot 10^{-3} \pm 1.2 \cdot 10^{-4}$ |
| Intercept ± S.D. | $6.8 \cdot 10^{-3} \pm 8 \cdot 10^{-3}$ | $1.94 \cdot 10^{-2} \pm 7.4 \cdot 10^{-3}$ |
| $r$ | 0.9996 | 0.9997 |
| $t$ (0.95) | 0.85 | 2.64 |
| | (3.18)* | (3.18)* |

*Significant level.

TABLE II

PRECISION AND RELATIVE RECOVERY IN THE DETERMINATION OF PYRAZOLOIMIDAZOLE IN SPIKED HUMAN PLASMA

| Pyrazoloimidazole added (ng/ml) | Mean amount recovered (ng/ml) ($n$ = 5) | Precision (C.V.) (%) | Relative recovery (%) |
|---|---|---|---|
| 1000 | 1000.18 | 0.83 | 100.02 |
| 500 | 491.11 | 1.71 | 98.22 |
| 200 | 199.77 | 1.30 | 99.89 |
| 77 | 74.86 | 1.52 | 97.22 |
| 35 | 36.36 | 1.50 | 103.85 |
| 16.2 | 16.05 | 5.90 | 99.10 |

TABLE III

PRECISION AND RELATIVE RECOVERY IN THE DETERMINATION OF PYRAZOLOIMIDAZOLE IN SPIKED HUMAN URINE

| Pyrazoloimidazole added (ng/ml) | Mean amount recovered (ng/ml) ($n$ = 5) | Precision (C.V.) (%) | Relative recovery (%) |
|---|---|---|---|
| 1200 | 1211.64 | 0.32 | 101.64 |
| 1000 | 995.23* | 1.08 | 99.52 |
| 500 | 485.16 | 1.12 | 97.03 |
| 112 | 108.82 | 1.21 | 97.16 |
| 35 | 35.15 | 1.23 | 100.42 |
| 16.2 | 16.10 | 3.11 | 99.40 |

*Mean of four determinations.

imidazole. This is exemplified by typical chromatograms resulting from blank plasma and plasma with added pyrazoloimidazole and the internal standard, and typical chromatograms resulting from blank urine and urine with added pyrazoloimidazole and the internal standard, carried through the extraction procedure (Figs. 3 and 4).

The proposed technique permits the determination of pyrazoloimidazole in human plasma and urine at concentrations down to 16.2 ng/ml. The method is rapid, reproducible and sensitive.

ACKNOWLEDGEMENT

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REFERENCE

1 National Cancer Institute, U.S. Pat., 2 989 537 (1961).