Analysis of 4-Methylpyrazole in Plasma and Urine by Gas Chromatography with Nitrogen-Selective Detection

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Abstract □ A simple, sensitive, and specific gas chromatographic method for the quantitation of 4-methylpyrazole in plasma and urine is described. Samples containing 4-methylpyrazole, with 3-methylpyrazole as the internal standard, are extracted into ether and the concentrated ethereal extracts are chromatographed on a Carbowax 20M column using nitrogen-selective detection. Standard curves are linear and reproducible over the range of 25–1000 ng/mL for plasma and 0.5–5 μg/mL for urine. Recovery of 4-methylpyrazole is complete from plasma and urine, and the overall between-day coefficient of variation is within 6.0%. No interference is observed from the extractive constituents of plasma and urine. The assay method is suitable for an examination of 4-methylpyrazole disposition in animals and humans.

Keyphrases □ 4-Methylpyrazole—analysis in urine and plasma, gas chromatography with nitrogen-selective detection □ Gas chromatography—analysis of 4-methylpyrazole in plasma and urine, nitrogen-selective detection.

Pyrazole and several of its derivatives have been shown to be inhibitors of the alcohol dehydrogenase enzyme system (1). Because of its apparent low systemic toxicity, 4-methylpyrazole (I) is of particular interest. This compound inhibits the *in vitro* metabolism of alcohols in various animal species (1–3), and it reduces the rate of ethanol elimination in animals (4, 5) and humans (6–8). Several studies have also indicated its effectiveness in reducing the rate of metabolism of methanol (9, 10) and ethylene glycol (11, 12). As a result, 4-methylpyrazole may be a promising alternative to ethanol in treatment of poisoning with alcohols whose toxicity is associated with metabolites formed *via* alcohol dehydrogenase. There have been relatively few studies, however, where plasma concentrations of the compound have been determined (9, 10, 13, 14).

The methods currently available for the determination of I in biological fluids are quite limited. Rydberg and Buijten (15) described potential gas chromatographic (GC) conditions for I which simply involved injecting aqueous solutions of the compound into the column and identifying the chromatographic peak by mass spectrometry. Furthermore, a flame-ionization detector (FID) was employed. With FID, we found that the broad solvent front severely limits the lower measurable concentrations. The GC–MS method (16) of determining I in plasma, although specific, is time consuming and lacking in sensitivity. To examine the disposition of I in animals there is need for a simple, sensitive, and reproducible analytical method for plasma and urine. The present report describes such an assay method, utilizing GC and nitrogen-selective detection, for 4-methylpyrazole (I) using 3-methylpyrazole (II) as an internal standard.

EXPERIMENTAL

Chemicals—4-Methylpyrazole[1] and 3-methylpyrazole[1], the internal standard, were used as supplied. All organic solvents were HPLC grade[2]. Sodium carbonate, sodium chloride, and hydrochloric acid were reagent grade[3].

I II

Gas Chromatography—Samples were analyzed using a gas chromatograph[4] equipped with a nitrogen–phosphorus detector. A 122.0 cm × 2 mm i.d. glass column packed with 5% Carbowax 20M + 2% KOH on 80–100 mesh Chromosorb G (HP)[5] was used. Hydrogen and air flow rates were 3 and 50 mL/min, respectively. Helium was used as a carrier gas at a flow rate of 30 mL/min. The collector voltage was adjusted for adequate sensitivity. The column oven was operated isothermally at 135°C, injection port and detector temperatures were 250°C and 300°C, respectively.

Stock Solutions—A stock solution of I was prepared by dissolving 20 mg in 100 mL of methanol. Working standards were prepared by serial dilution of the stock solution with methanol to encompass the appropriate concentration ranges for plasma and urine. Concentrations of the standards were such that 50 μL added to 1 mL of biological fluid would produce the desired concentration. Solutions of the internal standard were prepared in methanol at a concentration of 4 μg/mL for plasma and 20 μg/mL for urine. All solutions were stored in the refrigerator (0–4°C).

Extraction of Plasma—One milliliter of plasma (containing I) was transferred into a glass culture tube with a polytef-lined screw cap. Fifty microliters (200 ng) of the internal standard solution, 1 g of NaCl, and 0.5 mL of 2 M HCl were added. After addition of 6 mL of ether, the tube was shaken gently for 5 min in a mechanical shaker, centrifuged at 3000 rpm (1000×*g*) for 5 min, and the upper organic layer was withdrawn and discarded. Five hundred microliters of 1 M sodium carbonate was added to the aqueous phase, mixed, and extracted as above with 6 mL of ether. After centrifugation, the clear ether layer was transferred into a 13 × 100-mm disposable glass culture tube containing 30 μL of *n*-butyl alcohol. A boiling chip was added to the tube which was placed in a water bath (45°C). The tube was removed from the water bath after most of the ether had evaporated. If necessary, the extract can be concentrated further by leaving the tube in the water bath for a few minutes after the boiling has ceased. Three microliters of the concentrated ethereal extract was injected into the column. The peak height ratio of I to the internal standard was calculated and the concentration of I was determined with reference to a standard curve.

Extraction of Urine—To 1.0 mL of urine in a glass culture tube (with a screw cap) were added 50 μL (1 μg) of internal standard solution, 1 g of NaCl, and 6 mL of ether. The tube was shaken gently for 5 min in a mechanical shaker and centrifuged for 5 min. The clear organic layer was transferred into a 13 × 100-mm glass culture tube containing 30 μL of *n*-butyl alcohol and treated as described above for the extraction of plasma.

Hydrolytic Treatment of Urine—A 1.0-mL sample of urine was placed into a glass culture tube, 0.5 mL of 2 M HCl was added, and the contents were mixed. The tube was capped tightly and placed in an oven at 115°C. After 1 h the tube was removed from the oven and cooled. One gram of sodium chloride, 50 μL (1 μg) of internal standard solution, and 0.5 mL of 1 M sodium carbonate were added. After addition of 6 mL of ether, the sample was carried through the procedure outlined above.

Standard Curves—Duplicate standards were prepared by transferring 50 μL of the working standards of I and 1.0 mL of blank plasma or urine to glass culture tubes to give final concentrations in the ranges of 25–1000 ng/mL and 0.5–5 μg/mL for plasma and urine, respectively. After addition of 50 μL of internal standard solution, the samples were treated as described above. Standard curves were prepared by plotting peak height ratios of I to the internal standard *versus* concentrations of I.

Extraction Efficiency (Analytical Recovery)—Analytical recoveries of I

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] Burdick and Jackson, Muskegon, Mich.
[3] Fisher Scientific Co., Fair Lawn, N.J.
[4] Model 5710A; Hewlett-Packard, Palto Alto, Calif.
[5] Supelco Inc., Bellefonte, Pa.

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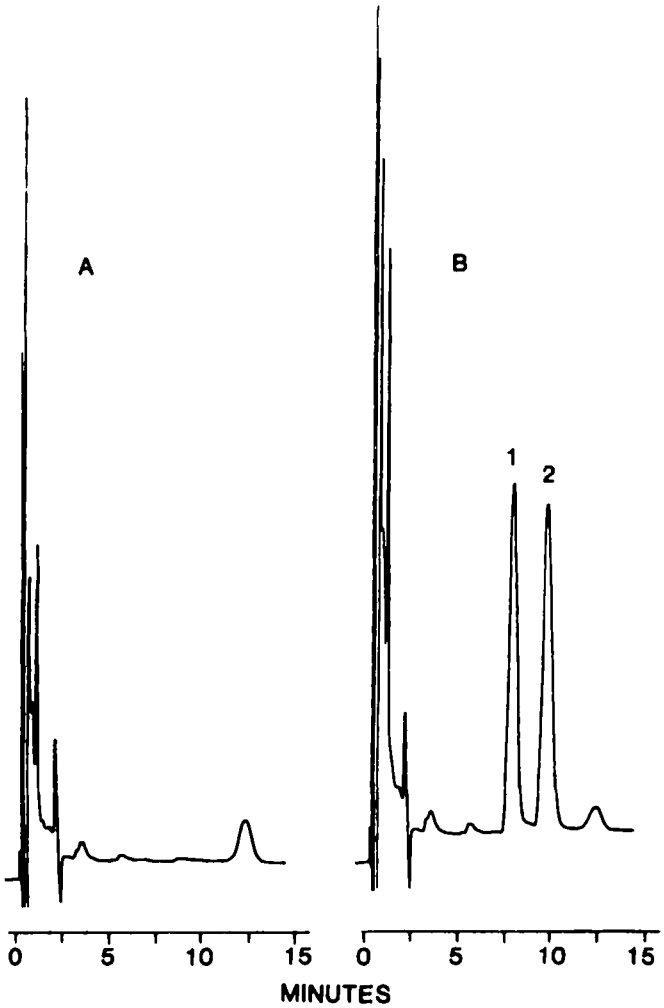

Figure 1—*Chromatograms of ethereal extracts of human plasma. Key: (A) plasma blank; (B) plasma spiked with 200 ng each of 3-methylpyrazole (1) and 4-methylpyrazole (2) (attenuation: 1 × 32).*

and the internal standard from plasma and urine were determined by comparing, respectively, the peak heights of I and internal standard obtained from the extracted spiked plasma and urine samples to those obtained from direct injection of methanolic standards containing equivalent amounts of the compounds. Recoveries of I were also calculated by comparing the peak height ratios of I to the internal standard in the extracted standards to those in the methanolic standards. Three different concentrations of I were used.

RESULTS AND DISCUSSION

A number of different solvents were used for the extraction of I and II from plasma and urine. Hexane and *n*-pentane did not extract either compound. Chlorinated solvents were not suitable for the nitrogen–phosphorus detector. Ethyl acetate gave good extraction of I, but the plasma extracts were not clean and, furthermore, ethyl acetate has a high boiling point. Ether gave the best extraction of I and II; both compounds were completely recovered with a single ether extraction. However, to obtain cleaner extracts an acid-wash step was used for the plasma samples.

Bjorkhem *et al.* (16) also used ether for extraction of I from plasma. However, recovery and reproducibility data were not presented. The ethereal extract was concentrated to ~50-μL under nitrogen. This method of concentrating the ethereal extract would require constant attention since, if the solvent was allowed to evaporate to dryness, almost complete (>90%) loss of I would occur. In our procedure we added 30 μL of *n*-butyl alcohol to the ethereal extract, and the ether was allowed to boil-off in a water bath (45°C). The main advantage of this technique is that the tubes can be left unattended in the water bath without the risk of losing the compound. When ether is evaporated, the compound is retained in *n*-butyl alcohol. However, nitrogen cannot be used to aid solvent evaporation as this will cause the tube to dry and result in almost complete loss of both I and the internal standard (II).

Although I extracts well in the pH range of 4.5–11.5, at higher pH values a small interfering peak was present in the extracts of dog plasma but not in the extracts of human plasma. In the present procedure, plasma was extracted at pH 6–7, which was achieved by adding 1 M sodium carbonate to the acidified plasma.

Table I—Within-Day and Between-Day Reproducibility of the Plasma Assay [a]

Amount Added, ng/mL	Within-Day Found, ng/mL	Within-Day *CV*, %	Between-Day Found, ng/mL	Between-Day *CV*, %
40	41.1 ± 0.45	1.1	40.8 ±0.77	1.9
160	158.5 ± 2.6	1.6	158.7 ± 4.8	3.0
800	805.1 ± 14.4	1.8	790.5 ± 24.3	3.0

[a] Mean ± *SD*; $n = 5$.

Typical chromatograms obtained from the ethereal extracts of human blank plasma and plasma spiked with 200 ng each of I and II are shown in Fig. 1. The column used gave sharp and symmetrical peaks for both compounds. The internal standard (II) eluted close to I, but was well resolved. Under the conditions employed, the retention times for I and II were 10 and 8 min, respectively. Extracts of both dog and human blank plasma showed a small and broad peak at 24 min at high sensitivity (attenuation <1 × 64). By carefully spacing the sample injections this peak can be eluted in the region between the solvent front and sample peaks.

Analytical recoveries (mean ± *SD*) of I and internal standard using peak height comparison were 102.6 ± 2.6, and 101.6 ± 1.9%, respectively. Peak heights from the extracted standards were consistently greater than those from the methanolic standards. This would result from the smaller volume of the concentrated extracts in the extracted standards compared with the volume (0.1 mL) of the methanolic standards. This discrepancy could be obviated if the sample extracts could be evaporated to dryness and reconstituted to the

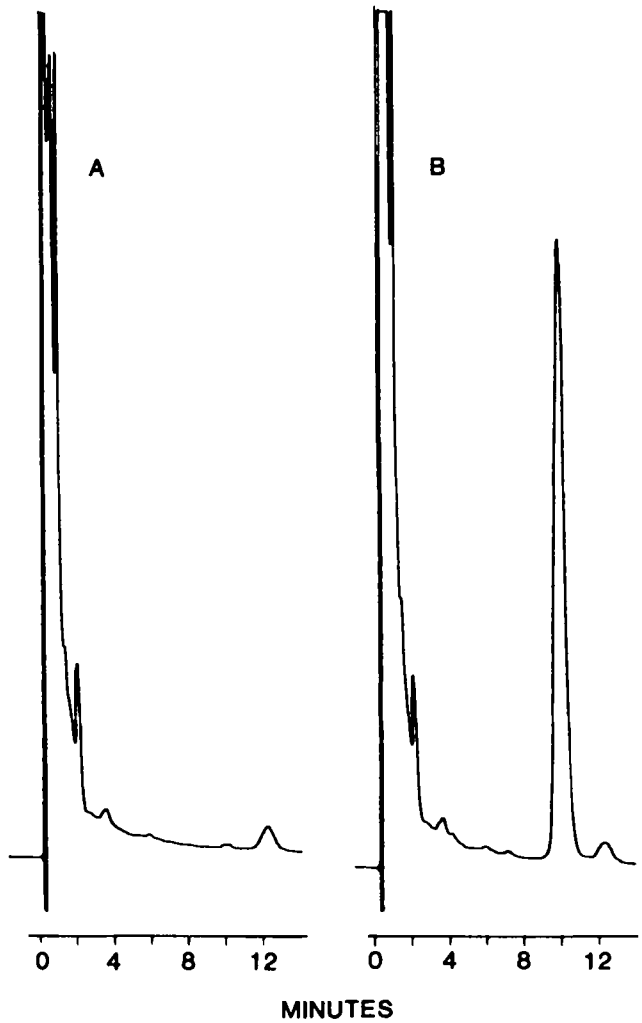

Figure 2—*Chromatograms of ethereal extracts of dog plasma before (A) and 16 h after (B) a 10-mg/kg iv dose of 4-methylpyrazole. The peak corresponds to a concentration of 640 ng/mL (attenuation: 1 × 64).*

Table II—Within-Day and Between-Day Reproducibility of the Urine Assay [a]

Added, μg/mL	Within-Day Found, μg/mL	Within-Day CV, %	Between-Day Found, μg/mL	Between-Day CV, %
1.6	1.62 ± 0.03	1.8	1.62 ± 0.066	4.0
2.7	2.75 ± 0.04	1.5	2.78 ± 0.114	4.1
3.9	4.15 ± 0.14	3.4	4.18 ± 0.194	4.6

[a] Mean ± *SD*; $n = 4$.

same volume as the methanolic standards. Peak height ratio, however, would not be affected by volume difference. Since the extraction efficiencies of I and of the internal standard were independent of each other and the internal standard was completely recovered, recovery of I calculated from peak height ratio measurement would provide a more meaningful value. Using peak height ratio measurement, mean (±*SD*) recoveries of I (six samples) at 100, 500, and 1000 ng/mL were 99.0 ± 1.0, 98.5 ± 1.8, and 99.0 ± 1.5%, respectively.

Standard curves were linear and reproducible over the concentration range of 25–1000 ng/mL. Linear regression statistics for five standard curves prepared on different days over a period of 2 weeks produced a slope of 0.0044 (±0.0001), an intercept of −0.024 (±0.032), and a mean coefficient of determination (r^2) of 0.999.

Assay reproducibility was determined by repeated analysis of plasma samples containing I. Three different concentrations other than those used in the preparation of the standard curves were used. The mean concentrations, standard deviations, and coefficients of variation for within-day and between-day analysis are presented in Table I. These data indicate excellent assay reproducibility, which is further supported by the reproducible nature of the standard curves as shown above.

Plasma spiked with I (1 μg/mL) was stored in the refrigerator (0–4°C), and 1-mL aliquots were analyzed once a week for 6 weeks. Recoveries of I were similar over the 6-week period indicating good stability of I in plasma.

Recovery of I from urine was similar to that from plasma. Standard curves were linear and reproducible over the concentration range of 0.5 to 5 μg/mL with $r^2 > 0.999$. No interference was observed from the extractive constituents of blank urine. Both within-day and between-day assay reproducibility was excellent (Table II). The procedure can be used for concentrations down to 0.1 μg/mL with good reproducibility (coefficient of variation, 4.5%).

The lower sensitivity limit of the GC–MS method (16) was only ~100 ng/mL when 2 mL of plasma was used. Our GC method is simple, rapid, able to quantitate I down to 25 ng/mL with good precision (coefficient of variation, 6.0%), and requires only 1 mL of plasma. This is advantageous, since with greater assay sensitivity plasma concentrations can be monitored for a longer period of time and a smaller dose can be used. The versatile nature of the present method makes it particularly suitable for pharmacokinetic studies of I.

To further evaluate the usefulness of the assay procedure, a 14.5-kg dog was given a 10-mg/kg iv dose of I, and blood and urine samples were obtained. Figure 2 shows the chromatograms of ethereal extracts of dog plasma before and after the administration of I. After hydrolytic treatment of urine samples, the total recovery of I was 2.3% over a period of 96 h. Disposition studies in the dog are currently in progress.

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[^{14}C]Normacromerine Fate in the Rat

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Abstract □ The biological fate of [^{14}C]normacromerine, a dimethoxylated phenethylamine derivative with putative hallucinogenic properties, was evaluated in male Sprague–Dawley rats at 100 mg/kg po. Urine was the primary elimination route accounting for 50% of administered carbon-14 after 24 h. Of this urine radioactivity, normacromerine comprised 30% at 8 h decreasing to nondetectable levels at 24 h. Carbon-14 in feces represented <10% of the administered dose at 24 h, and $^{14}CO_2$ expiration was not detected. Studies of normacromerine fate in comparison with previously studied phenethylamines may enhance evaluation of hallucinogenic potential of normacromerine.

Keyphrases □ Normacromerine—biological fate in the rat, hallucinogenic potential □ Biological fate—[^{14}C]normacromerine, rat, hallucinogenic potential □ Hallucinogens—normacromerine, biological fate in the rat

Normacromerine (*N*-methyl-3,4-dimethoxy-β-hydroxyphenethylamine), the major alkaloid in extracts of the Dona Ana cactus [*Coryphantha macromeris* (Engelm.) Br. and R.], has been implicated as the chemical responsible for hallucinogenic effects experienced by humans ingesting the cactus (1, 2). The potency of the Dona Ana cactus has been estimated to be one-fifth that of the peyote cactus [*Lophophora williamsii* (Lem.) Coult.] which contains mescaline, another natural hallucinogen (2, 3).

Laboratory studies of the behavioral effects of normacromerine in rats have provided both positive (4) and negative (5) results in support of the hallucinogenic properties of the drug. Absent from these studies has been a thorough evaluation of the mammalian disposition of normacromerine after ingestion. Studies of biological fate of the compound are unavailable in the literature.

Patterns of absorption, distribution, metabolism, and excretion of chemicals determine their extent and duration of action within biological systems (6). Such studies of norma-

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