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Note

Determination of 3-amino-1,2,4-triazole (amitrole) in urine by ion-pair high-performance liquid chromatography

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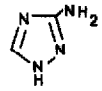
3-Amino-1,2,4-triazole (amitrole) (Fig. 1a) is a non-selective herbicide introduced in 1954[1]. Little is known of the human metabolism of the compound but experiments with [^{14}C]amitrole orally administered to rats showed that 80–90% of the compound was excreted unchanged in the urine[2]. In a reported[3] case of non-fatal suicidal ingestion of amitrole, 50% of the amitrole taken was excreted in the urine within a few hours of ingestion; the amitrole concentration in the urine was 1 mg/ml. To monitor the absorption of amitrole by workers exposed to the compound, a method was required to determine low concentrations of amitrole in urine. A paper chromatographic method has been described[3] for the estimation of elevated levels of amitrole in urine; after separation the amitrole was extracted from the paper and estimated colorimetrically. Procedures based on azo dye formation have been described for the estimation of low levels of amitrole in foods[4,5] and soils[6]. Diazotised amitrole has been coupled with N-(1-naphthyl)-ethylene-diamine[4–7], 1,8-dihydroxy-



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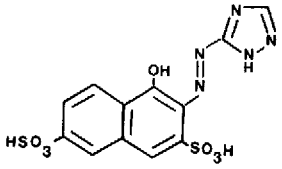

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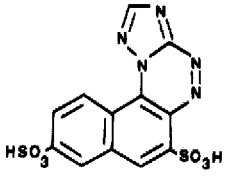

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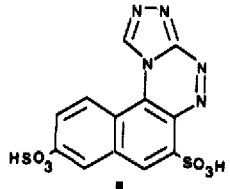

d

Fig. 1. a, 3-amino-1,2,4-triazole; b, 2-[3-(1,2,4-triazolylazo)]-1-hydroxy-naphthalene-3,6-disulphonic acid; c, naphtho[1,2-*e*]-(1,2,4)-triazolo[5,1-*c*]-(1,2,4)-triazine-6,9-disulphonic acid; d, naphtho[1,2-*e*]-(1,2,4)-triazolo[3,4-*c*]-(1,2,4)-triazine-5,8-disulphonic acid.

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naphthalene-3,6-disulphonic acid (chromotropic acid)[3,8,9] and 8-amino-1-hydroxynaphthalene-3,6-disulphonic acid (H acid)[5,8,10]. This last compound gives with amitrole an azo dye which is amphoteric and when separated by ion-pair high-performance liquid chromatography produces at least two peaks[5]. In the method described in this paper, diazotised amitrole is coupled with the de-amino analogue of H acid, 1-naphthol-3,6-disulphonic acid, to produce a compound suitable for separation and quantitation by ion-pair liquid chromatography.

EXPERIMENTAL

Chromatography

The apparatus used consisted of an Altex Model 321 liquid chromatograph with a Rheodyne 7125 sample injector fitted with a 100-μl loop, and an Altex-Hitachi variable-wavelength detector set at 440 nm and 0.05 absorbance units. An ASIμC_{18} reversed-phase column (300 × 3.9 mm I.D.; Analytical Sciences) was used with a flow-rate of 1.5 ml/min. The mobile phase was prepared from anhydrous sodium acetate (6 g) and tetrabutylammonium phosphate solution (Ajax Chemicals, Sydney, Australia) dissolved in 580 ml of water and mixed with 420 ml of methanol to give a mobile phase of 42% methanol in 0.073 *M* sodium acetate and 0.005 *M* tetrabutylammonium phosphate.

Reagents

All reagents were prepared in aqueous solution. Amitrole, technical grade (Fairmount Chemical, Newark, NJ, U.S.A.), was recrystallised from ethanol (5 g/50 ml) to give colourless needles, m.p. 151–152 (lit.[11] 150–153°C). Hydrochloric acid, 5 *M*; sodium nitrite, 10 g/100 ml; 1-naphthol-3,6-disulphonic acid, di-sodium salt (Violet acid, Rudolf Guercke acid) (Tokyo Kasei Kogyo), 1 g/100 ml; potassium permanganate, 2 g/100 ml; sodium acetate, anhydrous, 20 g/100 ml; amitrole, recrystallised, 10 mg/100 ml.

Procedure

Prepare a standard urine containing 1 μg/ml of amitrole by adding 20 μl of amitrole solution to 2 ml of urine containing no amitrole. Add to this solution and to 2 ml of sample urine, 0.2 ml 5 *M* hydrochloric acid, 0.2 ml of potassium permanganate solution and mix. Add to each mixture 0.5 ml of sodium nitrite solution, mix and add 0.2 ml of naphthol disulphonic acid solution and mix. Allow to stand at room temperature for 15–30 min and then add to each mixture 0.5 ml of sodium acetate solution and mix. Inject 100 μl of the resultant yellow solutions within 45 min after the addition of the sodium acetate solution. Measure the peak height of the amitrole derivative (peak C, Fig. 2) and calculate the concentration of amitrole in the urine sample.

RESULTS AND DISCUSSION

Typical chromatograms from a reagent blank in water, a urine sample containing no amitrole and a urine containing 1 μl/ml of amitrole are shown in Fig. 2. The yellow colour produced in both sample and blank (peak A, Fig. 2) is due to products

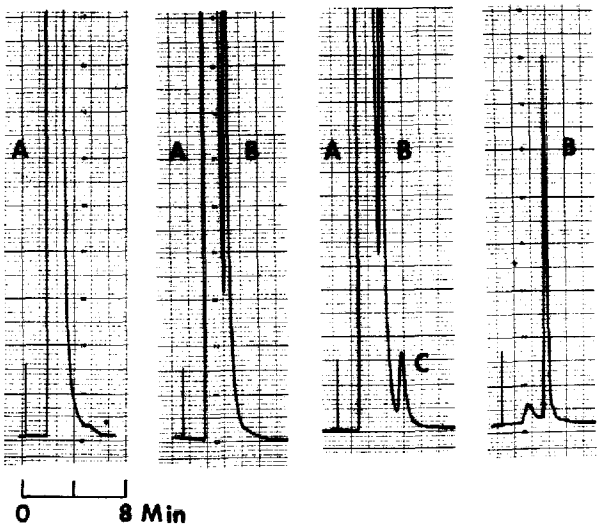

Fig. 2. HPLC chromatograms of (left to right): reagent blank in water; urine (2 ml) with reagents; urine (2 ml) containing 1 μg/ml amitrole, with reagents; urine (20 μl), untreated. Peak A: reaction by-products; peak B: urine pigment; peak C: amitrole reaction product. Conditions: mobile phase, methanol–water (42:58); 0.005 *M* tetrabutylammonium phosphate, 0.073 *M* sodium acetate; flow-rate, 1.5 ml/min; detector wavelength, 440 nm.

from the reaction of nitrous acid with the naphthol disulphonic acid. Peak B (Fig. 2) is a yellow pigment present in all urines in varying concentrations and is unaffected by treatment with potassium permanganate or nitrous acid. Peak C (Fig. 2) is the compound produced from amitrole, nitrous acid and the naphthol disulphonic acid and is absent if any one of these three compounds is omitted from the reaction mixture.

The concentrations of reagents and the lengths of reaction times were varied and modified to produce the maximum response for a given amitrole concentration and the methanol concentration of the mobile phase was varied to give a reasonable separation of the amitrole peak with a minimum retention time. Diazotised amitrole was reported[12] to decompose to the corresponding 3-chloro derivative in the presence of chloride ions; urine contains a significant amount of chloride ion (about 6 mg/ml) but no difference in peak height was found if the reaction was carried out in either urine or water, each containing equivalent quantities of either hydrochloric or sulphuric acid. Sulphamic acid is often used in colorimetric procedures to remove excess nitrous acid from diazonium salt solutions[10] but if sulphamic acid was added to the reaction mixture after diazotisation, a reduced peak height was obtained for the amitrole peak; if the diazotised mixture was allowed to stand with sulphamic acid no peak for amitrole was obtained. Similar instability in the presence of sulphamic acid is reported[13] for the diazotised carboxylic acid derivative of amitrole, 5-amino-1,2,4-triazole-3-carboxylic acid. The strongly acid reaction mixture was neutralised with sodium acetate before injection into the chromatograph to reduce decomposition of the reversed-phase column packing.

The detector wavelength was varied from 400 to 505 nm to determine the wavelength of maximum absorption for the amitrole derivative; maximum absorp-

tion occurred at 440 nm. The amitrole peak height varied linearly with the urine amitrole concentration over the range 0.5–5.0 μg/ml and the average recovery of amitrole added to ten different blank urine samples, at the 1 μg/ml level, was 92% (range 83–106%). The minimum detectable concentration of amitrole in urine was 0.2 μg/ml. All the urine specimens examined so far have shown amitrole concentrations of less than 0.2 μg/ml.

Tryptophan was reported[10] to interfere in the coupling of diazotised amitrole with H acid; in the present work tryptophan (2 μg/ml) prevented the coupling of dizotised amitrole with 1-naphthol-3,6-disulphonic acid in aqueous solution. The addition of potassium permanganate removed this interference and it was found that any excess permanganate could be conveniently removed with sodium nitrite; if sodium sulphite was used for this purpose, any residual sulphite interfered in the subsequent diazotisation reaction. In aqueous solution, not containing tryptophan, the addition of potassium permanganate was not required but with urine the omission of potassium permanganate resulted in decreased peak height for the amitrole derivative. At least 0.2 ml of 2% potassium permanganate solution was required for 2 ml of urine; a variation in reaction time from 10 sec to 10 min produced no significant variation in peak size. Urea present in urine reacts with nitrous acid to give nitrogen and at least 0.5 ml of 10% sodium nitrite solution was required to give the maximum peak size for the amitrole derivative. When the 1-naphthol-3,6-disulphonic acid was replaced by the isomeric 2-naphthol-3,6-disulphonic acid (R acid) no amitrole derivative could be separated by liquid chromatography.

The exact nature of the compound produced from diazotised amitrole and 1-naphthol-3,6-disulphonic acid is not certain. Diazotised amitrole when treated with excess sodium nitrite forms 3-nitro-1,2,4-triazole[14] but this compound has no absorption at 440 nm[14]. The azo dye (Fig. 1b) prepared from amitrole and the naphthol disulphonic acid on the 0.05 *M* scale was obtained as a dark red powder, soluble in water to give a yellow solution which turned violet in the presence of traces of copper ion. This compound, dissolved in the mobile phase, had maximum absorption at 505 nm and a retention time of 3.0 min whereas the compound obtained in the analytical procedure gave no absorption at 505 nm and had a retention time of 5.0 min. Diazotised amitrole couples with 2-naphthol to give the 1-azo derivative which is readily cyclised to either of two isomeric naphtho-triazolo-1,2,4-triazines; this cyclisation produces a decrease in the wavelength of maximum absorption of 62 and 64 nm for the two isomers when compared with original azo compound[15]. This decrease is similar to the difference in maximum absorption wavelengths for the azo dye (prepared from amitrole and 1-naphthol-3,6-disulphonic acid) and the compound obtained from amitrole in the analytical procedure, 65 nm, and it is suggested that the compound obtained from amitrole is the naphtho-triazolo-1,2,4-triazine (Fig. 1c) rather than the isomeric compound (Fig. 1d) in which there can be steric interaction between the protons on C_{10} and C_{12}.

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