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Letter to the Editor

Modified high-performance liquid chromatographic assay for antipyrine and its three major metabolites in urine

Sir,

Multiple methodologies exist for the characterization of antipyrine and its metabolites in biologic fluids [1–5] A recently developed, precise, single-extraction, reversed-phase high-performance liquid chromatographic (HPLC) method was chosen as suitable for our use in clinical pharmacology studies [5] However, this method could not be replicated in our laboratory Chromatographic conditions (i e , same column, mobile phase, temperature, flow-rate, and extraction) yielded run times in excess of 40 min and incomplete resolution between the internal standard and antipyrine The method utilized a very high flow-rate (3 5 ml/min) [5]

Modifications to this procedure yielded more reasonable run times with optimal resolution of antipyrine, its three major metabolites, and the internal standard Specifically, the modifications included use of a shorter, commonly available column, addition of an ion-pairing agent (diethylamine), an alternate internal standard (phenacetin), and use of a lower flow-rate

This report outlines the results of modifications to a method for the simultaneous assay of antipyrine (AP), norantipyrine (NORA), hydroxymethylantipyrine (HMA), and 4-hydroxyantipyrine (OHA) in urine

EXPERIMENTAL

Instrumentation

Liquid chromatographic separation of antipyrine and its major urine metabolites was carried out using an HPLC system consisting of a Model 6000A solvent delivery system (Waters Chromatography, Division of Millipore, Milford, MA, U.S A), a Model 712 WISP autosampler (Waters), and a Model SP4290 plotting integrator (Spectra-Physics, Autolab Division, San Jose, CA, U S A) Ultraviolet detection at 254 nm was accomplished with a Model 153

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single-wavelength detector (Altex Scientific, Berkeley, CA, U S A.) The column was a prepacked 15 cm×3 9 mm I D μBondapak® C_{18} column (10 μm average particle size) (Waters) A prepacked 2 cm×4 6 mm Supelguard® LC18 disposable column (5 μm average particle size) (Supelco, Bellefonte, PA, U S.A) was used as a guard column

Chemicals

Water was purified by reverse osmosis Acetonitrile, methanol, and ethyl acetate were HPLC grade (Fischer Scientific, Fairlawn, NJ, U S A) Antipyrine, phenacetin and diethylamine were obtained from Sigma (St Louis, MO, U S A) NORA, OHA, and sodium metabisulfite were purchased from Aldrich (Milwaukee, WI, U S A) Glusulase® was obtained from EI duPont de Nemours (Garden City, NY, U S A) HMA was synthesized by and received as a gift from D D Breimer (Leiden, The Netherlands)

Chromatographic conditions

Separation was carried out at room temperature An ion-pairing agent was added to reduce peak tailing The mobile phase components were similar to that used by Mikati et al [5] with the addition of an ion-pairing agent 7 5% of acetonitrile, 92 5% of 0 1 *M* sodium acetate and 0 5 m*M* diethylamine (v/v) The final pH of the mobile phase was adjusted to 6 6 with glacial acetic acid The mobile phase solution was filtered through a 0 22-μm Nylon-66 membrane filter and degassed for 15 min The mobile phase was run at a flow-rate of 2 0 ml/min and recirculated with constant stirring.

Extraction procedure

The enzyme hydrolysis and extraction procedures used by Mikati et al [5] were utilized with minor modification Briefly, sample urine (1 ml) was added to 100 mm×13 mm glass tubes which contained 50 mg of sodium metabisulfite Sodium acetate solution (1 ml of 1 *M*, pH 5 0) and Glusulase (50 μl) were added to each tube and thoroughly vortex-mixed Samples were then incubated for 3 h at 35–37°C After incubation, 0 5 ml of 1 *M* sodium acetate (pH 5 0), 20 μl of 1 mg/ml phenacetin as internal standard, and 5 ml of ethyl acetate were added to each tube Vortex-mixing produced an emulsion which rapidly resolved Samples were centrifuged for 10 min at 400 *g* The upper organic phase (~ 4 5 ml) was removed and evaporated under nitrogen at a temperature less than or equal to 37°C Samples were reconstituted with methanol (200 μl), vortex-mixed, and 20-μl aliquots were injected onto the HPLC system

RESULTS AND DISCUSSION

Total run times at a flow-rate of 2 0 ml/min were approximately 7–8 min longer than the method by Mikati et al [5] because of the slower elution time

of phenacetin However, more complete resolution of AP was obtainable by using phenacetin instead of the internal standard used by Mikati et al [5] (Fig 1) Increasing flow-rates to 3 5 ml/min as used by Mikati et al [5] reduced total run times to 14–15 min but increased HPLC system pressures to greater than 137 bar

Within-day assay variation was assessed by multiple measures of quality controls ($n=8$) at two concentrations (AP, NORA, OHA, 60 and 6 μg/ml, HMA, 30 and 6 μg/ml) Coefficients of variation for AP, NORA, OHA, and HMA at high and low concentrations were 6 3 and 4 0%, 4 9 and 4 9%, 4 1 and 3 0%, and 5 6 and 8.6%, respectively Between-day ($n=4$) coefficients of variation based upon duplicate high and low samples ranged from 5 4 to 12 0% for

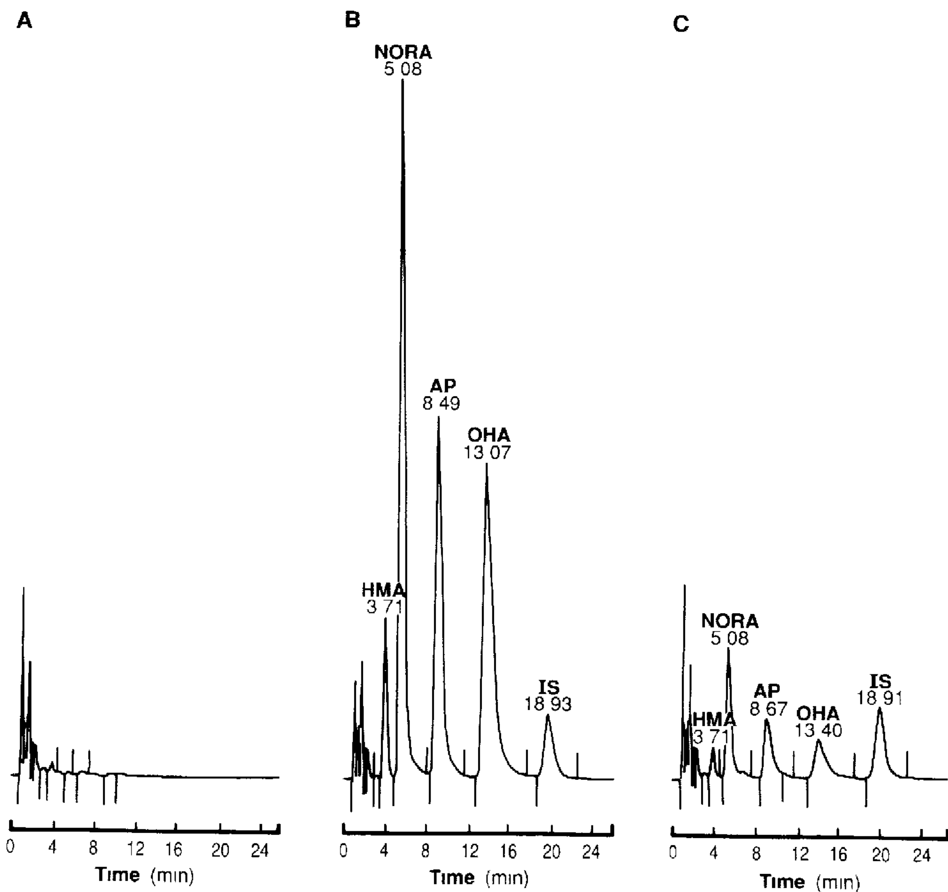

Fig 1 Chromatograms of (A) blank urine, (B) urine sample containing 80 μg/ml hydroxymethylantipyrine (HMA), 150 μg/ml each of norantipyrine (NORA), antipyrine (AP), and 4-hydroxyantipyrine (OHA), and 20 μg of phenacetin (IS), and (C) urine sample containing 10 μg/ml HMA, 25 μg/ml each of NORA, AP, and OHA, and 20 μg of IS

all compounds Accuracy was estimated as percentage recovery and ranged from 83 to 103% for all compounds Average extraction efficiency for AP, NORA, OHA, and HMA was 63, 86, 79, and 37%, respectively Correlation coefficients for all standard curves during assay validation were between 0 997 and 0 999

AP, NORA, and OHA were easily quantifiable to a concentration of 5 μg/ml However, the HMA metabolite was consistently quantifiable to 8 μg/ml due to an unknown interfering peak which appeared in most urine samples after the addition of Glusulase Measurement of urine metabolite concentrations from normal human subjects who had received 1200 mg of oral AP showed concentrations of AP, NORA, OHA, and HMA ranging from 10 to 250 μg/ml Percentages of each metabolite and parent compound measured in the urine of these subjects were comparable to values reported by other investigators [1–3]

This modified HPLC method is sensitive and specific for application to clinical pharmacology studies

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