Journal of Chromatography, 433 (1988) 305–311
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4439

Note

Improved high-performance liquid chromatographic assay of antipyrine, hydroxymethylantipyrine, 4-hydroxyantipyrine and norantipyrine in urine

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(First received April 27th 1988, revised manuscript received August 18th, 1988)

Antipyrine (AP), an analgesic, has been extensively used as a marker drug to study changes in hepatic microsomal system activity. The total metabolic clearance of AP, measured in the vast majority of these studies, quantitates a mixture of oxidative reactions. AP is simultaneously metabolized via two distinct hepatic microsomal enzyme systems: (i) N-demethylation leading to norantipyrine (NORA) and (ii) aliphatic hydroxylation leading to 4-hydroxyantipyrine (4OHA) and hydroxymethylantipyrine (HMA). More recently the quantitation of AP and of its metabolites has been used to better understand the mechanisms

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0378-4347/88/$03.50

of drug metabolism [1–8]. The same approach may prove to be helpful for prediction of drug interactions. The availability of reliable assay procedures for AP and its metabolites is thus of considerable importance for clinical pharmacology.

Six high-performance liquid chromatographic (HPLC) assays of AP and its metabolites have been reported [9–14]. However, upon review of these methods we found none of them to be completely optimal for clinical pharmacology studies. Precision testing with control samples (samples not used as points on the calibration curve) was not reported for two of these methods [9,10] and was incomplete and/or showed relatively high coefficients of variation (>5% and occasionally >10%) in the other four [11–14]. Additionally, two [9,10] of the extraction procedures described were of multiple steps and three [10,11,13] required cumbersome silanization of the glassware. Finally, in three methods [10,12,14], the internal standard used was phenacetin which is a medication frequently found in over the counter analgesic preparations in some countries. Because minimal amounts of phenacetin are excreted in the urine as the unmetabolized drug, if inadvertently taken by a patient, this medication could potentially interfere with the above-mentioned assays. In this article we describe an improved method to assay AP and its metabolites in urine with none of the above-listed drawbacks.

EXPERIMENTAL

Instrumentation

The following apparatus from Waters Assoc. (Milford, MA, U.S.A.) was used: Model ALC/CPC 204/6000A HPLC system, WISP Model 710 B automatic injection system and Model 450 variable-wavelength detector. A CRI reversed-phase, 10 μm irregular particle size, 30 cm × 3.9 mm stainless-steel C_{18} column (Column Resolution, Santa Clara, CA, U.S.A.) was used. Column temperature was controlled by a water circulator (Model FE, Haake Instruments, Saddle Brook, NJ, U.S.A.). The recorder was a dual-pen Omniscribe B5000 (Houston Instruments, Austin, TX, U.S.A.). The data integration system consisted of a Canon AS100 personal computer with a Quasitronics Q3024 analog to digital interface (Houston, PA, U.S.A.). Software was produced by Binary Systems (Newton, MA, U.S.A.).

Mobile phase

The mobile phase consisted of 7.5% acetonitrile in 0.1 *M* sodium acetate, adjusted with glacial acetic acid to a pH 6.6 and run at a rate of 3.5 ml/min. The temperature of the column was maintained at 35°C. The mobile phase was degassed by passing it through a 0.45-μm GN-6 Metricel cellulose ester filter membrane (Gelman Instruments, Ann Arbor, MI, U.S.A.).

Reagents

AP, NORA and 4OHA were obtained from Aldrich Pharmaceuticals (Milwaukee, WI, U.S.A.). Aminoantipyrine (amino-AP) was obtained from Kodak (Rochester, NY, U.S.A.). HMA was the generous gift of Dr. D.D. Breimer (Lei-

den University, Leiden, The Netherlands). Glusulase (a commercial mixture of β-glucuronidase and sulfatase) was obtained from Dupont Pharmaceuticals (Wilmington, DE, U.S.A.). HPLC-grade acetonitrile and methanol were used (Fisher Scientific, Pittsburgh, PA, U.S.A.).

Analytical procedures

Standard solutions of AP, its metabolites and the internal standard were prepared by dissolving 10 mg of each compound in 10 ml of methanol. During assays for the calibration (standard) curves known amounts of AP and its metabolites were added to a series of 13-ml round-bottom tubes. The organic solvent was then evaporated under a gentle stream of nitrogen with temperatures not exceeding 35°C. Drug-free urine (1 ml) was then added to each tube together with 50 mg sodium metabisulfite (as an antioxidant to prevent degradation of the metabolites especially of NORA), 1.0 ml of buffer (1 *M* sodium acetate pH 4.8–5.0) and 50 μl of Glusulase. Glusulase was added because AP metabolites are predominantly excreted in the urine as the glucuronides and to a much smaller extent, the sulfate metabolites [12]. During regular assays on unknown urines, 1 ml of the unknown urine was combined with 50 mg sodium metabisulfite, 1 ml of buffer and 50 μl of Glusulase. All samples were incubated in a shaker bath at a temperature of 37°C for 3 h. Then to each tube 0.5 ml of the buffer and a fixed amount of internal standard (usually 20 μg) was added and the samples were subsequently extracted with 5 ml of ethyl acetate by shaking them on a horizontal mixer for 10 min at 90 cycles/min. Vortex-mixing was not used because it frequently produced emulsification. After centrifugation at 400 *g* for 10 min an aliquot (approximately 4.5 ml) of the organic phase was transferred to a 13-ml tapered glass centrifuge tube. The organic solvent was again evaporated to dryness under a gentle stream of nitrogen with temperatures not exceeding 35°C. The samples were then reconstituted with 300 μl of methanol. Samples of 10–20 μl were injected onto the HPLC column.

Calibration graphs

Calibration graphs (standard curves) were prepared by injecting sequential samples from urine spiked with known amounts of AP and its metabolites as described above. Five-point calibration graphs were obtained for each compound. AP ranged from 3 to 45 μg/ml, NORA and 4OHA ranged from 10 to 150 μg/ml and HMA from 8 to 120 μg/ml. Linearity of calibration curves was determined by linear least-squares regression analysis of known spiked concentration values versus ratio of peak height to internal standard for each compound.

Accuracy and precision

Precision was evaluated by serially assaying four to seven identical control urine samples spiked with known amounts of AP and its metabolites. Low, intermediate and high concentrations were used for each compound. HMA was tested at 16, 48 and 96 μg/ml, NORA at 20, 60 and 120 μg/ml, AP at 3, 18 and 36 μg/ml and 4OHA at 20, 60 and 120 μg/ml. Mean, standard deviation and coefficient of variation (C.V.) for each concentration were calculated. Additionally, absolute

and relative recoveries were calculated. Absolute recovery were calculated by dividing the extracted drug peak height by the peak height obtained by injecting equivalent amounts of unextracted standard solution. The relative recovery was defined as and calculated by dividing the measured concentration values obtained for drug-supplemented urine with the actual known added concentration.

RESULTS AND DISCUSSION

Analytical variables

The following reversed-phase HPLC columns were tried sequentially: a 30 cm×3.9 mm stainless-steel CRI C_{18} column (Column Resolution), a Waters 10 cm×8 mm radial pack compression μBondapak C_{18} column and a 30 cm×3.9 mm Waters C_{18} μBondapak stainless-steel column (Waters Assoc.). The CRI column produced the best separation and resolution of peaks.

Using the CRI column, nine different potential internal standards were tried. These included 3,4-dimethyl-1-phenyl-3-pyrazoline-5-one, 4-(3-methyl-5-oxo-pyrazoline-1-yl)benzoic acid, 4-methylphenazone, phenacetin, alphenal, 4-dimethylantipyrine, 3-methyl-1-phenyl-2-pyrazolin-5-one, antipyric acid and amino-AP. Amino-AP and phenacetin were the most suitable internal standards because the other compounds either eluted too late or coeluted and interfered with the metabolites being measured. Amino-AP was found to be superior to phenacetin because amino-AP eluted much earlier than phenacetin and because the retention of amino-AP could be controlled by modifying the pH of the mobile phase. Amino-AP, a structural analogue of AP, is not one of its several known urinary metabolites [1–8]. To further document this finding we tested serial urine samples (0–12, 12–24 and 24–48 h urine collections) of each of five volunteers who had taken 1000 mg of AP orally (at the time=0 h). None of these samples had any amino-AP peaks. Using our assay method the elution times of AP, amino-AP and phenacetin were 9.1, 11.5 and 33.0 min, respectively. Under the same conditions HMA eluted at 4.0 min, NORA at 7.0 min and 4OHA at 13.5 min (Fig. 1). The elution time (14 min) required by our method resulted in a significant advantage by achieving good peak separation with optimal resolution.

The ionic strength and composition of the mobile phase (0.05–0.15 *M* sodium acetate and 5–15% acetonitrile) were modified systematically. The best baseline and peak resolution was obtained at 0.1 *M* sodium acetate and 7.5% acetonitrile.

The effect of the pH of the mobile phase was systematically studied between pH 5.2 and 7.0. Increasing the pH caused NORA to elute earlier and amino-AP to elute later. Retention time of the other peaks remained essentially unchanged. A pH of 6.6 provided optimal resolution of peaks in the following order: HMA, NORA, AP, amino-AP, 4OHA. All peaks eluted within 15 min. This contrasts with over 33 min needed if phenacetin was used as the internal standard. The effect of the temperature of the column was studied between 25 and 45°C. A temperature of 35°C produced the shortest run time possible with optimal resolution.

The temperature used in the evaporation of the organic phase during the extraction of procedure was found to be critical. Temperatures above 35–40°C pro-

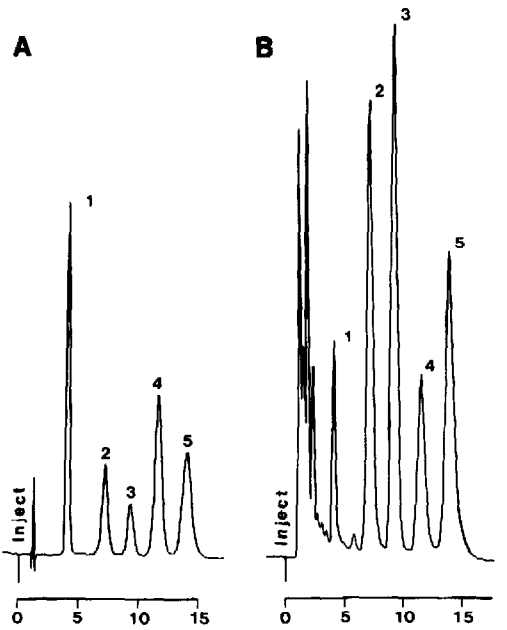

Fig. 1. Liquid chromatograms of antipyrine and its metabolites. (A) Chromatogram of a 10-μl injection of methanol standard with the following drugs: (1) hydroxymethylantipyrine, (2) norantipyrine, (3) antipyrine, (4) aminoantipyrine (internal standard) and (5) 4-hydroxyantipyrine. (B) Chromatogram of a 10-μl injection of a urine sample spiked with the same drugs and subsequently extracted as described in the text.

duced erratic results in the assays of 4OHA and NORA. Similarly, these two compounds proved to be unstable if stored in methanol standard solutions in a warm place for over 24–48 h. The use of nitrogen rather than air in evaporation of the organic phase also improved the precision of our method.

Using plastic tubes or silanized glass tubes during the extraction, presumably to avoid adsorption, proved to be of no additional value.

Different UV wavelengths (200–500 nm) were tried to obtain optimal peaks. A wavelength of 254 nm produced the best peaks.

We also studied the stability of the four compounds and of the internal standard after extraction, reconstitution in methyl alcohol and storage at room temperature. Under these conditions NORA was unstable and showed a degradation of 6% within 40 min, 18% within 125 min and 26% by 260 min. HMA was stable for 90 min but showed 12% degradation by 260 min. AP, amino-AP and 4OHA showed no degradation even by 260 min.

Evaluation of method

Correlation coefficients for the standard curves (Fig. 2) were 0.9996, 0.9998, 0.9999 and 0.9998 for HMA, NORA, AP and 4OHA, respectively. Coefficients of variation for low, middle and high range values were calculated for AP and each of its metabolites and were less than 4.2% in all cases (range 1.2–4.1%). The previously reported methods had coefficients of variation as high as 5.5–10.2%

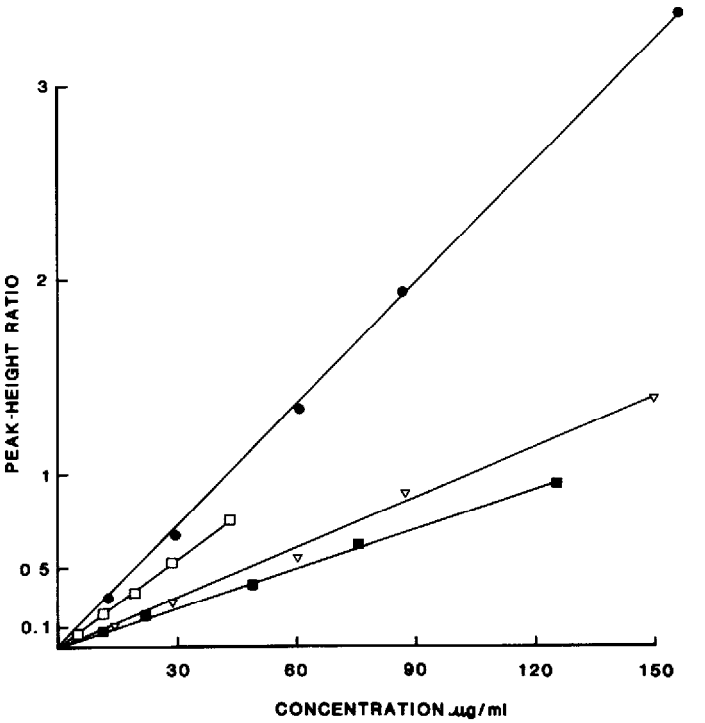

Fig. 2. Peak-height ratio of drug to internal standard versus concentration. (■) Hydroxymethylantipyrine ($r=0.9996$); (●) norantipyrine ($r=0.9998$); (□) antipyrine ($r=0.9999$); (▽) 4-hydroxyantipyrine ($r=0.9998$).

TABLE I

ACCURACY IN THE DETERMINATION OF ANTIPYRINE AND ITS METABOLITES

HMA		NORA		AP		4OHA	
Added concentration (µg/ml)	Relative recovery* (%)	Added concentration (µg/ml)	Relative recovery (%)	Added concentration (µg/ml)	Relative recovery (%)	Added concentration (µg/ml)	Relative recovery (%)
8	93	10	105	3	97	10	113
24	100	30	98	9	99	33	101
48	101	60	101	18	100	60	94
72	102	90	99	27	101	90	102
120	99	150	100	45	102	150	100

*See text for definition.

[9–14]. Absolute recovery was 20, 89, 66, 69 and 87% for HMA, NORA, AP, amino-AP and 4OHA, respectively. Relative recoveries for HMA, NORA, AP and 4OHA at all ranges of assay are shown in Table I. The relative recoveries were within ±5% of 100% in seventeen out of the twenty samples. Of the previously published methods only one article [11] reported relative recovery data (as de-

fined in the Experimental section). In this method relative recovery values fell outside ±5% limit in approximately 50% of the assays performed on AP, 4OHA, NORA and HMA. Relative recoveries as high 127% and as low as 88% of actual added concentrations were also obtained [11].

When compared with the above methods our method has the following advantages: (1) it utilizes a simple, single-step extraction; (2) it does not require silanization; (3) it utilizes amino-AP as an internal standard; (4) it has the highest documented accuracy of the reported methods; (5) it has higher precision than the previously documented methods.

ACKNOWLEDGEMENTS

Dr. Mikati was supported by the Merritt-Putnam fellowship of the Epilepsy Foundation of America. Supported in part by the Veterans Administration.

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