Journal of Chromatography, 426 (1988) 212–215
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4066

Note

Quantitation of acetaminophen and its metabolites in rat plasma after a toxic dose

VIJAY O. BHARGAVA*, STEVEN EMODI and JUNJI HIRATE

Department of Pharmaceutical Sciences, School of Pharmacy, University of Nebraska Medical Center, 42nd and Dewey Avenue, Omaha, NE 68105 (U.S.A.)

(First received July 21st, 1987; revised manuscript received November 25th, 1987)

Acetaminophen (A), N-acetyl-*p*-aminophenol, is a widely used analgesic and antipyretic drug. Large doses of A cause acute dose-dependent hepatic necrosis in rats, mice, hamsters and man [1–4]. Damage to the liver caused by A is thought to be a result of the conversion of a fraction of the dose to chemically reactive metabolites which can bind to cellular macromolecules [5]. It has been observed that reactive metabolites of A are detoxified by conjugation to glutathione and cause extensive depletion of glutathione in the liver of susceptible animal species [3,6,7]. Drug glutathione conjugates are excreted intact or after metabolism in vivo to mercapturic acids [8]. Hence, the extent of conversion of A to the mercapturic acid should be an index of the activity of the metabolic pathway leading to the formation of the toxic metabolite.

A number of high-performance liquid chromatographic (HPLC) methods for measuring A and its two major metabolites, acetaminophen glucuronide (AG) and acetaminophen sulfate (AS) [9–11], have appeared in the literature. A few assays are also available to quantitate the mercapturic acid metabolite of acetaminophen (AM) [8,14,15]. The assays reported by Colin et al. [14] and Jollow et al. [8] are applicable only for urine samples. Fisher et al. [15] used radioactivity to quantitate A and did not have adequate sensitivity to carry out detection at low levels of mercapturic acid metabolite in plasma. In view of these factors, we describe the development of a rapid and sensitive method for the detection of A and its major metabolites in plasma after administration of high doses that can cause hepatic necrosis. This assay can also be used when working with the neonatal and pediatric population or in small laboratory animals (e.g. rats).

0378-4347/88/$03.50 © 1988 Elsevier Science Publishers B.V.

EXPERIMENTAL

Materials

A and the internal standards (theophylline and theobromine) were obtained from Sigma (St. Louis, MO, U.S.A.). AG and AS were generously donated by Dr. Ray Galinsky of the University of Utah (Salt Lake City, UT, U.S.A.) AM was generously donated by Dr. Sid Nelson of the University of Washington (Seattle, WA, U.S.A.) Methanol, HPLC grade, was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). All the other reagents, analytical grade or better, were purchased from commercial sources and were used without further purification.

Instrumentation

The liquid chromatograph (Model M-45, Waters Assoc.) was equipped with a variable-wavelength UV detector (Model 481, Waters Assoc.) and separation was achieved using a C_{18} reversed-phase column (250 mm × 4.6 mm I.D., particle size 5 μm, Ultrasphere ODS, Beckman) with a C_{18} guard column (32 mm × 4 mm I.D., particle size 7–50 μm, Waters Assoc.). The mobile phase was composed of 0.75% (v/v) glacial acetic acid and 5% (v/v) methanol in a 0.05 *M* potassium dihydrogenphosphate buffer (pH of the mobile phase was adjusted to 5.5 with 10% potassium hydroxide). Samples were injected into a Rheodyne injector (Model 7125). The separation was carried out at ambient temperature at a flow-rate of 2.0 ml/min and a chart speed of 2.0 cm/min. The effluent was monitored at 242 nm.

Analysis of plasma samples

To 50 μl of plasma were added 10 μl of 25 μg/l theophylline solution and 80 μl of a 0.236 μg/l theobromine solution in 10% perchloric acid. The mixture was made up to 300 μl with 10% perchloric acid. The sample was vortexed for 30 s and then centrifuged at 13 000 *g* for 2 min to pellet the precipitated proteins. A 200-μl aliquot of the supernatant was then injected into the chromatographic system.

Calibration curves

Calibration curves were constructed by using blank plasma to which increasing quantities of A and its metabolites were added. The concentration range for A was 2.5–250 μg/ml, for AG and AS 2.5–150 μg/ml and for AM 2.5–80 μg/ml. Standards stored in the refrigerator were found to be stable for a period of up to six weeks. Peak-height ratios of A, AG and AS to the internal standard, theobromine, and peak-height ratio of AM to the internal standard, theophylline, were used to construct the standard curve. All standard curves were calculated by the least-squares regression analysis of peak-height ratios versus drug concentration.

RESULTS AND DISCUSSION

Using the described chromatographic conditions A, AG, AS, AM and the internal standards, theobromine and theophylline, yielded sharp and well resolved peaks with no interference from endogenous compounds at 8.3, 3.4, 6.3, 23.0, 12.1

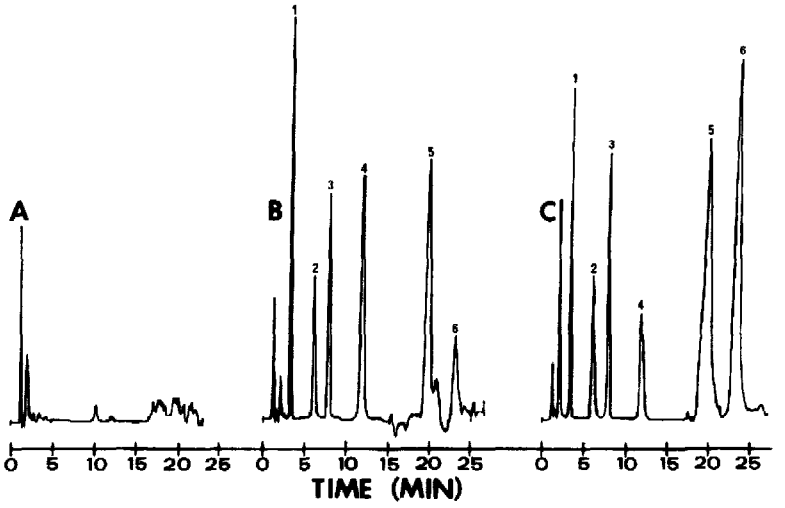

Fig. 1. Chromatogram of (A) blank plasma, (B) plasma spiked with AG (1), AS (2), A (3), theobromine (4), theophylline (5) and AM (6) and (C) rat plasma 40 min following intravenous injection of 400 mg/kg A in the rat. Concentrations are 35.7, 50 7, 52.3 and 72.3 μg/ml for AG, AS, A and AM, respectively.

and 20.3 min, respectively. Representative chromatograms of plasma spiked with A and its metabolites and of plasma taken before and after intravenous injection of A to a rat are shown in Fig. 1.

The standard curves, based on peak-height ratios of the drug and its metabolites to the internal standards, were all linear and highly reproducible. The intra-assay statistics for the standard curves generated are reported in Table I.

The correlation coefficients for all standard curves were 0.985 or better. The within-day coefficient of variation performed on eight samples at two different drug and metabolite concentrations were 6.0, 2.8, 1.8 and 5.1% for A, AG, AS and AM, respectively. (The two concentrations for A, AG and AS were 10 and 150 μg/ml, respectively, and 10 and 80 μg/ml for AM). The day-to-day coefficients of variation performed on five consecutive days for these concentrations of drug

TABLE I

INTRA-ASSAY STATISTICAL EVALUATION

Compound	Concentration range (μg/ml)	Coefficient of variation (%)		Regression equation*
		Mean	Range	
A	2.5–250	5.25	4.5- 6.0	$y=0.05x-0.132$
AG	2.5–150	4 05	2.8- 5.3	$y=0.08x+0.233$
AS	2.5–150	2.10	1.3- 2.3	$y=0.03x+0.048$
AM	2.5- 80	8.10	5.1–10.3	$y=0.03x+0.041$

*x = concentration of compounds; y = peak-height ratio.

and metabolites were 4.5, 5.3, 2.2 and 4.6% for A, AG, AS and AM, respectively. The minimum detectable concentration, determined by injecting serial dilutions of stock solutions, was 0.5 μg/ml for A, AS and AG and 5.0 μg/ml for AM.

It was decided to use two different internal standards for the assay because the peak height of AM was much smaller than the peak heights of the other drugs at similar concentrations. In order to keep the peak-height ratios of drug to the internal standard comparable, we used theobromine as the internal standard for A, AG and AS and theophylline as the internal standard for AM.

Consideration of the time course and mechanism of A-induced hepatic necrosis is hampered by a lack of knowledge concerning the disposition of a toxic dose of the drug. Concentrations of the mercapturic acid metabolite of A in plasma at various times after a toxic dose have not been reported. Knowing the disposition kinetics of the mercapturic acid metabolite, it may be possible to predict its accumulation in various tissues such as the liver and kidney. Knowledge of the relative predominance of drug and individual metabolites in hepatic and renal tissues may be important because these substances may influence development of the metabolically linked necrosis which occurs after a time in the A-poisoned animal.

The HPLC assay procedure developed for the determination of A and its metabolites, AG, AS and AM, in plasma is simple, rapid and reproducible for use in routine drug monitoring and pharmacokinetic studies. Detection of the mercapturic acid metabolite in plasma would help in elucidating the hepatotoxic effects of A at high doses.

REFERENCES

1 E.M. Boyd and G.M. Bereczky, Br. J. Pharmacol., 26 (1966) 606.
2 J.R. Mitchell, D.J. Jollow, W.Z. Potter, D.C. Dairs, J.R. Gillette and B.B. Brodie, J. Pharmacol. Exp. Ther., 187 (1973) 185.
3 W.Z. Potter, S.S Thorgeirsson, D.J. Jollow and J.R. Mitchell, Pharmacology, 12 (1974) 129.
4 L.F. Prescot, N. Wright, P. Roscoe and S.S. Brown, Lancet, i (1971) 519.
5 D.J. Jollow, J.R. Mitchell, W.Z. Potter, D.C. Dans, J.R. Gillette and B.B. Brodie, J. Pharmacol. Exp. Ther., 187 (1973) 195.
6 D.C. Dairs, W.Z. Potter, D.J Jollows and J.R. Mitchell, Life Sci., 14 (1974) 2099.
7 J.R. Mitchell, D.J. Jollow, W.Z. Potter, J.R. Gillette and B.B. Brodie, J. Pharmacol. Exp. Ther , 187 (1973) 211
8 D.J. Jollow, S.S. Thorgeirsson, W.Z. Potter, M. Hashimoto and J.R. Mitchell, Pharmacology, 12 (1974) 251.
9 D. Howie, P.I. Adriaenssens and L.F. Prescot, Br. J. Clin. Pharmacol., 29 (1977) 235.
10 J.M. Knox and J. Jurand, J. Chromatogr., 149 (1978) 297.
11 P.I. Adriaenssens and L.F. Prescot, Br. J. Clin. Pharmacol., 6 (1978) 87.
12 J.M. Wilson, J.T. Slattery, A.J. Forte and S.D. Nelson, J. Chromatogr., 227 (1982) 453.
13 D. Jung and N.U. Zafar, J. Chromatogr., 339 (1985) 198.
14 P. Colin, G. Sirois and S. Chakrabarti, Arch. Int. Pharmacodyn. Ther., 281 (1986) 281.
15 L.J. Fischer, M.D. Green and A.W. Harman, J. Pharm. Exp. Ther., 219 (1981) 281.