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Note

Micro high-performance liquid chromatographic assay of acetaminophen and its major metabolites in plasma and urine*

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Acetaminophen, N-acetyl-*p*-aminophenol, is a commonly used analgesic and antipyretic substitute for aspirin. Acute acetaminophen overdose has been associated with hepatoxicity [1, 2]. Recent studies [3, 4] on the metabolism of acetaminophen have shown that the adverse effects are due to a quantitatively minor reactive metabolite which is formed in parallel with the major metabolites, acetaminophen glucuronide and acetaminophen sulfate. The pharmacokinetics of acetaminophen and its subsequent formation to these two conjugates may affect the rate of formation of the reactive metabolite and thus, the potential toxicity of acetaminophen.

Recently, a number of high-performance liquid chromatographic (HPLC) methods for measuring acetaminophen in biological fluids have appeared in the literature [5—10]. However, only a few are available for simultaneously measuring acetaminophen and its two major metabolites, acetaminophen glucuronide and acetaminophen sulfate [11—15]. Since all of these procedures require at least 1 ml of plasma or are applicable only for urine samples, pharmacokinetic and metabolic studies in acute acetaminophen overdosage have not been completed in the pediatric population since plasma samples of 1—2 ml cannot be repeatedly obtained from these subjects unlike the situation in adults [1, 11, 16, 17]. Recently, an ion-pair reversed-phase method requiring 0.1 ml plasma or urine has been described [18]. In view of these factors, we describe the development of a rapid, sensitive and selective method for the detection of acetaminophen and its major metabolites in plasma and urine which may be

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used when working with the neonatal and pediatric population or in small laboratory animals (e.g. rats).

EXPERIMENTAL

Materials

Acetaminophen and the internal standard, theophylline, were obtained from Sigma (St. Louis, MO, U.S.A.). Acetaminophen glucuronide and acetaminophen sulfate were generously donated by Dr. Josiah Tam of McNeil Consumer Products (Fort Washington, PA, U.S.A.). All other reagents, analytical grade or better, were purchased from commercial sources and were used without further purification.

Apparatus

A Beckman (Berkeley, CA, U.S.A.) liquid chromatograph equipped with a Model 100 A solvent delivery system, a Model 160 ultraviolet detector and a Model 210 injector with a 20-μl sample loop was used. Separations were performed at ambient temperature on a 25 cm × 4.5 mm I.D. octadecyl (5 μm particle size) column (I.B.M., Danbury, CT, U.S.A.). The mobile phase consisted of 7% acetonitrile—0.05 *M* sodium sulfate buffer adjusted to pH 2.2 with 85% phosphoric acid. All water was Milli-Q grade (Millipore, Bedford, MA, U.S.A.). A flow-rate of 1.5 ml/min was used and the eluent monitored at 254 nm at attenuations between 0.05 and 0.2 a.u.f.s. Detector output was quantitated using a strip chart recorder (Model 261, Linear Instruments, Irvine, CA, U.S.A.) set at 10 cm/h.

Analysis of plasma samples

To 50 μl plasma were added 100 μl of a 20 mg/l theophylline (as internal standard) solution in 6% perchloric acid in 0.5-ml polypropylene microcentrifuge tubes. The mixture was vortex-mixed for 5 sec and centrifuged at 13 000 *g* for 2 min to pellet the precipitated proteins. A 5-μl aliquot of the supernatant is injected into the chromatographic system.

Analysis of urine samples

Urine samples were diluted ten-fold with distilled water prior to analysis. To 50 μl urine were added 20 μl of an aqueous 200 mg/l theophylline solution. The mixture was treated in the same fashion as for the plasma samples and injected into the same chromatographic system.

Calibration curves

Standard curves were prepared by using plasma or diluted urine to which known amounts of acetaminophen and its metabolites were added to yield final concentrations between 5 and 200 mg/l. The standards were stored at −70° C and used for the next twelve weeks. Peak height ratios of acetaminophen and its metabolites to the internal standard, theophylline, were used to construct the standard curves. All standard curves were calculated by a least-squares linear regression analysis of peak height ratios versus drug concentration.

RESULTS AND DISCUSSION

Using the described chromatographic conditions, acetaminophen, acetaminophen glucuronide, acetaminophen sulfate and the internal standard, theophylline, yielded very sharp and well resolved peaks with no interference from endogenous compounds at 3.2, 4.2, 5.7 and 7.3 min, respectively. Representative chromatograms of plasma and urine taken before and after intravenous injection of acetaminophen to a rat are shown in Fig. 1.

The standard curves based on peak height ratios of the drug and its metab-

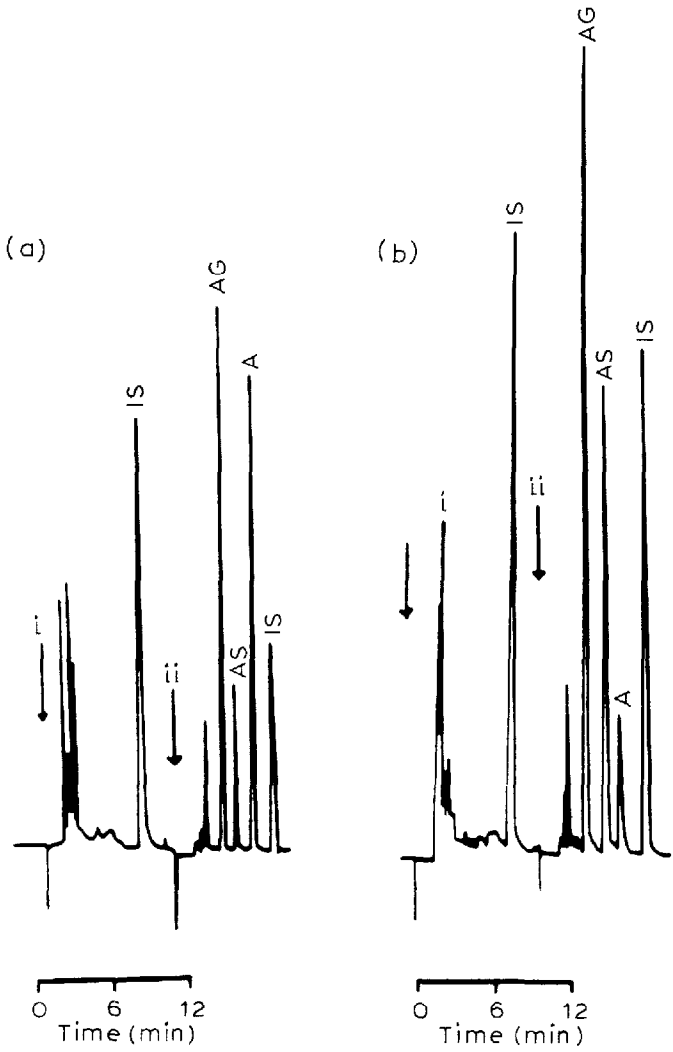

Fig. 1. (a) Chromatogram of acetaminophen (A) and its metabolites, acetaminophen glucuronide (AG) and acetaminophen sulfate (AS) in rat plasma. Range = 0.1 a.u.f.s. Arrow indicates when injection was made. (i) Blank rat plasma containing the internal standard (IS), theophylline; (ii) rat plasma 2 h following intravenous injection of 100 mg/kg acetaminophen. (b) Chromatogram of A, AG, AS and IS in ten-fold diluted rat urine; (i) blank rat urine; (ii) 24-h collection of rat urine.

olites to internal standard were all linear and highly reproducible. Eleven standard curves in plasma and eight in urine with acetaminophen and its metabolites were made at different times over a period of twelve weeks. The day-to-day coefficients of variation in the slope of the calibration curves were 4.1%, 11.5% and 7.7% for acetaminophen, acetaminophen glucuronide and acetaminophen sulfate in plasma, respectively. The respective day-to-day coefficients of variation in urine were 3.2%, 0.7% and 3.3%. The within-day coefficients of variation performed on six samples at three different drug and metabolite concentrations between 5 and 100 mg/l were 3.7%, 1.7% and 3.7% in plasma and 3.0%, 2.7% and 9.0% in urine for acetaminophen, acetaminophen glucuronide and acetaminophen sulfate, respectively.

Analytical recovery studies comparing spiked plasma and urine with aqueous standards over a concentration range of 5—200 mg/l showed an average recovery of 98.2%, 92.2% and 87.4% from plasma and 100.3%, 97.3% and 92.6% from urine for acetaminophen glucuronide, acetaminophen and acetaminophen sulfate, respectively.

Only a few methods are available for simultaneously measuring acetaminophen and its two major metabolites, acetaminophen glucuronide and acetaminophen sulfate in biological fluids [11—15]. Recently, a method [18] was reported which determined acetaminophen and its two major metabolites in plasma, urine and bile. Although that method uses a deproteinization procedure similar to that described herein, it requires 0.1 ml of biological fluid, the use of an ion-pair reagent (tetra-*n*-butylammonium hydroxide) and a variable-wavelength detector set at 250 nm. In our method, since only 5 μl of the supernatant solution (equivalent to 3 μl plasma or 0.35 μl urine) is injected onto the column, a guard column was not used. Over a period of more than a year, over 1000 plasma and urine samples have been assayed without significant deterioration of the column. With a proper column cleaning procedure, the column life is expected to last over a year with normal use.

The HPLC assay procedure developed for the determination of acetaminophen and its two major metabolites, acetaminophen glucuronide and acetaminophen sulfate in plasma and urine is simple, rapid, selective and reproducible for use in routine drug monitoring and pharmacokinetic studies. In addition to its applicability to biological fluids from the rat, the method described has been used to determine the concentrations of acetaminophen and its major metabolites in plasma and urine following single 600-mg oral doses of acetaminophen to normal human subjects [19]. Essentially no interference from endogenous compounds has been observed in chromatograms from human urine or plasma. Practically, it is possible to determine acetaminophen and its metabolites in as little as 5 μl of plasma or urine if the concentration is above 1 mg/l. The small sample size required for this method is especially important when dealing with neonates or small animals (e.g. rats) where samples are difficult to obtain and volume is limited. As a result of the choice of internal standard, the assay described herein can be used to determine theophylline concentrations in plasma and urine without an extraction procedure using acetaminophen as the internal standard.

REFERENCES

1 L.F. Prescott, N. Wright, P. Roscoe and S.S. Brown, Lancet, i (1971) 519.
2 E.M. Boyd and G.M. Bereczky, Brit. J. Pharmacol., 26 (1966) 606.
3 J.R. Mitchell, S. Thorgeirsson, W.Z. Potter, D.J. Jollow and H. Keiser, Clin. Pharmacol. Ther., 16 (1974) 676.
4 J.A. Hinson, Rev. Biochem. Toxicol., 2 (1980) 103.
5 C.G. Fletterick, T.H. Grove and D.C. Hohnadel, Clin. Chem., 25 (1979) 409.
6 L.Y. Lo and A. Bye, J. Chromatogr., 173 (1979) 198.
7 D. Blair and B.H. Rumack, Clin. Chem., 23 (1977) 743.
8 B. Ameer, D.J. Greenblatt, M. Divoll, D.R. Abernethy and L. Shargel, J. Chromatogr., 226 (1981) 224.
9 R.A. Horvitz and P.I. Jatlow, Clin. Chem., 23 (1977) 1596.
10 L.T. Wong, G. Solomonrajal and B.H. Thomas, J. Pharm. Sci., 65 (1976) 1064.
11 D. Howie, P.I. Adriaenssens and L.F. Prescott, J. Pharm. Pharmacol., 29 (1977) 235.
12 J.H. Knox and J. Jurand, J. Chromatogr., 149 (1978) 297.
13 J.E. Mrochek, S. Katz, W.H. Christie and S.R. Dinsmore, Clin. Chem., 20 (1974) 1086.
14 P.I. Adriaenssens and L.F. Prescott, Brit. J. Clin. Pharmacol., 6 (1978) 87.
15 J.M. Wilson, J.T. Slattery, A.J. Forte and S.D. Nelson, J. Chromatogr., 227 (1982) 453.
16 M. Davis, C.J. Simmons, N.G. Harnson and R. Williams, Q.J. Med., 45 (1976) 181.
17 J.T. Slattery and G. Levy, Clin. Pharmacol. Ther., 25 (1979) 185.
18 N. Watari, M. Iwai and N. Kaneniwa, J. Pharmacokin. Biopharm., 11 (1983) 245.
19 T. Ahmad, personal communication.