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Determination of Acetaminophen and Phenacetin in Plasma by High-Pressure Liquid Chromatography

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We describe a sensitive and precise high-pressure liquid chromatographic method in which acetoacetanilide is used as the internal standard to simultaneously determine acetaminophen and phenacetin in plasma. Therapeutic as well as toxic concentrations can be determined on as little as 0.1 ml of plasma. Sample preparation is rapid and chromatography is complete in 5 min. Quantitation is accurate at 0.5 mg/liter concentration for both drugs. Day-to-day precision within 5% is attainable. Of 36 other drugs tested, only theophylline interfered, with the determination of acetaminophen.

Acetaminophen and phenacetin are commonly used aspirin alternatives. Although either drug is usually well tolerated when used at the recommended dose, large doses have been associated with lethal hepatic necrosis and renal failure (*1, 2*). In Great Britain, acetaminophen poisoning represents one of the most common causes of hepatic failure (*2*).

In 1948 Brodie and Axelrod (*3*) described the first practical spectrophotometric method for the determination of acetaminophen. Since then, other spectrometric methods for acetaminophen have been described (*4, 5*). Spectrophotometric methods, however, are subject to interference by other drugs. Gas–liquid chromatographic methods have been described in an attempt to increase specificity (*6, 7*). Recently, high-pressure liquid chromatographic (HPLC) methods have been introduced. Burtis et al. (*8*) in 1970 described a method in which anion-exchange chromatography is used to study the metabolites of phenacetin in urine. Similarly, Mrochek et al. (*9*) in 1974 studied acetaminophen metabolites in blood and urine. These studies, however, were intended for metabolic investigations and were not proposed for routine use, inasmuch as specialized instrumentation was developed for both methods. Riggin et al. (*10*) described a HPLC method for use with biological fluids; however, this method involves a lengthy extraction procedure requiring 3 ml of serum and makes use of a less common electrochemical detector. Wong et al. (*11*) developed a HPLC method for determining concentrations of acetaminophen in blood, but it requires 1 ml of plasma, does not include internal standardization, does not measure phenacetin, and potential interference by other drugs was not considered. Duggin (*12*) described an HPLC method to measure phenacetin in blood, however, acetaminophen was not measured simultaneously, internal standardization was not used, and potential interference from other drugs was not discussed.

We describe here an HPLC method that simultaneously determines unconjugated acetaminophen and phenacetin in serum or plasma. The procedure requires as little as 0.1 ml of sample, the extraction technique is rapid, and internal standardization is incorporated. Therapeutic and toxic concentrations of both drugs in blood can be accurately and precisely measured. Interference by other drugs was investigated.

Materials and Method

Instrumentation

We used a Model 601 liquid chromatograph with a Model LC-55 variable wavelength detector (Perkin-Elmer Corp., Norwalk, Conn. 06856) with a 1-mV recorder. We used 30 cm × 4 mm (i.d.) column of silica beads chemically bonded with octadecyl trichlorosilane (μ Bondapack C18; Waters Associates Inc., Milford, Mass., 01757). The detector was set at 254 nm. The column was eluted with acetonitrile/phosphate buffer (pH 4.4) mixture (19/81 by vol) at a flow rate of 3 ml/min. Oven temperature was maintained at 50 °C.

Reagents

Acetonitrile, "ultraviolet" grade (Burdick and Jackson Laboratories Inc., Muskegon, Mich. 49442.)

Phosphate buffer (pH 4.4) was prepared by adding 0.3 ml of 1 mol/liter potassium dihydrogen phosphate and 0.05 ml of 4.4 mol/liter phosphoric acid to 1800 ml of distilled water.

Drug standard. The standard solution was prepared by dissolving acetaminophen (200 mg), acetoacetanilide (400 mg), and phenacetin (200 mg) in 1 liter of methanol.

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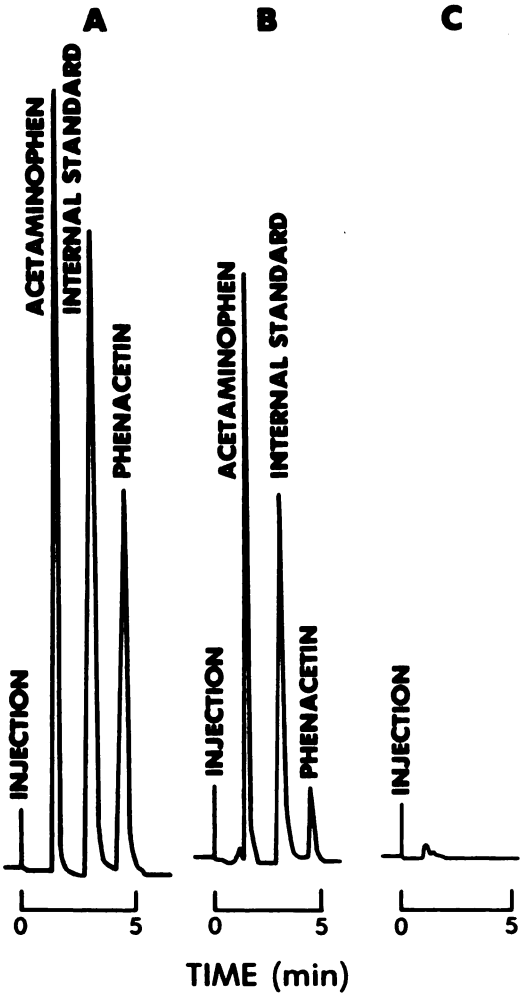

Fig. 1. Some representative chromatograms

A, the drug reference standard; *B*, plasma containing 32 mg of acetaminophen and 3 mg of phenacetin per liter; *C*, a drug-free plasma

Table 1. Recovery of Acetaminophen and Phenacetin from Blood Plasma

Drug added	Drug recovered	Recovered	Recovery range
mg/liter		%	
	Acetaminophen		
200	197.8	98	97–102
100	99.8	100	96–109
50	49.5	99	94–109
20	19.6	97	94–102
10	10.0	100	98–106
5	5.1	102	94–102
	Phenacetin		
20	20.6	103	99–110
10	10.2	102	98–103
5	4.9	98	92–104
2	1.9	95	92–100
1	0.9	96	90–100

Table 2. Precision of Plasma Analysis

	Acetaminophen		Phenacetin	
	Within run			
Mean, mg/liter	22.1	46.1	2.1	5.1
SD, mg/liter	0.41	1.5	0.11	0.17
CV, %	1.9	3.4	5.4	3.5
n	10	10	10	10
	Day-to-day			
Mean, mg/liter	10.1	49.2	2.2	5.2
SD, mg/liter	0.58	1.71	.075	0.17
CV, %	5.7	3.5	3.5	3.3
n	17	17	17	17

Internal standard. A stock solution was prepared by dissolving 500 mg of acetoacetanilide in 1 liter of methanol. A working internal standard was prepared daily by diluting the stock standard 10-fold in water.

All drugs were purchased from Sigma Chemical Co., St. Louis, Mo. 63178.

Procedure

Transfer 0.5 ml of plasma, 0.5 ml of working internal standard, and 0.5 ml of 1 mol/liter phosphate buffer (pH 7.0) to a 12-ml stoppered tube. Add 7 ml of ethyl acetate and shake the tube for 10 min on a mechanical shaker. Centrifuge the tube for 5 min. Decant the ethyl acetate and evaporate it at 70 °C under reduced pressure in a rotary evaporator. Dissolve the residue in 50 μl of methanol and inject 10 to 20 μl onto the column.

Results

Figure 1*A* illustrates a typical chromatogram for the drug standard. Figure 1*B* illustrates a plasma containing acetaminophen (32 mg/liter) and phenacetin (3 mg/liter). Figure 1*C* illustrates a typical chromatogram of a drug-free plasma.

Sensitivity. Acetaminophen and phenacetin are detected and reproducibly quantitated at concentrations of 0.5 mg/liter of blood.

Linearity. Acetaminophen and phenacetin were added to a drug-free plasma in amounts equivalent to 0.5 mg to 400 mg/liter for acetaminophen and 0.2 mg to 40 mg/liter for phenacetin. A constant amount of internal standard was added to each sample and processed as described. Concentration and peak height were linearly related over the stated ranges.

Analytical recovery. Known amounts of acetaminophen and phenacetin in methanol were added to drug-free plasma and processed as described. At least five samples were processed at each concentration over a range of 0.5 to 200 mg/liter for acetaminophen and 0.5 to 20 mg/liter for phenacetin. Analytical recoveries are tabulated in Table 1. Absolute recovery exceeded 80% for both drugs.

Precision. Within-run precision was evaluated by processing aliquots of a pooled plasma containing acetaminophen and phenacetin at two concentrations for each drug. Day-to-day precision was similarly evaluated on consecutive days. Precision data are tabulated in Table 2.

Table 3. Retention Times for Selected Drugs

Drug	Retention time, min	Drug	Retention time, min
Acetaminophen	1.5	Salicylamide	2.6
Acetoacetanilide	3.4	Nicotine	2.6
Phenacetin	5.0	Theophylline	1.6
Phenytoin	11.3	Glutethimide	11.4
Carbamazepine	13.4	Methaqualone	19.3
Mesantoin	6.7	Codeine	N.D.[a]
Phensuximide	5.0	Methapyrilene	N.D.
Methsuximide	8.4	Phenylpropanol-amine	N.D.
Ethosuximide	2.2	Quinidine	N.D.
Primidone	2.8	Chlorophenaramine	N.D.
Phenobarbital	4.4	Propoxyphene	N.D.
Amobarbital	10.1	Carisoprodal	N.D.
Butabarbital	5.0	Pseudoephredrine	N.D.
Pentobarbital	9.4	Scopalamine	N.D.
Secobarbital	12.9	Diazepan	N.D.
Mephobarbital	9.6	Oxazepam	N.D.
Salicylate	1.6	*N*-Desmethyldi-azepam	N.D.
Caffeine	2.1	Chlorodiazepoxide	N.D.

[a] Not detected.

Interference. Potential interference caused by other drugs was studied by chromatographing each drug in methanol. Any drug that eluted sufficiently close to acetaminophen, phenacetin, or the internal standard was further studied by extracting known amounts of the interfering drug from plasma and evaluating the quantitative effect. Table 3 lists retention times for the drugs studied. Only two drugs, salicylate and theophylline, interfered. Salicylate co-elutes with acetaminophen, but insignificant amounts of salicylate are extracted by the procedure described (a plasma salicylate concentration of 333 mg/liter will increase the apparent plasma acetaminophen concentration by 1 mg/liter). Theophylline also co-elutes with acetaminophen. Theophylline presents a potential interference because a therapeutic concentration of theophylline (23 mg/liter) will increase the apparent acetaminophen concentration by 7 mg/liter. Butabarbital and phensuximide co-elute with phenacetin, but neither of these drugs have a significant effect on the phenacetin quantitation (a 200 mg/liter plasma concentration of either drug will increase the apparent plasma phenacetin concentration by no more than 20 μg/liter).

Comparison with a colorimetric method. As an assessment of the suitability of this HPLC method for the determination of acetaminophen, it was compared to the method of Glynn and Kendal (*13*). The regression analysis of the two methods were: $r = 0.989$, slope = 0.996, the y-intercept was 7.17, and n = 30. The y-axis was this HPLC method and the x-axis the method of Glynn and Kendal.

Discussion

This method easily provides the necessary sensitivity to quantitate therapeutic as well as toxic blood concentrations of acetaminophen and phenacetin simultaneously. High-pressure liquid chromatography appears to be more suitable than gas–liquid chromatography for measurement of these drugs because derivatization is not necessary. Sample processing is rapid; it involves few steps and requires as little as 0.1 ml of serum or plasma. Chromatography is complete in 5 min; the entire procedure requires no longer than 40 min. Other drugs commonly used in combination with acetaminophen and phenacetin do not interfere.

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References

1. McJunkin, B., Barwick, K. W., Little, W. C., and Winfield, J. B., Fatal massive hepatic necrosis following acetaminophen overdose. *J. Am. Med. Assoc.* **23,** 1874 (1976).

2. Boyer, T. D., and Rouff, S. L., Acetaminophen-induced hepatic necrosis and renal failure. *J. Am. Med. Assoc.* **218,** 440 (1971).

3. Brodie, B. B., and Axelrod, J., The estimation of acetanilide and its metabolic products, aniline, *N*-acetyl-*p*-aminophenol and *p*-aminophenol (free and conjugated) in biological fluids and tissues. *J. Pharmacol. Exp. Ther.* **94,** 22 (1948).

4. Knepil, J., A sensitive, specific method for measuring *N*-acetyl-*p*-aminophenol (Paracetamol) in blood. *Clin. Chim. Acta* **52,** 369 (1974).

5. Routh, J. I., Sane, N. A., Arredondo, E. G., and Paul, W. D., Determination of *N*-acetyl-*p*-aminophenol in plasma. *Clin. Chem.* **18,** 882 (1968).

6. Street, H. V., Estimation and identification in blood of paracetamol (*N*-acetyl-*p*-aminophenol) in the presence of barbiturates. *J. Chromatogr.* **109,** 29 (1975).

7. Dechtiaruk, W. A., Johnson, G. A., and Solomon, H. M., Gas-chromatographic method for acetaminophen (*N*-acetyl-*p*-aminophenol) based on sequential alkylation. *Clin. Chem.* **22,** 879 (1976).

8. Burtis, C. A., Butts, W. C., and Rainey, W. T., Jr., Separation of the metabolites of phenacetin in urine by high-resolution anion exchange chromatography. *Am. J. Clin. Pathol.* **53,** 769 (1970).

9. Mrochek, J. E., Katz, S., and Dinsmore, S. R., Acetaminophenmetabolism in man, as determined by high-resolution liquid chromatography. *Clin. Chem.* **20,** 1086 (1974).

10. Riggin, R. M., Schmidt, A. L., and Kissinger, P. T., Determination of acetaminophen in pharmaceutical preparations and body fluids by high-performance liquid chromatography with electrochemical detection. *J. Pharm. Sci.* **64,** 680 (1975).

11. Wong, L. T., Solomonra, J. G., and Thomas, B. M., High-pressure liquid chromatographic determination of acetaminophen in biological fluids. *J. Pharm. Sci.* **65,** 1064 (1976).

12. Duggin, G. G., Phenacetin estimation from biological samples by high-performance liquid chromatography. *J. Chromatogr.* **121,** 156 (1976).

13. Glynn, J. P., and Kendal, S. E., Paracetamol measurement. *Lancet* **i,** 1147 (1975).