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Note

Improved acetaminophen assay sensitivity by modification of a high-performance liquid chromatography technique

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Several assays for acetaminophen using high-performance liquid chromatography (HPLC) have been described. Some are suitable for analysis in pharmaceutical preparations, and others are suitable for analysis in biological fluids, but only at clinical to toxic concentrations of the drug [1—4]. This paper gives detailed performance specifications for a modification of the method of Fletterick et al. [1] which extends the assay sensitivity at least ten-fold to allow measurement of concentrations necessary for pharmacokinetic studies.

EXPERIMENTAL

Materials

Ethyl acetate, chloroform and heptane were from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water was distilled from an all-glass still. Acetaminophen (4-acetaminophenol) was obtained from Sigma (St. Louis, MO, U.S.A.) and 3-acetaminophenol from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals were of analytical reagent grade.

Sample preparation

One ml of plasma and 1 ml of 0.5 *M* phosphate buffer at pH 7.0 (KH_2PO_4—Na_2HPO_4) were added to test tubes containing 25 μg of 3-acetaminophenol (internal standard) and for standard curve calibrators, appropriate amounts of acetaminophen, in 550 μl of water. To each tube, 10 ml of ethyl acetate were added before vortexing for 1 min and then centrifuging for 5 min at 500 *g*. The organic phases were transferred to fresh test tubes and evaporated to dryness at 45°C under a nitrogen stream. These extracts were reconstituted in 200 μl of ethanol and vortexed for 30 sec before transfer to disposable poly-

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ethylene limited-volume inserts (Brinkman, Westbury, NY, U.S.A.) for the injection vials of the HPLC automatic sampler.

A slightly modified method for concentrations below 0.2 μg/ml uses 5 μg of internal standard, reconstitution in 50 μl of ethanol, and glass limited-volume inserts (Waters Assoc., Milford, MA, U.S.A.) for the automatic sampler vials.

Chromatography

The mobile phase was water-saturated chloroform—heptane—ethanol—acetic acid (225:700:75:1). Prior to use, it was degassed in an ultrasonic bath for 5 min. The column was washed with and stored in heptane when not in use.

The chromatographic system consisted of a 6000A pump with eluent flow-rate at 1.6 ml/min, a 710B automatic sample injector (both Waters Assoc.), a UV-50 ultraviolet detector set at 248 nm (Varian, Palo Alto, CA, U.S.A.) and a SP4100 computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.). The column was Spherisorb SI, 5 μm, 250 × 4.6 mm (Altex, Berkeley, CA, U.S.A.). The operating pressure was about 60 bar. The injection volume was 25 μl.

Quantitation

The standard curve was computed from samples spiked with acetaminophen concentrations of 0, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 μg/ml. An unweighted least-squares linear fit of peak area ratio versus concentration was used to estimate sample concentrations. Samples at 20 and 50 μg/ml have been run to confirm the linearity of the assay at higher concentrations. When samples between 0.1 and 0.2 μg/ml have been assayed, an additional standard at 0.1 μg/ml has been used. For samples containing well below 0.2 μg/ml, however, it is preferable to use the modified method described, which employs standards at 0, 0.02, 0.05, 0.1, 0.2 and 0.5 μg/ml of acetaminophen.

RESULTS

Table I shows linearity, precision and accuracy specifications for both of the procedures described. Fig. 1 shows chromatograms from plasma samples of a subject (a) before dosing and (b) 13 h after the last of a series of thirteen 6-hourly 650-mg oral doses of acetaminophen. These samples were processed by the normal range method and the chromatogram in Fig. 1b corresponds to a concentration of 0.17 μg/ml. Retention times for internal standard and acetaminophen are 6.2 and 8.8 min, respectively. Also shown in Fig. 1 are (c) a chromatogram from drug-free plasma processed without internal standard and (d) the same plasma spiked with 0.02 μg/ml of acetaminophen and processed with internal standard. Both of these latter samples were processed by the method for lower concentrations.

In drug-free plasma samples from twenty five different people, no peaks in the position of acetaminophen were observed at attenuations giving well defined, quantitative peaks from 0.1 μg/ml spiked samples. In addition, no interference with the assay was caused by aspirin or its metabolites in plasma after a 650-mg dose of aspirin, but higher levels of salicylate (>20 μg/ml) than encountered might interfere.

TABLE I

LINEARITY, PRECISION AND ACCURACY SPECIFICATIONS

Range*	Linearity**	Concentration of samples spiked for precision/bias tests*** (μg/ml)	Precision§	Bias§§
Normal	6.8 ± 1.8	0.2	8.1 (n=5, on 5 days)	−6.1 (n=5, on 5 days)
	Mean ± S.D.	1.0	3.6 (n=5, on 5 days)	−0.1 (n=5, on 5 days)
	(n=5 curves)	10.0	1.1 (n=5, on 5 days)	−0.0 (n=5, on 5 days)
Low	10.3	0.02	25.0 (n=4)	−6.3 (n=4)
	(n=1 curve)	0.05	8.6 (n=5)	−6.9 (n=5)
		0.1	5.2 (n=5)	+2.9 (n=5)

*Low range refers to the assay procedure modified for low concentrations.
**Coefficient of variation of concentration—normalized peak area ratios (%).
***Not standard curve calibrators but additional controls.
§ Coefficient of variation of determined concentration (%).
§§ Percent deviation of mean determination from amount spiked.

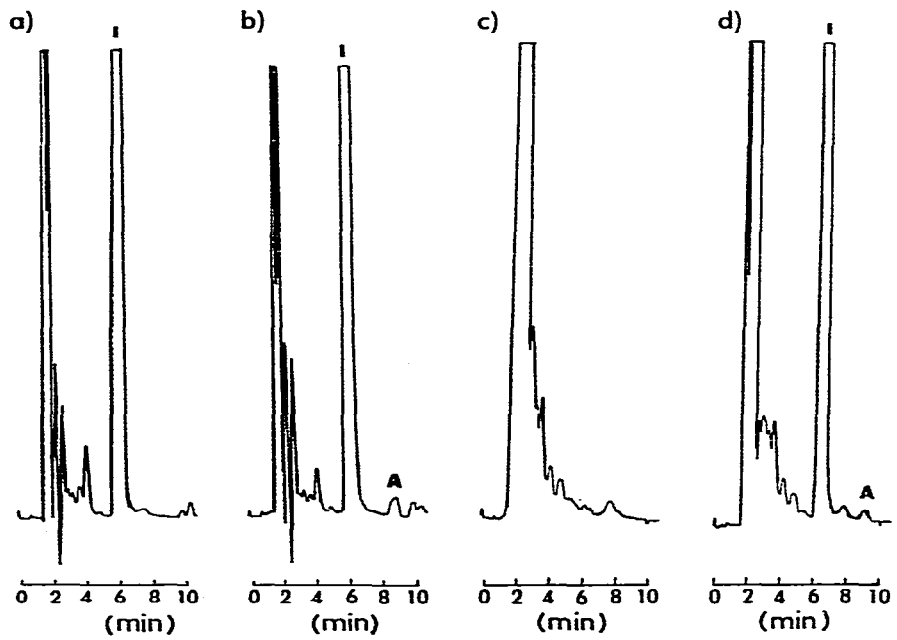

Fig. 1. Chromatograms from a subject (a) before dosing and (b) 13 h after the last of thirteen 6-hourly 650-mg acetaminophen doses, assayed by the first method described; and (c) from blank plasma without internal standard and (d) a 0.02 μg/ml spiked standard, assayed by the more sensitive method. The concentration of the sample yielding chromatogram (b) was 0.17 μg/ml. Peaks: I = 3-acetaminophenol, the internal standard; A = acetaminophen.

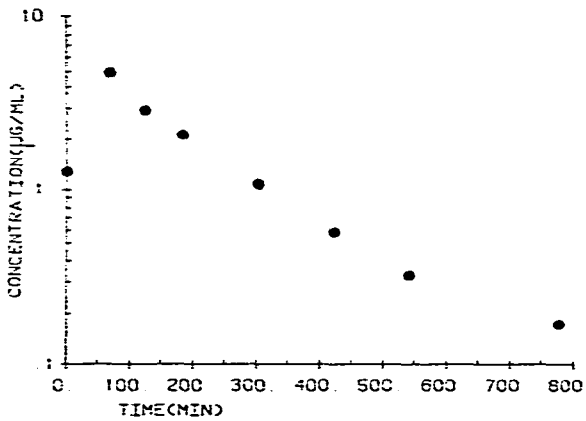

Fig. 2. Time course of plasma acetaminophen concentrations for 13 h following the last of thirteen 6-hourly 650-mg acetaminophen doses.

Fig. 2 shows a profile of plasma concentrations during the 13 h following the last acetaminophen dose referred to above. All samples were assayed by the normal range method.

DISCUSSION

The assay of Gotelli et al. [2] is sensitive enough to monitor acetaminophen at therapeutic levels. However, while the authors state that standards ranging from 0.5—400 µg/ml gave a linear response, no specifications are given relating to samples less than 5 µg/ml. Fletterick et al. [1] described their method also as being linear down to 0.5 µg/ml. However, the only specification including concentrations any lower than 5 µg/ml, is a correlation coefficient of 0.990 for samples ranging from 0.5—300 µg/ml when assayed by the HPLC method and an undescribed colorimetric method. Furthermore, there does appear to be significant HPLC interference from endogenous compounds, as Fletterick et al. [1] report that five pre-dose samples from five volunteers had a mean assayed value of 0.0 ± 0.1 µg/ml. At the time of submission of the current paper, another [5] appeared describing a reversed-phase method for which precision specifications were given for samples at 0.25 µg/ml and above, although no accuracy specifications were given. As Ameer et al. [5] stated, however, routine quantitation of plasma concentrations as low as 0.1—0.2 µg/ml is required for meaningful interpretation of single-dose acetaminophen pharmacokinetic studies.

We give detailed performance specifications for a method of acetaminophen assay which is rapid, convenient, selective, precise and more sensitive (0.05 µg/ml) than the other methods available [1—5]. The five-fold (or more) advantage in sensitivity makes this assay particularly attractive for pharmacokinetic studies. No loss in performance has been observed after well over 500 plasma assays, despite avoiding the time-consuming column regeneration procedures required by the assay according to Fletterick et al. [1].

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