Trace Determination of Acetaminophen in Serum

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Abstract □ A simple and accurate method for measuring acetaminophen in serum was developed using liquid chromatography with electrochemical detection. Acetaminophen can be quantitated in 100 μl of serum over the range of 20 ng/ml–20 μg/ml. The method is linear (r = 0.9997) and reproducible (RSD = 3.0% at 2 μg/ml, RSD = 5.1% at 200 ng/ml, n = 6). An internal standard (N-propionyl-p-aminophenol) is used, and a single extraction is performed.

Keyphrases □ Acetaminophen—liquid chromatographic analysis in serum □ Liquid chromatography—analysis, acetaminophen in serum □ Analgesics—acetaminophen, liquid chromatographic analysis in serum

The determination of free acetaminophen and its metabolites in biological fluids is a subject of continuing interest. Therapeutic and overdose levels are easily measured by several techniques including spectrophotometry, GLC, TLC, and liquid chromatography. Current methods are often inadequate, however, for microliter samples from newborns and experimental animals. Subtherapeutic levels, which are of interest in drug metabolism (pharmacokinetic) studies, also push most methods to their detection limits where data are subject to poor precision and specificity. The most sensitive methods are based on liquid chromatography with electrochemical detection (1), liquid chromatography with UV detection (2–5), or GLC (6, 7). The detection limit of these methods is roughly 100 ng/ml with 1 ml of serum.

Liquid chromatography with electrochemical detection has steadily gained acceptance for clinical determinations (8). This paper reports improvements in an earlier procedure (1) that allow accurate quantitation at levels lower than those previously reported (20 ng/ml) while requiring 10-fold less serum and little sample preparation.

EXPERIMENTAL

Reagents—Acetaminophen[1] was obtained commercially. Standard solutions were prepared in 1 M phosphate buffer (pH 7). The internal standard, N-propionyl-p-aminophenol, was synthesized by a standard method (9). Methanol[2] was distilled once prior to use. Tap water was deionized and distilled prior to use.

Instrumentation—A stainless steel liquid chromatograph[3] was equipped with a 20-μl injection loop and a carbon paste electrochemical detector. The working electrode potential was +0.65 v *versus* a silver-silver chloride reference electrode. The column (15 cm × 4.6 mm) was slurry packed with microparticulate reversed-phase packing material[4].

The mobile phase was prepared by mixing 390 ml of water, 34 ml of 1 M ammonium acetate, 70 ml of methanol, and 9 ml of 1 M acetic acid. A flow rate of 1.17 ml/min was used. All data were manually recorded from peak height measurements. Culture tubes were silanized with trimethylchlorosilane by a gas phase technique analogous to that described by Fenimore *et al.* (10).

Analysis—Solid sodium chloride (~0.07 g), 100 μl of serum, and 100 μl of 1.0 M phosphate buffer (pH 7) (saturated with sodium chloride and containing 2 mg of N-propionyl-p-aminophenol/liter) were added to a silanized 3-ml culture tube. Two milliliters of dichloromethane–2-propanol–ether (5:1:2) was added, and the tubes were capped and shaken for 10 min on a reciprocal shaker. Then the tubes were centrifuged briefly, and the organic layer was removed and evaporated at 40° under dry nitrogen. The residue was reconstituted in 500 μl of mobile phase. Standards for calibrating instrumental response were prepared by spiking drug-free serum and processing it with each set of samples.

RESULTS AND DISCUSSION

A typical chromatogram is shown in Fig. 1C for serum containing 200 ng of acetaminophen/ml. The detection limits of previously published methods were roughly at this level. Since one complete chromatogram requires 12 min, five samples can be processed per hour. The time required per sample can be significantly reduced when only therapeutic levels are measured and/or if the internal standard is not used (with some sacrifice in precision).

With the electrochemical detector operating at high sensitivity, a chromatogram for drug-free serum could be obtained as illustrated in Fig. 1A. The chromatographic conditions were chosen to minimize interferences so that low levels of acetaminophen could be measured (Fig. 1B). With the specified chromatographic conditions, no significant endogenous interferences were found in nine drug-free serum samples tested. Ultimately, however, the method became blank limited. The minimum detectable concentration of acetaminophen was 3 ng/ml of serum. In the absence of the serum matrix, a minimum of 2 pg injected in the 20-μl loop could be detected (S/N = ~2). Since the method is not detector limited, further sample cleanup procedures or more efficient chromatography would undoubtedly improve the detection limits.

Other drugs were tested as possible interferences, but none interfered. Barbiturates, theophylline, theobromine, salicylamide, caffeine, and aspirin were not electrochemically active at the electrode potential employed. Salicylic acid was poorly extracted and detectable with much less sensitivity than acetaminophen at the potential employed. Phenacetin was more strongly retained than N-propionyl-p-aminophenol and could be quantitated by increasing the working electrode potential.

The organic extractant used (dichloromethane–2-propanol–ether) gave slightly cleaner extracts than did ethyl acetate. Ether was also tried, but,

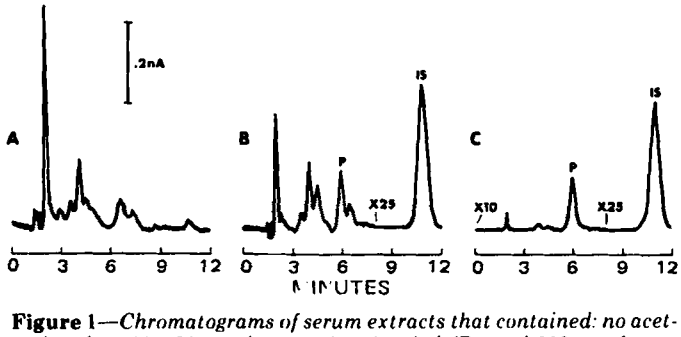

Figure 1—*Chromatograms of serum extracts that contained: no acetaminophen (A), 20 ng of acetaminophen/ml (B), and 200 ng of acetaminophen/ml (C). Key: P, acetaminophen; and IS, internal standard.*

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Technical grade.
[3] LC-50, Bioanalytical Systems, Lafayette, Ind.
[4] μBondapak, Waters Associates, Milford, Mass.

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while it gave cleaner extracts, the efficiency of extraction for acetaminophen was significantly lower. Acetaminophen and the internal standard partitioned nearly identically to one another. Absolute recovery of acetaminophen was 80–90%, and recovery relative to a matrix-free standard was 109%.

Linearity was evaluated by spiking drug-free serum with 10 standard solutions over the range of 20 ng/ml–20 μg/ml. The correlation coefficient obtained was 0.9997. Reproducibility was determined by spiking six different drug-free serum samples. The relative standard deviation at 2 μg/ml was 3.0%; at 200 ng/ml, it was 5.1%.

Reversed-phase chromatography with electrochemical detection is a useful means of quantitating acetaminophen metabolites in blood, urine, and liver homogenates. Since all major metabolites are more polar than the free drug or oxidize at significantly more positive potentials, they do not interfere in the present method.

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Extraction and TLC Separation of Food, Drug, and Cosmetic Dyes from Tablet-Coating Formulations

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Abstract □ A rapid method for extraction of dyes from tablet-coating formulations is described. The dyes were released from their lakes by treatment with concentrated phosphoric acid and dissolved in methanol. After being made alkaline with ammonium hydroxide, the mixture was centrifuged to obtain a clear supernate for application to the TLC plate. With ethyl acetate–methanol–water–concentrated ammonium hydroxide (150:40:35:5) on silica gel, 20 dyes were separated sufficiently to confirm their presence in the coating formulation.

Keyphrases □ Dyes, various—TLC determination in tablet-coating formulations □ TLC—determination, various dyes in tablet-coating formulations □ Tablet-coating formulations—TLC determination of various dyes

The widespread use of coloring agents in pharmaceutical preparations, combined with stringent and changing regulations on their use, makes a simple and rapid method for identifying the components of a colorant mixture highly desirable. TLC has been used extensively for this purpose, and several systems for the separation of dyes were published (1–4). If the dyes are components of a formulation, a suitable preparative scheme must be employed before the sample is applied to the thin-layer plate. Since tablet-coating formulations are relatively simple[1], published systems for extracting dyes from foods and drugs (5–7) are unnecessarily complex.

The aim of this work was to develop a combination of preparative scheme and TLC system that could be applied uniformly to various formulations.

[1] Formulations contained one or more lakes, titanium dioxide, and one or more of the following: sucrose, povidone, hydroxypropylcellulose, sodium benzoate, alcohol, and water.

Table I—Summary of R_f Values

Dye	R_f
FD&C Yellow No. 5	0.06
FD&C Green No. 3	0.07
FD&C Red No. 2[a] (amaranth)	0.07
FD&C Blue No. 1	0.16
D&C Blue No. 4	0.17
FD&C Yellow No. 6	0.22
FD&C Red No. 40	0.22
FD&C Red No. 33	0.23
FD&C Red No. 4	0.24
FD&C Violet No. 1[a]	0.26
FD&C Red No. 7	0.27
D&C Green No. 5	0.31
FD&C Red No. 3 (erythrosine sodium)	0.31
FD&C Red No. 28	0.33
FD&C Orange No. 4	0.36
D&C Red No. 19	0.36
D&C Yellow No. 10	0.37
D&C Yellow No. 11	0.77
D&C Red No. 36	0.81
D&C Green No. 6	0.85

[a] Use no longer permitted in the United States.

EXPERIMENTAL

Extraction of Dyes—Ten drops (~0.4 ml) of phosphoric acid (85%) were placed in a 50-ml centrifuge tube, and an appropriate amount of tablet-coating liquid (up to 1 ml), pure dye or lake, was added. The contents of the tube were mixed by swirling intermittently for 5 min. Methanol, 10 ml, was added, and the tube was shaken for 1 min. Then 1 ml of concentrated ammonium hydroxide (29%) was added, and the tube again was shaken for 1 min. Solids were removed by centrifuging, and 10 μl of the supernate was applied to the thin-layer plate.

TLC Plates—Silica gel G[2] plates, 20 × 20 cm, 0.25 mm thick, were

[2] E. M. Reagents, E. Merck, Darmstadt, West Germany.

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