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Quantitative Gas Chromatographic Determination of Acetaminophen Using Trimethylanilinium Hydroxide as the Derivatizing Agent

Pharmaceutical preparations of acetaminophen, an analgesic, are marketed and extensively used in the single drug form such as Tylenol® and Datril® and in combination with other analgesics such as aspirin and salicylamide in Excedrin®, Excedrin P.M.®, and others. Acetaminophen overdosage can result in centrilobular hepatic necrosis or hepatic failure [*1*] with severe overdosage resulting in a high fatality rate [*2*]. Workers have investigated and related severity of liver damage to drug serum concentration [*1,3,4*]. Initial concentrations relative to time of ingestion and drug half-life are two criteria in the early diagnosis of possible liver damage or necrosis [*4*]. Administration of cysteamine soon after ingestion has been advocated [*3*] after successful use in severe overdosages, but its use is a risk to the patient and warranted only in cases of probable severe or fatal hepatic necrosis. Consequently, for intelligent therapy of suspected overdose cases and legal protection of physicians, rapid and accurate serum acetaminophen levels must be available throughout the first few critical hours after ingestion of acetaminophen.

Many types of serum acetaminophen analyses are extensively described in the literature [*5-13*], but each is suited to a particular situation or suffers a drawback. There is a need for a more simple, quick, and accurate method using gas chromatography in the case of an overdose of acetaminophen (10 to 50 mg/dl) in the possible presence of barbiturates and salicylates. Therefore the following procedure was developed and compared against a modified colorimetric procedure of Glynn and Kendall [*7*].

Acetaminophen is extracted twice into ether from a buffered sample of sodium chloride-saturated serum with aqueous internal standard added. The ether is dried on a steam bath and the residue reconstituted in methanolic trimethylanilinium hydroxide before injection into a gas chromatograph with a 3.8% SE-30 column. Standards are processed simultaneously, and quantitation of unknowns is calculated in a standard manner.

Experimental Procedure

Gas Chromatography

Materials—A Perkin-Elmer 3920 gas chromatograph fitted with a 3.8% SE-30 Chromasorb (AW-DMCS) column, 80-100 mesh, 180 cm by 4.0 mm inside diameter, with a flame ionization detector was used. The helium carrier gas flow rate was 40 ml/min with in-

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jector and detector temperatures both at 300 °C, column temperature at 145 °C, attenuation at 4, and range 100.

All reagents were reagent grade.

Acetaminophen was obtained from Applied Science, State College, Pa., and 100 mg was placed in a 100-ml volumetric flask, dissolved in a minimum amount of ethanol, and made up to volume with distilled water. When it is kept under refrigeration (40 °C) its shelf life is six weeks. Working standards of 50, 40, 30, 20, and 10 mg/dl were made by aqueous dilution of the stock 100 mg/dl solution.

The internal standard, *N*-butyryl-*p*-aminophenol, was obtained from K&K Laboratories, Plainview, N.Y., and 25.0 mg was dissolved in a small amount of ethanol in a 100-ml volumetric flask and made up to volume with distilled water.

The serum acetaminophen standards were made by dissolving 50 mg acetaminophen in 50 ml of drug-free serum and diluting to appropriate concentrations with serum.

The buffer, pH 7.4, was made from dry buffer salt, pH 7.41, obtained from Fisher Scientific Co., Fair Lawn, N.J.

Trimethylanilinium hydroxide (TMAH), 0.2*M*, was prepared according to the method of Brochmann-Hanssen and Oke [*14*].

Method—Into 50-ml ground-glass stoppered centrifuge tubes pipette 0.5 ml of serum, standards, and serum blank. To each add 1.0 ml of internal standard, 1.0 ml of pH 7.4 buffer, and approximately 2.0 g of sodium chloride crystals. Mix thoroughly to assure saturation. Add 10 ml of diethyl ether to each and gently mix on a tilt extractor for 5 min. Centrifuge for 30 s and decant the top ether layer into labeled 20-ml or larger test tubes. Add another 10 ml of diethyl ether to each centrifuge tube and shake and centrifuge as before. Decant the ether again, combining extracts. Filter the ether through medium-porosity sintered-glass filters (Corning #32960) into 20-ml beakers; and, with a stream of air, take to dryness on the steam bath. Reconstitute the residue with 200 μl of TMAH and inject 1 μl.

Colorimetric Procedure

A modified procedure of Glynn and Kendall is used [*7*]. To small test tubes add 2 ml of serum, blank, and aqueous standards. To each of the tubes add 2 ml of 20% (w/v) trichloroacetic acid, mix well, and centrifuge briefly. Filter the supernate through filter paper (Eaton-Dikeman fluted filter paper, grade 513) into a small test tube. Nitrous acid is prepared in a fume hood by combining one volume of 6*N* hydrochloric acid with two volumes of freshly prepared 10% sodium nitrite (w/v). (The sodium nitrite solution must be fresh since it decomposes in less than 24 h.) Add 1 ml of filtrate to 3 ml of nitrous acid, mix thoroughly, and allow to react for 2 min. Add 2 ml of 15% ammonium sulfamate (w/v) to each test tube, followed by 1 ml of 50% sodium hydroxide (w/v). Vortex briefly to release the nitrogen gases from the solution. Scan the blank, standards, and samples versus water in the range 500 to 380 nm with the absorbance of the standards at the maximum, 430 nm, for quantitation.

Results and Discussion

Basic to the history of gas chromatographic analysis of acetaminophen are two problems: poor recovery of drug from serum and broad tailing peaks of underivatized drug. This procedure uses the best aspects of other techniques and combines them into a single method that exhibits good recovery, excellent chromatographic sensitivity, and linearity.

Analysis of underivatized acetaminophen, *N*-acetyl-*p*-aminophenol, with a 3.8% SE-30 column (common in toxicology laboratories), results in peak broadening and severe tailing because of the parahydroxy function of the molecule reacting with active sites in the liquid

phase and solid support. Phenacetin, *p*-ethoxyacetanilide, does not exhibit this tendency: it yields sharp peaks under the same conditions. Therefore, derivatization of this phenolic moiety should result in sharp symmetric peak responses.

It is possible to form *O*-methyl-*N*-methyl acetaminophen with on-column methylation with TMAH. Methylation of phenolic hydroxy functions of alkaloids and xanthine derivatives was demonstrated by Brochmann-Hanssen and Oke [*14*]. Methylation of the metabolite of phenytoin 5-(*p*-hydroxyphenyl)-5-phenylhydantoin to 1,3-dimethyl-5-(*p*-methoxyphenyl)-5-phenylhydantoin was demonstrated by Midha et al [*15*], who used mass spectrophotometry and chromatography.

Analysis of acetaminophen, phenacetin, and the internal standard, *N*-butyryl-*p*-aminophenol, reconstituted in TMAH by a 3.8% SE-30 column, is shown in Fig. 1. The methoxy

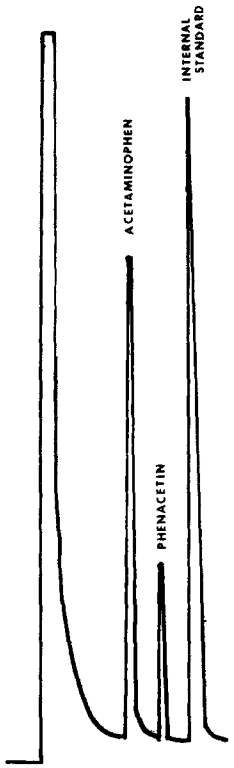

FIG. 1—*Gas chromatograph of derivatized acetaminophen, phenacetin, and* N-*butyryl*-p-*aminophenol.*

derivative of acetaminophen elutes just before the phenacetin, possessing an ethoxy function, as would be expected when both are derivatized to the *N*-methyl derivative. The internal standard *N*-butyryl-*p*-aminophenol, proposed by Street [*11*], was used because of the obvious similarity to acetaminophen, and when derivatized it follows the phenacetin peak (Fig. 1). The addition of internal standard at the onset assures better quantitation because of similar recovery characteristics. Saturation of the sample with sodium chloride reduces the solubility of the compounds in serum, and double extraction for 5 min with ether is an efficient method of extraction. Figure 2 illustrates a chromatograph of a standard and an actual patient's sample.

The peak height ratios of drug and internal standard were plotted versus drug con-

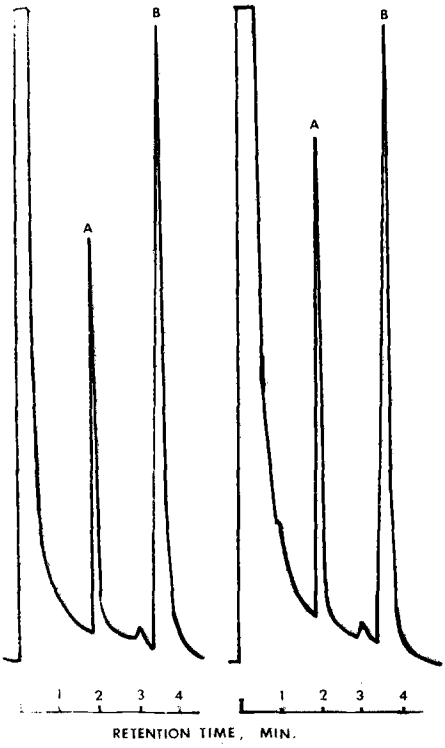

FIG. 2—*Chromatographs showing acetaminophen* (A) *and internal standards* (B). *The first is an aqueous standard; the second is an actual patient's sample.*

centrations (Fig. 3). Linearity is excellent between 10 and 50 mg/dl with standard points falling directly on the line between those limits. The line passes not through the origin but slightly above the origin. This is due to a small peak (apparently from the internal standard) that also falls under the acetaminophen peak. It is reproducible, affects every sample equally, and raises the graph slightly. Clearly it gives no false positives in the range of interest, above 10 mg/dl, especially when compared to the standard curve. Possibly it is due to incomplete *N*-methylation of internal standard caused by the bulky *N*-butyryl moiety.

Recovery of drug from spiked serum was tested at two levels, 20 and 40 mg/dl, after single, double, and triple extraction. Samples were extracted as per the procedure except for substituting water only for the aqueous internal standard. Internal standard in ether was added to the final ether extracts and dried. Ether samples equivalent to 100% drug extraction were prepared, internal standard was added, and then the mixture was dried. Percentage of recovery was based on comparison of the two levels. A similar method was used to study internal standard recovery.

Drug recovery after a single extraction was 64%. Good efficiency is realized after the second extraction, 91%, with a third producing only a comparatively small increase in yield, 92 to 95%. The internal standard showed a good yield also after two extractions, 94%.

Within-run precision was tested with 10 samples of 10 mg/dl. The coefficient of variation was 5.2%, with the average calculated as 10.5 mg/dl. Day-to-day precision was tested by using 60 separate frozen aliquots from drug-free serum pools spiked to 30, 40, and 50 mg/dl acetaminophen, with the averages being 29.6, 40.1, and 50.3 mg/dl, respectively. The coefficients of variation were 2.7, 3.1, and 3.0%, respectively.

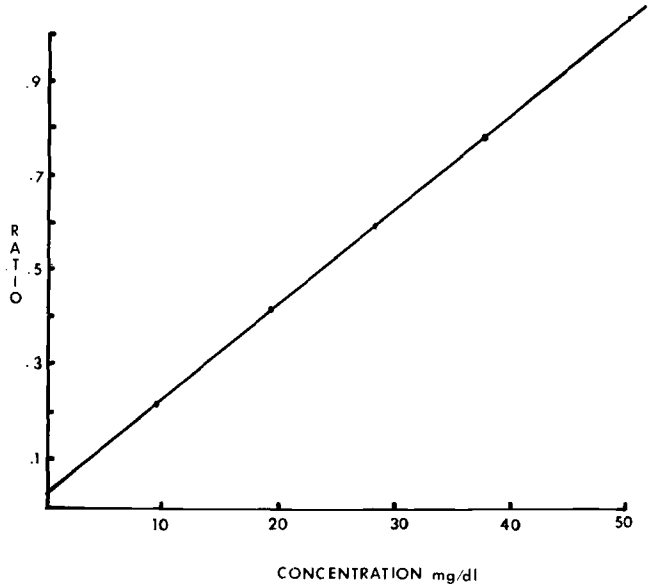

FIG. 3—*Graph of the ratio (acetaminophen peak height divided by internal standard peak height) versus the concentration of acetaminophen.*

Fifty samples ranging from 5 to 50 mg/dl acetaminophen were made in drug-free serum and subjected to gas chromatographic and colorimetric quantitations. The actual average value of the random spiked samples was 19.7 mg/dl. The average values for gas chromatographic and colorimetric analyses were 19.7 and 19.8 mg/dl, respectively. With nonparametric statistics for this non-Gaussian distribution the Spearman's rank correlation coefficient is 0.990228, showing excellent correlation between the two methods. With the *t* test for method comparison a value of 0.142 is obtained, indicating similarity of methods. Actual patients' samples were subjected to both analyses and also compared favorably.

Interference from other common drugs with both methods of analysis is insignificant (Table 1). The listed levels yield no greater than 4 mg/dl as acetaminophen in either analysis.

However, salicylamide interferes slightly with both analyses. Its retention time under the stated conditions is identical to acetaminophen and even when singly present results in a double peak. A Trinders test on serum confirms its presence. Salicylamide can be separated, however, on a 3% OV-17 column at 135 °C, thereby eliminating the problem when it exists.

In the colorimetric procedure the sample should be scanned between 500 and 380 nm. A positive sample showing the peak maximum at a wavelength shorter than the standard 430 nm indicates the presence of salicylamide and should be read at 460 or 480 nm, where salicylamide does not add absorbance (Fig. 4). However, in nine months this laboratory has only encountered one patient's sample that exhibited strong salicylamide interference, reinforcing the belief that salicylamide is rapidly absorbed and metabolized so quickly that free salicylamide is very hard to detect in the blood [*16*].

Conclusion

This study has presented a gas chromatographic technique for the analysis of acetaminophen that is accurate and precise, with good drug recovery. This analysis is particularly well suited to overdose cases where an accurate, rapid result is necessary.

TABLE 1—*Serum drug levels that do not interfere with acetaminophen analysis.*

Drug	Level, mg/dl	Drug	Level, mg/dl
Aminophylline	2.0	Meperidine	0.6
Amitriptyline	0.4	Meprobamate	5.0
Amobarbital	2.0	Mescaline	0.2
Amphetamine	0.2	Methadone	0.2
Benzoylecognine	0.2	Methamphetamine	0.2
Butabarbital	2.0	Methapyrilene	1.0
Carbamazepine	3.0	Methaqualone	0.6
Caffeine	5.0	Methyprylon	4.0
Carisoprodol	5.0	Morphine	0.2
Chloral hydrate	5.0	Pentazocine	0.4
Cocaine	0.2	Pentobarbital	2.0
Codeine	0.2	Phenobarbital	6.0
Desipramine	0.6	Phenytoin	5.0
Diphenhydramine	1.0	Primidone	2.0
Diazepam	2.0	Promazine	0.2
Ephedrine	0.2	Propoxyphene	0.3
Ethosuximide	2.0	Quinine	0.2
Flurazepam	0.2	Quinidine	0.2
Glutethimide	2.0	Secobarbital	2.0
Imipramine	0.4	Thorazine	0.4

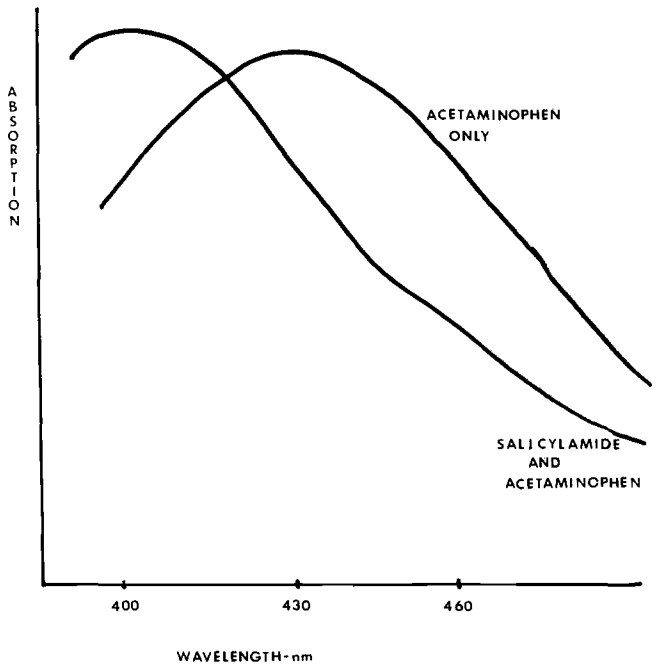

FIG. 4—*Spectrophotometric scan of the acetaminophen chromaphore showing the typical trace and change in the presence of salicylamide.*

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