Gas-Chromatographic Method for Acetaminophen (N-Acetyl-p-Aminophenol) Based on Sequential Alkylation

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A gas-chromatographic procedure for acetaminophen is described in which the drug is chromatographed as the O-heptyl-\(N\)-methyl derivative. This derivative is prepared by a sequential alkylation procedure in which the phenolic hydroxyl group of the parent compound is alkylated off-column with heptyl iodide and the amide group is derivatized on-column by reaction with trimethylanilinium hydroxide. The internal standard, \(N\)-propionyl-\(p\)-aminophenol, is subjected to the same derivatization procedure. This gas-chromatographic procedure correlates well with conventional colorimetric and spectrophotometric procedures for acetaminophen and is more sensitive. Within-run precision (CV) was 2.0% at a serum concentration of 10.0 mg/liter (\(n = 10\)) and between-run precision was 4.0% over a period of eight months. This method is particularly applicable to studies of the pharmacokinetics of acetaminophen.

Additional Keyphrases: pharmacokinetics • saliva, serum

In man, large doses of acetaminophen (\(N\)-acetyl-\(p\)-aminophenol) can produce severe liver necrosis and occasionally cause fatal hepatic coma. Prescott et al. reported that intravenous cysteamine protected patients against hepatic necrosis following massive overdosage with acetaminophen (1). The decision whether to administer cysteamine to patients who ingested large doses of acetaminophen was based on the concentration of this drug in the plasma. Thus measurements of acetaminophen in plasma are critical in selecting patients at risk of potentially serious liver injury and also in determining those for whom treatment with cysteamine is unnecessary.

Numerous methods for the determination of acetaminophen in various biological fluids have been reported, including a colorimetric procedure (2); differential ultraviolet spectrophotometry (3); and gas-chromatography of the free drug (4), the mono- and di-trimethylsilyl derivatives (5), the acetylated derivative (6), and the \(O\)-benzoyl, \(N\)-trimethylsilyl derivative (7). Recently a high-resolution liquid-chromatographic method has been described that permits determination of free acetaminophen as well as seven of its metabolites (8). However, this method was used for studies of the metabolism of acetaminophen and was not proposed for routine use in diagnosis and management of intoxication.

Of the various procedures described, the differential ultraviolet spectrophotometric method of Routh et al. (3) and the colorimetric procedure of Glynn and Kendal (2) are probably most applicable to the laboratory diagnosis of massive overdosage with acetaminophen, as they are relatively easy to perform and can be provided without delay. In contrast, the published gas-chromatographic procedures are more complex and take considerably longer to perform. Moreover, undervatized acetaminophen is strongly adsorbed to most chromatographic columns, the trimethylsilyl derivatives are unstable, and the extent of formation of the benzoyl \(N\)-trimethylsilyl derivative of acetaminophen is incomplete (7).

The gas-chromatographic method we described for acetaminophen is based on the formation of the stable \(O\)-heptyl-\(N\)-methyl derivative of the drug, which shows a symmetrical chromatographic peak without evidence of adsorption on the column. This procedure was used in our laboratory to validate both the differential spectrophotometric and the colorimetric method for the emergency determination of acetaminophen. Our gas-chromatographic procedure is sensitive and specific and is particularly suited to pharmacokinetic studies of acetaminophen.

Materials and Methods
Reagents and Comparison Materials

Acetaminophen, \(N,N\)-dimethylacetamide, iodobutane, iodoheptane, and propionic anhydride were obtained from Aldrich Chemical Co., Milwaukee, Wis. 53233. A stock solution of acetaminophen (100 mg/liter) was prepared by dissolving 10 mg in 100 ml of ethanol.
4-Aminophenol was purchased from the Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y. 11514. N-Propionyl-p-aminophenol, the internal standard, was prepared by reacting 4-aminophenol with propionic anhydride in ethyl ether at 25 °C for 1 h, similar to the procedure of Stewart and Willis for synthesis of N-butyryl-p-aminophenol (9). The product was recrystallized three times from ethanol by gradual addition of water until crystallization commenced. A stock solution of the internal standard (100 mg/liter) was prepared by dissolving 10 mg in 100 ml of ethanol. A working solution of the internal standard (1 mg/liter) was prepared by evaporating 10 ml of the stock solution to dryness under nitrogen in a water bath at 40 °C and then dissolving the residue in 1 liter of ether/toluene (40/60 by vol). Trimethylanilinium hydroxide (TMAH), 25 g/liter, in methanol. This reagent was prepared by reacting trimethylanilinium iodide (Eastman Organic Chemicals, Rochester, N. Y. 14650) with silver oxide (Matheson, Coleman and Bell, East Rutherford, N. J. 07073) according to the method of Osiewicz et al. (10).

Methanol and toluene ("ChromatoQuality") and tetramethylammonium hydroxide (TMH) 240 g/liter, in methanol were purchased from Matheson, Coleman and Bell. The TMH was diluted 10-fold with methanol and stored at 4 °C.

Pentyl iodide and hexyl iodide were obtained from Eastman Organic Chemicals.

Procedure for Extraction and Alkylation before Gas Chromatography

Serum (1 ml) or saliva (1 ml) was combined in a 16 × 125 mm test tube (Teflon-lined screw cap) with 1 ml of distilled water, 1 g of NaCl, and 10 ml of ethyl ether/toluene (40/60 by vol) containing 10 μg of the internal standard, N-propionyl-p-aminophenol. The mixture was shaken for 15 min in an Eberbach shaker at 250 oscillations/min and centrifuged at 2500 rpm for 10 min. The organic (top) phase was transferred to 16 × 125 mm test tubes (Teflon-lined screw cap) containing 2 ml of 0.1 mol/liter NaOH. The tubes were shaken for 10 min and centrifuged for 5 min. The organic phase was aspirated and discarded. One-half milliliter of phosphate buffer (1 mol/liter, pH 6.8), 1 g of NaCl, and 10 ml of ether were added to the aqueous solution. The mixture was shaken for 10 min and centrifuged for 5 min. The ether was transferred to tubes with constricted tips ("Concentratubes"; Laboratory Research Co., Los Angeles, Calif. 90036) and evaporated under nitrogen in a water bath at 40 °C. Eighty microliters of N,N-dimethylacetamide, and 20 μl of dilute tetramethylammonium hydroxide (TMH in methanol 24 g/liter) was added to the residues. The tubes were mixed on a vortex-type mixer for 5 s after each addition. Iodoheptane, 20 μl, was added and the tubes were again mixed on a vortex-type mixer for 5 s and allowed to stand at room temperature for 10 min. Then the tubes were centrifuged for 1 min at 2000 rpm to sediment the tetramethylammonium iodide. The clear supernate was transferred to 1.0 ml "Reacti-Vials" (Pierce Chemical Co., Rockford, Ill. 61050) and evaporated under nitrogen in a water bath at 40 °C. Twenty-five microliters of dilute trimethylanilinium hydroxide (25 g/liter of methanol) was added to the residues and the contents of the tubes were mixed on a vortex-type mixer for 10 s. Of this solution, 1 μl was injected into the gas chromatograph.

Serum-based standards (5, 10, 15, and 20 mg/liter) were prepared by evaporating to dryness under nitrogen 50, 100, 150, and 200 μl of a solution of acetaminophen in ethanol (100 μg/ml) and adding 1 ml of serum. Similar standards were prepared with saliva.

Gas Chromatography

A Model 5710A (Hewlett Packard, Avondale, Pa. 19311) dual-column gas chromatograph equipped with flame ionization detectors was used. The glass columns (122 cm × 6 mm, 2 mm i.d.) were packed with 3% OV-17 on 100/120 mesh Gas Chrom Q (Applied Science Labs., Inc., State College, Pa. 16801). The injector temperature was 250 °C; the detector 300 °C; and the oven temperature was programmed at a rate of 16 °C/min from 150 to 260 °C. The flow rate of nitrogen was 40 ml/min.

Differential Spectrophotometric Method for Acetaminophen

The differential spectrophotometric absorbance method of Routh et al. (3) was used for the determination of acetaminophen in serum and saliva. One milliliter of serum or saliva was extracted with 10 ml of diethyl ether, and 8 ml of the organic phase was removed and extracted with 2.5 ml of NaHCO3 (4 g/liter). One milliliter of the aqueous layer and 1 ml of water were pipetted into a silica cuvette (reference cell), 1 ml of the aqueous layer and 1 ml of 0.25 mol/liter NaOH into the other cuvette (sample cell). Absorbance was measured.
at 266 nm with a Beckman DB-GT spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif. 92634).

Colorimetric Method for Acetaminophen

Acetaminophen in serum was determined by a modification of the method of Glynn and Kendal (2) based on the reaction of this drug with nitrous acid to yield the chromophore, 2-nitro-4-acetamidophenol (10).

To a 16 x 100 mm glass screw-cap culture tube, add 1 ml of serum and 2 ml of trichloroacetic acid (100 g/liter). Mix on a vortex-type mixer for 30 s and then centrifuge at 2000 rpm for 2 min. Decant the supernatant fluid into a 50-ml culture tube containing 1 ml of hydrochloric acid (6 mol/liter) and 2 ml of freshly prepared sodium nitrite (100 g/liter). Allow the tubes to stand for 2 min at room temperature. Then add 2 ml of ammonium sulfamate (150 g/liter) followed by 5 ml of NaOH (100 g/liter); mix thoroughly. Using a Beckman DB-GT Spectrophotometer, measure the absorbance at 430 nm of the solution against a blank, prepared with 1 ml of drug-free serum.

Results and Discussion

In preliminary experiments, we synthesized the O-methyl derivative of acetaminophen according to the classical method of Claisen (11), which is known to alkylate phenolic hydroxyl groups (12). The retention time of this compound on OV-17 was the same as that observed when acetaminophen was reacted with methyl iodide according to our modification of the method of Greeley (13). However, when acetaminophen was methylated on-column with trimethylanilinium hydroxide, a new chromatographic peak was observed with a different retention time than O-methyl acetaminophen, which suggests that under these conditions the amide nitrogen as well as the phenolic hydroxyl group of this drug might be alkylated. This alkylation would be analogous to that observed by Tanaka and Wien (14) in which on-column alkylation of substituted phenyl ureas with trimethylanilinium hydroxide yielded the 3-N-methyl analogs. When we subjected O-methyl acetaminophen to on-column methylation, we observed a single chromatographic peak, which had the same retention time as that noted when acetaminophen was methylated on-column. Subsequently, we studied the effect of on-column N-methylation on the gas-chromatograph characteristics of a series of O-alkyl derivatives of acetaminophen prepared with the corresponding alkyl iodide according to our modification of the method of Greeley (13). Retention times for the O-alkyl derivatives of acetaminophen and N-propionyl-p-aminophenol (the internal standard) were linearly related to the number of carbon atoms in the alkyl iodide used for derivatization (Figure 1). The difference between the retention times of the derivatives of acetaminophen and the internal standard remained constant as the alkyl chain length increased.

While the preparation of mixed derivatives for gas-chromatography is well known, the principle of sequential alkylation has to our knowledge not been applied previously to derivatization of drugs. Our studies with acetaminophen indicate the potential versatility provided by sequential alkylation when the compound has multi-functional groups, some of which cannot be alkylated by the mild procedure of Greeley but can be derivatized by on-column alkylation. By varying the chain length of the alkyl iodide used in the initial alkylation, it was possible to vary the retention time of the final product in order to minimize interference from background peaks. Although we used trimethylanilinium hydroxide for the on-column alkylation step in our procedure, it is apparent that other quaternary alkylating agents such as triethylphenylammonium hydroxide and tetrabutylammonium hydroxide could be used to further increase the versatility of sequential alkylation.

Chromatograms of serum specimens extracted and derivatized according to our procedure are presented in Figure 2. Chromatogram A, with internal standard omitted, was obtained from serum not containing acetaminophen. Chromatogram B, with the internal standard included, represents serum from an individual prior to receiving acetaminophen. Chromatogram C was obtained from the same individual 0.5 hour after an oral dose of 900 mg. The serum concentration of acetaminophen in this sample was 26.0 mg/liter.

Figure 3 represents chromatograms of salivary specimens extracted and derivatized according to our procedure. Chromatogram A, with internal standard omitted, was obtained from saliva that did not contain acetaminophen. Chromatogram B, with the internal standard included, represents saliva from an individual prior to dosing with acetaminophen. Chromatogram C was obtained from the same individual 1 h after a oral
dose of 900 mg. The concentration of acetaminophen in the specimen of saliva was 16.4 mg/liter.

Figure 4 represents a typical standard curve obtained with our procedure for acetaminophen. There was a linear relation between the ratio of peak height of drug to internal standard and the concentration of drug in serum over the range of 0–20 mg/liter. Identical curves were obtained with standards prepared in serum and saliva. The lower limit of detection for acetaminophen based on twice the standard deviation of baseline noise was 0.5 mg/liter when the sample volume was 1 ml.

Within-run reproducibility for our assay was 2.0% at a serum concentration of 10.0 mg/liter (n = 10) and 3.0% at this concentration in saliva (n = 10). Between-run reproducibility during eight months was 4.0% for serum and 3.4% for saliva.

Other drugs, including sodium salicylate, p-amino-phenol, p-acetophenetidin, phenobarbital, primidone, and diphenylhydantoin, that may be present in serum or saliva were investigated for possible interference with our procedure for acetaminophen. None of these interfered except primidone, which had the same retention time as acetaminophen.

It is known from the work of Mrochek et al. (8) that large amounts of the glucuronide of acetaminophen and smaller quantities of acetaminophen sulfate are present in the serum of subjects receiving this drug. Our assay is designed to measure unconjugated acetaminophen. The stability of ether glucuronides and sulfate esters is well known (15). Because hot concentrated acid is required to hydrolyze an ether glucuronide, the mild conditions of our assay (pH 6.8) are unlikely to produce detectable hydrolysis of this metabolite. Moreover, the polar nature of the conjugates would not lead to their extraction during our procedure.

Five-nine serum specimens were analyzed for acetaminophen by the differential spectrophotometric method and by our procedure. Concentrations of the drug ranged from 0.0 to 31.7 mg/liter. The mean value observed for the spectrophotometric method was 10.5 mg/liter and with our procedure was 10.6 mg/liter. When values for the gas-chromatographic procedure were regressed against values for the differential spectrophotometric method, the slope of the non-weighted linear least-squares regression line was 1.03; the intercept was –0.26 mg/liter, and the coefficient of correlation was 0.99.

Similar comparative studies were conducted with 52 specimens of saliva containing acetaminophen in concentrations ranging from 1.4 to 27.5 mg/liter. The mean value observed for the differential spectrophotometric method was 8.8 mg/liter and with our procedure was 10.1 mg/liter. The slope of the non-weighted linear least-squares regression line was 1.00; the intercept was 1.17 mg/liter, and the coefficient of correlation was 0.99.

Five clinical specimens were analyzed for acetaminophen by our gas-chromatographic procedure and by the colorimetric method of Glynn and Kendal (2). The mean value as determined by the gas-chromatographic assay was 24.3 mg/liter and by the colorimetric assay was 27.2 mg/liter. The maximum difference observed between the two methods was 6.6 mg/liter. The colori-

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**Table 1. Concentrations of Acetaminophen in Serum and Saliva after an Oral Dose of 900 mg**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Serum (A)</th>
<th>Saliva (B)</th>
<th>Ratio A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time, h</td>
<td>mg/liter</td>
<td>mg/liter</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>—</td>
</tr>
<tr>
<td>0.5</td>
<td>25.7</td>
<td>27.5</td>
<td>0.93</td>
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<tr>
<td>1.0</td>
<td>20.7</td>
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<td>0.99</td>
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<td>2.0</td>
<td>15.3</td>
<td>15.4</td>
<td>0.99</td>
</tr>
<tr>
<td>3.0</td>
<td>11.1</td>
<td>10.7</td>
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</tr>
<tr>
<td>4.0</td>
<td>8.3</td>
<td>8.4</td>
<td>0.99</td>
</tr>
<tr>
<td>6.0</td>
<td>4.6</td>
<td>5.3</td>
<td>0.87</td>
</tr>
</tbody>
</table>
metric procedure was less sensitive than either the gas-chromatographic method or the differential spectrophotometric procedure but is adequate for analysis of the toxic concentrations of acetaminophen.

The application of our gas-chromatographic procedure to pharmacokinetic studies with acetaminophen is demonstrated in Table 1. In this study, a healthy volunteer received 900 mg of acetaminophen orally after an overnight fast and serum and saliva were collected during the following six hours. Within 0.5 h of administration of the dose, the serum and salivary concentrations of acetaminophen were 25.7 and 27.5 mg/liter, respectively. Thereafter concentrations in serum and saliva declined exponentially with a half-life of 2.3 h, which is similar to that reported in normal subjects (16, 17). At the pH values normally observed for human serum and saliva, 7.4 and 6.8 to 7.2, respectively, acetaminophen (pKₐ = 9.51) is uncharged, hence its distribution ratio between these fluids should reflect only the extent to which it binds to serum proteins. The observed equivalence of serum and salivary concentrations of acetaminophen in this subject indicates no significant binding of this drug.

References