High-Performance Liquid Chromatographic Assay for *N*-Acetylcysteine in Plasma and Urine

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Abstract □ A sensitive high-performance liquid chromatographic (HPLC) assay for *N*-acetylcysteine in human plasma and urine has been developed. The method employs a prechromatographic stage to produce the dinitrophenyl derivative of *N*-acetylcysteine, which is chromatographed on a 5-μm C_{18} bonded reverse-phase column using an external standard method. The derivatized *N*-acetylcysteine molecule has a retention time of ≃13 min at a flow rate of 1.0 mL/min for a mobile phase of methanol–aqueous 0.05 M citrate–0.001 M EDTA buffer pH 7.0 (30:70). In a multistep extraction procedure, which is slightly modified for the *N*-acetylcysteine assay in urine, the limits of sensitivity for the plasma and urine assays were found to be ≃60 ng/mL and ≃200 μg/mL, respectively. Preliminary data from pilot studies in human subjects are presented.

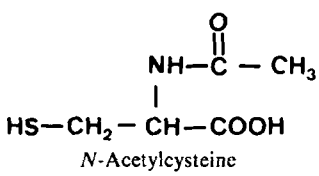
Keyphrases □ *N*-Acetylcysteine—HPLC, plasma and urine □ HPLC-*N*-acetylcysteine, plasma and urine

N-Acetylcysteine, when administered intravenously, has been shown to be effective as an antidote for the treatment of acetaminophen poisoning, giving protection against liver damage (1), and to influence the consistency and volume of sputum when administered orally to patients with chronic obstructive bronchitis (2). A further potential use of *N*-acetylcysteine is the inhibition of cyclophosphamide-induced bladder toxicity in rodents without adversely affecting the beneficial immunosuppressive action of cyclophosphamide (3).

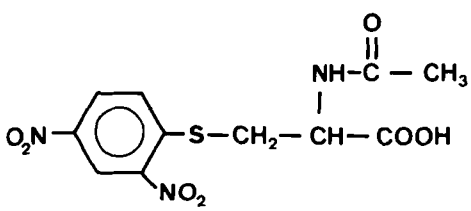
Pharmacokinetic studies with *N*-acetylcysteine are confined to studies (4) employing material labeled with sulfur-35 which measured total radioactivity. Studies with unlabeled *N*-acetylcysteine have been hampered by the lack of a suitable analytical procedure to determine the compound in plasma and urine.

A GC procedure (5) has been reported but is unsuitable for determinations in plasma. A high-performance liquid chromatographic (HPLC) method for determining the compound in plasma ultrafiltrates has been published (6), but this method failed to detect *N*-acetylcysteine on every occasion and was unable to determine total plasma levels. *N*-Acetylcysteine has been determined (7) as *N*-(1-pyrene)maleimide and *N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide derivatives, but the procedure can only be used for urinary measurements.

This paper describes a reliable and sensitive procedure for determining *N*-acetylcysteine in plasma and urine which is suitable for the study of the pharmacokinetics of the drug.



N-Acetylcysteine



2,4-Dinitrophenyl Derivative

EXPERIMENTAL SECTION

Materials—Pure authentic *N*-acetylcysteine[1] was used as received without purification, as a standard. All chemicals used were of "Analar" [2] grade. All solvents used were of glass-distilled[3] or HPLC[3] grade. The ultrafiltration cones[4] used were of a membrane type.

Apparatus—The high-performance liquid chromatographic pump[5] was fitted with a loop injector[6] with a 50-μL loop and a variable-wavelength detector[7] set at 360 nm, attenuated at 0.02 AUFS for plasma and 0.5 AUFS for urine. The signal was recorded on a 10-mV recorder[8]. An "in-house" packed 25 cm × 0.46-mm i.d. column containing a reverse-phase (C_{18}) chemically bonded to 5-μm silica[9] was employed at ambient temperature.

Mobile Phase—The mobile phase was prepared by mixing an aqueous solution of 0.05 M trisodium citrate and 0.001 M EDTA, adjusted to pH 7.0 with a citric acid solution, with HPLC-grade methanol in a ratio of 70:30. (When performing the assay on urine samples, this solution may be modified by mixing the aqueous buffer with methanol in a 65:35 ratio.) This solution was filtered through a membrane filter[10] and deaerated by sonication in an ultrasonic bath[11]. Immediately prior to use the solution was purged with pure helium. The mobile phase was pumped isocratically through the column at ambient temperature with a flow rate of 1.0 mL/min, giving a back-pressure of ≃1000 psi.

Standard Solutions—Aqueous stock solutions of *N*-acetylcysteine were prepared daily and stored at 4°C. Plasma standard solution was prepared by dissolving authentic *N*-acetylcysteine in the mobile phase solution (25 mg/100 mL). Urine standard solution was prepared by dissolving authentic *N*-acetylcysteine in 2.0% sodium hydrogen carbonate solution (2.0 g/100 mL).

Plasma standards were produced by spiking 3.0-mL aliquots of fresh human plasma to give a range of calibrators with concentrations of *N*-acetylcysteine as follows: 0, 0.25, 0.50, 0.75, 1.0, 1.25, 1.50, 2.0, and 2.5 μg/mL. These standards were vortex-mixed for 30 s and then allowed to stand at ambient temperature for 15 min to equilibrate. Similarly, urine calibration standards were produced by spiking 100-μL aliquots of fresh human urine to give a range of concentrations of *N*-acetylcysteine as follows: 0, 0.25, 0.50, 0.75, 1.0, 1.25, and 1.50 mg/mL.

Each range of calibrators were taken through the appropriate complete extraction procedure as described below. In each instance, calibration curves were constructed by plotting the integrated peak area against the corresponding *N*-acetylcysteine standard concentration. Plasma and urine samples were similarly treated, and the measured peak areas (referred to the appropriate calibration curve) were used to determine the concentration of *N*-acetylcysteine present.

Chromatographic Method—Aliquots of plasma (3.0 mL) or urine (100 μL) diluted in 1 mL of glass-distilled water were pipetted into 10-mL polytef-lined screw-capped glass tubes. A 500-μL aliquot of 5-mg/mL dithiothreitol solution was added; the tubes were vortex-mixed for 10 sec, capped, and incu-

[1] Laboratories Bristol, France.
[2] BDH Ltd., Poole, Dorset, England.
[3] Rathburn Chemicals Ltd., Walkerburn, Peebleshire, Scotland.
[4] CF5OA Centriflo Cones; Amicon Ltd., Stonehouse, Gloucestershire, England.
[5] LDC Constametric III HPLC Pump; Milton Roy Corp., Fla.
[6] Rheodyne type 7125; Rheodyne Inc., Calif.
[7] LDC Spectromonitor III variable-wavelength detector; Milton Roy Corp., Fla.
[8] Rikadenki Mitsui Ltd., Machinery Sales, Chessington, Surrey, England.
[9] Hypersil ODS 5 μm, Shandon Ltd., Runcorn, Cheshire, England.
[10] Whatman PTFE Membrane Filters 0.5 μm; Whatman LabSales Ltd., Maidstone, Kent, England.
[11] Baird and Tatlock Ltd., Romford, Essex.

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Table I—Replicate Calibration Data for Plasma and Urinary *N*-Acetylcysteine by HPLC, Showing Linear Regression Analysis

Assay	Calibration Range	*n*	Slope	Intercept	*r*
Plasma	0.25–2.5 μg/mL	4	8.07	0.82	0.996
Urine	0.25–1.5 mg/mL	5	152.7	11.5	0.997

bated for 30 min at 37°C in a water bath. Following incubation 1.0 mL of 2% sodium hydrogen carbonate in water and 350 μL of 5.0% 2,4-dinitro-1-fluorobenzene in ethanol, which had been recrystallized from ether, was added to all tubes. The tubes were vortex-mixed for 10 s and incubated for 30 min at 60°C in a water bath.

The contents of each tube were then transferred to the membrane centrifuge cones, conditioned before use by immersion in double-glass-distilled water for 60 min and centrifuging for 5 min at 1000×*g* to remove excess water. The samples in the ultrafiltration cones were placed in labeled 20-mL glass vials and centrifuged at 1000×*g* for 20 min at 20°C. Aliquots (1.0 mL) of the ultrafiltrates were transferred to 25-mL extraction tubes, and 5 mL of water was added. The ultrafiltration step was omitted for the urine sample assays. Ether (10 mL) was added to all tubes, which were then capped and shaken mechanically[12] at 250 cpm for 5 min in the dark. Following centrifugation at 1000×*g* for 5 min at 10°C, the ether layers were transferred to clean glass vials and the solvent was evaporated to dryness under a gentle stream of nitrogen at room temperature. The dried residues were stored in capped vials at −20°C until just prior to chromatography, when the plasma and urine residues were reconstituted with 75 μL and 1.0 mL of mobile phase, respectively.

RESULTS AND DISCUSSION

The chromatographic conditions described were implemented in order to provide the best compromise between chromatographic resolution and peak symmetry and relatively short retention times, permitting the analysis of a large number of samples with good accuracy and precision. Under these HPLC conditions, the *N*-acetylcysteine derivative gives a retention time of ≃13 min, with a run time between injections of 20 min.

Figures 1 and 2 illustrate typical plasma and urine chromatograms, respectively, of spiked material, drug-free control material, and of material following dosing of the compound. Standard calibration lines were constructed by plotting the peak areas of *N*-acetylcysteine against the concentration of the compound in spiked standards. Typical calibration curve data is presented in Table I, showing linearity over the ranges employed with coefficients of variation of 12.6% at 0.25 mg/mL and 3.8% at 1.5 mg/mL for the urinary range and 11.9% at 0.25 μg/mL and 9.7% at 2.5 μg/mL for the plasma calibrations. These data show good precision despite the lack of an internal standard. A number of candidate substances were investigated for suitability as an internal standard, but none were acceptable for a variety of reasons.

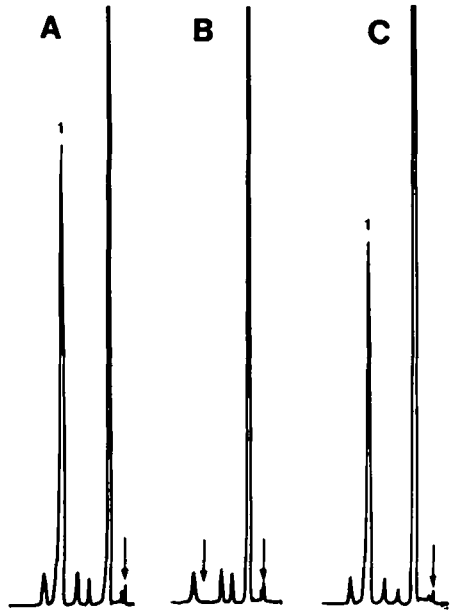

Figure 1—*Typical chromatograms of* N-*acetylcysteine in human plasma. Key: (A) a spiked plasma extract containing 2.0 μg/mL of* N-*acetylcysteine (1); (B) a drug-free control plasma extract; (C) a plasma extract from a subject receiving oral* N-*acetylcysteine.*

[12] Braun Lab-Shaker LSL; FT Scientific Ltd., Tewkesbury, England.

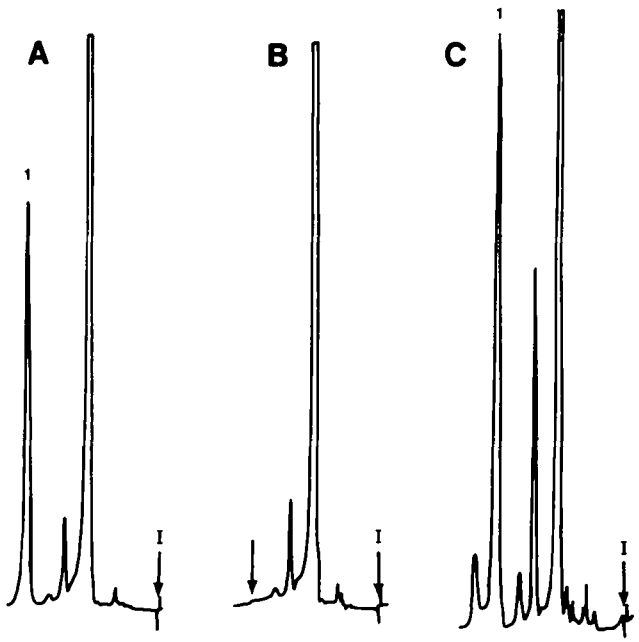

Figure 2—*Typical chromatograms of* N-*acetylcysteine in human urine. Key: (A) a spiked urine extract containing 1.5 mg/mL of* N-*acetylcysteine (1); (B) a control drug-free urine extract; (C) a test urinary collection 4–6 h following intravenous administration of* N-*acetylcysteine.*

Within- and between-batch data showed generally very favorable reproducibility. Ten replicate plasma controls each at 0.4, 1.1 and 1.9 μg/mL assayed the same day gave mean values of 0.43 ± 0.07 (*SD*), 0.99 ± 0.06 (*SD*), and 1.95 ± 0.22 (*SD*) μg/mL, respectively. Between-batch reproducibility ranged from 0.6 to 17.3% (mean 7.9%) for a series of seven control samples; however, occasional outliers were encountered, *e.g.*, a control spiked at 0.83 μg/mL gave a result of 1.15 μg/mL, a 38% error. Investigations of the stability of *N*-acetylcysteine in plasma on storage at −20°C lend further evidence of good overall reproducibility for the procedure. Plasma from a subject in receipt of a 1.0-g iv dose of *N*-acetylcysteine was assayed freshly and at 4, 5, 6, and 11 d of storage at −20°C. Samples withdrawn at 0.5, 1, 1.5, and 2.0 h postdose gave concentrations of 58.9, 25.0, 15.4, and 12.0 μg/mL when analyzed freshly, and the levels did not deteriorate on storage, giving *SD* values of 2.41, 2.48, 1.46, and 1.52, respectively, over the period.

Levels as low as 50 ng/mL can be seen over a baseline signal-to-noise ratio of 2.1. Although accurate detection limits have not been determined, sensitivity for the plasma and urine levels encountered were not a problem.

The measurement of free levels of *N*-acetylcysteine in plasma was investigated by omitting the dithiothreitol stage in order to produce ultrafiltrates containing free, unbound compound. Following derivatization and HPLC, no discernible free levels could be detected, even after oral doses of 800 mg of *N*-acetylcysteine. These data call into question that published by other workers who have reported free *N*-acetylcysteine in plasma. Our investigations demonstrate that a reduction employing dithiothreitol is essential to release *N*-acetylcysteine from binding sites on protein and to release the compound from disulfides, *e.g.*, bis-*N*-acetylcysteine. There is evidence to suggest that the latter is present, since our investigation of urinary *N*-acetylcysteine levels shows that very much lower levels of the compound are found when omitting the dithiothreitol stage. An ultrafiltration stage is incorporated in the plasma assay to remove excess 2,4-dinitro-1-fluorobenzene, which binds to plasma proteins and interferes with the chromatography.

The procedure described herein is being used to investigate the pharmacokinetics of *N*-acetylcysteine in adult healthy human subjects in receipt of intravenous and oral dosage forms. Preliminary data indicate that a rapid, specific, and precise procedure for determining *N*-acetylcysteine in biological fluids has been developed.

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Comparative Aspirin Absorption Kinetics after Administration of Sodium- and Potassium-Containing Buffered Solutions

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Received March 28, 1983, from the *Pharmacokinetics Laboratory, University of Missouri-Kansas City, Schools of Pharmacy & Medicine, Kansas City, MO 64108.* Accepted for publication June 29, 1983.

Abstract □ Twelve fasting normal volunteers received three aspirin dosage forms in a single 325-mg dose in a complete crossover study; the plasma aspirin and salicylic acid levels and the urine salicylic acid and salicyluric acid levels were measured over 10 h. The three dosage forms included an unbuffered tablet and two effervescent solutions, one with sodium bicarbonate-citrate buffer and the other with potassium bicarbonate-citrate buffer. A significantly faster absorption rate was observed with the sodium bicarbonate-citrate buffer, when compared with the potassium bicarbonate-citrate buffer and the unbuffered tablets, which were equivalent. These differences were attributed primarily to gastric emptying rate differences. Urine pH and salicylate renal clearance were significantly affected by the single dose of antacid buffer. The area under the curve and urine accumulation comparisons suggested that ~25% more aspirin reaches the general circulation intact after administration of the unbuffered tablet than the two solutions, but that the total salicylate absorbed is equivalent for all three dosage forms. This difference in aspirin bioavailability is probably due to the fact that the two buffered solutions are predominantly absorbed through the intestine, in which presystemic hydrolysis occurs, whereas a significant portion of the tablet dose is absorbed through the gastric mucosa.

Keyphrases □ Aspirin—absorption kinetics after administration of sodium- and potassium-containing buffered solutions □ Salicylic acid—absorption kinetics after administration of sodium- and potassium-containing buffered solutions □ Absorption kinetics—aspirin after administration of sodium- and potassium-containing buffered solutions

Aspirin is the drug of choice when a mild analgesic-antipyretic effect is required. It is also a primary agent used in the chronic management of rheumatoid arthritis and osteoarthritis. After oral administration for pain, rapid absorption is desirable to provide the rapid onset of effects and to reduce contact time with the gastric mucosa. The potential influence of potassium ion *versus* sodium ion on the absorption kinetics of aspirin from two different effervescent solutions is the subject of this report.

BACKGROUND

A recent report (1) from this laboratory compared the absorption kinetics of aspirin from three commercially available dosage forms. Two of the dosage forms were extemporaneously prepared effervescent solutions containing either 1.825 or 3.808 g of sodium bicarbonate as a component of the buffer. It was concluded that a primary mechanism for the more rapid absorption of the solutions than the unbuffered tablet was more rapid gastric emptying. Also, the lower area under the aspirin curve for the solutions was attributed to a greater portion of the aspirin being absorbed through the intestine (*i.e.*, rather than through the stomach wall) and thus being subjected to a greater presystemic hydrolysis.

Patients on sodium-restricted diets may wish to take aspirin as a buffered effervescent solution. Absorption kinetics of the commercially available buffered effervescent solution was compared with that of a solution in which the sodium was replaced with potassium. A commercially available unbuffered tablet was also studied for comparison.

EXPERIMENTAL SECTION

Dosage Forms—Three dosage forms were used to provide equal doses of aspirin: a plain tablet containing 325 mg of aspirin[1]; an effervescent tablet containing 324 mg of aspirin, 1,904 g of sodium bicarbonate, and 1 g of citric acid[2]; and a prepared effervescent tablet containing 324 mg of aspirin, 2.26 g of potassium bicarbonate, and 1 g of citric acid.

Subjects—Twelve healthy volunteers (eight males and four females; ages 21–37 years; weight, 53.6–94.5 kg) were evaluated by a comprehensive physical examination, blood chemistry profile (including blood count and differential), and complete urinalysis. None of the subjects had a history of gastrointestinal disease or surgery. All subjects were free of any active disease process, and none had used any medication for 14 d before the study.

Method—A Latin-square design for three treatments in 12 subjects was employed. A 10-h fast preceded dosing and continued for 4 h postdose. At ~7 a.m., predose urine and blood samples were obtained, and a single dose of aspirin with 240 mL of water was administered. The effervescent tablets were dissolved in 140 mL of water 3 min before dosing and the mixture was then swallowed. The glass was rinsed with 100 mL of water, which also was swallowed. After dosing, 100 mL of water was administered at 1, 2, and 3 h, and a uniform meal was served after the 4-h sample was obtained, after which water was allowed *ad libitum*. Subjects remained standing or sitting through the day, and exercise was limited to walking about the room.

Blood was drawn into chilled vacuum containers[3] *via* an indwelling catheter at 5, 10, 15, 20, 30, and 45 min and 1, 1.5, 2, 3, 4, 6, 8, and 10 h. Plasma was separated by centrifugation at 1764×*g* at 4°C within 20 min of collection. All urine was collected at 2-h intervals over 10 h, the pH and volume were measured, and an acidified aliquot was saved for analysis. All samples were frozen at −30°C and assayed within 2 weeks of collection by the HPLC method described previously (2).

RESULTS

As shown in Tables I and II, both plasma aspirin and salicylic acid levels rise more rapidly after administration of the solution with sodium than the potassium solution or the tablet. This rank order and profile reflect the values for 11 of the 12 subjects and is statistically significant ($p < 0.05$). Plasma aspirin levels rise rapidly to a mean peak of 7.20 μg/mL at 17.5 min with the single 324-mg dose in a sodium-containing effervescent solution, whereas the potassium-substituted solution reaches only 5.26 μg/mL at 30.4 min, which is not significantly higher ($p < 0.05$) than for the tablet. The ultimate exposure

[1] Bayer Aspirin; Glenbrook Laboratories -Division of Sterling Drug Inc., New York.
[2] Alka-Seltzer; Miles Laboratories, Inc., Elkhart, Ind.
[3] Vacutainer BD, 278-069, 7.0 mL, containing 14 mg of potassium oxalate and 17.5 mg of sodium fluoride.

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