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Journal of Chromatography B, 732 (1999) 323–329

JOURNAL OF CHROMATOGRAPHY B

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Simple and rapid assay for acetaminophen and conjugated metabolites in low-volume serum samples

Lane J. Brunner*, Shuang Bai

Pharmaceutics Division, College of Pharmacy, The University of Texas at Austin, Austin, TX 78712-1074, USA

Received 1 February 1999; received in revised form 27 May 1999; accepted 22 June 1999

Abstract

The use of marker compounds for estimating drug metabolic capacity or pharmacokinetic parameters is common in the biological sciences. Often small laboratory animals are used and thus sample size is a limiting concern. In this report, we describe an assay we developed for measuring the concentration of acetaminophen and its conjugated metabolites in low-volume serum samples. Acetaminophen and metabolites were removed from 10 μl serum samples by a single-step 6% (v/v) perchloric acid deproteination using theophylline as internal standard. Samples were separated in a pH 2.2 sodium sulfate–acetonitrile mobile phase at a flow-rate of 1.5 ml/min on a 15 cm octadecylsilyl column at room temperature. Analytes were detected at a wavelength of 254 nm. The resulting chromatograms showed no interfering peaks from endogenous serum components. The concentration ranges measured were 1.56–200 μg/ml for acetaminophen and acetaminophen sulfate and 3.91–500 μg/ml for acetaminophen glucuronide. The assay was linear in the range of concentrations analyzed. The intra-day and inter-day coefficient of variation ranged from 0.4 to 8.2% and 0.2 to 12.3% for acetaminophen, 0.5 to 12.9% and 0.3 to 16.1% for acetaminophen glucuronide, and 0.4 to 8.1% and 0.2 to 14.3% for acetaminophen sulfate, respectively. Results from the experiments show that acetaminophen and its conjugated metabolites can easily and reproducibly be measured in low-volume serum samples and thus may offer an additional method to measure these compounds when the volume of biological samples may be limited.

Keywords: Acetaminophen

1. Introduction

Acetaminophen is a widely used analgesic and antipyretic agent available alone or in combination with other drugs [1]. In addition to its use as a therapeutic agent, acetaminophen is also used as an in vivo and in vitro probe to measure conjugative (Phase II) metabolism [2]. Acetaminophen is primarily eliminated from the body through the formation of glucuronide and sulfate conjugates [3]. Since oxidation metabolism by cytochrome P450 plays only a minor role in the clearance of acetaminophen [4], the drug is often used as a marker of Phase II metabolism in humans and animals.

Studies that examine the pharmacokinetics of a drug substance often require frequent blood sampling to adequately describe the disposition characteristics. Usually frequent blood sampling does not lead to significant concerns, such as hypotension or anemia, with adults or larger animal models. However,

*Corresponding author. Tel.: +1-512-471-0942; fax: +1-512-471-7474.

E-mail address: ljb@mail.utexas.edu (L.J. Brunner)

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00290-X

frequent blood sampling in children or small animals may result in the removal of excessive amounts of blood, which may alter hemodynamics or affect the study outcome. Thus, assays that use a minimal amount of blood to measure drug concentrations would be a significant advantage.

To date, several high-performance liquid chromatographic methods for determining acetaminophen concentration in plasma, serum, and blood have been reported. However, relatively few could simultaneously separate and measure acetaminophen and its sulfate and glucuronide conjugated metabolites. In addition, current methods available may not be adequate for the analysis of small volume serum samples. Often, methods for the analysis of acetaminophen and conjugated metabolites require complex sample preparation steps [4,5] or a long retention time [6]. Furthermore, current assays often necessitate the use of large amounts of sample [7,8]. Assays do exist which require a relatively low sample volume of 50–100 μl of serum or plasma [9–11]. This sample volume may be adequate for isolated serum samples; however, serial sampling as often used in pharmacokinetic studies may result in excessive blood loss in small laboratory animals. The purpose of the present study was to develop a simple and rapid high-performance liquid chromatography (HPLC) assay for the Phase II metabolic probe, acetaminophen, which required a minimal sample volume, and was also able to separate and detect both the sulfate and glucuronide conjugates as well as the parent drug.

2. Experimental conditions

2.1. Materials

All chemicals were analytical grade reagents and used as received without further purification. Acetaminophen and theophylline were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Perchloric acid, sodium sulfate, phosphoric acid, and acetonitrile (HPLC grade) were purchased from EM Science (Gibbstown, NJ, USA). Acetaminophen sulfate and acetaminophen glucuronide were kindly donated by Dr. Mark Gemborys of McNeil Consumer Products (Fort Washington, PA, USA).

2.2. Animals

Eight adult male Sprague-Dawley rats (250–300 g, Harlan Sprague Dawley, Indianapolis, IN, USA) were used during the study. Prior to the start of the study, all procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas at Austin. All procedures are in accordance with the guidelines established by the National Institutes of Health for the humane treatment of animals.

2.3. Instrumentation

A Shimadzu HPLC system (Shimadzu Scientific Instrument, Inc. Columbia, MD, USA), equipped with an automatic injection system (Model SIL-10A), dual solvent delivery pumps (Model LC-10AS), a system controller (Model SCL-10A), and a variable visual/ultraviolet wavelength detector (Model SPD-10AV). Column temperature was maintained with the use of a column oven (Eppendorf Model CH-30, Brinkmann Instruments, Westbury, NY, USA) and column temperature controller (Eppendorf Model TC-50, Brinkmann Instruments, Westbury, NY, USA). Data was collected and analyzed using a commercial computer software package (CLASS-VP, Shimadzu Scientific Instrument, Inc. Columbia, MD, USA).

2.4. Rat samples

Rats were housed in a 12 h light/dark cycle with free access to standard laboratory rodent chow (Harlan, Indianapolis, IN, USA) and deionized water. Rats were acclimated to the animal care facility for three days prior to drug administration. Following the acclimation period, a silicone and polyethylene cannula was implanted into the right jugular vein during surgical plane anesthesia. Cannulas were tunneled subcutaneously and brought to the exterior at the base of the skull. The rats were placed into individual cages and allowed to recover for 1 day following cannula placement. Cannulas were flushed once daily with 0.5 ml normal saline containing 20 U/ml sodium heparin. Following the recovery period, rats were administered a single 1 ml/kg intravenous dose of acetaminophen solution (25 mg/

ml in 20% propylene glycol in normal saline, v/v) via the jugular vein cannula and then the cannula was immediately flushed with 1 ml of normal saline. Serial blood samples (50 μl) were obtained from the catheter prior to and at 10, 20, 30, 45, 60, 75, 90 min following drug administration. Blood samples were allowed to clot at ambient temperature and serum separated by centrifugation (17 000 *g* for 5 min at 4°C). Samples were stored at −80°C until analyzed (within 1 week).

2.5. Sample preparation

Standards were prepared using a concentration range of 1.5 to 200 μg/ml for acetaminophen and acetaminophen sulfate, and 3.9 to 500 μg/ml for acetaminophen glucuronide. The quality control samples were prepared independently. A low concentration quality control sample was composed of acetaminophen 8.3 μg/ml, acetaminophen sulfate 8.3 μg/ml, and acetaminophen glucuronide 20.8 μg/ml. A high concentration quality control sample was composed of acetaminophen 66.7 μg/ml, acetaminophen sulfate 66.7 μg/ml, and acetaminophen glucuronide 166.7 μg/ml. An aliquot of each sample was placed into a polypropylene centrifuge tube and stored at −80°C. Standards were analyzed within two weeks. Samples were stable for up to three months when stored at −80°C (data not shown). Prior to extraction, samples were allowed to equilibrate at room temperature for 60 min. Serum samples from rats administered acetaminophen were treated the same as spiked serum samples.

2.6. Extraction procedure

Serum aliquots (10 μl) were deproteinated by the addition of 20 μl 6% (v/v) perchloric acid in water containing 10 mg/ml theophylline as the internal standard. The tubes were mixed on a vortex for 5 s and then centrifuged (17 000 *g*×5 min at 4°C). The clear supernatant was removed and placed into a glass vial. A 10 μl aliquot was injected into the HPLC system via the autosampler.

Separation of acetaminophen and conjugated metabolites from extracted serum components was achieved using an isocratic mobile phase consisting of 7% acetonitrile in 93% 0.05 m*M* sodium sulfate (pH 2.2, adjusted with phosphoric acid) buffer. The flow-rate was constant at 1.5 ml/min throughout the separation. Acetaminophen and conjugated metabolites were resolved at 30°C on a 150 mm×4.6 mm C_{18} column (Supelco, Bellefonte, PA, USA) with a 5 μm particle diameter and a 120 Å pore size. The column was preceded by a 10 mm×4.3 mm C_{18} guard column with a 5 μm particle diameter and a 50 Å pore size (Upchurch Scientific, Oak Harbor, WA, USA). Detection of analytes was achieved using ultraviolet absorbance at 254 nm. A total of 4 sets of standards were analyzed for both intra-day and inter-day assay variability. Low concentration and high concentration of quality control samples were run approximately every twelve samples.

2.7. Data analysis

Peak areas of acetaminophen, conjugated metabolites, and the internal standard were measured. Peak area ratios of acetaminophen, and conjugated metabolites to that of the internal standard were calculated and used for the construction of the standard curves. These peak area ratios were plotted against the added concentration of each of the standards. Least-squares linear regression was then used to determine the linearity of the curves and calculate the slope, intercept, and the correlation coefficient of the line for each of the analytes. The concentration of each sample was calculated based on the standard curve. Intra-day and inter-day variability were measured by using one factor analysis of variance (SuperANOVA; Abacus Concepts, Berkeley, CA, USA). All data is represented as mean±SD (%RSD).

3. Results

3.1. Concentration range and limits of detection and quantification

A wide range of concentrations was used for the analysis; 1.5–200 μg/ml for acetaminophen and acetaminophen sulfate, and 3.9–500 μg/ml for acetaminophen glucuronide. These concentration ranges represent blood concentrations commonly measured in pharmacokinetic studies and are also

relevant to the clinical laboratory setting. Within each range, eight different concentrations were measured. The limit of detection was 0.05 μg/ml. The limit of quantification was 0.15 μg/ml for all of the three compounds at a signal-to-noise ratio of 3:1.

3.2. Interference and retention time

Using the described chromatographic method, acetaminophen, acetaminophen glucuronide, acetaminophen sulfate, and the internal standard, theophylline, yielded sharp and well resolved peaks with no interference from endogenous compounds at 4.0, 2.3, 3.1, and 5.1 min, respectively. Fig. 1 shows representative chromatograms from a blank serum sample containing the internal standard 20 μg/ml only and a serum sample containing acetaminophen 3.1 μg/ml, acetaminophen glucuronide 7.8 μg/ml, acetaminophen sulfate 3.1 μg/ml, and internal standard 20 μg/ml. Separation of these three compounds was achieved within 6 min. Total time of analysis is 10 min. The additional peak at 9.5 min is unknown and was present in all serum samples from rats, but not seen in human samples (data not shown). Variability in retention time was determined by dividing the standard deviation of the retention time by the mean of the retention time. The %RSD of the retention time for acetaminophen, acetaminophen glucuronide, and acetaminophen sulfate was 0.20%, 0.20%, and 0.47%, respectively for 40 consecutive samples.

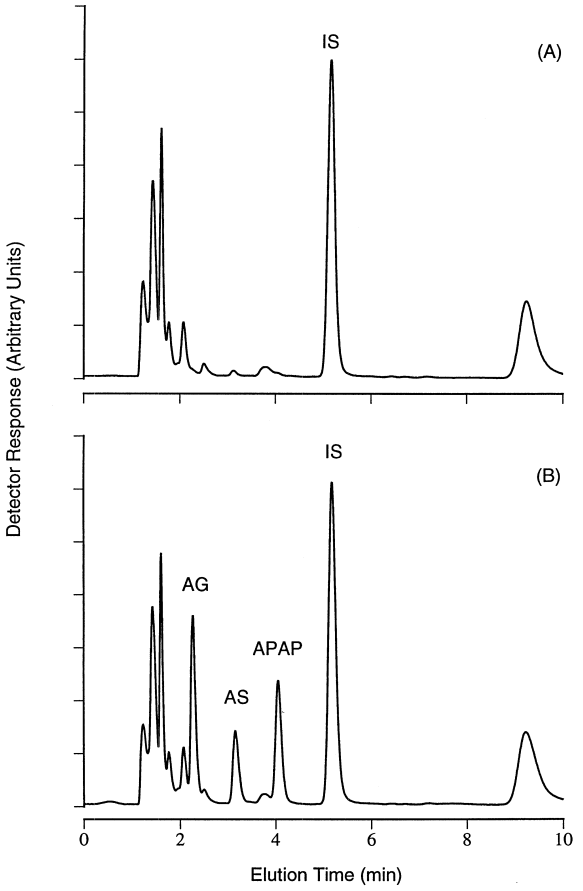

Fig. 1. Representative chromatograms of rat serum containing internal standard (I.S.) 20 μg/ml only (A) and acetaminophen (APAP) 3.1 μg/ml, acetaminophen glucuronide (AG) 7.8 μg/ml, acetaminophen sulfate (AS) 3.1 μg/ml, and internal standard (I.S.) 20 μg/ml (B).

3.3. Linearity

The assay was validated using eight point calibration curves in the concentration ranges outlined above. The standard curves of the peak area ratios of acetaminophen and its metabolites to internal standard were all linear. The correlation coefficients (r^2) were 0.9998±0.0002 (0.02%), 0.9997±0.0003 (0.03%) 0.9998±0.0002 (0.02%) for the standard curves of acetaminophen, acetaminophen glucuronide, and acetaminophen sulfate, respectively. Equations of the standard curves were:

Acetaminophen: $y = (0.1012 \pm 0.0014)x - (0.0345 \pm 0.0299)$,

Acetaminophen glucuronide:

$y = (0.0429 \pm 0.0009)x + (0.0068 \pm 0.0394)$

and

Acetaminophen sulfate: $y = (0.0561 \pm 0.0016)x - (0.0065 \pm 0.0196)$

where y represents the peak area ratio of analyte to internal standard and x represents the added concentration of the analyte.

Fig. 2 shows the concentration versus time profiles for acetaminophen, acetaminophen glucuronide, and

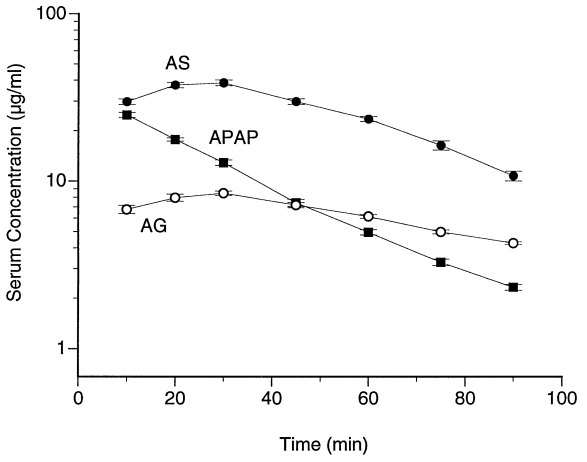

Fig. 2. Representative standard curves for acetaminophen (APAP, 1.5–200 μg/ml, –■–), acetaminophen glucuronide (AG, 3.9–500 μg/ml, –○–), and acetaminophen sulfate (AS, 1.5–200 μg/ml, –●–). The different slopes of the standard curves underscore the difference in response of the assay for each analyte over the concentration range examined.

acetaminophen sulfate following the administration of a single 25 mg/kg intravenous dose of acetaminophen to eight rats.

3.4. Repeatability

The quality control (QC) samples with low and high concentrations were prepared as the other samples and run within each batch. The repeatability of each quality control sample was calculated by the standard deviation over the mean of measured concentration. For acetaminophen, the intra-day relative deviations (RSDs) of high and low concentrations of quality control samples were 1.3 and 3.2%, the inter-day RSDs were 2.2 and 4.0%. For acetaminophen glucuronide, the intra-day RSDs of high and low concentrations of quality control samples were 0.9 and 3.6%, the inter-day RSDs were 2.3 and 5.7%. For acetaminophen sulfate, the intra-day RSDs of high and low concentrations of quality control samples were 2.4 and 4.0%, the inter-day RSDs were 2.3 and 4.3%. The repeatabilities of all quality control samples were below 6%, indicating a reliable measurement using this method.

3.5. Precision

The intra-day variability was determined by analyzing four sets of standards on the same day. For the determination of inter-day variability, a single set of standards was assayed on four consecutive days. Both the intra-day and inter-day variability were evaluated by spiking drug free rat serum with eight different concentrations of acetaminophen, acetaminophen glucuronide, and acetaminophen sulfate, and by calculating the coefficient of variation for each measurement. Over the range of concentrations of each compound, the intra-day relative standard deviations (RSDs) were 0.4 to 7.9%, 0.5 to 13%, and 0.4 to 8.1% for acetaminophen, acetaminophen glucuronide, and acetaminophen sulfate, respectively. The inter-day RSDs were 0.2 to 12.4%, 0.3 to 16.1%, 0.2 to 14.3% for acetaminophen, acetaminophen glucuronide, and acetaminophen sulfate, respectively.

3.6. Accuracy

Analytical accuracy was evaluated by measuring the variation between the added concentration and the measured concentration for the three analytes. The intra-day accuracies over the range of concentrations were 99.2 to 122.2%, 95.3 to 100.6%, and 99.1 to 109.7% for acetaminophen, acetaminophen glucuronide, and acetaminophen sulfate, respectively. The inter-day accuracies were 95.4 to 114.4%, 96.3 to 101.6%, and 96.3 to 106.7% for acetaminophen, acetaminophen glucuronide, and acetaminophen sulfate, respectively.

3.7. Absolute recovery

Absolute recovery was measured at concentrations of 12.5, 50.0 and 200.0 μg/ml for acetaminophen and acetaminophen sulfate, and 31.25, 125.0, and 500.0 μg/ml for acetaminophen glucuronide. Serum samples ($n=4$) at each concentration containing acetaminophen, acetaminophen glucuronide, and acetaminophen sulfate were extracted and injected. Four samples of the same amount of compound in water were directly injected and the concentrations were calculated. The absolute recovery was calculated by comparing the concentrations for direct

Table 1
Absolute recovery of serum acetaminophen, acetaminophen glucuronide, and acetaminophen sulfate. Values are mean±SD (%RSD) of $n=4$ each

Compound	Concentration (μg/ml)	Absolute recovery (%)
Acetaminophen	12.5	102.8±2.4 (2.3)
	50.0	97.9±2.3 (2.3)
	200.0	100.0±0.7 (0.7)
Acetaminophen glucuronide	31.25	101.2±1.6 (1.6)
	12.5	99.4±3.0 (3.0)
	500.0	99.7±0.7 (0.7)
Acetaminophen sulfate	12.5	101.9±2.4 (2.4)
	50.0	99.7±3.7 (3.7)
	200.0	99.8±0.7 (0.7)

injection of pure compounds with those for serum samples. The absolute recoveries are shown in Table 1.

4. Discussion

Acetaminophen is primarily metabolized via conjugation to form acetaminophen sulfate and glucuronide. Acetaminophen and its conjugated metabolites do not significantly bind to plasma proteins and their renal excretion is independent of urinary pH. Thus, acetaminophen is a good marker of phase II metabolism. However, the elimination of acetaminophen is dose-dependent [12]. Large dose administration of acetaminophen would result in concentration-dependent pharmacokinetics, by which the half-life increases with increasing dose [12]. Thus, in order to avoid nonlinearity in elimination, a low dose of acetaminophen is commonly used. However, when smaller volume samples are used, such as in pharmacokinetic studies in rodents, a more sensitive analytical method is often required. The present study describes the assay developed and routinely used in our laboratory for drug metabolic studies. We used mobile phase at pH 2.2. According to the literature, acetaminophen is very stable and the half-life at pH 2 is 0.73 years [13], so acetaminophen was considered stable during HPLC analytical process.

In order to accurately estimate pharmacokinetic parameters, serial blood samples need to be taken. Ideally, blood samples should be collected from the earliest time point, such as immediately after drug administration, until the blood concentration is below the detection limit of the assay. The more frequent the samplings are, the more accurately one is able to estimate the pharmacokinetic parameters. However, frequent blood sampling often requires large volumes of blood to be removed during the entire study. In the present study, small volumes of serum were used for the measurement of acetaminophen and conjugated metabolites. For each sample, 10 μl of serum was requires and thus as little as 25 μl of whole blood would be necessary for each sample.

As can be seen from the results of the intra-day and inter-day analyses, the present assay provides reasonable repeatability, precision, accuracy and recovery over the concentration range tested. In addition, this concentration range corresponds to those levels often seen in pharmacokinetic studies. Application of the present assay for the determination of acetaminophen and conjugated metabolite pharmacokinetics is given in Fig. 2. All of the standard curves show linearity with a correlation coefficient above 0.999 for each analyte whether measured within days or between days. The slope of the peak area ratio versus concentration curve showed little variability with a %RSD of less than 3%, indicating a good reproducibility. In addition, the retention time %RSD for all three analytes was between 0.20% and 0.47%.

We report a simple and rapid HPLC method to separate and quantify acetaminophen and its two conjugated metabolites, glucuronide and sulfate in serum samples. This method is characterized by the use of 10 μl serum samples and resolution of all analyte peaks within six min. Sample preparation consisted of a single perchloric acid deproteination, not requiring further purification. Thus, this assay provides a simple and rapid method to measure acetaminophen and conjugated metabolite concentrations where sample volumes may be limited.

Acknowledgements

The authors gratefully acknowledge the technical assistance of Habiballah Abdus-Salaam. This work was supported, in part, by an unrestricted research

grant from The R.W. Johnson Pharmaceutical Research Institute.

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