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Development and validation of a UPLC-MS/MS method for the quantification of acetaminophen in human plasma and its application to pharmacokinetic studies

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We developed an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for the determination of acetaminophen concentration in human plasma. Following protein precipitated extraction, the analytes were separated and analyzed using an UPLC-MS/MS in the multiple reaction monitoring (MRM) mode with the respective $[M+H]^+$ ions, *m/z* 152.06 → 110.16 for acetaminophen and *m/z* 180.18 → 138.12 for phenacetin (internal standard, IS). The method showed a linear response from 1 to 100 μg/mL ($r > 0.9982$). The limit of quantitation for acetaminophen in plasma was 1 μg/mL. The intra- and inter-day accuracy ranged in the ranges of 94.40–99.56% and 90.00–99.20%, respectively. The intra- and inter-day precision ranged in the ranges of 2.64–10.76% and 6.84–15.83%, respectively. This method was simple, reliable, precise and accurate and can be used to determine the concentration of acetaminophen in human plasma. Finally, this fully validated method was successfully applied to a pharmacokinetic study of acetaminophen in healthy volunteers following oral administration.

Introduction

Acetaminophen (or paracetamol) is a widely used over-the-counter analgesic and antipyretic agent. Acetaminophen has been commonly used for the relief of headaches and other minor aches and pains, and it is a major ingredient in numerous cold and flu remedies.[1,2]

A lot of methods have been reported for the determination of acetaminophen concentrations in human plasma. Although liquid chromatography (LC) with ultraviolet or fluorescence detection has been well established, LC method is limited by its poor sensitivity and long analysis times.[3-7] Improvements in sensitivity, analysis time, and specificity have been achieved using LC-tandem mass spectrometry (LC-MS/MS) methods. [8-10] Ultra performance liquid chromatography (UPLC) techniques offer efficient chromatography with improved sensitivity by taking advantage of smaller particle size and higher operating pressures than conventional high performance liquid chromatography (HPLC).[11-15] UPLC techniques have been explored for applications in pharmacokinetic analysis, drug metabolism, and metabolite profiling.[16-18] For example, Tonoli D et al.[19] and Qiu X et al.[20] investigated a more rapid and sensitive UPLC-MS/MS method. Thus, we also attempted to validate a new UPLC-MS/MS method for quantification of acetaminophen concentration in human plasma in this study. The validated method was applied to a pharmacokinetics study in healthy volunteers after oral administration.

Methods

Chemical and reagents

Acetaminophen and phenacetin (internal standard, IS) were

purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile and other solvents were purchased from Merck (Darmstadt, Germany). American Chemical Society (ACS) reagent-grade formic acid and other chemicals were purchased from Sigma Aldrich. Blank human plasma samples were obtained from healthy Korean male volunteers. Water for chromatography was purified through a Milli-Q water® purification system (Millipore, Bedford, MA, USA).

Liquid chromatography

An UPLC system, equipped with a micro-vacuum degasser, thermostated autosampler, binary gradient pumps, and thermostated column compartment was purchased from Waters Corp. (Milford, MA, USA). The analytes were separated on an ACQUITY UPLC BEH Shield RP18 column (2.1 mm × 100 mm, 1.7 mm, Waters Corp.) maintained at 40°C. Mobile phase A was purified using water containing 0.1% formic acid and mobile phase B was acetonitrile containing 0.1% formic acid. Mobile phase B was increased from 10% to 90% with a flow rate of 0.2 mL/min, and then maintained at 90% for 1 min, followed by a rapid return to the initial condition (10% mobile phase B), which was held for 2 min. The autosampler temperature was kept at 5°C and the samples were injected onto the column with an injection volume of 5 µL (partial loop in needle overfill mode). The data acquisition run time was kept at 7 min for the mass spectrometer (MS). All data were collected and processed using MassLynx™ software with QuanLynx™ (Waters Corp., Milford MA, USA).

Mass spectrometry

A Quattro Premier XE™ micro mass triple quadrupole mass spectrometer (Waters Corp., Milford MA, USA) was interfaced with an electrospray ionization (ESI) probe in positive ionization mode. Nitrogen was used as the cone and desolvation gas with flow rate 50 L/h and 900 L/h. Argon was used as the collision gas, maintained at 6.87×10^{-4} mbar. The optimal MS conditions were the following: capillary voltage of 3.5 kV, cone voltage of 25 kV, source temperature 80°C, and desolvation temperature 250°C. The optimized collision energy for both and IS was 15 eV. The MS was operated in multiple reaction monitoring (MRM) mode and the MRM transition sets for acetaminophen and IS were *m/z* 152 → 110 and *m/z* 180 → 138, respectively, with a dwell time of 0.10 s per transition and a m/z tolerance of ± 0.1 Da.

Preparation of standards and quality controls

Stock solutions (1 mg/mL, 1,000 ppm) of acetaminophen and IS were prepared separately with acetonitrile. Working standard solutions containing 10, 20, 100, 200, 500, and 1,000 µg/mL of acetaminophen and a 10 µg/mL of IS solution were prepared by serial dilutions from the stock solution. Drug-free blank plasma (45 µL) was spiked with working standard solutions (5 µL) to prepare plasma calibration standards with final concentrations of 1, 2, 10, 20, 50, and 100 µg/mL of acetaminophen. Similarly, quality control (QC) samples were prepared in four concentrations: 1 µg/mL (lower limit of quantitation, LLOQ), 2 µg/mL (low, LQC), 50 µg/mL (middle, MQC), and 80 µg/mL (high, HQC) of acetaminophen. Calibration standard solution and quality controls were extracted daily before analysis using the procedure described below.

Plasma sample preparation

Samples were prepared by precipitating protein with acetonitrile. After transferring 50 µL aliquot of plasma into a 1.75 mL microtube, 10 µL of IS solution (10 µg/mL of phenacetin) and 940 µL of acetonitrile were added and vortexed for 5 min. After centrifugation at 13,200 rpm for 5 min at 4°C, 100 µL of the supernatant was diluted with 400 µL of mobile phase (50% mobile phase A, 50% mobile phase B). The mixture was vortexed for 2 min and 200 µL of the supernatant was filtered through a nylon filter (Conning Spin-X®, 0.22 mm). Filtered supernatant was transferred into glass vials and 5 µL of this solution was directly injected into the UPLC-MS/MS system.

Method validation

The newly developed UPLC-MS/MS method was validated with regard to linearity, specificity, accuracy, precision, percent recovery, and stability as suggested in the guideline of Ministry of Food and Drug Safety (MFDS).[21,22]

Linearity

Calibration curves were prepared daily prior to sample analysis by analyzing calibration standards ranging in concentration from 1 to 100 µg/mL. Calibration curves were plotted using the peak area ratio of acetaminophen to the IS as a function of the nominal concentration. Continuous calibration curves were fitted to the raw data using a weighted (1/x) least squares regression. Sensitivity was defined by the LLOQ, which was the concentration of acetaminophen at which the signal to noise (S/N) ratio was greater than 10 with an acceptable accuracy and precision. This value was set as the lowest concentration in calibration curves.

Specificity

The specificity was assessed by comparing chromatograms of the respective drug-free blank plasma from six healthy individuals with blank plasma spiked with acetaminophen (1 µg/mL) and IS (1 µg/mL). The retention times of endogenous compounds in the matrix were compared with that of acetaminophen and IS. Plasma was spiked with acetaminophen (1 µg/mL) and the IS (1 µg/mL).

Accuracy and precision

Intra-day accuracy and precision were assessed by replicate analysis (n = 5) of four QC plasma samples each containing 1 (LLOQ), 2 (low), 50 (middle), and 80 (high) µg/mL acetamino-

phen on a single day. Inter-day accuracy and precision were determined by replicate analysis (n = 5) of the same QC samples on five different days. Accuracy, expressed as the percent deviation (% DEV), was calculated as (mean measured concentration/nominal concentration) × 100. Precision, expressed as the relative standard deviation (RSD), was calculated as (standard deviation, SD/mean measured concentration) × 100.

Extraction recovery

The extraction recovery was determined by triplicate analyses of three QC concentrations (LQC, MQC, and HQC). The percent recovery was calculated as the peak area ratio of acetaminophen before and after extraction.

Stability

The stability of plasma QC samples at low (2 μg/mL) and high (80 μg/mL) concentrations was determined in triplicate by comparing the mean measured concentrations of freshly prepared samples with those obtained after stability testing. The stability of acetaminophen was evaluated under four different conditions: post-preparative stability at 5°C for 24 h, freeze-thaw stability for three cycles, and short-term temperature stability at room temperature for 4 hours and long-term temperature stability at -70°C for 132 days. The stability of stock solutions of acetaminophen and IS at -20°C for 30 days was evaluated after diluting each with mobile phase to a concentration of 1 μg/mL and comparing their peak areas to those of freshly prepared samples at the same nominal concentration.

Pharmacokinetic applications

The validated quantification method was applied to samples obtained from a pilot pharmacokinetic study to determine the plasma concentrations of acetaminophen after administration of multiple oral doses in eight healthy volunteers. All volunteers received 2 tablets of 500 mg acetaminophen 3 times a day for 7 days. The study protocol was approved by the institutional review board of Kyungpook National University Hospital, Daegu, Korea. During the study period, volunteers were prohibited from taking any other medications and were hospitalized as inpatients. After giving their written informed consent, all volunteers were orally administered with 2 tablets of 500 mg dose of acetaminophen. Blood samples (7 mL) were collected in heparinized tubes before (0 h) and 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, and 12 h after study drug administration. Plasma from all blood samples was immediately separated by centrifugation at 3,000 rpm for 10 min. All plasma samples were stored at -70°C until analysis.

The pharmacokinetic parameters for acetaminophen in plasma were determined using a non-compartmental model implemented in WinNonLin Pro 5.3 (Certara L.P., St. Louis, MO, USA).

Results

Optimization of UPLC-MS/MS

Measurements of acetaminophen and IS levels in human plasma samples were conducted using a UPLC-MS/MS instruments in MRM scan mode. Solutions of acetaminophen and IS (1 μg/mL) were directly infused into mass spectrometer along with mobile phase (0.2 mL/min) and MS parameters were optimized to increase sensitivity for respective product ions. The optimized MS spectra of acetaminophen and IS showed intense $[M+H]^+$ ions at *m/z* 152 and 180, respectively. When these parent ions underwent fragmentation, the resulting mass spectra showed intense product ions at *m/z* 110 and 138 for acetaminophen and IS, respectively (Fig. 1). Therefore, the corresponding transitions associated with these product peaks were selected for MRM analysis.

Although solid-phase extraction procedures can considerably reduce the matrix effect, it increases overall time and cost of analysis. In this study, we optimized simple protein precipitation with acetonitrile procedure which is fast enough for high-throughput analysis.

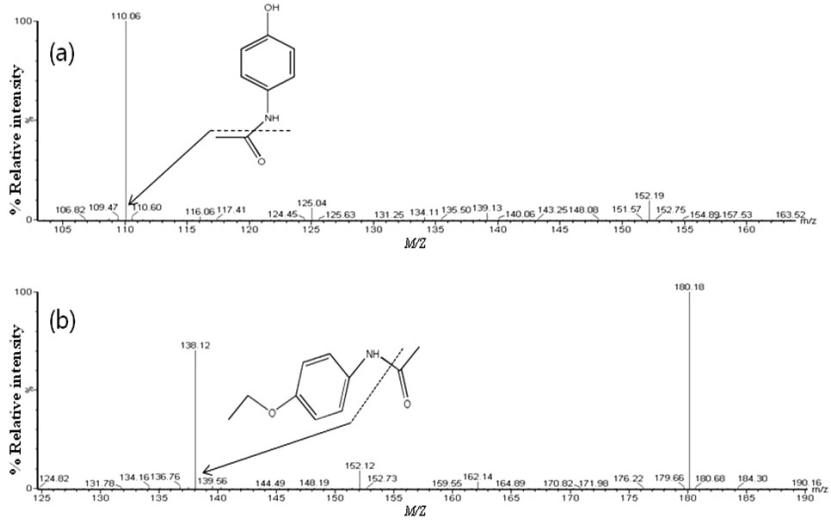

Figure 1. Product ion mass spectra are given for (a) acetaminophen and (b) phenacetin (IS).

Method validation and linearity of calibration

Typical MRM chromatograms obtained from the drug-free plasma, and plasma spiked with standard acetaminophen (1 μg/mL) and IS (1 μg/mL) are shown in Figure 2. No endogenous or background was observed at the retention times of acetaminophen or IS in plasma samples collected from six healthy volunteers

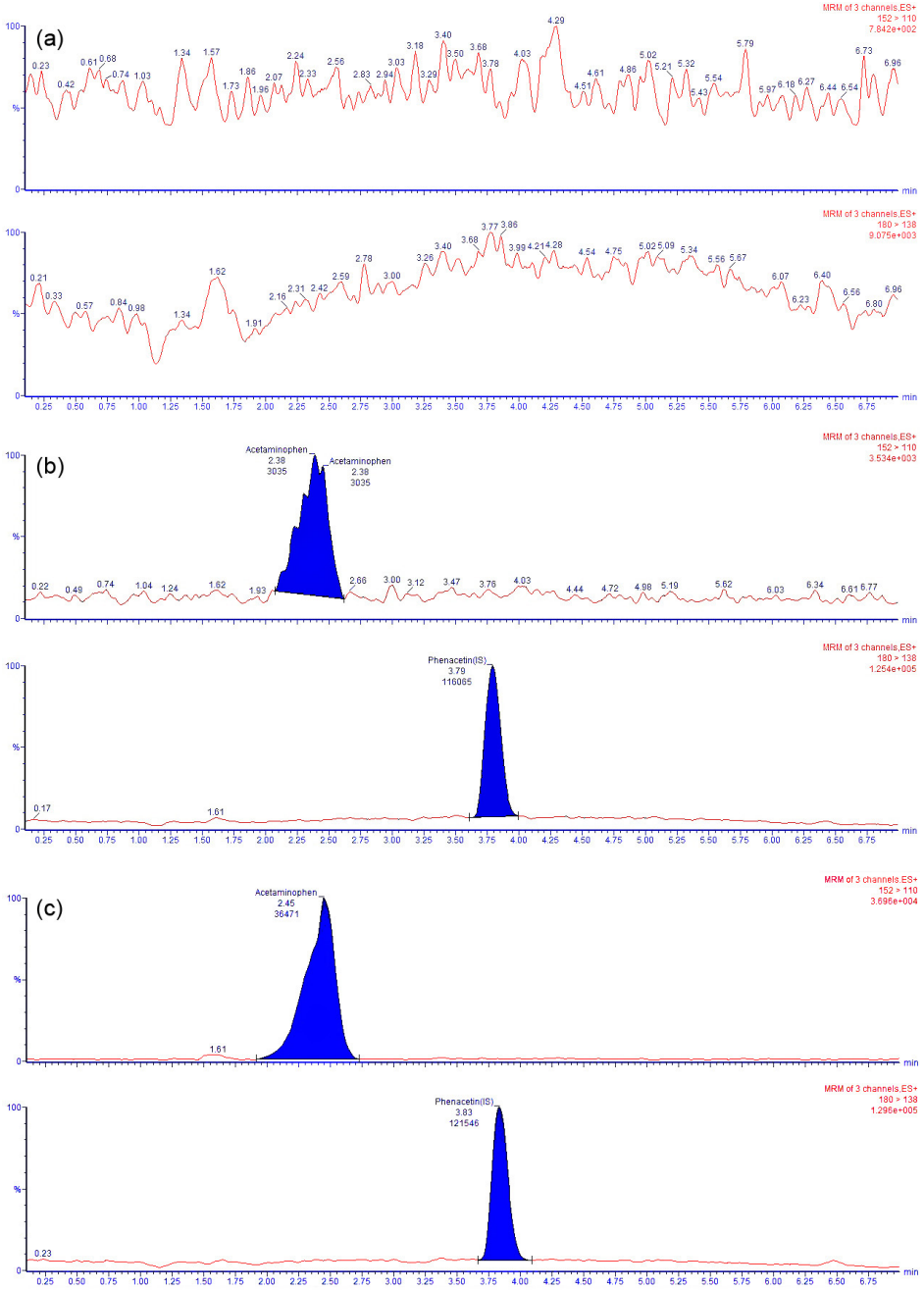

Figure 2. Typical MRM chromatograms of acetaminophen (upper panel) and phenacetin (lower panel) in human plasma samples obtained from (a) a drug-free blank plasma sample, (b) a plasma sample spiked with acetaminophen at the LLOQ (1 μg/mL) and 1 μg/mL phenacetin and (c) plasma from a volunteer 1 h after oral administration of a 1,000 mg dose of acetaminophen spiked with phenacetin (experimental conditions were same as in text).

(Fig. 2a, c). Retention times of acetaminophen and the IS were 2.38 min and 3.79 min, respectively (Fig. 2b). In addition, each respective peak was distinct, and no peaks significantly interfering with the analytes were detected for acetaminophen and IS from six individual human plasma samples. Calibration curves for acetaminophen in plasma were linear from 1 to 100 μg/mL, and a representative calibration curve is shown in Figure 3. The regression equation for this calibration curve was y = 0.0438x + 0.0013, where y is the peak area ratio of acetaminophen to the IS and x is the concentration of acetaminophen. The mean

correlation coefficient (r) of the calibration curve were 0.9982 (n = 5). The mean percent recovery (n = 3) of acetaminophen from plasma at LQC, MQC, HQC was 91.70, 89.40, 89.00%, respectively and the mean percent recovery of the IS at a concentration 10 μg/mL was 10.25% with an acceptable precision (% RSD < 10). All the results about recovery are summarized in Table 1. Intra- and inter-day accuracy and precision for the four QC solutions analyzed in triplicate are summarized in Table 2. The intra- and inter-day accuracy values (%) ranged from 94.40% to 99.56% and from 90.00% to 99.20%, respectively. The intra- and inter-day precision values (% RSD) for the various concentrations ranged from 2.64% to 10.76% and from 6.84% to 15.83%, respectively. Both accuracy and precision was found to be acceptable in this study. The stability of acetaminophen in human plasma is summarized as % relative concentration in Table 3. Stock solution stored for up to 30 days at -20°C showed no significant change in chromatographic peak areas (data not shown).

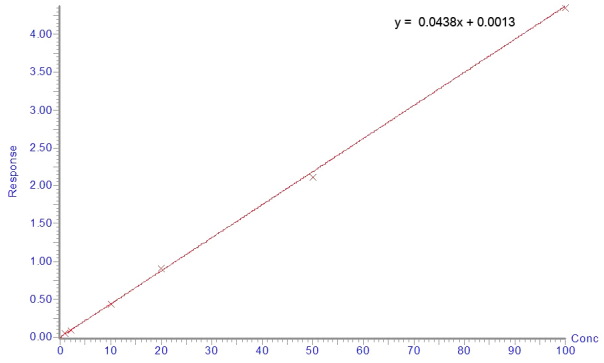

Figure 3. Calibration curve of acetaminophen.

Table 1. Recovery of acetaminophen preparation and extraction

Nominal concentrations (μg/mL)	Recovery (mean±SD[a], %)	RSD[b] (%)
	Acetaminophen	
2	91.70±0.69	8.26
50	89.40±0.16	1.59
80	89.00±0.17	1.56
10	Phenacetine (IS[c])	
	10.25±0.34	3.30

[a]Standard deviation (SD), [b]Relative standard deviation (RSD), [c]Internal standard (IS).

Pharmacokinetic application

The validated UPLC-MS/MS method was successfully used in a pharmacokinetic study of acetaminophen. Plasma concentrations of acetaminophen were determined after oral administration of a 1,000 mg dose in 8 healthy Korean male volunteers. The volunteers received 2 tablets of 500 mg acetaminophen 3 times a day for 7 days. Almost all of the samples obtained at 0 h administration had a small amount of acetaminophen, which was less than LLOQ. The mean plasma concentration-time profile for acetaminophen is summarized in Figure 4. After 8

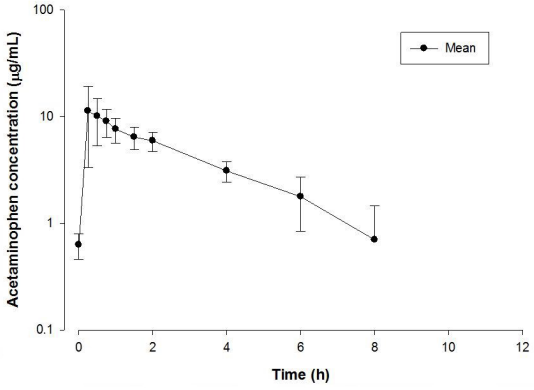

Figure 4. Mean plasma concentration-time profile of acetaminophen after a 1,000 mg oral dose in 8 healthy volunteers. The vertical bars show the standard errors.

Table 2. The intra- and inter-day precision and accuracy of quality-control samples containing acetaminophen at four concentrations (1, 2, 50, and 80 mg/mL) in plasma (weight: 1/x)

Nominal concentration (μg/mL)	Intra-day (n = 5)		Inter-day (n = 5)	
	Accuracy (%)	Precision (%RSD[a])	Accuracy (%)	Precision (%RSD)
1	96.00	10.76	90.00	15.83
2	99.00	7.06	95.00	8.15
50	99.56	3.04	99.20	6.84
80	94.40	2.64	96.53	8.44

[a]Relative standard deviation (RSD)

Table 3. Stability of acetaminophen under four different conditions (n = 3)

Storage condition		LQC[a] (2 μg/mL)		HQC[b] (80 μg/mL)	
		Test[c]	Reference[d]	Test	Reference
Post-preparation	Mean (mg/mL)	2.00	2.07	85.63	75.70
	SD[e] (±)	0.20	0.12	1.86	4.36
	RSD[f] (%)	10.00	5.60	2.20	5.80
Freeze-thaw cycles (for 3 cycles)	Mean (mg/mL)	1.73	1.83	66.77	75.83
	SD (±)	0.21	0.23	3.03	0.99
	RSD (%)	12.00	12.60	4.50	1.30
Short-term	Mean (mg/mL)	2.20	2.07	79.60	82.97
	SD (±)	0.00	0.23	1.22	3.04
	RSD (%)	0.00	11.200	1.50	3.70
Long-term	Mean (mg/mL)	2.60	2.40	76.47	81.03
	SD (±)	0.10	0.27	3.79	4.02
	RSD (%)	3.80	11.00	5.00	5.00

[a]Low quality control (LQC), [b]High quality control (HQC), [c]Concentration of the samples stored under different storage conditions, [d]Concentration of freshly prepared samples, [e]Standard deviation (SD), [f]Relative standard deviation (RSD).

h administration, the plasma concentration of acetaminophen was dropped to zero. The pharmacokinetic parameters were estimated using WinNonlin 5.3 software. The mean (± SD) of $AUC_{0\to8}$ (area under plasma concentration–time curve) and maximum concentration (C_{max}) was 31.6 (± 6.6) μg·h/mL and 13.0 (± 6.2) μg/mL, respectively, the latter of which was seen at 0.6 h (T_{max}). These pharmacokinetic parameters were in accordance with those reported previously.[23]

Discussion

A simple and suitable method for determination of acetaminophen concentrations in human plasma was successfully developed and validated, which significantly increased column efficiency of UPLC. Leading to a complete analysis within 7 min with an LLOQ of 1 μg/mL. The protein precipitation sample preparation method in the present was more simplistic than those reported previously. This validated method was successfully used in a pharmacokinetic analysis of acetaminophen with human plasma samples, confirming its sensitivity for bioanalysis. Thus, this fully validated method can be an appropriate tool for high-throughput analyses of clinical samples that are a critical component of pharmacokinetic studies and other clinical trials.

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Conflict of interest

The authors have no conflicts of interest with regard to this article.

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