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Rapid Determination of Acetaminophen levels in Human plasma by High performance liquid chromatography

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ABSTRACT

A simple, precise, and rapid high performance liquid chromatography (HPLC) method for the determination of acetaminophen level in human plasma using caffeine as an internal standard (IS) was developed and validated. 0.5 ml plasma samples containing acetaminophen were mixed with 50 µg of the IS. After adding 30 µl of 50% perchloric acid, the mixture was vortexed for one minute and then centrifuged for 5 minutes at 13200 rpm. The clear supernatant was transferred into an auto-sampler vial and 100 µl was injected into the HPLC system with a run time of 7.0 min. The compounds of interest were efficiently separated on Symmetry C18 (4.6 x 150 mm, 5-µm) column, and were detected with a photodiode array detector set at 245 nm. The mobile phase consisted of water, methanol, and acetonitrile (80:10:10, v:v:v) and was delivered at a flow rate of 0.9 ml/min. No interference in blank plasma or by commonly used drugs was observed; and the detection limit of acetaminophen was 0.05 µg/ml. The relationship between acetaminophen concentration in plasma and peak area ratio of acetaminophen /IS was linear ($r^2 \geq 0.9991$) in the range of 0.1– 40 µg/ml. Intra- and inter-day coefficient of variations (CV) and biases were ≤11.6% and ≤10.8%, and ≤≤14.0 and ≤12.8, respectively. Extraction recovery of acetaminophen and the IS from the plasma samples was ≥99% and 86%, respectively. Using the method, acetaminophen was found to be stable under conditions generally encountered in the clinical laboratory (≥99% and 91% in processed and unprocessed samples, respectively). Further, the method was successfully used to measure acetaminophen level in plasma samples from a healthy volunteer.

Keywords: Acetaminophen, Caffeine, Human plasma, HPLC

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INTRODUCTION

Acetaminophen (CAS: 103-90-2, N-acetyl-p-aminophenol) is an antipyretic and analgesic, non-steroidal drug, that is commonly available over-the-counter in different formulations. It lowers the body temperature and reduces pain by inhibiting the enzyme, cyclooxygenase (COX)[1]. Its absolute bioavailability is in the range of 70-90%, and it has a peak plasma concentration of 5.2 -8.9 ng/ml within one hour after the ingestion of a 500 mg therapeutic dosage [2,3].

Several analytical methods have been reported in the literature for determination of acetaminophen in pharmaceutical preparations and human serum/plasma samples. These includes titrimetry [4,5], ultraviolet-visible spectrophotometry [6,7], spectrofluorometry [8,9], gas chromatography-mass spectrometry (GC-MS) [10,11], high-performance liquid chromatography (HPLC) [12-16], and HPLC with tandem mass spectrometry (LCMS/MS) [17,18]. In general, HPLC with UV detection is the preferred method for quantification of analytes in biological matrix. However, due to endogenous plasma component interference which is mostly observed at lower wavelength, a higher wavelength has to be used. Some of the reported HPLC-UV methods for the determination of acetaminophen level in human plasma adopted complex procedures which include on-line post-column photochemical derivatization [14] or used ion-pair reversed phase chromatography [16].

In this paper, we describe a precise and accurate HPLC method for acetaminophen level determination in human plasma. The method uses 0.5 ml plasma and simple extraction method was fully validated, and successfully used to determine acetaminophen level in plasma samples from a healthy volunteer and to acetaminophen stability.

MATERIALS AND METHOD

Apparatus

Chromatography was performed on a Waters Alliance HPLC 2695 (Waters Associates Inc., Milford, MA, USA) consisting of a quaternary pump, autosampler, column thermostat, and photodiode array detector. A reversed phase Symmetry C18 (4.6 x 150 mm, 5-µm particle-size) column and a guard pre-column Nova-Pak C18, 4-µm insert were used for the separation. Data were collected with a Pentium IV computer using Empower Chromatography Manager Software.

Chemical and reagents

All reagents were of analytical grade unless stated otherwise. Acetaminophen USP reference standard from Sigma-Aldrich Co., St. Louis, MO, USA. Caffeine, acetonitrile, methanol, and perchloric acid (HPLC grade) were purchased from Fisher Scientific, Fairlawn, NJ, USA. HPLC grade water was prepared by reverse osmosis and was further purified by passing through a

Synergy water purification System (Millipore, Bedford, MA, USA). Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital & Research Centre (KFSHRC) Riyadh, Saudi Arabia. Samples from healthy volunteers were collected after obtaining approval of the Research Ethical Committee of KFSHRC.

Chromatographic conditions

The mobile phase was composed of water, methanol, and acetonitrile (80:10:10, v:v:v) and was delivered at a flow rate of 0.9 ml/min. The analysis was carried out under isocratic condition maintaining column temperature at 40°C and sample compartment temperature at 8ºC. A photodiode array detector set at 245 nm was used for recording chromatograms.

Preparation of standard and quality control samples

Stock solutions (1mg/ml) of acetaminophen and caffeine (internal standard, IS) were prepared in methanol. They were diluted with blank human plasma or mobile phase, respectively, to produce working solutions of 40 µg/ml for acetaminophen and 1 mg/µl for the IS. Calibration curve standards (nine concentrations) in the range of 0.1 – 40 µg/ml were prepared in human plasma. Four quality control (QC) samples (0.1, 0.3, 20, and 36 µg/ml) were also prepared in human plasma. 0.5 ml aliquots in 1.5 ml eppendorf micro-centrifuge tubes (Fisher Scientific Co., Fairlawn, NJ, USA) were stored at –20ºC until used.

Sample preparation

Aliquots of 0.5 ml of calibration curve samples, QC samples, and volunteer samples in 1.5 ml micro-centrifuge tubes were allowed to equilibrate to room temperature. To each tube, 50 µl of the IS working solution (1mg/µl in mobile phase) were added and vortexed for 10 seconds, then 30 µl of 50% perchloric acid were added and the mixture was vortexed again for 1 minute and then centrifuged for 5 minutes at 13200 rpm at room temperature. The supernatant layer was carefully transferred into an auto-sampler vial, and 100 µl were injected into the HPLC system. The run time was 7.0 minutes.

Stability studies

Three QC samples (concentration 0.1, 0.3, and 36 µg/ml) were used for acetaminophen stability studies: five aliquots of each QC sample were extracted and immediately analyzed (baseline), five aliquots were allowed to stand on the bench-top for 24 hours at room temperature before being processed and analyzed, five aliquots were stored at -20ºC for 7 weeks before being processed and analyzed, and five aliquots were processed and stored at room temperature for 24 hours or -20 ºC for 48 hours at before analysis. Fifteen aliquots of each QC sample were stored at -20ºC for 24 hours. They were then left to completely thaw unassisted at room temperature. Five aliquots of each sample were extracted and analyzed and the rest returned to -20°C for another 24 hours. The cycle was repeated three times.

Method validation

The method was validated according to standard procedures described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance [19]. The validation parameter included: specificity, linearity, accuracy, precision, recovery and stability.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Optimal experimental conditions consisted a mobile phase composed of water, methanol, and acetonitrile (80:10:10 v:v:v) and was delivered at a flow rate of 0.9 ml/min. Under these conditions acetaminophen, caffeine, and components of plasma exhibited a well-defined separation within a 7 minute run. The retention times of acetaminophen and caffeine were around 4.3 and 5.7 minutes, respectively.

Specificity

Specificity is defined as the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Potential interfering substances in plasma samples include endogenous components, metabolites, and decomposition products. We screened six batches of blank human plasma and six frequently used medications (ranitidine, omeprazole, ibuprofen, nicotinic acid, ascorbic acid, and diclofenac) for potential interference. No interference was found in plasma and none of the drugs co-eluted with acetaminophen or the IS. **Figure 1** depicts a representative chromatogram of drug free human plasma used in preparation of standard and QC samples.

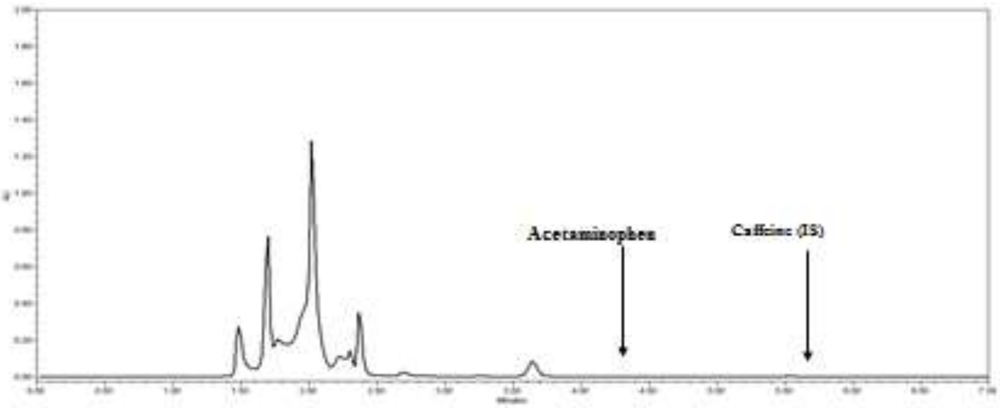

Figure 1: Representative chromatogram of a drug-free human plasma. The arrows indicate retention times of acetaminophen and the internal standard (caffeine, IS)

Limit of detection & quantification and linearity

The limit of quantification was defined as the lowest concentration on the calibration curve that can be determined with acceptable precision and accuracy (i.e., coefficient of variation and bias ≤15%). The limit of quantification of acetaminophen in human plasma was 0.1 µg/ml. The limit of detection (≥3 signal to noise-ratio) was 0.05 µg/ml. Linearity of acetaminophen was evaluated by analyzing ten curves of nine standard concentrations (plus zero concentration) prepared in human plasma. Figure 2 depicts an overlay of chromatograms of a typical calibration curve. Mean (SD) of slope, intercept, and coefficient of determination (R^2) of the ten curves were 0.0502 (0.0013), -0.0029 (0.0066), and 0.9991 (0.0004), respectively. The suitability of the calibration curves was confirmed by back-calculating the concentration of acetaminophen from the calibration curves (Table 1). All calculated concentrations were well within the acceptable limits.

Table 1: Back calculated acetaminophen concentrations from ten calibration curves

Nominal level(µg/ml)	Measured level (µg/ml) Mean (SD)	CV (%)	Bias (%)
0.1	01001 (0.0115)	11.5	0
0.2	0.2118 (0.0089)	4.2	5.9
0.4	0.3912 (0.0305)	7.8	-2.2
0.8	0.7963 (0.0813)	10.2	0
4.0	4.0335 (0.2571)	6.4	0.8
12	11.5157 (0.7595)	6.6	-4.0
24	23.6661 (0.6599)	2.8	-1.3
32	32.4118 (0.4560)	1.4	1.3
40	39.8468 (0.2773)	0.7	0

SD, standard deviation. CV, standard deviation divided by mean measured concentration x100

Bias = (mean measured concentration – nominal concentration divided by nominal concentration) × 100.

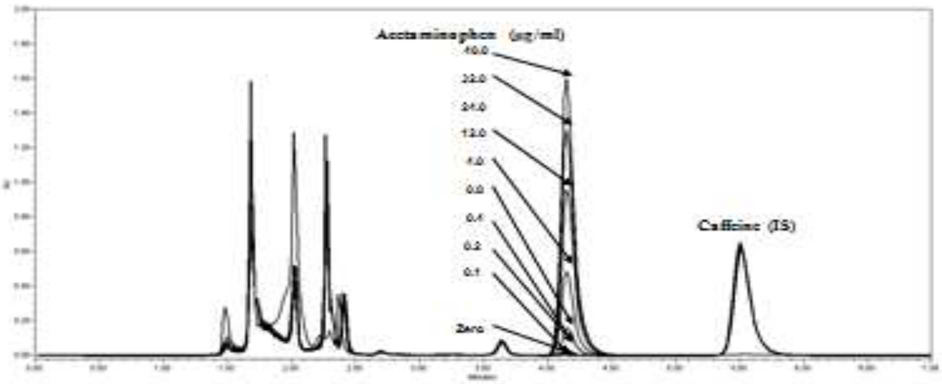

Figure 2 Overlay of chromatograms of extracts of 0.5 ml human plasma spiked with the internal standard (IS) and zero or one of nine concentrations of acetaminophen.

Precision and bias (inaccuracy)

The intra-day and inter-day precision and bias of the method were evaluated by analyzing four QCs concentration (0.1, 0.3, 20, and 36 µg/ml). Intra-day precision and bias (n = 10) ranged from 3.3% to 11.6% and from –7.6% to –14%, respectively. Inter-day precision and bias were determined over three different days (n = 20) ranged from 6.1% to 10.8% and from –5.3% to 12.8%, respectively. The results are summarized in Table 2.

Table 2: Intra and inter-day precision and bias of acetaminophen assay

Nominal level (µg/ml)	Measured level (µg/ml) Mean (SD)	CV (%)	Bias (%)
Intra-day (n= 10)			
0.1	0.114 (0.013)	11.6	14
0.3	0.275 (0.027)	10.0	-8.3
20	18.197 (1.124)	6.2	-.9.0
36	33.243 (1.090)	3.3	-7.6
Inter-day (n= 20)			
0.1	0.112 (0.010)	9.4	12.8
0.3	0.299 (0.032)	10.8	0
20	18.918 (1.243)	6.6	-5.4
36	34.098 (2.094)	6.1	-5.3

SD, standard deviation. CV, standard deviation divided by mean measured concentration x100

Bias = (mean measured concentration – nominal concentration divided by nominal concentration) × 100.

Recovery

Absolute recovery of acetaminophen was assessed by direct comparison of peak areas from plasma and mobile phase samples, using five replicates for each of four QCs (0.1, 0.3, 20, and 36 µg/ml). Similarly, the recovery of the IS was determined by comparing peak areas of the IS in five aliquots of human plasma spiked with 100 µl of 50 µg/ml IS solution with the peak areas of equivalent samples prepared in the mobile phase. The results are presented in Table 3.

Table 3: Recovery of acetaminophen and the internal standard from 0.5 ml human plasma

Concentration (µg/ml)	Human plasma *Mean (SD)	Mobile phase *Mean (SD)	Recovery (%)
Acetaminophen			
0.1	390350 (2971)	391030 (420)	100
0.3	436182 (4997)	394357 (1135)	111
20	8320460 (85997)	8422254(16399)	99
36	15300041(44813)	15437661(12692)	99
Internal standard			

0.2	571645 (12624)	661584(3921)	86

*Mean peak area (standard deviation), n = 5. Recovery is calculated as mean peak area in human plasma divided by mean peak area in mobile phase x 100.

Robustness and Ruggedness

The robustness of a method is a measure of its capacity to remain unaffected by small variations in analysis conditions. The robustness of the current assay was evaluated by slightly altering proportions of methanol and acetonitrile (±2.0%) in the mobile phase. No significant changes were observed. Ruggedness was tested by conducting split sample test. Two split samples (concentration 27 and 35 µg/ml) were analyzed by two blinded technologists on two different instruments. The accuracy of the reported concentrations was within the acceptable limits (bias ≤5.0%).

Stability

Stability of analytes in biological matrices is an important pre-analytical variable. It is necessary to perform stability studies of the analyte and IS to determine the range of appropriate conditions and time of storage. Acetaminophen and IS stability in processed and unprocessed plasma samples was investigated using three QCs (0.1, 0.3, and 36 µg/ml). Acetaminophen in processed samples was found to be completely stable for 24 hours at room temperature and 48 hours at −20 °C (104%). Acetaminophen in unprocessed samples was stable for at least 24 hours at room temperature (≥99%), 7 weeks at −20 °C (91%), and after three freeze-and thaw cycles (≥95%). **Table 4** summarizes the results of stability studies.

Table 4: Stability for acetaminophen in human plasma

Stability (%)							
Nominal level (µg/ml)	**Unprocessed**		**Processed**		**Freeze-Thaw Cycle**		
	24 hrs RT	**7 wks 20 °C**	**24 hrs RT**	**48 hrs 20 °C**	1	2	3
0.1	105	106	105	116	116	95	104
0.3	99	103	99	104	111	100	103
36	103	91	103	109	110	100	95

Stability (%) = mean measured concentration (n=5) at the indicated time divided by mean measured concentration (n=5) at baseline x 100. Spiked plasma samples were processed and analyzed immediately (baseline, data not shown), after 24 hours at room temperature (24 hrs RT), or after freezing at –20 ºC for 7 weeks (7 wks –20 ºC), or processed and then analyzed after storing for 24 hours at room temperature (24 hrs RT) or 48 hours at –20 ºC (48 hrs -20 ºC). Freeze-thaw (FT); samples were frozen at -20 ºC and thaw at RT.

Application to a volunteer sample

Figure 3 depicts an overlay chromatogram of samples collected from a volunteer before and 3.0 hours after the ingestion of a single dose of 500 mg acetaminophen. The measured levels of acetaminophen were zero and 4.1 µg/ml, respectively.

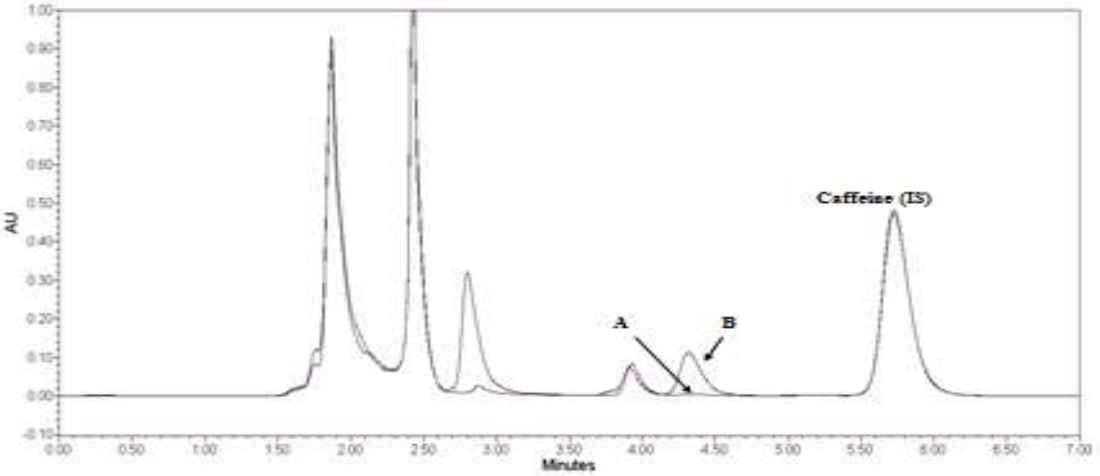

Figure 3 An overlay of chromatograms of plasma samples obtained from a healthy volunteer before (A) and 3 hours after (B) a single oral 500 mg acetaminophen dose.

CONCLUSION

The described HPLC assay is precise, simple, accurate and rapid. It requires only 0.5 ml plasma and utilizes a simple protein precipitation procedure. The assay was successfully applied to monitor stability of acetaminophen under various conditions encountered in the clinical laboratories and to determine the level of acetaminophen in plasma samples of a healthy volunteer.

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