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Journal of Pharmaceutical and Biomedical Analysis
xxx (2003) 487–493

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

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Development and optimization of a reversed-phase high-performance liquid chromatographic method for the determination of acetaminophen and its major metabolites in rabbit plasma and urine after a toxic dose

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Received 31 July 2002; accepted 29 December 2002

Abstract

A reversed-phase high-performance liquid chromatographic method with detection at 242 nm was developed, optimized and validated for the determination of acetaminophen (A) and its major metabolites glucuronide (AG) and sulfate (AS) conjugates in rabbit plasma and urine after a toxic dose. *m*-Aminophenol was used as internal standard (IS). A Hypersil BDS RP-C_{18} column (250 × 4.6 mm), 5 μm particle size, was equilibrated with a mobile phase composed of aqueous buffer solution of KH_2PO_4 0.05 M containing 1% CH_3COOH (pH 6.5) and methanol (95:5, v/v). Its flow rate was 1.5 ml/min. Calibration curves of A, AG and AS were linear in the concentration ranges of 0.5–250, 1–200, 0.5–100 μg/ml in plasma and 1–200, 0.5–150, 0.5–100 μg/ml in urine matrix, respectively. Limits of detection and quantitation were calculated in all cases and extensive recovery studies were also performed. Intra-day relative standard deviation (R.S.D.) for A, AG and AS in plasma was less than 5, 4, 2% and in urine less than 4, 7, 4%, respectively, while the corresponding inter-day values were 7, 6, 4% and 5, 8, 6%, respectively.
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Keywords: Acetaminophen; Acetaminophen glucuronide; Acetaminophen sulfate; Reversed-phase HPLC with UV detection; Rabbit plasma and urine

1. Introduction

Acetaminophen, paracetamol or *N*-acetyl-*p*-aminophenol [1,2] is a mild, safe, widely used analgesic and antipyretic substitute of aspirin. However, it causes liver necrosis in humans and experimental animals when high doses are administered [1,3,4]. Studies on the metabolism of acetaminophen (A) [1,5,6] have shown two major pathways of elimination. One of these, glucuronidation and sulfation produce glucuronide (AG) and sulfate (AS) conjugates and the other, oxidation, forms cysteine (AC) and mercapturic acid

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0731-7085/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0731-7085(03)00246-2

(AM) conjugates. Evidence strongly implicates a role for minor oxidized metabolites in the hepatotoxic reaction caused by acetaminophen.

Many analytical methods exist for the assay of acetaminophen in biological fluids [7–14]. Moreover, a number of high-performance liquid chromatography (HPLC) methods [14–23] have been developed for measuring A and its major metabolites in biological samples. However, most of them are specific for certain type of sample or certain application. Some of them are complicated and many of them use internal standards (IS) eluted after the last chromatographic peak.

The scope of our work was to develop and optimize a simple, fast, easy to use, of low cost reversed-phase HPLC method for the determination of acetaminophen and its major metabolites in rabbit plasma and urine after a toxic dose. The ultimate goal was to use this method, along with other observations, to study the effect of chronic ethanol consumption and acute ethanol administration on acetaminophen-induced hepatotoxicity in rabbits and search for an antidote.

In this paper, development and optimization of such a method are presented.

2. Experimental

2.1. Instrumentation

The chromatographic system used, consisted of a Waters 600E multisolvent delivery system (a 600 controller, a pump and a U6K injector) and a Waters 486 tunable absorbance detector (Waters, Milford, MA, USA). The above system was controlled by the software package Millennium 2010. The pH of the mobile phase was measured with a pH Meter 3310 Jenway Ltd. (Gransmore Green, UK). A Hettich centrifuge Universal 32R (Tuttlingen, Germany) was utilized to centrifuge plasma samples.

2.2. Chemicals and reagents

All chemicals were of analytical purity grade. Methanol (MeOH) of HPLC grade was purchased from E. Merck (Darmstadt, Germany). Acetaminophen was of pharmaceutical purity grade and was donated by Rhone-Poulenc (Athens, Greece). Acetaminophen metabolites were of analytical purity grade; AG was purchased by Sigma Co. (St. Louis, MO, USA), while AS was kindly donated by SmithKline Beecham, Consumer Healthcare, Weybridge, Surrey, UK. *m*-Aminophenol was used as IS and was of analytical purity grade. Water purified with Milli-Q RG water purification system (Millipore Co., Bedford, MA, USA) was used in all procedures.

2.3. Chromatographic conditions

A reversed-phase Hypersil BDS-C_{18} column (250×4.6 mm, 5 μm particle size) equipped with a precolumn Hypersil BDS-C_{18} (10×4 mm, 5 μm particle size) was used. The mobile phase was composed of aqueous buffer solution of KH_2PO_4 0.05 M containing 1% CH_3COOH (pH 6.5) and methanol (95:5, v/v). The mobile phase was degassed for 10 min with Helium gas at a degassing rate of 20 ml/min. The flow-rate of the mobile phase was 1.5 ml/min. Injection volume was 20 μl. Experiments were performed at ambient temperature. Absorption was measured at 242 nm, wavelength that was optimum for A, AG, AS and satisfactory for IS. The elution times for A, AG, AS and IS were approximately 11.9, 3.2, 7.8 and 6.3 min, respectively, in both plasma and urine samples.

2.4. Solution preparation

2.4.1. Mobile phase

The aqueous phase was prepared in a 1 l volumetric flask, adding 500 ml KH_2PO_4 0.1 M, 10 ml of glacial CH_3COOH, 300 ml of water, adjusting the pH to 6.5 with 10% KOH and diluting to volume. Thus, the mobile phase was composed of aqueous phase–MeOH (95:5, v/v).

2.4.2. Stock solutions

Stock solutions of A, AG and AS were prepared by dissolving 50 mg of each compound in 50 ml of a mixture of water–MeOH (95:5 v/v). Stock solution of IS was prepared by dissolving 25 mg of *m*-Aminophenol in 25 ml solution of $HClO_4$

10% v/v. Thus, concentrations of all stock solutions were of 1 mg/ml. Stock solutions were stored at −20 °C and were stable for at least 1 month.

2.4.3. Standard solutions

Working standard solutions were prepared for the establishment of the linearity range, the construction of calibration curves and evaluation of the precision of the proposed method. The concentration range was for A 1-250, AG 1-200 and AS 1-100 μg/ml. All dilutions to volume were performed with mixture of water–MeOH (95:5, v/v). Calibration curves were performed in mobile phase, plasma and urine. Working solution of IS was of 200 μg/ml in $HClO_4$ 10% v/v.

2.5. Sample preparation

2.5.1. Treatment of samples

The quantity of $HClO_4$ solution (10% v/v) that should be added in each sample was examined. It was observed that during the treatment of plasma samples, quantities of $HClO_4$ solution between 100 and 226 μl were used without changing the analysis result. However, increase of the quantity of $HClO_4$ in samples, increased the distortion of chromatograms in the early times. Thus, in plasma samples 120 μl of $HClO_4$ were added.

Also, it was found that the addition of $HClO_4$ was not necessary in the urine samples.

2.5.2. Analysis of plasma samples

50 μl of sample were transferred to a microcentrifuge tube, 30 μl of the working solution of IS (200 μg/ml), 120 μl $HClO_4$ 10% v/v and 100 μl of water were added to a final volume of 300 μl. After vortexing for 1 min, the samples were centrifuged for 10 min in 11 000 rpm at room temperature. The clear supernatant was then injected to the HPLC system.

2.5.3. Analysis of urine samples

Urine samples were first diluted 1/20 v/v with H_2O. Then, 50 μl of the diluted sample were transferred to a microcentrifuge tube, 30 μl of IS (200 μg/ml) and 220 μl of H_2O were added, again to a total volume of 300 μl. This solution was vortexed for 1 min and then injected to the HPLC system.

2.6. Data analysis

Calibration curves of A, AG and AS were constructed, using peak-area ratios of each of them to IS. Regression equations were obtained through unweighted least square linear regression analysis, applied to peak-area ratios as a function of their concentration.

3. Results and discussion

3.1. Mobile phase

Several eluent mixtures were tried and it was noticed that increasing the amount of MeOH in the mobile phase, the total elution time of all components was decreasing. However, values of resolution of peaks became smaller. Especially, the chromatographic peak of AG was getting close to the plasma background ones and that of AS to that of IS. A compromise of the above situation was the choice of a mobile phase, which consisted of aqueous solution–MeOH (95:5, v/v). Addition of CH_3COOH was attempted to avoid peak tailing. The eluent mixture was buffered with KH_2PO_4–KOH because adjustment of the pH of the mobile phase was necessary. The examined pH region was 4.5–7. At pH lower than 5.0 resolution of AG from the plasma background was poor ($R_s < 1.5$). As the value of the pH became higher, resolution of peaks became better and the analysis time longer. pH value of 6.5 was chosen as the most appropriate for the rest of the experiments, because R_s was greater than 2.0 for every peak and the total elution time was less than 12 min. Further pH increase resulted in higher R_s values, which were not necessary and further increase in total elution time, which was not desirable. Optimization of the experimental conditions took place using plasma blank solutions because its background was richer than that of urine and then, the optimized experimental parameters were checked and used in urine samples, as well.

3.2. Choice of internal standard

Twenty-one substances were tried as IS. Among these, *m*-Aminophenol was chosen as the most appropriate one in the present analysis because it was eluted between the other chromatographic peaks (A, AG, AS) without interfering with them or with the matrix of plasma or urine samples on one hand and on the other without increasing the elution time. The last fact was the drawback of most of the other methods appeared in the literature as the proposed IS was eluted after the peaks of A, AG and AS, enhancing in this way the analysis time. This substance also met all the other typical requirements of a compound to be used as IS (e.g. stability, proper solubility etc.)

3.3. Selectivity

After the above optimization, typical chromatographs obtained under the optimized experimental conditions, are shown in Figs. 1 and 2. Good resolution for every peak and its nearest ones was assured by the values of R_s which were greater than 2.

3.4. Calibration curves

Linear calibration curves for A, AG and AS were obtained throughout the concentration ranges studied. Regression analysis was done on the ratios of peak-areas of A, AG and AS to that of the IS (y) versus (x). The results are tabulated in Table 1. In each case, the slope of the calibration curve obtained from standard solutions prepared in plasma matrix was compared with that obtained in mobile phase, using the t-test. The t experimental value (t_{exp}) was less than the theoretical one (t_{theor}) for 12 degrees of freedom. The same procedure was followed for the comparison of slopes between urine and mobile phase. Again, the results showed that the slopes were statistically the same.

3.5. Precision and accuracy

To verify the precision of the proposed HPLC method, precision of standards and samples was

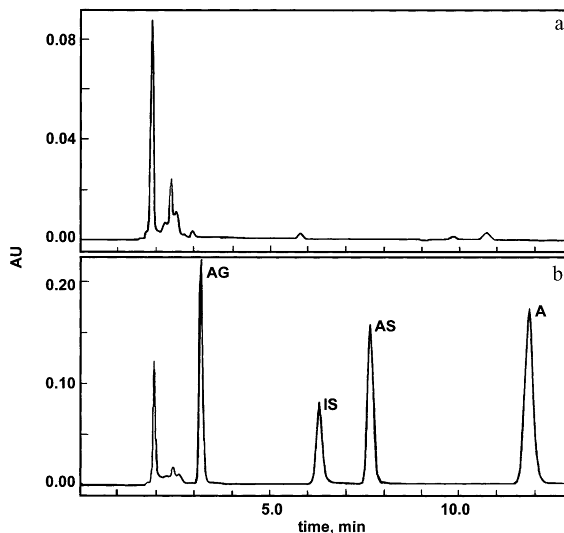

Fig. 1. Representative chromatograms of blank plasma solution (a) and spiked blank plasma solution (b) with A, AG, AS and IS, eluted at 11.9, 3.2, 7.8 and 6.3 min, respectively, where A is acetaminophen, AG is acetaminophen glucuronide, AS is acetaminophen sulfate and IS is internal standard. Concentrations of working standards of the above substances were 50, 50, 50 and 20 μg/ml, respectively.

obtained. Preparing and measuring standards of the same concentration of A, AG and AS three times each, intra- and inter-day relative standard deviation (R.S.D.) values were calculated in the concentration ranges used. R.S.D. values of A, AG and AS were also calculated in plasma and urine samples. The overall results showed that intra-day R.S.D. for A, AG and AS in plasma was less than 5, 4, 2% and in urine less than 4, 7, 4%, respectively, while the corresponding inter-day values were 7, 6, 4% and 5, 8, 6%, respectively.

The inter-day precision was an indication of the ruggedness of the developed method, because it included changes in reagents, chemicals and solvents. The robustness of the proposed method was assessed with respect to small alterations in several experimental parameters e.g. the pH and the % content of the mobile phase, the temperature of the analysis procedure, which was slightly different from day to day, the quantity of the solution of $HClO_4$ added in each sample etc. These deliberate

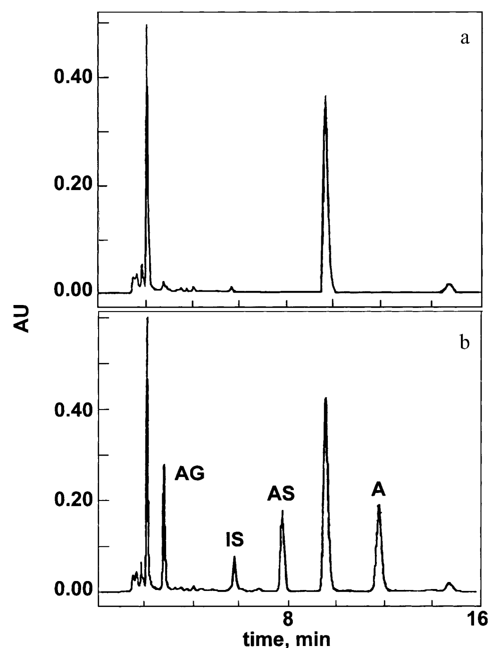

Fig. 2. Representative chromatograms of blank urine solution (a) and spiked blank urine solution (b) with A, AG, AS and IS, eluted at 11.8, 3.1, 7.8 and 5.9 min, respectively, where A is acetaminophen, AG is acetaminophen glucuronide, AS is acetaminophen sulfate and IS is internal standard. Concentrations of working standards of the above substances were 50, 50, 50 and 20 μg/ml, respectively.

changes did not increase the inter-day R.S.D. values that are above mentioned because they were anyway included in an every-day analysis procedure. Moreover, the use of IS solution in an analysis assures the ruggedness and the robustness of the used method.

The accuracy of the developed method was examined by recovery studies. These results are summarized in Tables 2 and 3.

Table 2
Recovery data for the determination of A, AG and AS in rabbit plasma

Concentration (μg/ml)			Mean % recovery ± S.D.[a]		
A	AG	AS	A	AG	AS
5	200	5	93 ± 2	100.6 ± 0.1	94 ± 5
10	10	10	104 ± 3	95 ± 5	96 ± 2
200	5	80	99 ± 4	107 ± 4	98 ± 3
50	50	50	102 ± 1	98 ± 3	106 ± 3
2	150	20	85 ± 1	99 ± 1	105 ± 1
100	100	100	101 ± 2	103 ± 3	99 ± 3

[a] S.D. is the standard deviation of the mean % recovery; standard solutions were prepared and measured three times each.

Table 1
Analytical parameters of the calibration curves of A, AG and AS

Compound	Matrix	Concentration range (μg/ml)	Regression equation[a]		
			Intercept, a ± S.D.[b]	Slope, b ± S.D.[b]	r (n)[c]
A	Mobile phase	0.5–250	0.04 ± 0.05	0.093 ± 0.004	0.99998 (7)
	Plasma	0.5–250	0.11 ± 0.06	0.0952 ± 0.0004	0.99995 (7)
	Urine	1–200	0.2 ± 0.1	0.094 ± 0.001	0.9996 (7)
AG	Mobile phase	0.5–200	0.1 ± 0.1	0.0471 ± 0.0007	0.9996 (7)
	Plasma	1–200	0.2 ± 0.1	0.048 ± 0.001	0.999 (7)
	Urine	0.5–150	0.23 ± 0.05	0.0490 ± 0.0007	0.9996 (7)
AS	Mobile phase	0.5–100	0.001 ± 0.01	0.060 ± 0.003	0.999 (7)
	Plasma	0.5–100	0.06 ± 0.07	0.064 ± 0.001	0.999 (7)
	Urine	0.5–100	−0.001 ± 0.05	0.065 ± 0.001	0.9992 (7)

[a] Linear unweighted regression analysis, with a regression equation $y = a + bx$, where x is concentration in μg/ml.
[b] S.D. is the standard deviation of intercept and slope.
[c] r is the correlation coefficient and n is the number of points in each calibration curve; each point is the mean of three experimental measurements.

Table 3
Recovery data for the determination of A, AG and AS in rabbit urine

Concentration (μg/ml)			Mean % recovery ± S.D.[a]		
A	AG	AS	A	AG	AS
5	20	5	108 ± 5	100 ± 2	105 ± 3
10	10	10	103 ± 2	98 ± 3	98 ± 1
200	5	80	97 ± 2	104 ± 5	96 ± 3
50	50	50	101 ± 2	97 ± 1	99 ± 2
2	150	20	115 ± 7	99 ± 2	101 ± 1
100	100	100	100 ± 1	103 ± 1	103 ± 1

[a] S.D. is the standard deviation of the mean % recovery; standard solutions were prepared and measured three times each.

3.6. Limits of detection (LOD) and quantitation (LOQ)

The LOD was defined as the analyte concentration that gives a signal equal to $y_b + 3.3s_b$, where y_b is the signal of the blank and s_b is its standard deviation. Similarly, the LOQ was defined as $y_b + 10s_b$. In the unweighted least-squares method is quite suitable in practice to use $s_{y/x}$ [24] instead of s_b and the value of the calculated intercept a instead of y_b. Thus,

$$\mathrm{LOD} = \frac{3.3s_{y/x}}{b} \quad \text{and} \quad \mathrm{LOQ} = \frac{10s_{y/x}}{b}$$

where b is the slope of the regression line.

Based on the above equations, the calculated LOD values for A, AG and AS in plasma and urine samples were 0.2, 0.33, 0.13 and 0.33, 0.17, 0.33 μg/ml, respectively, while the LOQ ones were 0.6, 1, 0.4, and 1, 0.5, 1 μg/ml, respectively.

4. Conclusion

In this work, a reliable, simple and fast reversed-phase HPLC method was developed, optimized and validated. This method was simpler than many other existing because it used isocratic elution versus gradient and avoided ion-pairing or other complicated techniques [16–18]. Also, the pH of the mobile phase was not harmful for a silica column, another advantage versus other works [25]. It also used a UV detection system, which is the most common one. Moreover, the IS used was reliable and its elution time was optimum. Since the ultimate goal of this developed method was studying acetaminophen-induced hepatotoxicity in rabbits when A was administered in toxic doses, as well as the effect of other factors as alcohol and antidotes on this hepatotoxicity, it was desirable to be able to determine the toxic metabolites e.g. cysteine and mercapturic acid conjugates, using the same method. Unfortunately, it was not possible to locate pure AC and AM compounds. However, from observations in the analyzed plasma and urine samples, when acetaminophen dose was higher than 1500 mg/kg, two more chromatographic peaks at elution times around 9 and 18 min were present. These peaks were probably due to AC and AM metabolites respectively, taking into consideration literature data [15,19], as well.

Concluding, this method developed for the determination of A, AG and AS can be used in routine drug monitoring and pharmacokinetic studies. It is also promising in elucidating hepatotoxic effects of A at high doses.

Acknowledgements

The authors are very grateful for the financial support given by the Special Research Account of Athens University, Athens, Greece. They also thank deeply SmithKline Beecham, Surrey, UK, for its kind donation of analytical purity grade acetaminophen sulfate. They also thank D. Dimitroulopoulos, Dr A. Papalois, and M. Vagena for their assistance supplying the rabbit blank samples. They also thank Associate Professor M. Parissi-Poulou and the chemists H. Melanitou and P. Sfaktos for preliminary work. Finally, they thank Dr M. Kondylis for his helpful suggestions.

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