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Acetic acid improves the sensitivity of theophylline analysis by gas chromatography–mass spectrometry

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Abstract

In the analysis of theophylline by gas chromatography–mass spectrometry (GC–MS), we found that the addition of acetic acid to the solvent (ethyl acetate) decreased the adsorption of theophylline to the glass wool packed into the inlet liner. The addition of acetic acid to ethyl acetate improved the sensitivity for theophylline (optimum concentration of 3%). This simple and sensitive method without derivatization can be applied to the quantification of theophylline in serum samples in clinical and toxicological practice.

Keywords: Theophylline; Acetic acid; Gas chromatography–mass spectrometry

1. Introduction

Theophylline is a popular broncho-dilator that is used to treat asthma. The therapeutic range for theophylline in the serum is 10–20 μg/mL, whereas the toxic range is 20 μg/mL and above [1,2]. Due to the narrow therapeutic range and individual differences in metabolism, analysis of theophylline levels is very important for therapeutic drug monitoring and determination of pharmacokinetics.

Theophylline (Fig. 1) has been measured by use of immunoassay [3], high-performance liquid chromatography [4,5], capillary electrophoresis [6], liquid chromatography–mass spectrometry [7–9] and gas chromatography–mass spectrometry (GC–MS) [10–15]. GC–MS analysis with derivatization of theophylline is one of the most sensitive methods, but the derivatization is a time-consuming step. Therefore, we wanted to improve the sensitivity of theophylline analysis by GC–MS without derivatization.

When we measured theophylline with 3-isobutyl-1-methylxanthine as an internal standard (IS), we noticed that the peak of the IS was much higher in the serum extract than the working solution, despite the fact that the same amount of IS had been added to both samples. The theophylline peak was also higher in the serum compared with the solution.

Since there were carboxylic acids in the serum extract, we hypothesized that carboxylic acids would improve the sensitivity. To test this hypothesis, we examined whether acetic acid, as a carboxylic acid, would improve the sensitivity of theophylline analysis by GC–MS, when added to the solvent. Additionally, we examined whether theophylline adsorbed to the glass wool packed into the inlet liner. On the basis of these observations, we developed a method to measure theophylline in serum by GC–MS without derivatization.

2. Experimental

2.1. Chemicals

Theophylline, 3-isobutyl-1-methylxanthine, acetic acid, ethyl acetate and methanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Normal human serum was purchased from Chemicon International (California, USA). Ultra-pure water was prepared using a Milli-Q purification system (Millipore, Tokyo, Japan). Oasis® HLB 3 cm^3 (60 mg) Extraction Cartridges were purchased from Waters (Milford, MA, USA).

Stock standard solutions of theophylline and 3-isobutyl-1-methylxanthine (IS) were prepared in methanol to

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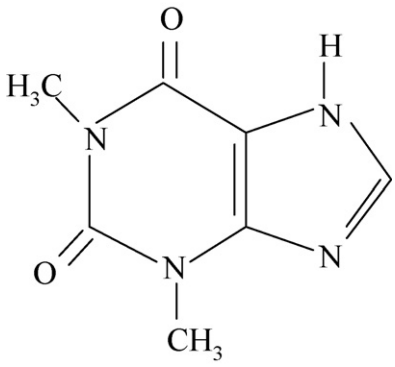

Fig. 1. Chemical structure of theophylline.

concentrations of 1 mg/mL. Working standard solutions were prepared by dilutions of these stock standard solutions to 10 and 100 μg/mL of theophylline and 100 μg/mL of IS. These standard solutions were stored at 4 °C.

2.2. Instrumentation and GC–MS conditions

GC–MS analysis was conducted using an HP 5973 mass selective detector interfaced to an HP 6890 gas chromatograph with an HP 7673C autosampler (Agilent Technologies, USA). Chromatographic separation was achieved on a J&W DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and using high-purity helium (99.9999%) as carrier gas at a flow-rate of 1.0 mL/min. The column oven temperature was programmed from 60 to 300 °C at 20 °C/min, with the initial temperature maintained for 1 min and the final temperature for 2 min. The solvent delay was 5 min. The inlet used a previously deactivated taper-liner with or without glass wool (Agilent, USA) at 270 °C and in a split-less mode for 1.0 min. The injection volume was 1.0 μL. The ionization was operated in electron impact mode at 70 eV. The transfer line, ion source and quadrupole temperature were 280, 230 and 150 °C, respectively. The MS was operated in selected ion monitoring (SIM) and scan mode, which were set to *m/z* 180.0, 95.0, 68.0 and *m/z* 50.0–400.0, respectively.

2.3. Extraction procedure

Twenty microliters of theophylline working solution (100 μg/mL) was transferred to a glass test tube, and dried under a stream of nitrogen at 45 °C. The dried residue was redissolved in 1 mL of serum and loaded on HLB cartridge pre-conditioned with 2 mL of methanol followed by 2 mL of water. The cartridge was washed with 3 mL of water followed by 3 mL of 5% methanol in water (v/v). Vacuum was applied for 10 min with Vac-Elut™ (Varian, USA). Analytes were eluted with 2 mL of ethyl acetate, and the eluate was evaporated to dryness under a stream of nitrogen at 45 °C. The dried residue was reconstituted in 100 μL of ethyl acetate, and compared with 2 μg theophylline (20 μL of 100 μg/mL working solution) dissolved in100 μL of ethyl acetate.

2.4. Samples of adding acetic acid

Twenty microliters of theophylline working solution (100 μg/mL) were transferred to glass test tubes, and dried under a stream of nitrogen at 45 °C. Acetic acid concentrations were adjusted to 0, 0.1, 1, 2, 3 and 4% in ethyl acetate (v/v), and the dried residues were redissolved in 100 μL of acetic acid in ethyl acetate for each concentration. One microliter of the solution (20 ng theophylline on column) was injected into the GC–MS, and the SIM ions were monitored. The peak area was integrated at *m/z* 180 + 95 + 68. The 0–4% samples were measured as a sequence of analysis. Between the samples, 1 μL of 2% acetic acid in ethyl acetate blank solution was injected into GC–MS to prevent carry-over of theophylline. The inlet liner with glass wool was exchanged for a liner without glass wool, and the same experiment was repeated.

2.5. Extracts of adding acetic acid

The extraction (described above) was repeated 10 times. Five samples of the dried residues were reconstituted in 100 μL of ethyl acetate, and the other five samples were reconstituted in 3% acetic acid in ethyl acetate. The peak area of the SIM ions was integrated at *m/z* 180 + 95 + 68.

2.6. Calibration curve

The calibration curve was obtained by measuring theophylline in the serum extracts. After 1 mL of serum samples were spiked with 1 μg of IS and 0.2, 0.5, 1, 2, 5 and 10 μg of theophylline, the analytes were extracted as described above and dissolved in 100 μL of 3% acetic acid in ethyl acetate. One microliter of the solution was injected into the GC–MS, and the SIM ions (*m/z* 180.0, 166.0, 122.0, 95.0) were monitored. The target ion and qualifier ion were *m/z* 180.0 and 95.0 of theophylline, *m/z* 166.0 and 122.0 of IS, respectively. The theophylline and IS retention times were 11.03 and 11.73 min, respectively.

3. Results and discussion

3.1. Matrix effect on theophylline

First, we show the higher sensitivity of GC–MS analysis for theophylline in the serum extract. Fig. 2 shows the SIM chromatograms for 2 μg theophylline (20 μL of 100 μg/mL working solution) (A) and the extract from a serum sample spiked with 2 μg theophylline (B), both of which were dissolved in ethyl acetate. The theophylline peak of the serum extract was sharper and higher than the peak from the working solution. We considered that this matrix effect was caused by carboxylic acids included in the serum extract. In support of this interpretation, the total ion chromatogram showed peaks for carboxylic acids, such as hexadecanoic acid and oleic acid (Fig. 3).

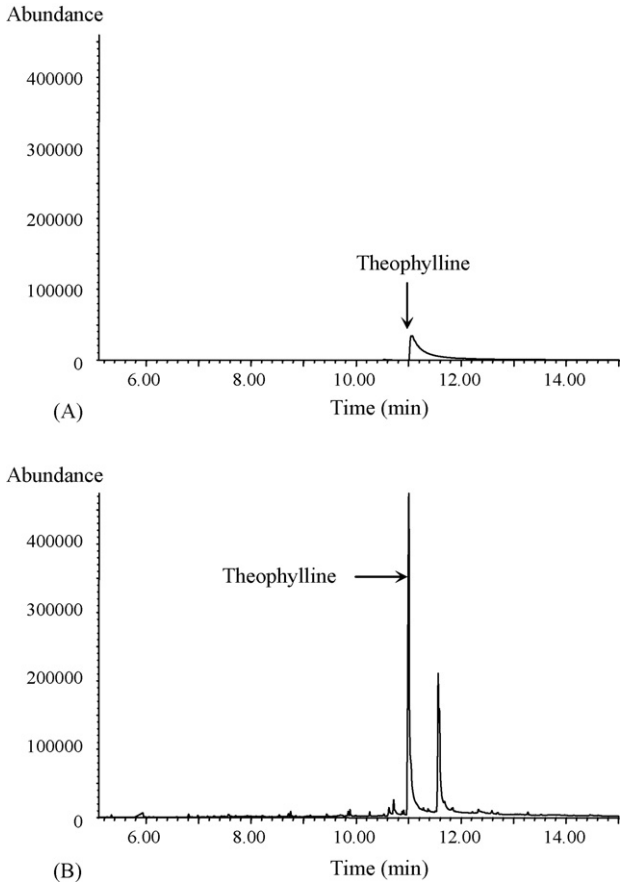

Fig. 2. SIM chromatograms of 2 μg theophylline (20 μL of 100 μg/mL working solution) (A) and the extract from a serum sample spiked with 2 μg theophylline (B), dissolved in ethyl acetate.

3.2. Effect of acetic acid on theophylline

To test the hypothesis that carboxylic acids improve the sensitivity of the theophylline analysis, we examined the effect of acetic acid, as a carboxylic acid, on the GC–MS. Acetic acid is a very popular reagent, and is less likely to cause carry-over compared with other larger molecular weight carboxylic acids.

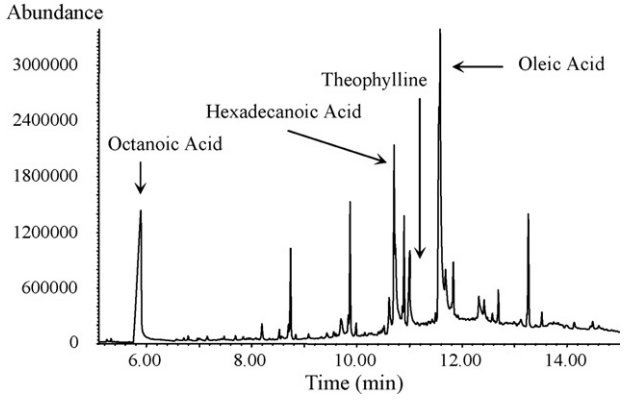

Fig. 3. Total ion chromatogram of the extract from serum sample spiked with 2 μg theophylline, dissolved in 3% acetic acid in ethyl acetate. Some of the carboxylic acids included in the extract are shown.

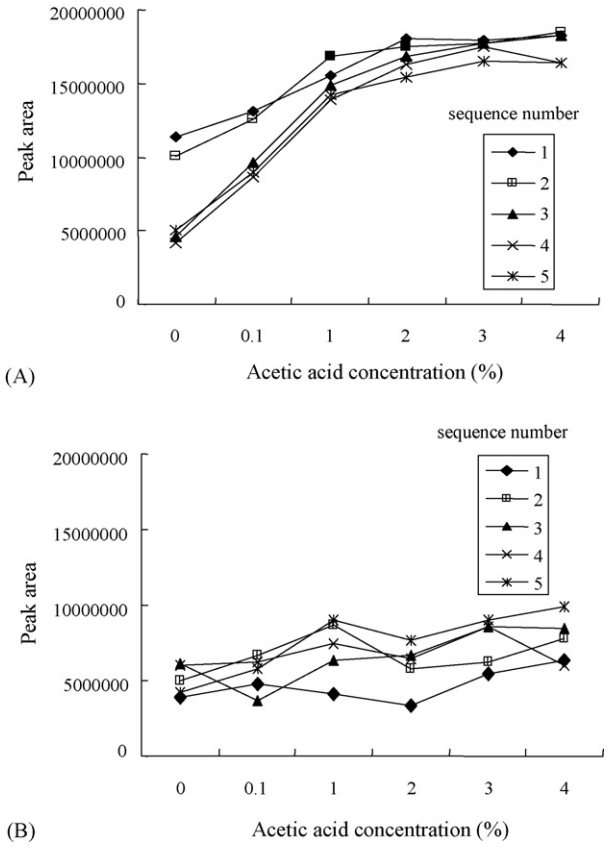

Fig. 4. The peak areas of 20 ng theophylline (on column) dissolved in 0, 0.1, 1, 2, 3 and 4% acetic acid in ethyl acetate, using liners with glass wool (A) and without glass wool (B). The 0–4% samples were measured in sequence ($n = 5$).

Theophylline was dissolved in 0, 0.1, 1, 2, 3 and 4% acetic acid in ethyl acetate. In the five measurements, samples with acetic acid from lower to higher concentrations were measured sequentially. The peak area increased with the increase in acetic acid concentration, attaining a maximum at around 3% (Fig. 4A). Furthermore, the peak was sharper (Fig. 5A) and coefficient of variation (CV) was smaller at 3% compared with the other concentrations (Table 1). The peak areas of 0 and 0.1% acetic acid decreased remarkably with repeated injections (Fig. 4A). However, the peaks were restored after the liner with the glass wool was replaced by a new one (data not shown). The theophylline peak appeared when 2% acetic acid in ethyl

Table 1
Mean and CV of the peak area of 20 ng theophylline (on column)

Acetic acid concentration (in ethyl acetate) (%)	Theophylline peak area ($n = 5$)			
	Liner with glass wool		Liner without glass wool	
	Mean	CV (%)	Mean	CV (%)
0	7050329	48.6	5034777	19.7
0.1	10590911	20.0	5433695	22.1
1	15040294	7.8	7120708	27.6
2	16828361	6.2	5960113	27.3
3	17460953	3.3	7592502	21.4
4	17570724	6.0	7667708	20.7

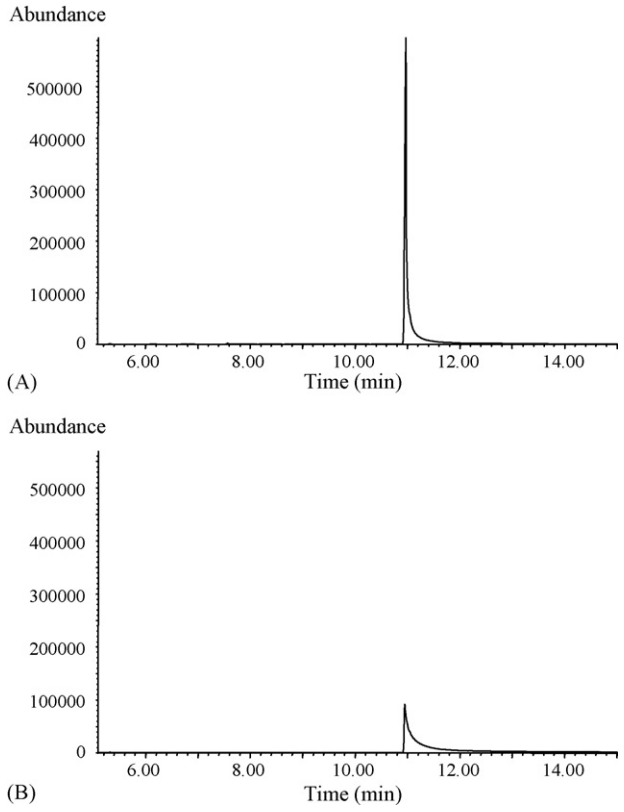

Fig. 5. SIM chromatograms of 20 ng theophylline (on column) dissolved in 3% acetic acid in ethyl acetate, using liners with glass wool (A) and without glass wool (B).

acetate blank solution was injected, especially between the samples of 0 and 0.1% acetic acid (Fig. 6). Moreover, when glass wool was removed from the liner, the peaks were broad and low, with little effect of acetic acid (Fig. 4B, 5B). These findings show that theophylline is adsorbed to the glass wool in the liner and that acetic acid decreases the adsorption (optimum concentration of 3%), and thereby improves the sensitivity of the theophylline analysis. In other words, acetic acid may decrease the availability of the active site on the glass wool. Perhaps the decrease in the peak areas by repeated injections (0 and 0.1% acetic acid) was caused by an increase in active site on the glass wool. However, the samples to which acetic acid (>2%) was added exhibited a small decrease. Therefore, in the glass wool that had been activated to some degree, the addition of acetic acid allowed more theophylline to reach the column.

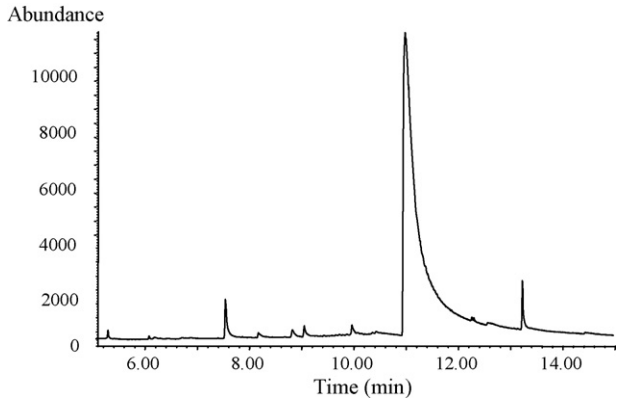

Fig. 6. SIM chromatogram of theophylline that appeared when 2% acetic acid in ethyl acetate blank solution was injected between the 0 and 0.1% acetic acid samples.

Table 2
Mean and CV of the peak area of 2 μg/mL theophylline in the serum extracts (liner with glass wool)

Acetic acid concentration (in ethyl acetate) (%)	Theophylline peak area ($n = 5$)	
	Mean	CV (%)
0	10131442	17.6
3	14874095	7.6

The limit of detection (signal-to-noise ratio of 3:1) was 1 ng on the column for theophylline dissolved in 3% acetic acid in ethyl acetate (liner with glass wool).

3.3. Effect of acetic acid on theophylline in serum samples

We then examined the effect of acetic acid on theophylline analysis in the serum samples (Table 2). The addition of acetic acid improved the mean and CV values as compared to that without the addition of acetic acid. Although the serum extract contained carboxylic acids, the addition of 3% acetic acid improved the sensitivity and precision of theophylline analysis more than the matrix effect by serum.

Fig. 7 shows the calibration curve of the theophylline extracted from the serum samples (0.2, 0.5, 1, 2, 5 and 10 μg/mL), with the use of 3-isobutyl-1-methylxanthine as IS and 3% acetic acid in ethyl acetate as solvent. The correlation coefficient was 0.998, and the linearity was confirmed within the range 0.2–10 μg/mL. Since the therapeutic range for theophylline in the serum is 10–20 μg/mL, 100 μL of serum would be sufficient for routine use. Additionally, the time-consuming derivatization step is not required. Thus, our GC–MS method with acetic acid addition has a great advantage for the quantification of theophylline in serum samples in clinical or toxicological practice.

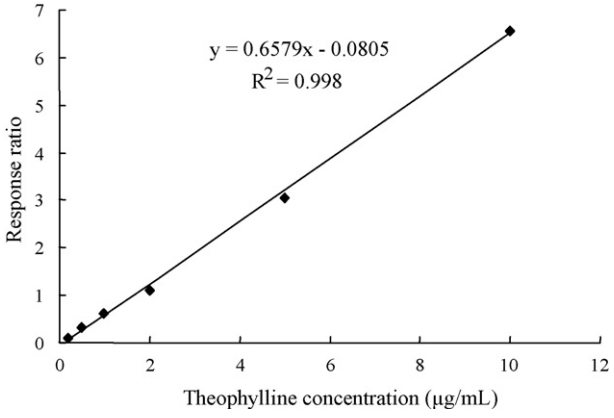

Fig. 7. Calibration curve of theophylline in the extracts from serum samples, dissolved in 3% acetic acid in ethyl acetate ($n = 6$).

4. Conclusion

In the theophylline analysis by GC–MS, adsorption of theophylline to the glass wool compromises the sensitivity. The addition of 3% acetic acid to the solvent improved the sensitivity by decreasing the adsorption. This simple and sensitive method without derivatization is favorable for the determination of theophylline in serum samples.

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