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ORIGINAL RESEARCH

Use of liquid chromatography–tandem mass spectrometry for the analysis of pentoxifylline and lisofylline in plasma

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ABSTRACT: A method was developed for the quantitation of pentoxifylline [1-(5-oxohexyl)-3,7-dimethylxanthine] and a primary active metabolite, lisofylline [1-(5-hydroxyhexyl)-3,7-dimethylxanthine], using high-performance liquid chromatography (HPLC)–tandem mass spectrometry. This method was developed in order to overcome problems encountered with HPLC–ultraviolet detection. The operating parameters of the electrospray interface (PE SCIEX, TurboIon Spray) and lens voltages of the triple-quadrupole detector (PE SCIEX 365) were optimized in positive ion mode to obtain the best sensitivity of the analytes. Collision-induced dissociation was used to produce fragment ions, and multiple reaction monitoring was used to quantitate pentoxifylline (*m*/*z* 279/181) and lisofylline (*m*/*z* 263/181). Dichloromethane was used to extract the drug, metabolite, and the internal standard (3-isobutyl-1-methylxanthine) from plasma. A reverse-phase C8(2) 150 × 1.0 mm HPLC column was used to resolve all three compounds in less than 6 min. Calibration curves were generated using peak area and were linear from 1 to 1000 ng/mL ($R^2 > 0.99$). The small sample volume, ease of extraction, and sensitivity provide advantages over more conventional methods of quantitation.

KEYWORDS: pentoxifylline; LC–MS; electrospray ionization

INTRODUCTION

Pentoxifylline [1-(5-oxohexyl)-3,7-dimethylxanthine] is a hemorrheologic agent used in the management of claudication (Muller, 1981). Pharmacokinetic studies have shown that pentoxifylline is metabolized to an initial active metabolite lisofylline [1-(5-hydroxyhexyl)-3,7-dimethylxanthine] from which other metabolites are produced (Baselt, 2002). A single 400 mg dose of pentoxifylline given to human adults produced average peak plasma concentrations of 1.6 and 2.0 mg/L, respectively, for pentoxifylline and lisofylline, whereas respective elimination half-lives averaged 0.8 and 1.0 h (Smith *et al*., 1986). Analytical methods using gas chromatography and high-performance liquid chromatography with ultraviolet detection (HPLC–UV) have been developed for the detection of pentoxifylline and its metabolites (Burrows, 1987; Grasela and Rocci, 1987; Mancinelli *et al*., 1992). In general, however, methods based on these techniques have been limited by poor chromatographic resolution and low sensitivity, requiring complex sample concentration techniques or derivitization of the compounds of interest.

The mass selective capability of high performance liquid chromatography–tandem mass spectrometry (LC–MS–MS) allows the user to specify a mass or range of masses to be detected. A compound having the specified mass is fragmented, and ion fragments characteristic of the parent compound are monitored. As a result, analysis by mass spectrometry results in lower background noise, higher sensitivity and fewer drug–metabolite interferences compared with other analytical methods (Turpeinen and Stenman, 2003; Wallemacq *et al*., 2003; Ballesteros *et al*., 2003). Analysis by LC–MS–MS generally exhibits sensitivity in the range of 10^{-6}–10^{-12} g/mL plasma (Xia *et al*., 1999; Zhu *et al*., 2002; Phillips *et al*., 2001). The utility of LC–MS–MS has been demonstrated in the analysis of many classes of drugs including antibiotics (Ballesteros *et al*., 2003), antidepressants (Naidong and Eerkes, 2004), and hypoglycemics (Lin *et al*., 2004).

Herein we describe the development of a sensitive method for the analysis of pentoxifylline and lisofylline using LC–MS–MS. This method was developed in order to overcome problems encountered with HPLC–UV analysis; namely, low sensitivity and co-elution of the parent and multiple metabolites. The small sample volume (0.5 mL), ease of extraction and high sensitivity

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Abbreviations used: CID, collision-induced dissociation; MRM, Multiple reaction monitoring.

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allow this method to be readily utilized for analysis of plasma samples from individual animals.

EXPERIMENTAL

Reagents. Pentoxifylline, lisofylline and 3-isobutyl-1-methylxanthine (purity >99% each as determined by HPLC) and ammonium acetate (purity ≥98%) were purchased from Sigma-Aldrich (St Louis, MO, USA). Optima-grade solvents were obtained from Fisher Scientific (Fair Lawn, NJ, USA). SeraPrep reagent and SeraPrep lithium diluent were obtained from Pickering Laboratories (Mountain View, CA, USA). Human donor plasma, which had passed its expiration date for human infusion, was obtained from an in-house blood bank. Rabbit plasma was prepared from blood obtained from anesthetized New Zealand white rabbits. All procedures were carried out under protocols approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee and conformed to NIH guidelines.

Instrumentation. Two Perkin Elmer Series 200 Micro LC pumps connected in parallel supplied mobile phase to a Perkin Elmer Series 200 autosampler equipped with a 20 µL sample loop. Mobile phase was composed of 50% 0.5 mM ammonium acetate (pH 3.5)–50% methanol (v/v) at a constant flow rate of 50 µL/min. Separation was achieved with a Phenomenex (Torrance, CA, USA) Luna C8(2), 5 µm, 150 × 1.0 mm column protected by a 0.5 µm in-line PEEK™ frit filter. A TurboIon Spray® ion source interfaced the API 365 (PE SCIEX, Foster City, CA, USA) triple quadrupole mass spectrometer operated in the positive ion mode. The nebulizer gas was set to 5 (instrument parameter) and the flow rate of nitrogen auxiliary gas was 300 mL/min at 300°C. The ion spray voltage was 5000 V, and the orifice voltage was 31 V. Collision-induced dissociation (CID) spectra were produced using nitrogen at –21 V collision energy. Selective ion monitoring was performed in the multiple reaction monitoring (MRM) mode with a dwell time of 300 ms for each ion. MacQuan software (PE SCIEX) was used to compare peaks and quantitate analytes on the basis of peak area using linear regression against authentic standards.

Preparation of standards. Pentoxifylline, lisofylline, and 3-isobutyl-1-methylxanthine (Fig. 1) were each weighed and dissolved in methanol to a concentration of 1 mg/mL. The 1 mg/mL solution of 3-isobutyl-1-methylxanthine was diluted to 1 µg/mL in water to make the internal standard solution. Equal volumes of the 1 mg/mL pentoxifylline and lisofylline solutions were combined and diluted with water to make a 10 µg/mL stock solution. An aliquot of the stock solution was then diluted with human or rabbit plasma to make plasma standards of 1000, 500, 100, 10 and 1 ng/mL by sequential dilution. Plasma standards were stored at 2–4°C and used within 2 weeks. These compounds were reported to be stable for 2 months in solutions of water or plasma (Chivers *et al.*, 1981).

Sample preparation. A 50 µL aliquot of the internal standard solution was added to 0.5 mL plasma in a 5 mL glass reaction tube. Proteins were removed via lithium precipitation by the addition of 0.5 mL SeraPrep reagent to the sample mixture. Precipitation of the proteins was performed to ensure a clean extraction by preventing the transferal of lipophilic proteins and lipids into the dichloromethane. The sample was vortexed and allowed to sit for 5 min. The pH was adjusted to approximately 2.5 by addition of 0.5 mL SeraPrep lithium diluent. The sample was then centrifuged at 3000 rpm for 5 min, and the supernatant was transferred to a 5 mL conical glass centrifuge tube. Five milliliters of dichloromethane were added, and the sample was mixed on a laboratory shaker for 5 min. The sample was then centrifuged at 3000 rpm for 5 min to separate the aqueous and organic phases. The lower (organic) layer was removed and evaporated to dryness using an evaporation apparatus set to 60°C and cool nitrogen gas. The best chromatographic peak shape and resolution were obtained by reconstituting the sample with water, which concentrated the sample on the front of the analytical column. Therefore, each sample was reconstituted by the addition of 100 µL water and then vortexed for 30 s.

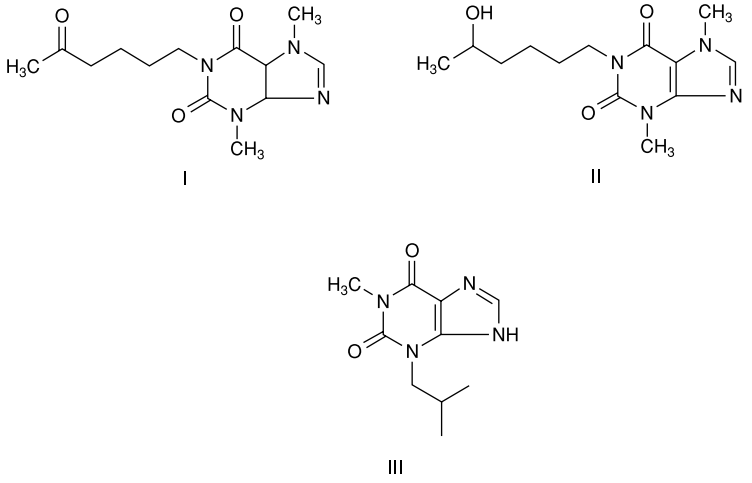

Figure 1. Structures of pentoxifylline (**I**), lisofylline (**II**) and 3-isobutyl-1–methylxanthine (**III**).

The sample was filtered using a Millex (Millipore, Bedford, MA, USA) 0.22 μm syringe filter before injection of a 15 μL aliquot onto the column for analysis.

Assay validation. The percentage recovery of each analyte was measured by comparing the response of extracted plasma standards with extracts of control plasma to which the analytes were added after extraction. The linear range of the method was established by measuring the concentration in human and rabbit plasma samples spiked with analyte concentrations from 1 to 1000 ng/mL. Mean slope values of the calibration curves were determined and reported with standard error. The intraassay (within-day) variation was determined by measuring five extracted samples from different batches of control plasma spiked to 10, 500 and 1000 ng/mL. The interassay (between day) variation was determined by measuring 10, 500 and 1000 ng/mL aliquots of spiked plasma extracted and injected over five non-contiguous days. The sample matrix may cause a profound increase or decrease on ionization of the analyte (Lindsey *et al*., 2001). Therefore, the effect of matrix on ionization was assessed by comparing the response of neat standards injected directly with extracted control plasma to which the analytes were added after extraction.

RESULTS AND DISCUSSION

LC–MS–MS

Pentoxifylline, lisofylline, and 3-isobutyl-1-methylxanthine, respectively, eluted at 3.4, 4.0 and 5.8 min (Fig. 2). The initial ionization of pentoxifylline and 3-isobutyl-1-methylxanthine, respectively, resulted in the formation of ions with mass to charge (m/z) ratios of 279.2 $[M + H]^+$ and 223.2 $[M + H]^+$. Lisofylline fragmented during the initial ionization and generated the product ion m/z 263.2, an event consistent with the loss of water $[M - H_2O + H]^+$, as commonly occurs during electrospray ionization (Reid *et al*., 2000; Kamel *et al*., 2002; Vazquez *et al*., 2001).

The CID of pentoxifylline produced a significant ion of m/z 181.0 (Fig. 3), which is consistent with the cleavage of its dimethylxanthine ring and oxohexyl moiety (Fig. 4). In fact, the CID of each analyte resulted in ions consistent with dissociation of the respective methylxanthine and aliphatic (chain) moieties. The CID of lisofylline produced significant ions at m/z 181.0

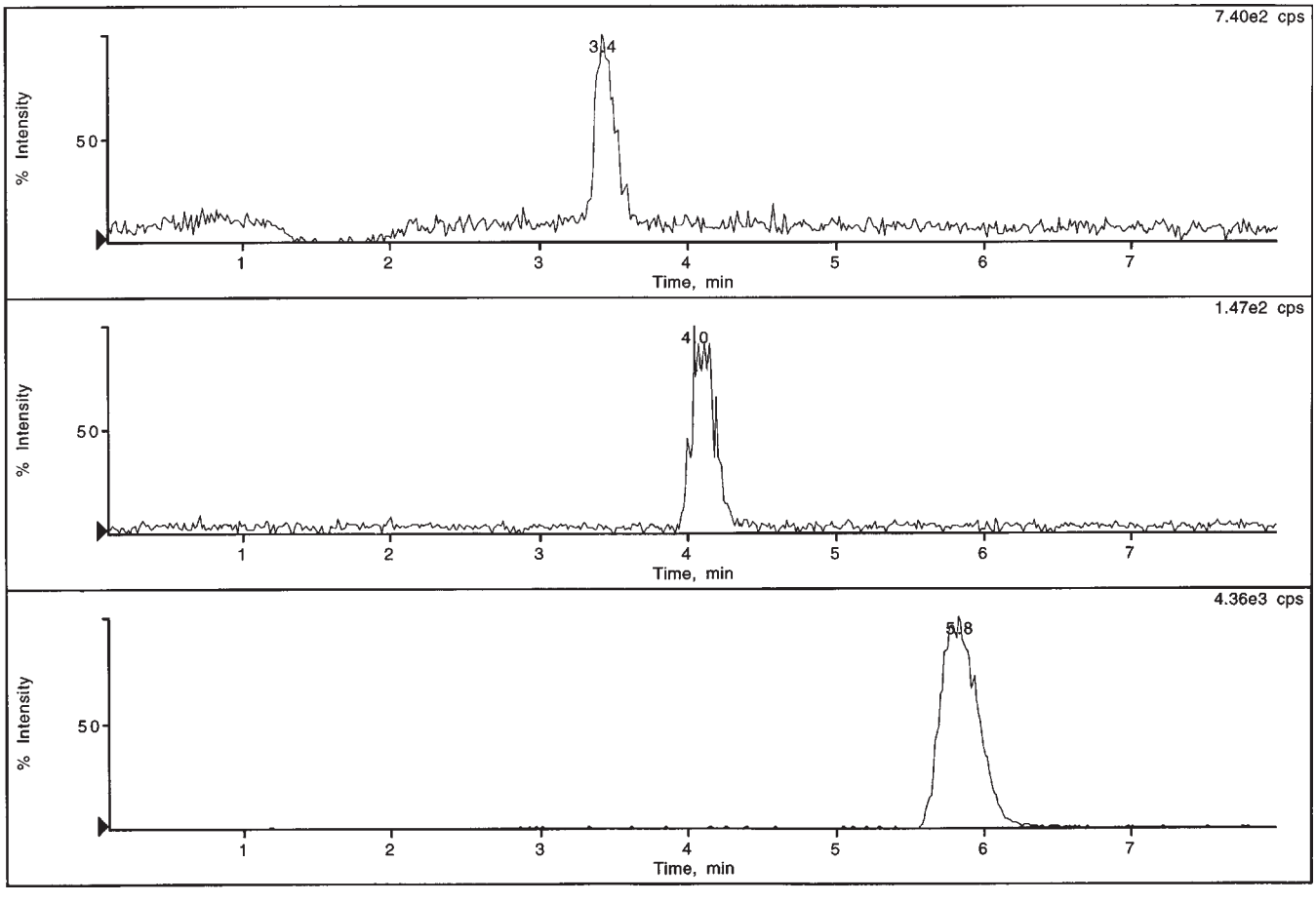

Figure 2. Ion chromatogram of pentoxifylline (3.4 minutes), lisofylline (4.0 minutes), and 3-isobutyl-1-methylxanthine (5.8 minutes). Plasma was spiked with pentoxifylline and lisofylline to 1 ng/mL, and 3-isobutyl-1-methylxanthine to 100 ng/mL prior to extraction.

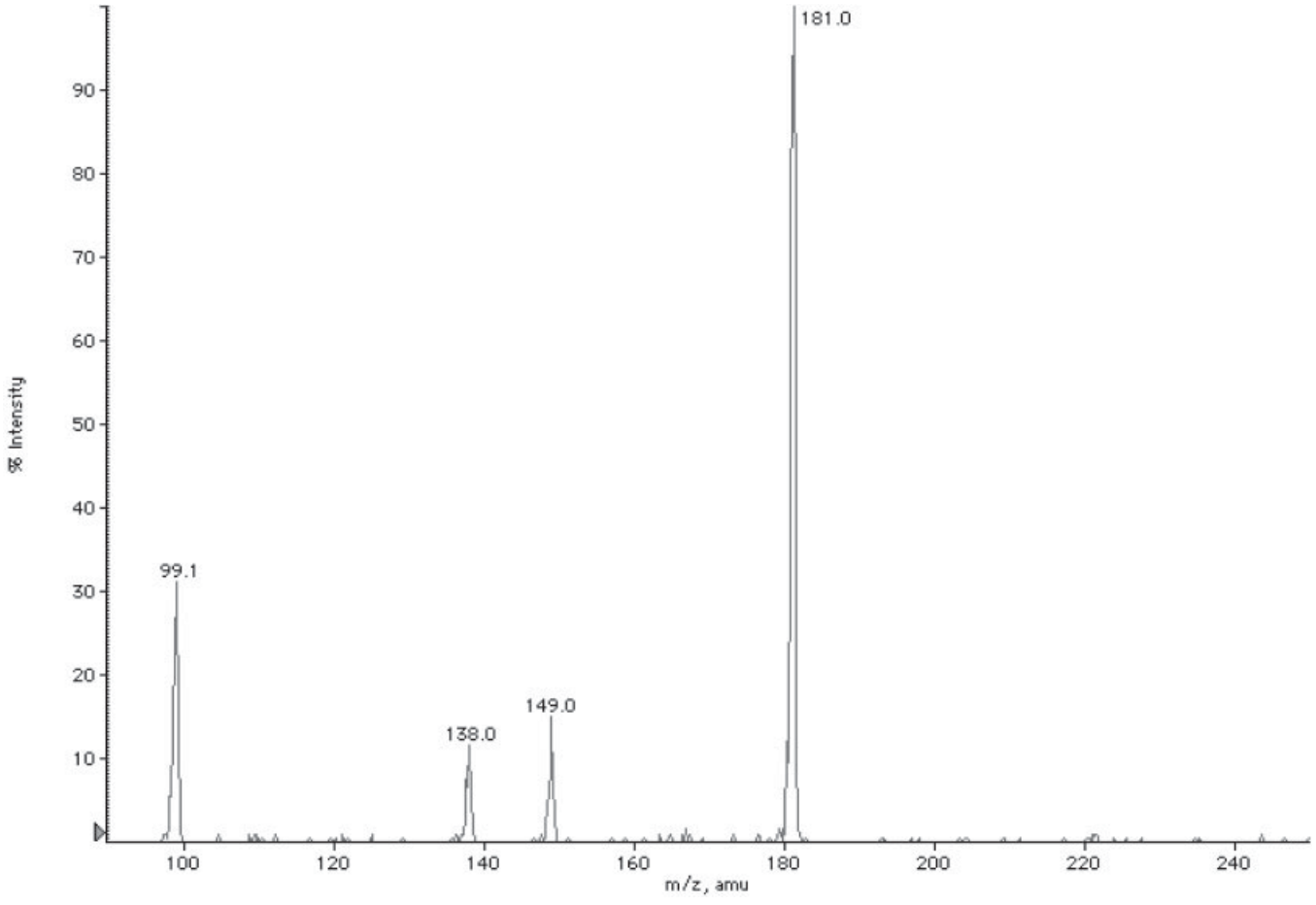

Figure 3. Fragment ions resulting from the collision-induced dissociation of pentoxifylline.

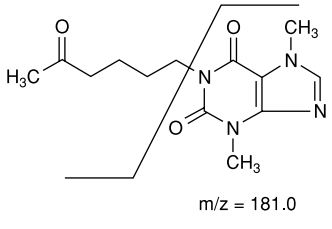

Figure 4. Proposed fragmentation of pentoxifylline during collision-induced dissociation.

and 193.0 (Fig. 5), whereas fragmentation of 3-isobutyl-1-methylxanthine produced a significant ion at *m/z* 167.0. In order to achieve the greatest sensitivity, the parent ion of each analyte was coupled with its dominant product ion to monitor pentoxifylline (279.2/181.0), lisofylline (263.2/181.0), and 3-isobutyl-1-methylxanthine (223.2/167.0).

Assay

The recoveries of pentoxifylline and lisofylline, respectively, were 82.0 ± 1.9 and 84.6 ± 1.7% from rabbit plasma and 82.3 ± 1.8 and 85.6 ± 2.0% from human plasma. The calibration curves of each analyte in human and rabbit plasma were linear from 1 to 1000 ng/mL ($r^2 > 0.99$). When multiple batches of human and rabbit plasma were analyzed, the slope of the calibration curves for pentoxifylline and lisofylline, respectively, were $1.21 \pm 0.08 \times 10^{-1}$ and $1.34 \pm 0.11 \times 10^{-2}$. The mean intraassay variations of pentoxifylline and lisofylline were 5% or less in human and rabbit plasma, while the interassay variations were less than 10% (Table 1). No significant matrix effect on analyte ionization was found after comparison of neat standards and multiple batches of human and rabbit plasma.

In conclusion, a rapid and sensitive method for the analysis of pentoxifylline and a primary active metabolite using LC–MS–MS is described. Analysis using LC–MS–MS offers several advantages compared with other chromatographic methods. High sensitivity is obtained due to the low background noise achieved when monitoring selected ion pairs during multiple reaction monitoring (MRM). The sensitivity of LC–MS–MS reduces the sample volume required and allows the analysis of plasma samples from small animals. High throughput can be realized using MRM because selective ion monitoring eliminates interference from other

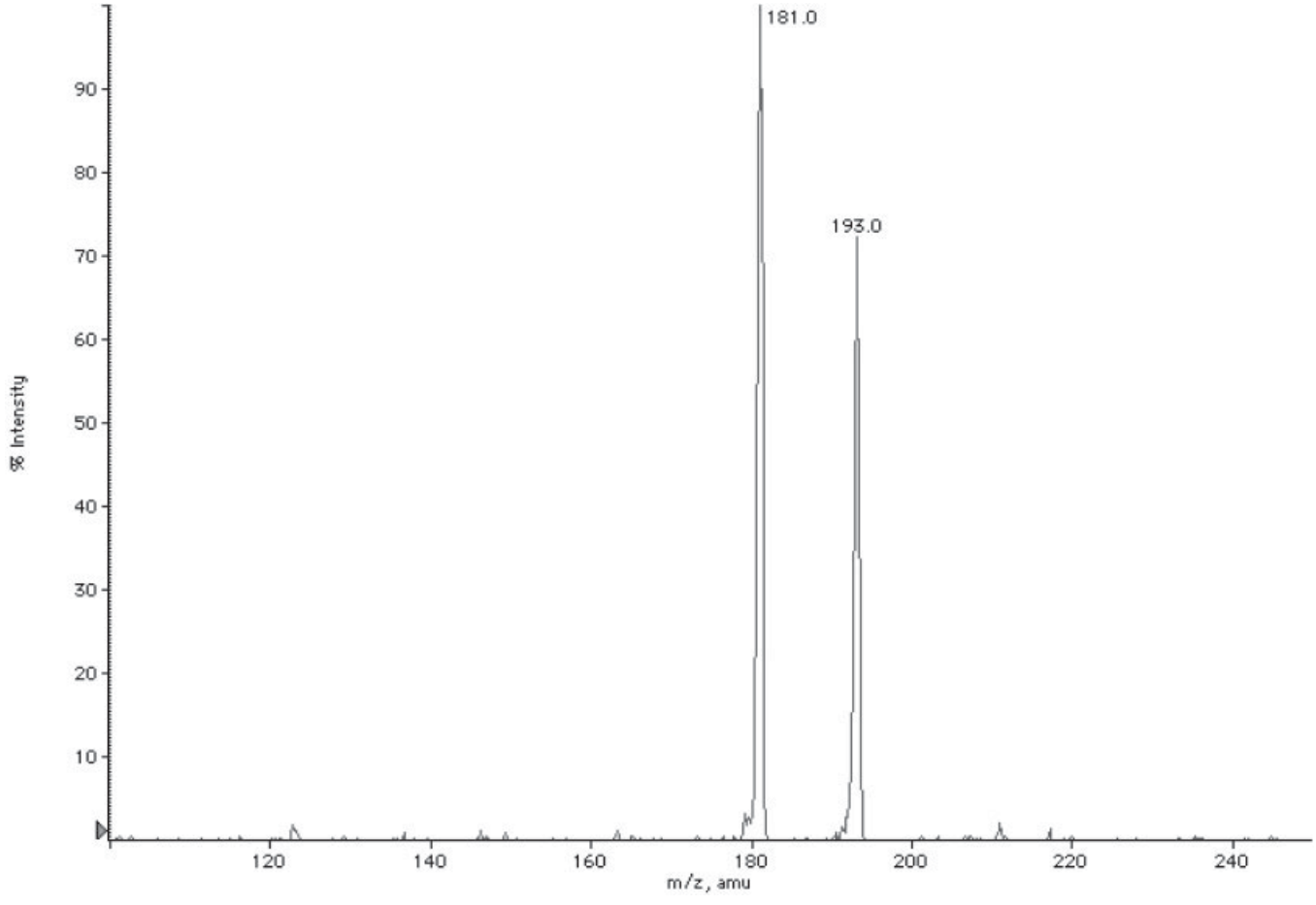

Figure 5. Fragment ions resulting from the collision-induced dissociation of lisofylline.

Table 1. Intra- and interassay variabilities of pentoxyfylline and lisofylline in human and rabbit plasma

	Intraassay variability (%)		Interassay variability (%)	
	Human	Rabbit	Human	Rabbit
Pentoxyfylline (*ng/mL*)				
10	2.2 ± 0.8	3.5 ± 1.5	8.8 ± 2.8	5.5 ± 2.6
500	3.7 ± 0.8	1.7 ± 0.7	2.2 ± 0.5	8.3 ± 2.3
1000	2.1 ± 0.8	1.9 ± 0.7	0.5 ± 0.1	7.4 ± 2.2
Lisofylline (*ng/mL*)				
10	3.8 ± 0.8	2.8 ± 0.7	5.0 ± 2.1	6.0 ± 1.2
500	5.0 ± 1.7	3.1 ± 1.2	1.6 ± 0.7	3.4 ± 1.1
1000	3.6 ± 1.0	2.6 ± 0.8	0.5 ± 0.2	9.8 ± 1.4

analytes and sample components. As a result, analysis times of 2–5 min may be achieved (Lin *et al*., 2004; Naidong and Eerkes, 2004; Wallemacq *et al*., 2003).

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