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Journal of Chromatography B, 712 (1998) 243–252

JOURNAL OF CHROMATOGRAPHY B

Screening of chlorpropamide in horse plasma by high-performance liquid chromatography with ultraviolet absorbance detection, and confirmation by gas chromatography–mass spectrometry

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Received 18 December 1997; received in revised form 10 March 1998; accepted 7 April 1998

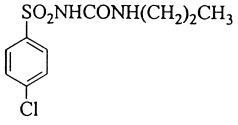
Abstract

A chromatographic method was developed to detect and confirm the presence of chlorpropamide (**I**) in horse plasma samples, for antidoping control. The plasma sample (1 ml) was extracted with dichloromethane and screened by high-performance liquid chromatography, and confirmation of the drug's presence was accomplished by using gas chromatography–mass spectrometry (GC–MS). The limit of detection was found to be 3.5 ng/ml at a signal-to-noise ratio of three. Derivatization of **I** with *N,O*-bis-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane allowed for highly stable, accurate and sensitive GC–MS analysis. Plasma samples collected after the administration of diabinese were positive for **I** (one–five days) in all samples analysed.

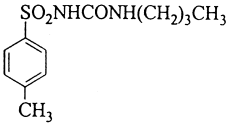
Keywords: Chlorpropamide

1. Introduction

Chlorpropamide (**I**), 1-(4-chlorobenzenesulphonyl)-3-propylurea, is a sulphonylurea derivative that is widely used as an oral hypoglycemic drug in the treatment of diabetes.



I



II

Klonoff [1] reported that excessive intake of **I** can produce hypoglycemic symptoms, coma, or even death. Compound **I** can stimulate pancreatic beta-cell insulin production. In toxic doses, the compound might be expected to stimulate excessive insulin production, which, in turn, results in the reduction of glucose levels in blood [2].

Several analytical techniques have been employed in the analysis of sulphonylurea compounds from biological samples such as human plasma, the most frequently used being high-performance liquid chromatography (HPLC) [3,4] and gas chromatography (GC) [5]. A micellar electrokinetic chromatographic (MEKC) method [6] has been described, which highlighted the ability of the technique to provide a rapid assay of the drug in human urine, with a detection sensitivity in the 50 ng/ml range. There are

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0378-4347/98/$19.00
PII: S0378-4347(98)00184-4

no literature references about identification procedures for **I** in horse plasma.

The aim of this paper is to establish an analytical procedure to screen and confirm the presence of **I** in horse plasma for pre- and post-race testing, by HPLC and gas chromatography–mass spectrometry (GC–MS). The need for such a procedure is driven by the necessity for anti-doping control in the horse-racing industry.

2. Experimental

2.1. Chemicals and reagents

Chlorpropamide (**I**) and tolbutamide (**II**) were purchased from Sigma (St. Louis, MO, USA). *N,O*-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was purchased from Pierce (Rockford, IL, USA). HPLC-grade methanol, acetonitrile, hydrochloric acid (36.5–38%) and acetic acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Sodium sulphate (anhydrous, granular, 12–60 mesh) was also purchased from J.T. Baker. Purified helium (99.995%) and oxygen-free nitrogen were from SOXAL (Singapore).

2.2. Standards

Stock solutions of **I** and **II** (1 mg/ml) were prepared weekly in methanol and stored at 4°C. Working solutions of the appropriate concentrations were prepared weekly by dilution in methanol.

2.3. Instrumentation

2.3.1. High-performance liquid chromatography

The Shimadzu (Kyoto, Japan) HPLC equipment consisted of an LC-6A solvent delivery unit, a manual injector [Rheodyne (Cotati, CA, USA) Model 7125 with a 20-μl sample loop and position sensing switch] and a variable wavelength UV detector (Shimadzu Model SPD-6A) set at a wavelength of 240 nm. Separations were achieved on a stainless steel column (Supelcosil 150 mm×4.6 mm I.D; packed with C_{18} stationary phase; 5 μm particle size; Supelco, Bellefonte, PA, USA), protected by a Shimpack G-ODS guard column (Shimadzu). The mobile phase consisted of acetonitrile–0.2% acetic acid (35:65, v/v) at a flow-rate of 1.2 ml/min. The mobile phase was degassed by ultrasonication and routinely filtered through 0.5 μm PTFE (polypropylene-lined) membrane filters (47 mm diameter, Whatman, Maidstone, UK). The column was maintained at ambient temperature using a Shimadzu Model CTO-6A column oven.

Peak recording and integration was performed with the Shimadzu Chromatopac CR-6A data processor.

2.3.2. Gas chromatography–mass spectrometry

GC–MS analyses were performed using an HP5989 MS Engine system (Hewlett-Packard, Palo Alto, CA, USA) consisting of an HP5890 Series II gas chromatograph connected to an HP5989A quadrupole mass spectrometer. The GC was equipped with an HP7673 autosampler and a HP-5MS column (crosslinked 5% diphenyl–95% dimethylsiloxane, 30 m×0.25 mm×0.25 μm film thickness). Helium was used as the carrier gas with a head pressure of 103 kPa. The injector temperature was maintained at 250°C and the transfer line was maintained at 280°C. The initial column temperature was set at 80°C, with a temperature increase of 15°C/min to 230°C, and then another increase at 20°C/min to 270°C. The final temperature was held for 12 min. The mass spectrometer conditions were as follows: electron-impact ionization, ion source temperature, 270°C; ionization voltage, 70 eV for both the scan mode (m/z 40–800) and selected ion monitoring (SIM) mode. In the SIM mode, the ions at m/z 320 and 248 (characteristic of chlorpropamide) were monitored.

2.3.3. Biological samples

Three thoroughbred mares, aged between five and seven years, and weighing between 386–438 kg, were used to demonstrate the applicability of the method.

A727 was used as a control, to which 1 l of water was administered. Y870 and A163 were treated with a single dose (10 mg/kg) of diabinese Pfizer (250 mg of **I** per tablet) by nasogastric intubation, with **I** dissolved in water (1 l). The horses were fasted for 18 h and **I** was administered. The animals were fasted for an additional 6 h and then fed. Blood samples were obtained in heparinized tubes at 0, 1,

2, 4 and 6 h after drug dosing, followed by one sample every 6 h for 48 h and, finally, one sample per day until the drug was non-detectable in the plasma. After sampling, the blood was centrifuged for 5 min at 3000 *g* at ambient temperature, and the plasma supernatant was separated and stored immediately at −10°C.

2.3.4. Procedures

Plasma (1 ml), 50 μl of **II** (50 μg/ml), which was added as the internal standard, and 0.1 *M* hydrochloric acid (800 μl) were mixed with dichloromethane (8 ml) using a rotorack for 15 min. The mixture was centrifuged at 3000 *g* for 5 min and the lower organic phase was transferred to a separate tube through sodium sulphate. Another 8 ml of dichloromethane were added and the mixture was rotoracked for 15 min. This organic phase was combined with the first extraction and was evaporated to dryness at 40°C under nitrogen. The dried residue was reconstituted in 100 μl of mobile phase and 10 μl of solution were injected into the column for HPLC analysis.

For the confirmation procedure by GC–MS, the organic phase (dichloromethane) was transferred to a reacti-vial and evaporated to dryness at 40°C under nitrogen. The dried residue was reconstituted in 20 μl of acetonitrile and was derivatized with 30 μl of BSTFA + 1% TMCS at 60°C for 30 min.

The efficiency of the extraction procedure was determined by adding known amounts of an aqueous solution of **I** to blank plasma samples and extracting as described above. The calibration standards were prepared by adding known amounts of **I** (0–50 μg/ml) and internal standard (50 μg/ml) to the blank plasma.

3. Results and discussion

3.1. Experiment optimization

Compounds **I** and **II** are weak acids and dichloromethane is a relatively polar solvent; it is possible that **I** and **II** will be extractable over a broad pH range [3]. A known amount of **I** was added to blank plasma to test the extractability and the background of the plasma under different conditions (500 μl to 1 ml of 0.1 *M* hydrochloric acid). For the convenience of extraction during pre-race testing where time is an important factor, the amount of hydrochloric acid used was evaluated rather than the pH value. The absolute recovery was calculated by the following formula:

$$\text{Percentage recovery} = \frac{\text{Response of analyte spiked into matrix (processed)} \times 100}{\text{Response of analyte of pure standard (unprocessed)}}$$

Adjustment of the pH with 500, 600 and 1000 μl of 0.1 *M* HCl had no effect on the percentage recovery of a 30-μg/ml spike (92.6–95.9%). However, adjustment of the pH with 800 μl of 0.1 *M* HCl resulted in 99.2% recovery.

Fig. 1 shows chromatograms corresponding to (a) plasma blank containing 50 μg/ml of **II** as the internal standard, (b) plasma spiked with **I** (20 μg/ml) and **II** (50 μg/ml), (c) extract of plasma sample obtained from horse Y870 and (d) extract of plasma obtained from horse A163, 18 h after administration of **I**. The selectivity for the analytes is shown by the sharp and symmetrical peaks, with no interference from normal endogenous plasma constituents.

The composition of the mobile phase was selected in order to optimize the peak shapes and capacity factors of the analytes. While a large capacity factor is desirable, the screening time is only 15 min for each sample during pre-race testing. Therefore, a mobile phase composition of 35% acetonitrile and 65% acetic acid (0.2%) was chosen, resulting in retention times of about 9 and 12 min for **I** and **II**, respectively.

The following co-administered drugs were tested for potential interference with **I** and **II**: furosemide, flufenamic acid, bumetanide, antipyrine, phenylbutazone, prednisolone, flunixin, tolfenamic acid, ketoprofen, hydrochlorthiazide, diclofenac, niflunic acid, naproxen, ibuprofen, diflunisal, meclofenamic acid, dexamethasone, indomethacin and oxyphenbutazone. None gave significant interfering peaks in the assay.

3.1.1. Calibration

A six-point calibration graph was obtained by plotting the peak height ratio between **I** and **II** versus the concentration of **I**, expressed in μg/ml. The difference between the observed *y*-values and the fitted *y*-value or residual was examined for four

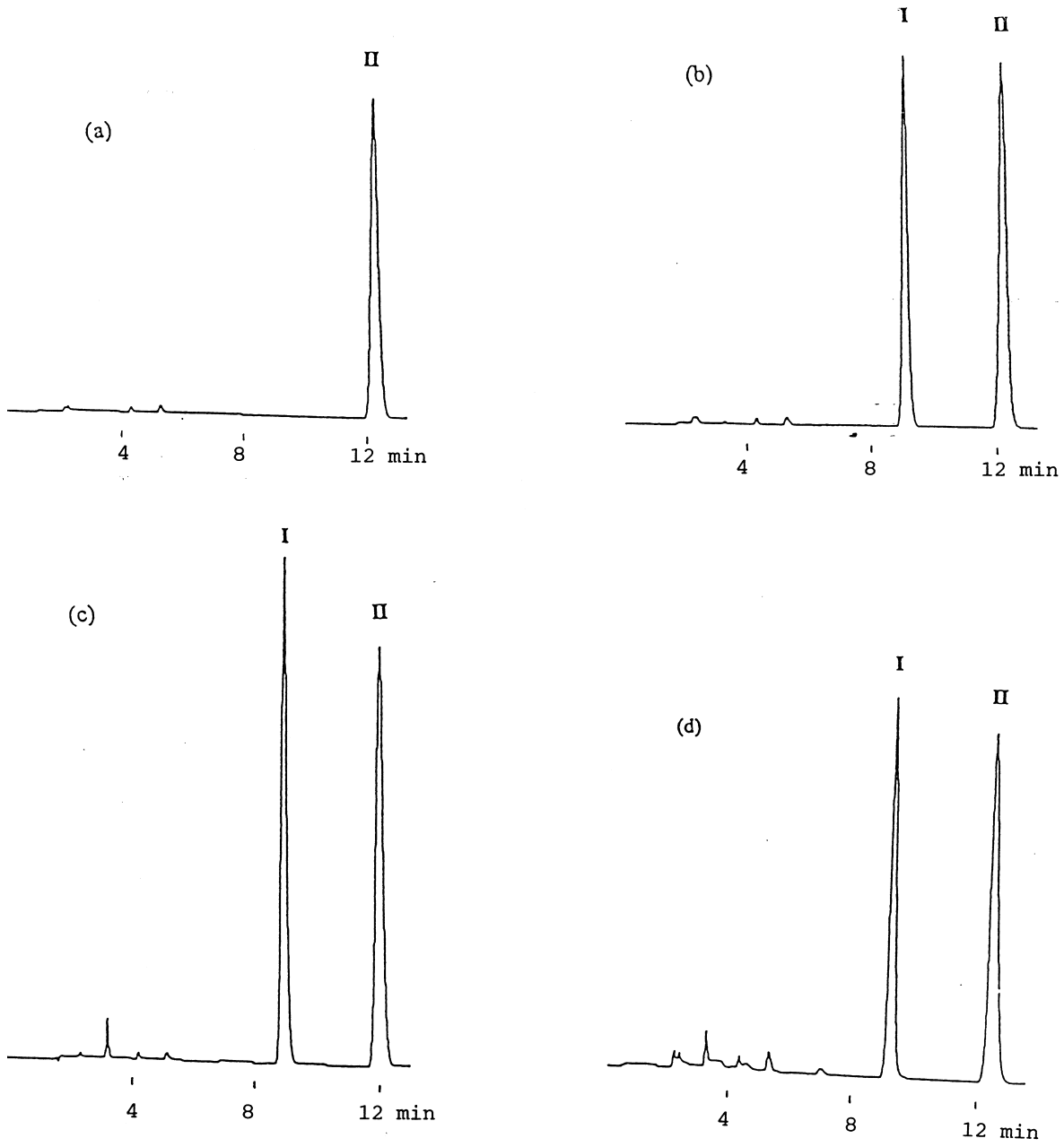

Fig. 1. Liquid chromatograms of (a) an extract of blank plasma containing **II** (50 μg/ml), (b) plasma spiked with **I** (20 μg/ml) and **II** (50 μg/ml), (c) an extract of plasma sample obtained from horse Y870 and (d) an extract of plasma sample obtained from horse A163, 18 h following the administration of **I**.

standard curves, with each constructed using six unique concentrations. A weighting factor of $1/y^2$ was selected by using a plot of studentised residual (raw residual/standard error) versus log concentration [7,8]. By using weighted linear regression analysis, the linearity was satisfactory ($y = 0.0364x +$

Table 1
Precision of the method at three different concentrations

Concentration (μg/ml)	Coefficient of variation (%)	
	Within-day ($n=6$)	Between-day ($n=6$)
1	2.5	3.6
10	1.7	2.0
30	1.2	1.4

0.0164, where x is the concentration of **I** and y is the peak-height ratio of **I** to **II**). The standard deviations for the slope and intercept were 0.00579 and 0.0172, respectively. The correlation coefficient (r^2) was >0.999. The UV detector response was linear over the range of 0–300 μg/ml, with a correlation coefficient exceeding 0.999.

3.1.2. Precision, accuracy and limit of detection

The precision of this method was determined by spiking known amounts of **I** into blank plasma. The limit of detection was found to be 3.5 ng/ml at a signal-to-noise ratio of three. The coefficient of variation (C.V.) was 9% ($n=6$) when analyses were performed on the same day and 11% ($n=6$) for different days. These data are better than those obtained in ref. [3] (10.2% on the same day and 12.5% on different days).

The lower limit of quantification was 0.005 ng/ml, with a C.V of 6% ($n=6$) when analyses were performed on the same day and 7% ($n=6$) for analyses performed on different days. The precision of the method was determined by spiking 1, 10 and 30 μg/ml of **I** into the blank plasma. The results are shown in Table 1. Accuracy was reported as percentage bias, calculated from the expression:

$$\%\ \text{Bias} = [(\text{measured value} - \text{true value})/\text{true value}) \times 100$$

Bias was not more than 5% for the concentrations tested.

3.1.3. Assay application

Plasma samples from the three horses (A727, Y870 and A163) were analysed. The pharmacokinetic profile over a 48-h period is shown in Fig. 2. The concentration of **I** in plasma reached a peak of 24.0 μg/ml at 6 h for A163 and of 42.9 μg/ml at 1 h for Y870. For each set of plasma

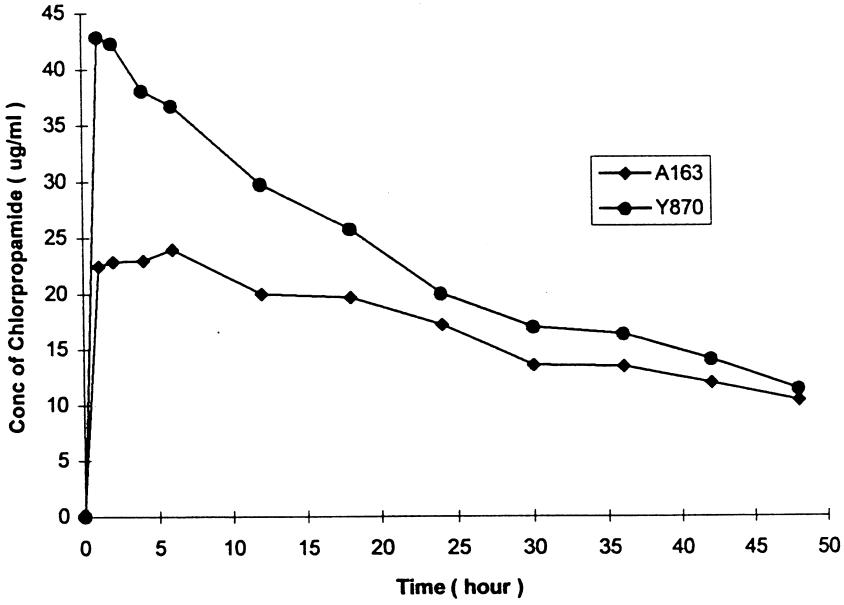

Fig. 2. Concentration of chlorpropamide in plasma from A163 and Y870 over a period of 48 h after the administration.

samples, a blank plasma was spiked with 30 μg/ml of **I** to act as a control. Glucose levels were monitored simultaneously by a one-touch glucose meter (Johnson and Johnson, USA). The glucose levels of the three horses over a period of 48 h are shown in Fig. 3. The glucose level of the administered horse was lower than that of the control horse (A727). By monitoring horses A163 and Y870, the effect of **I** on glucose level started to stabilize 12 h after the administration. An excellent correlation between the drug effect and drug level in the horses was observed in A163. Independent experiments showed that the rate of metabolism for **I** was different for the horses. It depended greatly on the dosage concentration and the activity level of the horse.

3.1.4. Confirmation of the drug

In the GC–MS technique, 1 μg of **I** was detected as a degradation product, 4-chlorobenesulphonamide, without the use of derivatization procedures. The compound decomposed on the column to give a tailing peak at high concentration. Midha et al. [9] indicated that methylation of **I** with diazomethane gave three peaks: *N*-Methyl-*p*-chlorobenzene-sulphonamide, *N*-methyl chlorpropamide and a methyl enol ether of **I**. An attempt was made to methylate **I** with MethElute reagent (trimethylanilinium hydroxide, 0.2 *M* in methanol, from Pierce). It was found that the methylated product was too unstable for analysis.

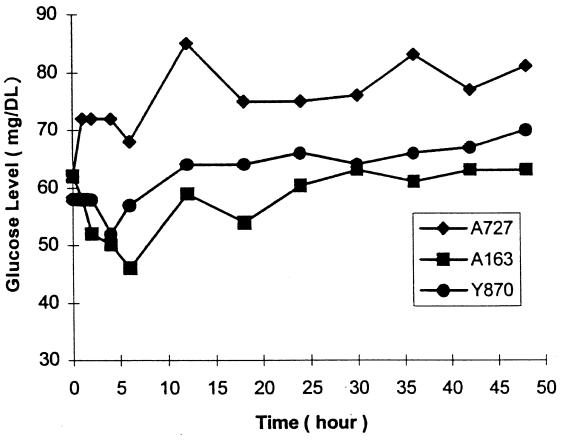

Fig. 3. Glucose levels in the three horses over a period of 48 h.

Representative ion chromatograms of (a) drug-free plasma, (b) pure **I** standard and (c) plasma extract from f A163, 24 h after administration, are shown in Fig. 4. Two products were observed in the chromatogram (peak A and peak B) when **I** was derivatized with BSTFA + 1% TMCS. Fig. 5 shows the mass spectra of trimethylsilyl (TMS) derivatives of **I**, corresponding to peaks A and B. When a compound has been trimethylsilylated, intense peaks are always observed at m/z 73, 75 and 147 [10,11]. With reference to the structure of **I**, there are two active hydrogen atoms that can be replaced by the TMS group. Therefore, it is possible to obtain mono-TMS and di-TMS derivatives [11,12]. However, the introduction of one silyl group hinders access of a second, which can be introduced only with difficulty. In this assay, the derivatization condition was chosen to accommodate a pre-race situation, and was not conducive to the formation of the di-TMS derivative. Thus, the derivatized products formed were due to the respective replacement by TMS of the hydrogen atoms at the two nitrogen sites. The ions at m/z 320 (peak A) and m/z 248 (peak B) are specific for chlorpropamide, as observed from our mass spectrometric studies. Depending on the location of the TMS group after derivatization of **I** (the respective molecular ions are at m/z 348), fragment ions will result in peaks exhibiting m/z values of 248 or 320.

A complete understanding of silylation and the mechanism of ion fragmentation are not of concern in this confirmation technique. Identification and confirmation are both based on the comparison of chromatographic retention times and on mass spectral fingerprinting with the pure standard. A scanned mass spectra of pure **I** was obtained and compared with the mass spectrum of the plasma extract. Fig. 6 shows reconstructed selected ion chromatograms of (a) drug-free plasma, (b) plasma spiked with **I** and (c) a plasma extract of A163 obtained 24 h after administration. In this region, there is no significant interference from blank plasma. It is difficult to monitor the rate of formation of peaks A and B. The presence of peaks A, B or both can confirm the presence of the drug.

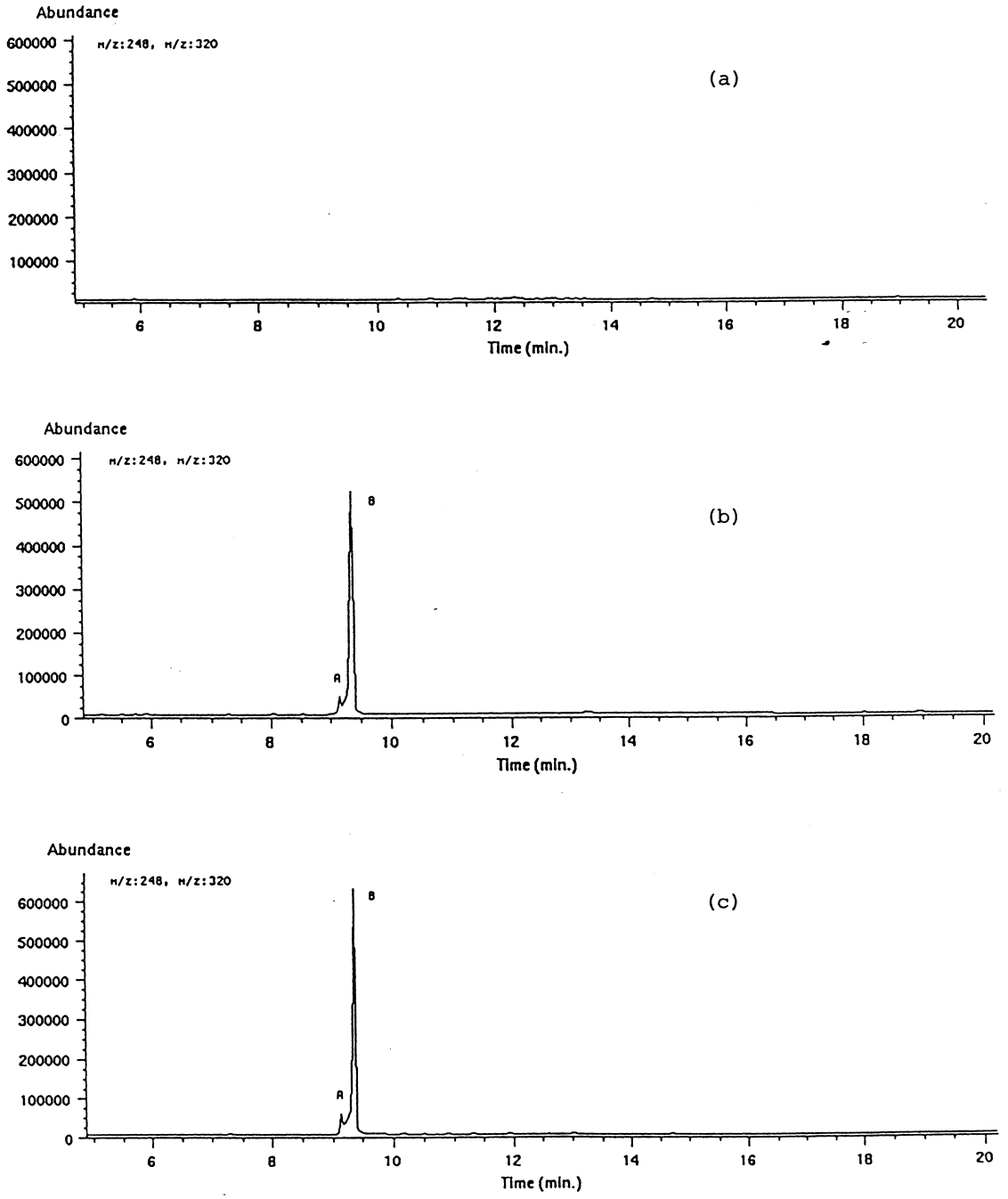

Fig. 4. Selected ion monitoring (SIM) signal of (a) drug-free plasma, (b) pure **I** standard, (c) a plasma extract from A163, 24 h after the administration.

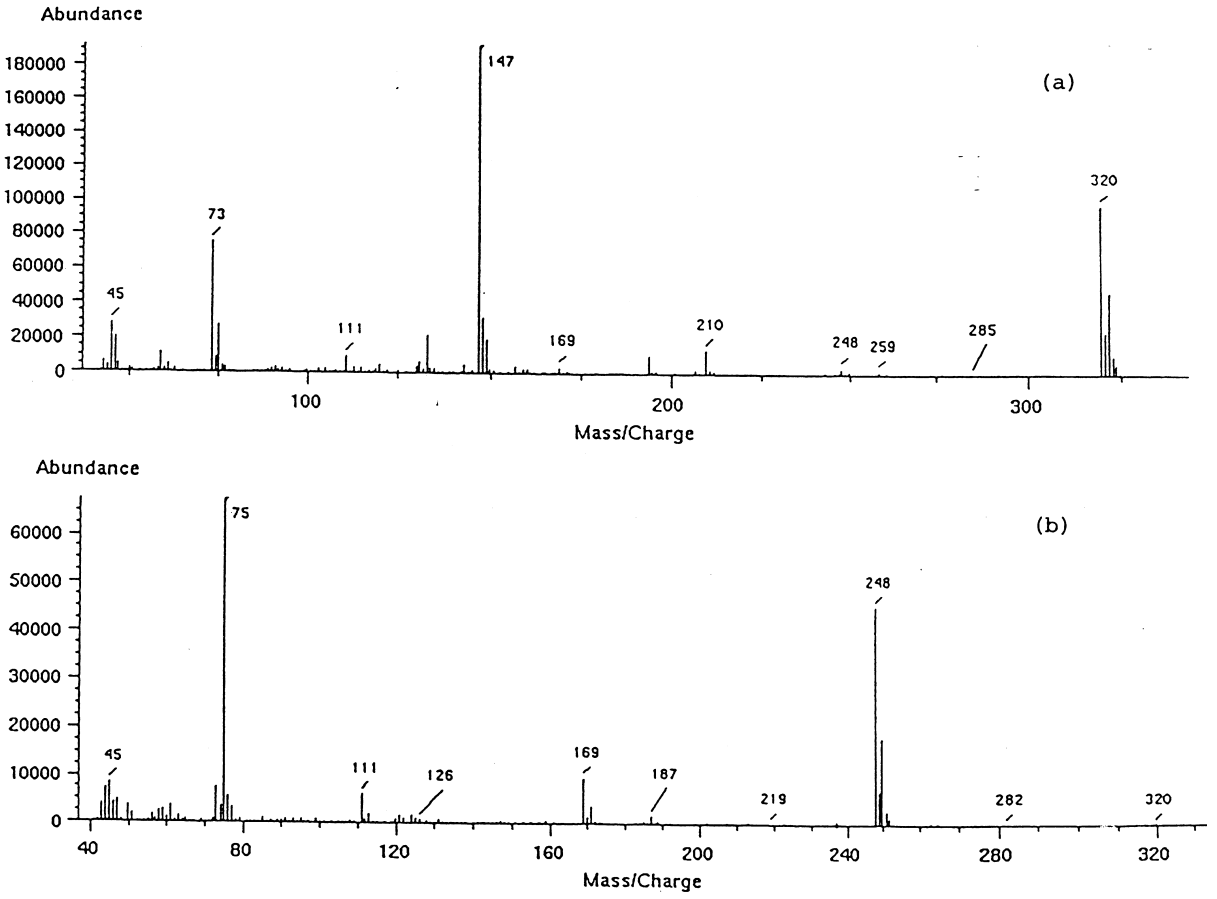

Fig. 5. Mass spectra of TMS derivatives of **I** corresponding to (a) peak A and (b) peak B, obtained using GC–MS with electron-impact (70 eV) ionization.

BSTFA + 1% TMCS was used because of its commercial availability and the stability of TMS derivatives. It was found that the TMS derivatives of **I** were stable for at least four days when kept in a sealed vial with excess trimethylsilyl reagent.

4. Conclusion

The proposed method using HPLC for drug screening and quantification and GC–MS for confirmation of the drug's presence proved to be satisfactory for the purpose of anti-doping control in horse-racing, an industry that plays an important role in the domestic economies of several countries in the world (e.g. Singapore, Malaysia, Hong Kong, Australia, New Zealand, the United Kingdom, France, etc.).

Acknowledgements

This research was supported by the Malayan Racing Association. The authors thank Dr. Deryck Tang and Dr. Tony Doherty for their assistance in plasma collection.

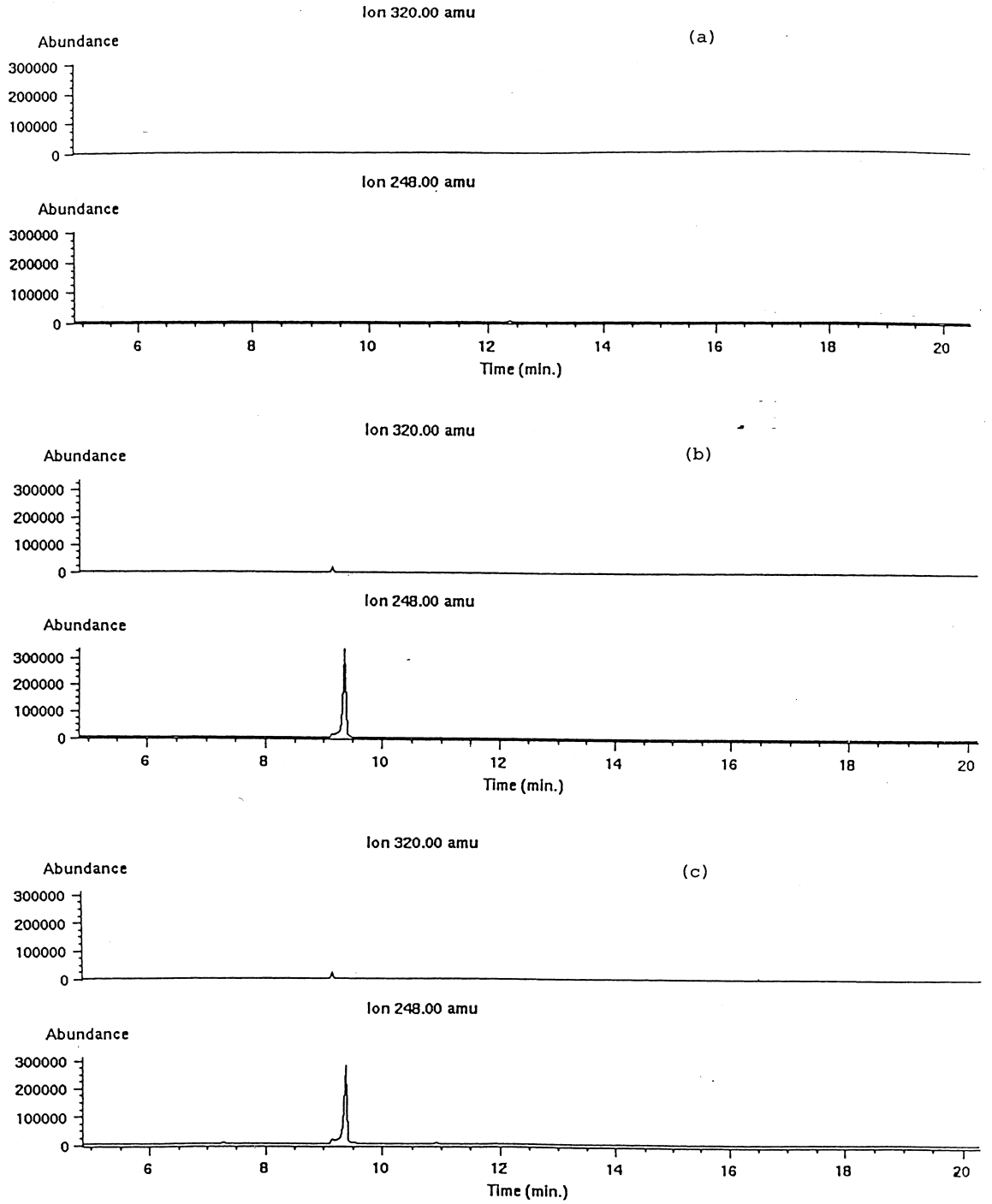

Fig. 6. Reconstructed selected ion chromatograms of (a) drug-free plasma, (b) plasma spiked with **I** (10 μg/ml) and (c) a plasma extract from A163, 24 h after administration.

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