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Determination and identification of amiloride in human urine by high-performance liquid chromatography and gas chromatography–mass spectrometry

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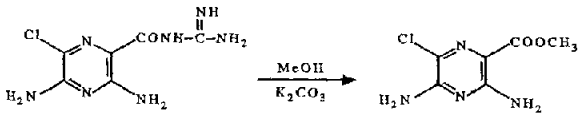
ABSTRACT

A simple and sensitive high-performance liquid chromatographic method was developed to screen and determine amiloride (I) in human urine. The detection limit of the method is 0.12 μg/ml and the recovery of amiloride from urine was 80.4–85.5% at different concentrations. The coefficients of variation were less than 2.8 and 4.4% for intra- and inter-assays, respectively. Total urinary excretion of I in 24 h after oral administration of 5 mg or 15 mg of I ranged from 22.0 to 33.3% of the total dose for three different subjects. I could be detected in urine up to at least 44 h after a 5-mg dose and 72 h after a 15-mg dose. A gas chromatographic–mass spectrometric (GC–MS) confirmatory method was established based on the methanolysis of I to methyl 3,5-diamino-6-chloropyrazinecarboxylate (II). The di-N-trimethylsilyl derivative of II showed very good GC–MS properties and provided reliable structure information for confirmation analysis of I. This is the first time that a reliable GC–MS method has been reported for the detection of urinary I.

INTRODUCTION

Amiloride, 3,5-diamino-N-(aminoiminomethyl)-6-chloropyrazinecarboxamide (I, Fig 1), is a potassium-sparing diuretic. It was first synthesized in the early 1960s by a research group at Merck [1]. It is either used alone or in combination with other major diuretics such as benzothiadiazides to treat hypertension and heart failure in clinical practice [2].

Diuretics have been banned at major sporting events since the 1988 Winter Olympic Games. Athletes often misuse diuretics for mainly two reasons [3,4]: (1) to reduce body weight prior to a competition in order to qualify for lower-weight classes and (2) to dilute a urine specimen to minimize the concentration of excreted doping agents or to inhibit their excretion in the urine. In the case of diuretic therapy, I is often administered to attenuate kaliuresis so as to maintain normal potassium level in body fluids. Therefore, the detection of I in urine specimens can be interpreted as a good indication of its misuse in combination with other major diuretics.



I II

Fig. 1. Structure of amiloride (I) and its methanolysis to form the methyl ester (II).

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The analysis of human urine for I has been widely investigated with high-performance liquid chromatography (HPLC) as the major tool [5–11]. As HPLC provides mainly quantitative information, reported methods cannot be applied to confirm the presence of I in urine specimens from athletes in real doping control practice. It is well known that gas chromatography–mass spectrometry (GC–MS) is the method of choice to provide definite proof of the presence of doping agents in urine samples. Owing to the poor GC behavior of I and great difficulties encountered in the derivatization of its very polar guanidine moiety, no GC–MS confirmatory method for the presence of I in biological fluids has yet been developed. HPLC–MS has been proposed as an alternative procedure [12,13], but it suffers from low sensitivity and a lack of characteristic mass fragment ions.

For the screening of I in biological samples, current HPLC methods have certain drawbacks. Owing to the poor lipophilicity of I, the liquid–liquid extraction recovery was only 25% at pH 9–9.5 [4]. Modifications of the sample preparation for urine specimens included direct urine analysis (after dilution) [7,9,11] and single solid-phase extraction [6,14]. As the urine samples were not subjected to extensive clean-up, these methods experienced overestimated recoveries (hidden interference peaks) and a shortening of the column life. Ion-pair extraction [10] seemed to be a better choice but it required several laborious steps to complete the sample preparation. Even with direct injection of urine, HPLC with UV detection did not give satisfactory detection limits for I [9]. Several methods using fluorescence detectors to achieve high sensitivity have been reported [5–8,10,11]. Nevertheless, with respect to real practice in routine doping control, universal UV detection is more suitable in a screening procedure that analyzes large varieties of compounds.

In this paper, we describe a simple and sensitive HPLC method with a common UV detector for the screening and quantification of I in urine samples, and an effective GC–MS procedure to confirm the presence of this diuretic. This method can be applied in routine doping control and in clinical laboratories.

EXPERIMENTAL

Materials and reagents

Amiloride (I) and its tablets (Midamor) were obtained from Merck Frosst Canada (Kirkland, Canada), triamterene (internal standard) from Smith Kline and French (Mississauga, Canada), N-methyl(trimethylsilyl)trifluoroacetamide (MSTFA) from Regis (Morton Grove, IL, USA), Dithioerythritol and trimethylsilyl iodide (TMSI) from Aldrich (Milwaukee, WI, USA) and Sep-Pak C_{18} cartridges from Waters Division, Millipore (Milford, MA, USA). All organic solvents (HPLC grade) were used as received (Caledon Labs, Georgetown, Canada). Inorganic salts were of analytical-reagent grade (J. T. Baker, Phillipsburg, NJ, USA, or Caledon Labs.). Distilled water was further treated with a four-stage Milli-Q water purification system (Continental Water System, Oakville, Canada) before use for HPLC (this is referred to simply as water hereafter).

Preparation of methyl 3,5-diamino-6-chloropyrazinecarboxylate (II) from the methanolysis of I (Fig. 1)

To 5 ml of a methanolic solution of I (100 mg, 0.38 mmol), 100 mg of potassium carbonate were added and stirred at 60°C for 3 h. After cooling to room temperature, the mixture was filtered. HPLC and GC–MS analysis showed that compound II was the sole product. Evaporation of solvent gave crude II. Recrystallization of the residue with methanol afforded 25 mg of II (28.3%), m.p. 214–215°C (literature [1] m.p. 212–213°C). The electron-impact (EI) mass spectra of II (underivatized and TMS derivative) are presented in Fig. 6.

Standard solutions

A stock standard solution of I was prepared in methanol at a concentration of 200 μg/ml. Working standard solutions in methanol contained 50 or 100 μg/ml I. A stock standard solution of the

internal standard (I.S., 150 μg/ml) was prepared in methanol. A working standard solution of I.S. (15 μg/ml) was prepared with 1% HCl in methanol water (1:1, v/v). All the stock and working standard solutions were sealed and stored at −20°C.

Human studies

After collecting control urine samples, three healthy volunteers (A, male, aged 55; B, female, aged 29; and C, male, aged 33) were given 5 mg of I in the morning whilst fasting. Post-administration urine samples were collected at certain time intervals up to 96 h to ensure the total excretion of I from the body. To the same subjects a 15-mg dose was administered ten days after the first 5-mg dose, and urine samples were collected accordingly.

Sample preparation for HPLC analysis

In a typical extraction procedure, 2.5 ml of urine were first basified to pH 12 with 100 mg of potassium carbonate. The resulting sample was extracted with ethyl acetate (2 × 5 ml). The combined organic layer was evaporated under a stream of nitrogen at 50°C. The residue was dissolved in 300 μl of the working standard solution of I.S. (15 μg/ml, 1% HCl in methanol–water solution) and 10 μl of this final solution were injected on to the HPLC column.

High-performance liquid chromatography

An HP 1090 liquid chromatograph was equipped with a diode-array UV detector and HP 79994 HPLC ChemStation data system (Hewlett-Packard, Palo Alto, CA, USA). Separation of I from the internal standard was carried out on an HP ODS Hypersil (C_{18}) column (200 mm × 4.6 mm I.D., 5 μm particle size) at ambient temperature. A laboratory packed HP ODS Hypersil precolumn (20 mm × 2.1 mm I.D., 30 μm particle size) was used to protect the analytical column. The mobile phase consisted of solvents A and B. Solvent A (0.02 *M* phosphate buffer, pH 3.2) was prepared by dissolving 6.9 g of monobasic sodium phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) and 1.59 g of propylamine hydrochloride in 1 l of water and adjusting the pH to 3.2 with concentrated phosphoric acid. It was filtered over a Millipore 0.45-μm HA filter and degassed with helium for 30 min before use. Solvent B was acetonitrile. The analysis was accomplished by gradient elution starting with 15% solvent B (held for 2 min) and then linearly increasing to 80% solvent B at 20 min. The flow-rate of the mobile phase was kept at 1 ml/min. The UV absorbance of the column effluent was monitored at 363 nm (pilot wavelength), 213 nm and 285 nm (band width of 4 nm for each wavelength) with the reference wavelength at 450 nm (band width of 20 nm). Spectral scanning was performed from 190 to 400 nm.

Calibration

Aliquots of 2.5 ml of urine were spiked with working solutions of I to obtain concentrations between 0.12 and 12 μg/ml. After being equilibrated for 1 h at 37°C, the urine samples were extracted as described under *Sample preparation for HPLC analysis*, and extracts were analysed by HPLC. For each concentration triplicate samples were prepared and duplicate injections were made for each sample. The peak-area ratios of I to the I.S. were measured for each analysis. The data were fitted by the linear regression equation $C_I = 0.73A_r - 0.039$, where C_I is the concentration of I in urine (μg/ml) and A_r is the peak-area ratio of I to I.S.

Recovery and precision

The extraction recovery of I from urine was assessed with spiked samples at three different concentrations, 0.48, 4.8 and 12 μg/ml. Calculation of the recovery was based on a comparison of the peak-area ratios of I to I.S. obtained from spiked sample extracts with the ratios from corresponding standard solutions containing I and I.S.

Intra- and inter-assay variabilities were determined by replicate analyses of amiloride-spiked urine samples (with the same concentration level as in the recovery test) on the day of preparation and on different days, respectively.

Sample preparation for GC–MS analysis

The solid-phase extraction method was adopted as described previously [15]. An aliquot of 2.5–10 ml of urine was passed through prewashed Sep-Pak C_{18} cartridges. After washing the cartridge with 5 ml of water and 2 ml of hexane, I was eluted with 4 ml of methanol. To the methanolic solution, 5 mg of K_2CO_3 were added and the resulting mixture was left at 60°C for 2.5 h. When the reaction was completed, the resulting solution was evaporated to dryness under nitrogen. The residue was dissolved in 1 ml of water and extracted with 2 × 5 ml of ethyl acetate. The extract was then evaporated to dryness under nitrogen. The residue was derivatized with 50–100 μl of MSTFA–TMSI (1000:4, v/v) [15] and 1 μl of the mixture was analysed by GC–MS.

Gas chromatography–mass spectrometry

A Mega Series gas chromatograph (Carlo Erba, Milan, Italy), equipped with an HP Ultra-2 (5% phenyl–methylsilicone cross-linked) capillary column (12 m × 0.2 mm I.D., 0.11 μm film thickness), was directly interfaced to an MS25RFA mass spectrometer (Kratos, Manchester, UK). The carrier gas (helium) flow-rate was about 0.8 ml/min. The oven temperature was programmed from 80°C (held for 1 min) to 200°C at 10°C/min and then to 280°C at 25°C/min (held for 5 min.). Injection was performed with a cold on-column injector. The temperatures of the transfer line and ion source were set at 310 and 200°C, respectively. Mass spectra were recored in the EI mode with an ionizing energy of 70 eV.

RESULTS AND DISCUSSION

Determination of I in human urine by HPLC

As the most common dose of I is 5 mg, sensitivity is a serious problem when establishing screening and quantitative methods. I is a weak organic base with a pK_a of 8.7, therefore liquid–liquid extraction should be carried out under basic conditions. It was suggested [4] that pH 9–9.5 be used in a screening procedure for the extraction of some basic and neutral diuretics, including I, but the recovery of I was only 25% using that procedure [4]. Solid-phase extraction methods have been reported to achieve higher recoveries [6,14]. Owing to the high cost and poor selectivity of the extraction cartridges, the use of these methods was limited in screening procedures. The present method uses common liquid–liquid extraction at pH 12, which increases the recovery efficiency to more than 80%. The detection limit of I is 0.12 μg/ml (at this concentration a recognizable UV spectrum can be obtained from the detected chromatographic peak). In practice with this method we were able to detect I in the urine at least 44 h after a 5-mg oral dose of I. Table I lists the recovery of I from spiked urine samples. The intra- and inter-assay results illustrate the accuracy and precision of the extraction method. The maximum intra- and inter-assay coefficients of variation (C.V.) are 2.8 and 4.4%, respectively. Fig. 2 presents the chromatograms obtained from analysis of a blank urine sample and a urine sample containing 0.84 μg/ml I, collected 11 h after a 5-mg oral dose of I. The chro-

TABLE I

RECOVERY OF AMILORIDE (I) FROM SPIKED HUMAN URINE

I added (μg/ml)	Intra-assay (n = 3)		Inter-assay (n = 9)	
	Recovery (mean ± S.D.) (%)	C.V. (%)	Recovery (mean ± S.D.) (%)	C.V. (%)
0.48	76.0 ± 2.1	2.8	81.6 ± 3.6	4.4
4.8	78.7 ± 1.7	2.2	80.4 ± 2.7	3.4
12	83.1 ± 0.3	0.4	85.5 ± 3.4	4.0

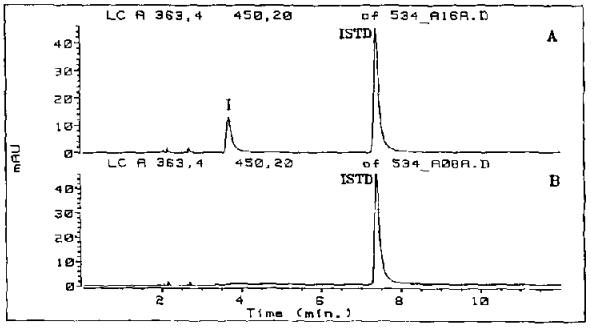

Fig. 2. HPLC of (A) a urine sample containing 0.84 μg/ml I, collected 11 h after oral administration of a 5-mg dose of I and (B) a blank urine sample. ISTD = internal standard (triamterene). For analytical conditions see Experimental.

matograms without background peaks show that the clean-up of urine specimens is very effective owing to extraction at high pH.

Human studies

Fig. 3 illustrates the excretion profiles obtained from the quantitative analysis of urine samples from three different subjects after administration of two different doses of I. The maximum excretion rate of I is at about 3–4 h (with one exception at 7 h). With the present method we can detect I in the urine at least 44 h after a 5-mg oral dose. In some subjects I can be detected for a longer time. With the 15-mg dose we can detect I in urine as long as 72 h after administration. This shows that the proposed method is very effective in doping control practice, as most diuretics are used prior to sports competition to lose body weight or as masking agents. Table II gives the total excretion data for different subjects at two dose levels. The total excretion of I within 24 h is 22–31.4 and 27.1–33.3% of the total oral dose after oral administration of 5 and 15 mg of I, respectively. This result is in good agreement with a previous study [9].

Conversion of I into methyl ester II

The difficulties in confirming I in urine by GC–MS in doping control laboratories arise because I contains a very polar guanidine chain so that certain derivatization steps are required before GC–MS analysis. The trimethylsilyl (TMS) derivative of I is very unstable and no molecular ion ($M^{+\cdot}$) could be observed in the mass spectrum of this derivative [16]. Although LC–MS could be an alternative method, the poor sensitivity and lack of fragment ions in the mass spectrum restrict its use.

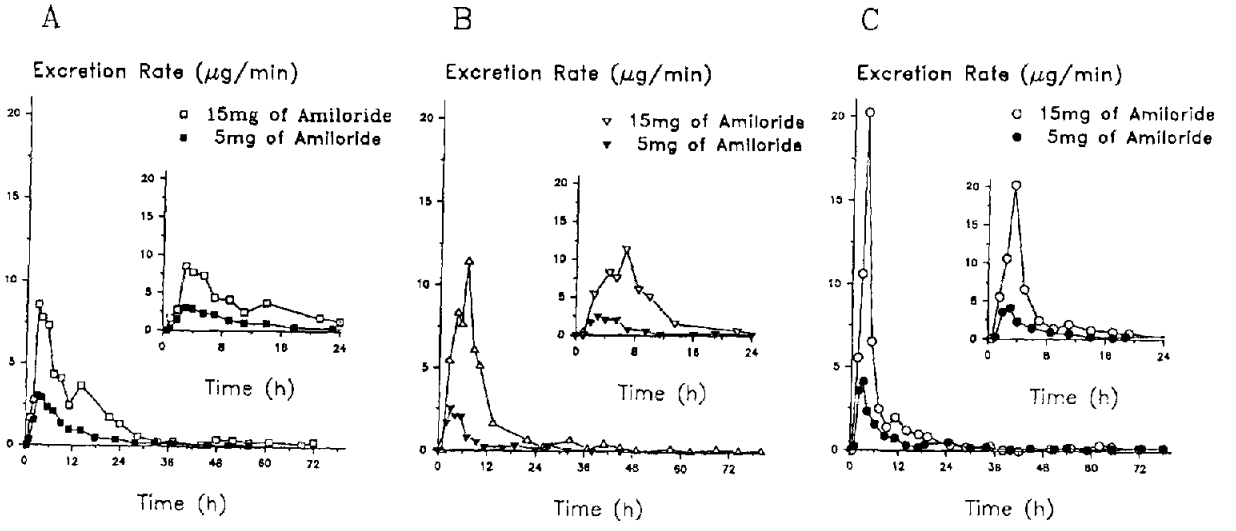

Fig. 3. Excretion rate profiles for three subjects after (closed symbols) 5- and (open symbols) 15-mg oral doses of amiloride. Insets are enlargements of the 0–24 h period.

TABLE II

EXCRETION OF AMILORIDE (I) IN HUMAN URINE

Time after administration of I (h)	5-mg dose		15-mg dose	
	Total excretion of I (mg)	% of dose	Total excretion of I (mg)	% of dose
Subject A				
4	0.46	9.2	1.19	7.9
14	1.33	26.6	3.70	24.7
24	1.57	31.4	4.44	29.6
Subject B				
4	0.49	9.8	1.70	11.3
14	0.91	18.2	4.58	30.5
24	1.10	22.0	4.99	33.3
Subject C				
4	0.61	12.2	2.18	14.6
14	1.09	21.9	3.68	24.5
24	1.34	26.9	4.07	27.1

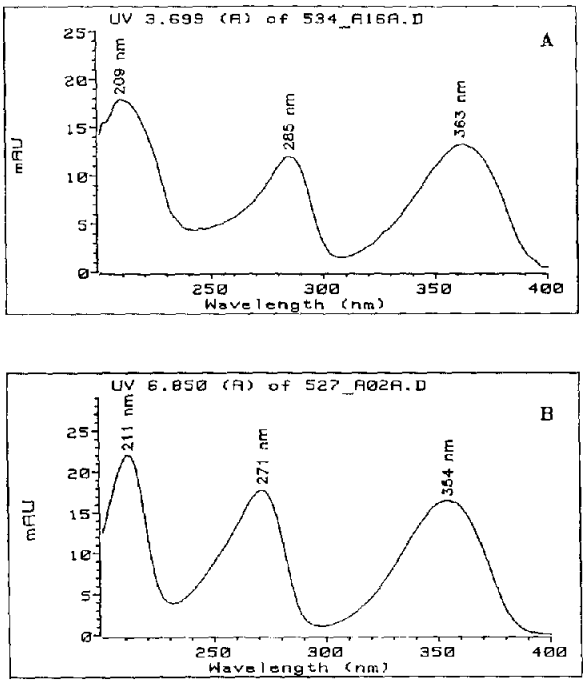

Fig. 4. UV spectra of (A) amiloride (I) and (B) its methyl ester (II).

In a previous study, an artifact of I was reported in basic methanolic solution which had a longer retention time than I and with a similar UV spectrum to that of I [4]. From the structure of I (Fig. 1) it is conceivable that under basic conditions, the attack of methoxide on the carbonylguanidine function could possibly replace the guanidine with a methyl ester. Indeed, after treating I with methanol–potassium carbonate, methyl ester II was successfully prepared. Fig. 4 shows the UV spectra of I and methyl ester II. The two spectra are very similar and the spectrum of II shows a hypsochromic shift at the maximum absorbance to the shorter wavelengths due to methylation. The conversion of I to II has changed the polarity of the molecule and also the fate of analysis.

As basic extraction is applied in the sample preparation, which co-extracts a certain amount of carbonate into the final extracts, it is inappropriate to dissolve the extracts directly with methanol. Therefore, the final samples for HPLC are prepared using the I.S. working standard solu-

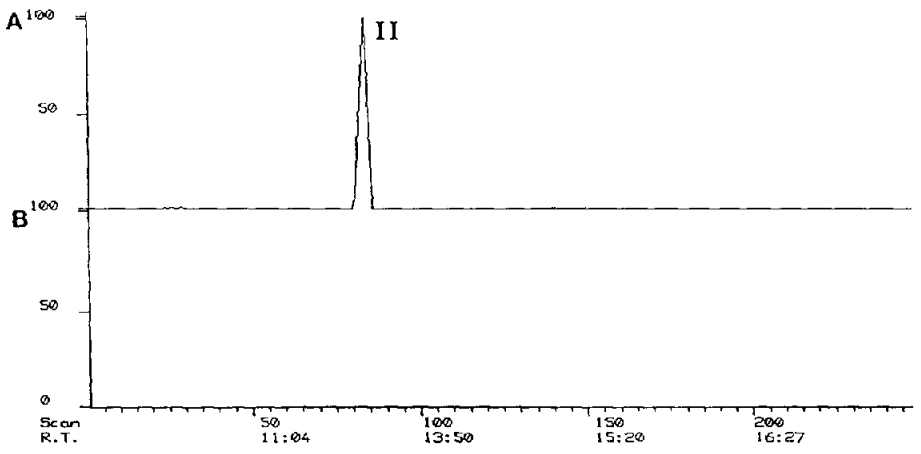

Fig. 5. Reconstructed ion (m/z 331) chromatograms of II from GC–MS analysis of (A) a urine sample containing 0.84 μg/ml I, collected 11 h after administration of a 5-mg dose of I and (B) a blank urine sample. For sample preparation, methanolysis of I and derivatization procedure see Experimental.

tion (1% HCl in methanol–water) to ensure the stability of I during the analysis.

GC–MS analysis of II

In order to achieve higher sensitivity and selectivity in the confirmatory analysis, a more comprehensive sample preparation method was adopted. Solid-phase extraction was used to enrich the concentration of I in the case of a diluted urine sample. After methanolysis of I, the resulting mixture was further purified by liquid–liquid extraction. This provided a much cleaner sample for GC–MS analysis. The chromatogram obtained from GC–MS analysis of a urine extract (Fig. 5) clearly demonstrates that the extraction is very effective and selective.

Owing to the presence of two polar amino functions, II can be detected by GC–MS at low sensitivity (4 μg/ml). The EI mass spectrum of underivatized II (Fig. 6A) shows that II has a very similar fragmentation pattern to I [17], as the fragmentation is focused on the carboxylate for both compounds. The major mass fragments of II arise from the cleavage of the ester moiety, whereas the fission of the guanidine chain forms major ions of I. The molecular ion at m/z 202 ($M^{+}\cdot$) of II shows the replacement of guanidine moiety with a methyl ester (Fig. 6A). The ions at m/z 171 ($[M - 31]^{+}$), m/z 144 ($[M - COOCH_3 + H]^{+}\cdot$) and m/z 142 ($[M - HCOOCH_3]^{+}\cdot$) confirm the presence of methyl carboxylate.

In order to achieve higher sensitivity in the confirmatory analysis, derivatization with MSTFA–TMSI was applied to afford the di-N-TMS derivative of II. This lowered the detection limit to 0.16 μg/ml. The EI mass spectrum of the TMS derivative of II is consistent with the proposed structure (Fig. 6B). With the TMS derivative, high intensities of the molecular ion ($M^{+}\cdot$, m/z 346) and the ion at m/z 331 ($[M - 15]^{+}$) were achieved. Other characteristic ions, such as those at m/z 315 ($[M - 31]^{+}$), m/z 274 ($[M - TMS + H]^{+}\cdot$) and m/z 259 ($[M - NHTMS + H]^{+}\cdot$), provided supporting evidence for structure confirmation.

CONCLUSION

We have presented a simple and sensitive HPLC method for the screening and quantification of amiloride in human urine. The method has a high extraction efficiency and precision. It can be easily adapted for use in routine doping control and in clinical laboratories. We have also reported a confirmatory method to identify urinary amiloride by GC–MS, which is based on the methanolysis of amiloride. This is the first reliable GC–MS method available for the confirmation of amiloride in human urine.

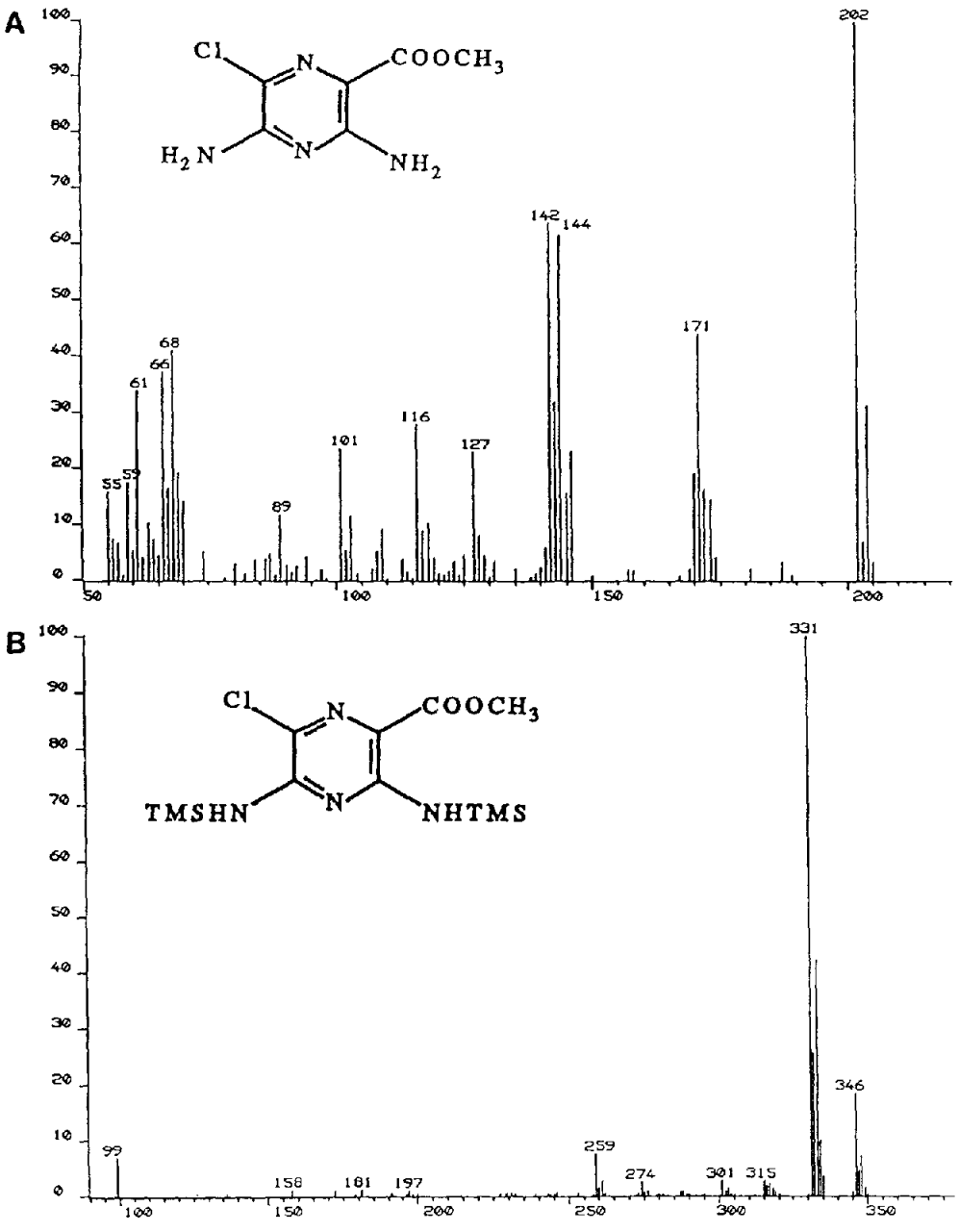

Fig. 6. EI mass spectra of (A) methyl ester II and (B) its di-N-TMS derivative.

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