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Short Communication

Gas chromatographic–mass spectrometric method for the determination of bifemelane in human plasma at therapeutic doses

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ABSTRACT

A gas chromatographic method with mass-selective detection for the determination of bifemelane in human plasma has been developed. The assay is based on a single hexane extraction and an efficient gas chromatographic separation on a capillary column. The assay has been validated and used to support clinical pharmacokinetic studies. The lowest limit of quantitation was found to be 1 ng/ml and allowed pharmacokinetic evaluation of the drug at doses down to 50 mg.

INTRODUCTION

Bifemelane–HCl [4-(*o*-benzylphenoxy)-N-methylbutylamine hydrochloride, BFM-HCl] (Fig. 1) is a compound with a new chemical structure developed by Mitsubishi [1] to improve cerebral functions. Its pharmacological effects are, in addition to improvement of cerebrometabolism and intracerebral blood flow, stimulation of the central nervous system, especially the noradrenaline and acetyl choline system [2–5].

A method with a limit of reliable quantitation (LQ) of at least 1 ng/ml was needed to support clinical studies employing 50–150 mg oral doses. In addition, in order to support large-scale pharmacokinetic studies, an efficient method capable of analysing more than 50 plasma samples a day was required. An assay with the 1 ng/ml LQ has been successfully developed. More than 1000

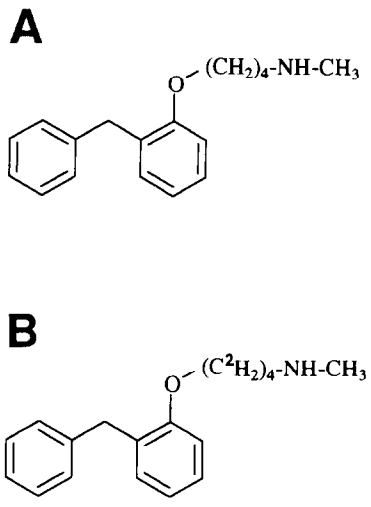

Fig. 1. Structures of (A) bifemelane and (B) [$^2H_8$]bifemelane (internal standard).

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plasma samples from various biopharmaceutical studies were analysed using the procedure described in this paper.

EXPERIMENTAL

Materials and reagents

Acetonitrile and water (all HPLC grade, J. T. Baker, Gross-Gerau, Germany), drug-free human control plasma (Blutspendedienst Hessen, Frankfurt, Germany), *n*-hexane, 10 *M* sodium hydroxide and 1 *M* hydrochloric acid (Merck, Darmstadt, Germany) were purchased from their suppliers. The plasma was stored frozen (nominal −20°C) prior to analysis.

Bifemelane–HCl and the internal standard (I.S.) [$^2H_8$]bifemelane–HCl were obtained from Mitsubishi Kasei (Tokyo, Japan).

Apparatus

A Hewlett-Packard GC–MS system equipped with a DOS HP G 1034 B MS ChemStation, an HP5890A Series gas chromatograph with automatic injection system HP 7673A and an HP 5971 mass-selective detector were used for all analyses.

GC–MS procedure

A capillary HP 1 column, 25 m × 0.2 mm I.D., 0.11 μm film thickness, was used under the following conditions: oven temperature programme, 1 min at 120°C, 20°C/min to 270°C, 10 min at 270°C; carrier gas, helium at a flow-rate of 1 ml/min (69 kPa); GC–MS interface maintained at 290°C. All injections were carried out using the splitless mode of injection with a purge-off time of 1.0 min (injector temperature 250°C, 4 mm split–splitless liner HP 19251-60540). The injection volume was 3 μl.

To perform mass fragmentography the ions *m/z* 269 for bifemelane and *m/z* 277 for [$^2H_8$]-bifemelane were recorded with a dwell time of 50 ms. The areas of the mass fragmentographic peaks were integrated using the data system with automatic selection of the baseline points (time programmed). The retention time of BFM and [$^2H_8$]BFM under the conditions described above were 8.01 and 8.05 min, respectively.

Standard solutions

Two stock standard solutions of BFM–HCl (4.2 and 2.1 mg/l) were prepared in water. These solutions were further diluted with human plasma to give two series of calibration standards of bifemelane–HCl. The concentrations were 1.05, 2.1, 5.25, 10.5, 16.8, 31.5 and 81.5, 52.5, 84.0, 105.0, 157.5, 210.0 ng/ml. The I.S. was also prepared as a stock solution (0.376 mg/l in water).

Quality control samples were prepared in bulk by fortifying control human plasma with the two stock solutions of BFM–HCl. They were divided into aliquots and stored in screw-cap vials at nominal −30°C prior to use.

Sample collection

Blood was collected in potassium EDTA-coated plastic tubes and the plasma was separated within 15 min and stored at −30°C until analysed.

Freeze–thaw stability was determined by examining quality control samples that had undergone one, two and three freeze–thaw cycles. There was no apparent degradation of the quality control samples after three freeze–thaw cycles and no apparent degradation of the sample extracts in the autosampler vial tray.

Sample preparation

For each sample, 1 ml of plasma, 0.5 ml of water, 0.2 of 1 *M* hydrochloric acid and 500 μl of I.S. solution were heated at 60°C for 15 min. After cooling to room temperature, 1.4 ml of *n*-hexane and 0.1 ml of 10 *M* sodium hydroxide were added, and extracted with a rolling-mixer for 30 min. The hexane phase was transferred to a GC vial with a screw cap and the solvent was evaporated at 85°C to dryness. After adding 75 μl of acetonitrile to the residue, the samples were vortex mixed and treated with the rolling-mixer for 10 min. After centrifugation (4000 *g*) the sample was transferred to a GC vial with microinsert (150 μl) and injected directly onto the GC–MS system.

Precision, accuracy, linearity and recovery

The precision of the method was determined by replicate analysis ($n = 6$) of human plasma containing bifemelane–HCl at the concentrations described in the *Standard solutions* section, and by multiple ($n = 4$) analysis of pooled plasma samples from patients ingesting BFM. The accuracy of the assays was checked by preparing qual-

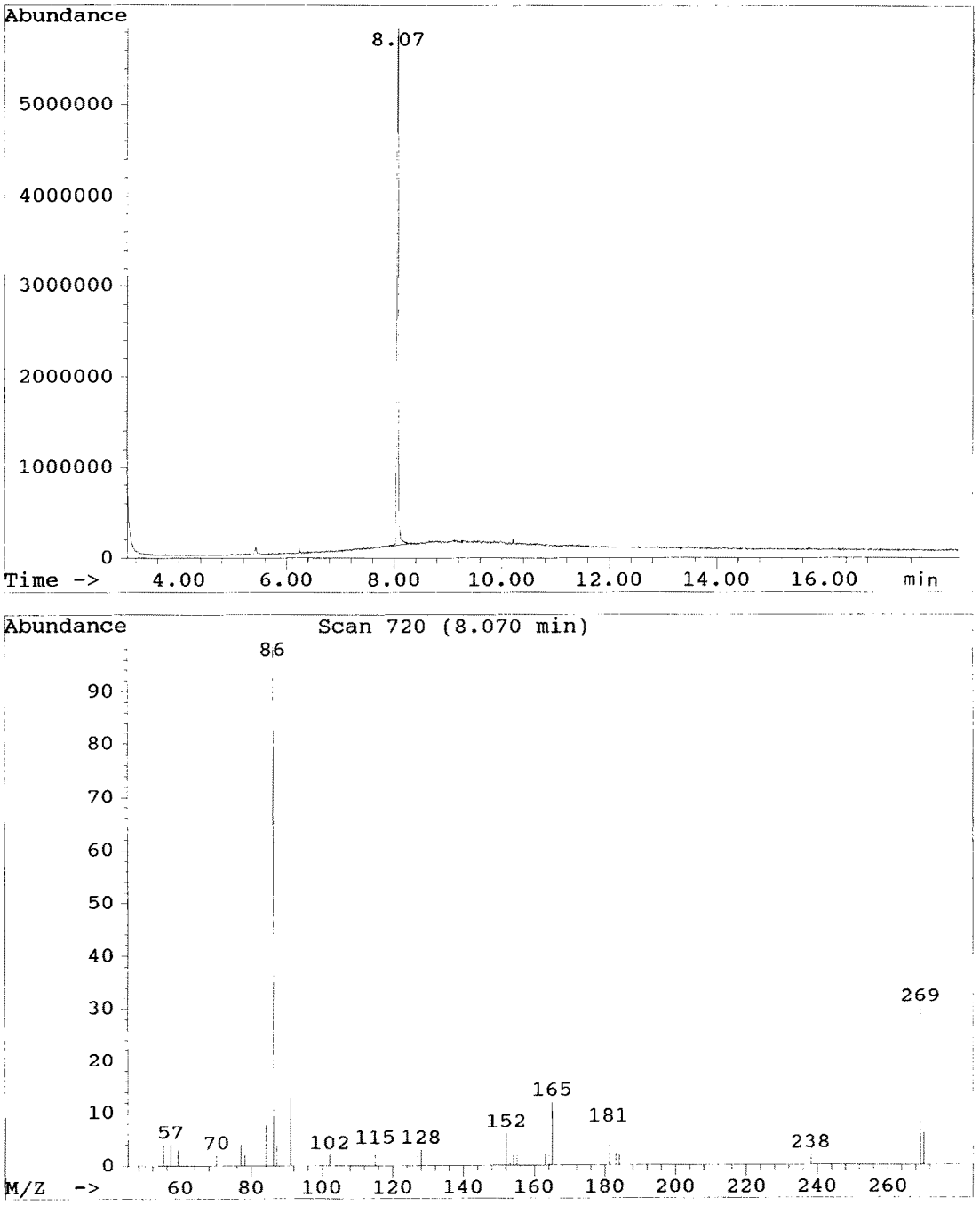


Fig. 2. Chromatogram and mass spectrum of bifemelane.

ity control samples at the start of clinical studies. Plasma samples containing known concentrations of BFM (4.2, 12.6, 23.8, 33.6, 100.8 and 168.0 ng/ml) were prepared and frozen at −30°C. These quality control standards were assayed with the unknown samples each day analyses were performed.

The calculated concentrations of the quality control samples were compared on a day-to-day basis. Recovery was calculated by comparing of the peak areas of BFM extracted from plasma with those of the injected standards.

The linearity of each standard curve was confirmed by plotting the ratio of drug to I.S. peak areas *versus* drug concentration. The standard curve was prepared and assayed daily with quality control samples and the unknown samples.

The specificity of the assay was checked by analysing blank samples and various patients' predose plasma samples.

TABLE I

INTER-DAY PRECISION AND RECOVERY FOR THE ANALYSIS OF BIFEMELANE IN PLASMA WITHIN THE STANDARD CURVE RANGE

Concentration (ng/ml)	C.V.[a] (%)	Recovery (%)
1.05	12.7	61
2.1	14.5	43
5.25	13.9	46
10.5	10.7	49
16.6	14.2	–
31.5	6.0	–
52.5	3.0	42
84	1.8	–
105	2.3	60
157.5	1.9	–
210	3.8	–

[a] Coefficients of variation of replicate analysis (n = 6).

RESULTS AND DISCUSSION

Initial studies at doses of 50–450 mg suggested that 24 h after oral administration of 50 mg of bifemelane–HCl to human subjects the plasma concentration would be in the 1–20 ng/ml range; thus, a reliable and efficient method to quantify bifemelane–HCl at these concentrations was required.

The mass spectrum of bifemelane (Fig. 2) showed two significant peaks: m/z 269 (molecular ion) and m/z 86 ($C_5H_{12}N^+$, N-alkyl chain) (analogues for I.S.: m/z 277 and m/z 94). In spite of the higher intensity of m/z 86 and m/z 94, we used m/z 269 (m/z 277 for I.S.) as detection ions, because of an interfering component when using the lower ions.

An extensive method validation required to support human pharmacokinetic studies was performed. The inter-day precision of the assay was less than 15% for all concentrations within the standard curve range (Table I). Recovery of the drug was 43–61% (I.S. 60–80%) and practically independent of concentration.

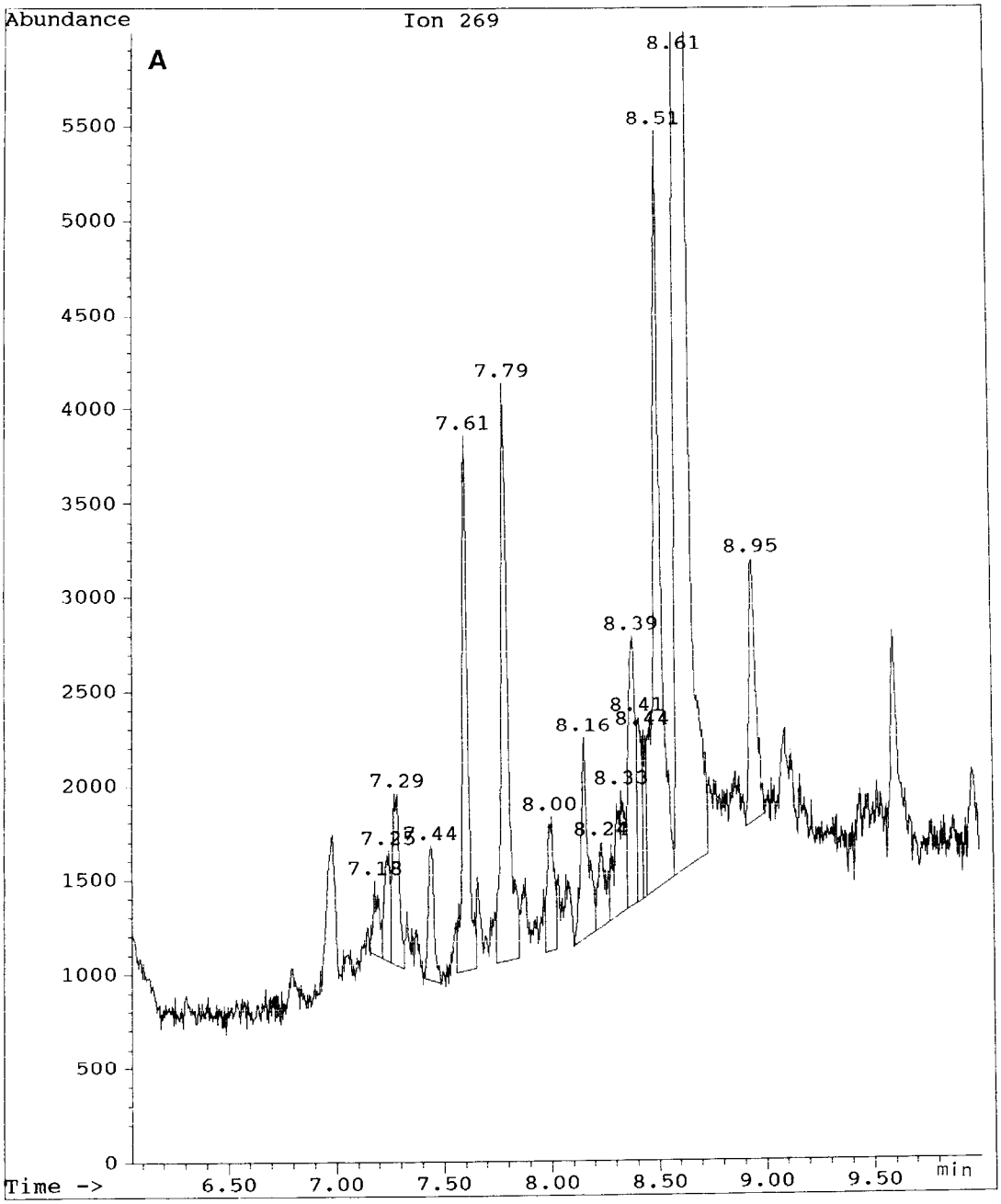
The inter-day variability of the assay was determined by analysing control samples in blank human plasma at six concentration within the standard calibration curve. The quality control samples were stored at −30°C under the same conditions as subject samples (Table II). The data in Table II indicate excellent inter-day assay precision and accuracy for all quality control concentrations. The precision of the method was also determined by multiple analyses (n = 4) of pooled plasma samples from patients ingesting

TABLE II

INTER-DAY VARIABILITY AND ACCURACY FOR THE ASSAY OF QUALITY CONTROL PLASMA SAMPLES SPIKED WITH BIFEMELANE–HCl

Spiked concentration (ng/ml)	n[a]	Found (ng/ml)	Accuracy (%)	C.V. (%)
4.2	7	4.29	113.2	9.8
12.6	7	13.2	113	6.2
23.8	7	23.9	101.3	7.4
33.6	6	32.9	97.9	1.2
100.8	6	101.0	100.2	6.3
168.0	6	166.7	99.2	6.7

[a] Assayed over a period of three days.


Abundance
Ion 269
A
8.61
8.51
7.79
7.61
8.95
8.39
8.41
8.44
8.16
7.29
8.33
8.00
8.24
7.44
7.18
5500
5000
4500
4000
3500
3000
2500
2000
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Time ->
6.50
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min

Fig. 3.

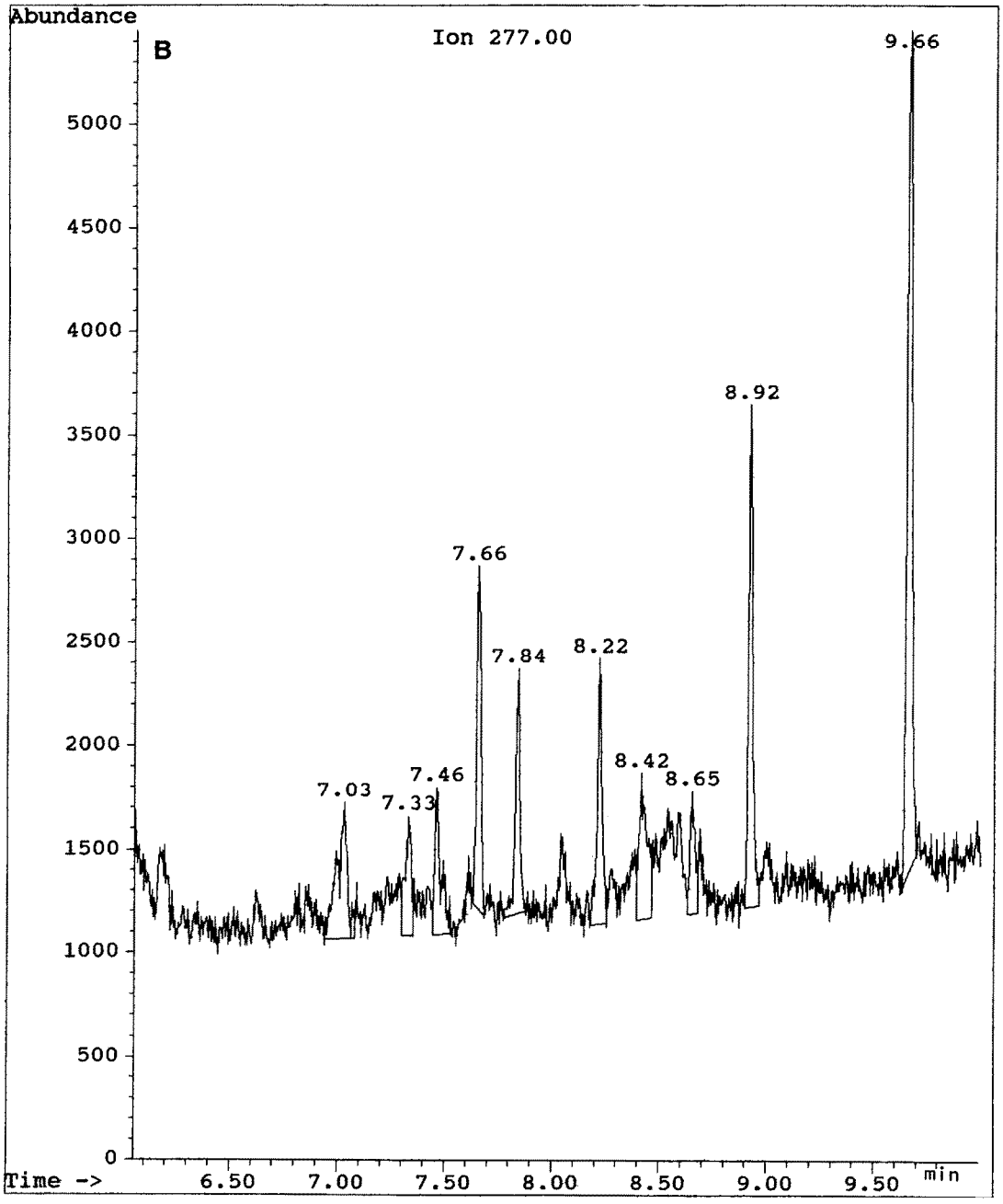

Fig. 3.

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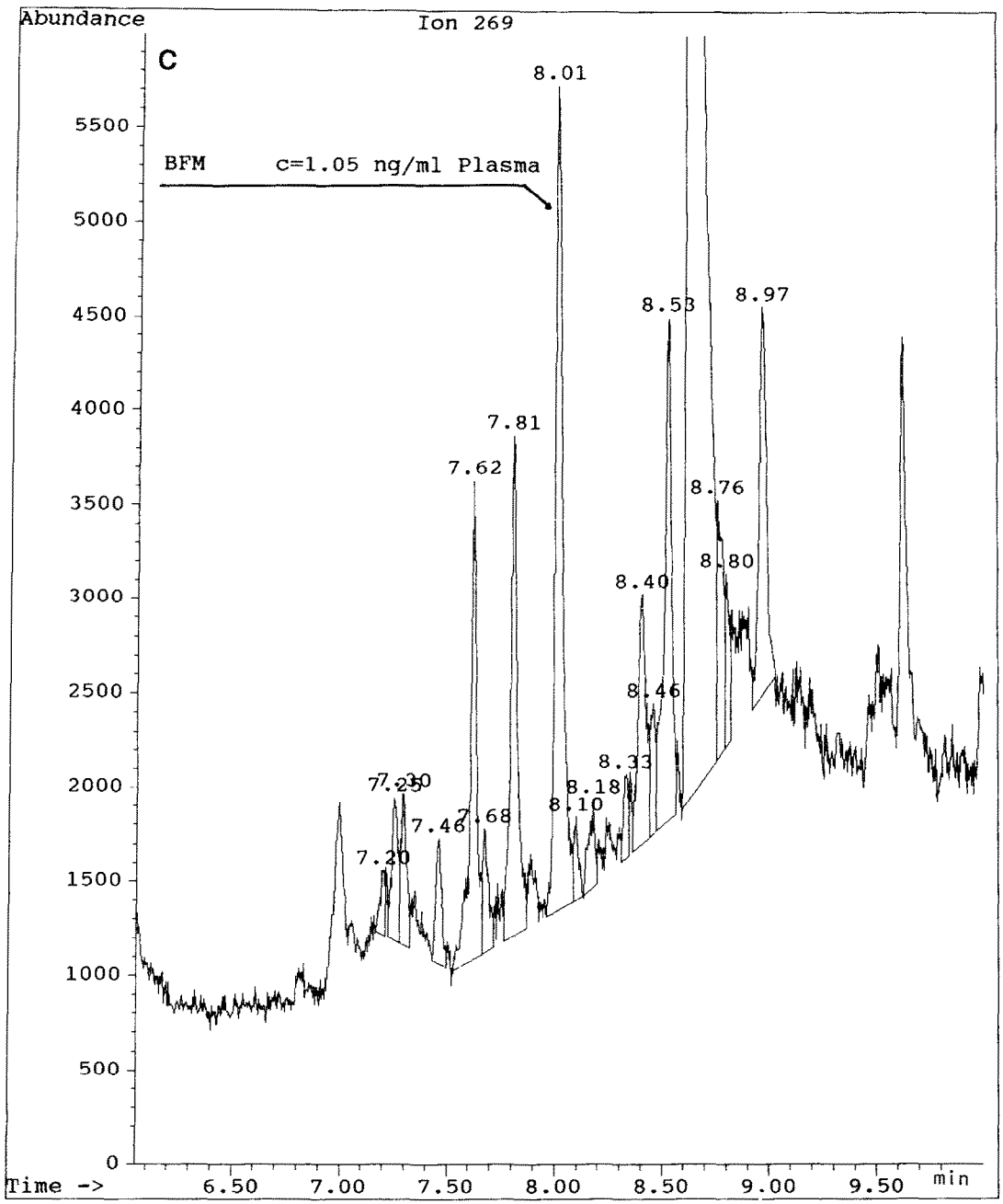

Fig. 3. Representative chromatograms of bifemelane in human plasma. (A) Predose plasma single-ion monitoring (SIM) *m/z* 269 (BFM). (B) Predose plasma SIM *m/z* 277 ([$^2H_8$]BFM). (C) Control plasma spiked with 1.05 ng/ml BFM.

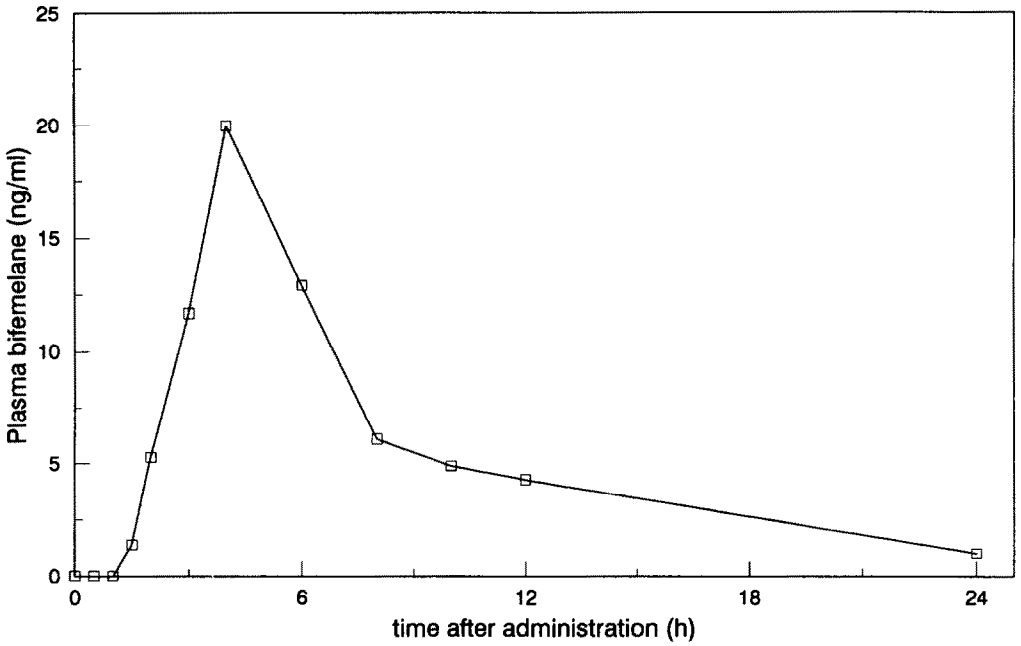

Fig. 4. Concentration of bifemelane in human subjects following a single 50-mg dose.

BFM–HCl. Both the quality control samples and genuine plasma samples exhibited a coefficient of variation of less than 10%.

The chromatograms of the predose samples of most volunteers showed a small peak (8.00 min) at the retenton time of BFM (8.01 min). We were not able to separate this peak, which is most probably caused by an endogenous compound. Calculated as BFM, this peak represents a concentration of 0.1 ng/ml. From this we have defined our LQ at a level of 1 ng/ml. Representative expanded chromatograms of the predose plasma and plasma spiked with 1.05 ng/ml bifemale–HCl are presented in Fig. 3.

The assay was utilized for the analysis of plasma samples from bioavailability and multiple-dose pharmacokinetic studies. Typical plasma concentration–time data for human subjects after a 50-mg single oral dose are presented in Fig. 4.

In conclusion, a simple and efficient method of assaying bifemelane on a single liquid extraction and an efficient GC separation has been developed. The method has been used routinely and has proved to be highly reliable and practical. More than 40 plasma samples per day have been assayed, with the lowest reliable limit of quantitation of 1 ng/ml. The method allows pharmacokinetic evaluation of the drug at doses down to 50 mg.

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