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Note

Determination of a new muscle relaxant (HY-770) in human serum by gas chromatography–mass spectrometry

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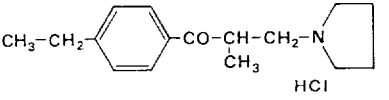
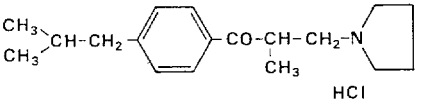
HY-770 [(±)-4′-ethyl-2-methyl-3-(1-pyrrolidinyl)propiophenone hydrochloride, I, Fig 1] is a newly synthesized central-acting muscle relaxant, which induces potent muscle relaxation and has relatively weak depressant action on the central nervous system (CNS) [1,2]

Studies on the disposition of I in rats indicated that the compound was well distributed into various tissues, including the CNS, and that the main metabolic pathway was oxidation of the 4′-ethyl side-chain of benzene ring and



I



Internal standard

Fig 1 Structures of I and the internal standard

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reduction of the ketone group [3] Because of the extensive metabolism of this compound, a sensitive and accurate analytical method is required for pharmacokinetic studies in human serum.

This paper describes a sensitive assay for determination of I in human serum by gas chromatography–mass spectrometry (GC–MS).

EXPERIMENTAL

Materials

Compound I and the internal standard (Fig. 1) were synthesized in our laboratory. Isobutane (purity > 99%), the reagent gas for GC–MS, was purchased from Seitetsu (Osaka, Japan). Other reagents were commercially available and of analytical-reagent grade.

Extraction procedure

To 5 ml of serum, 2.5 ml of internal standard solution (10 ng/ml in aqueous solution) and 5 ml of 0 5 *M* phosphate buffer (pH 7.0) were added. Samples were then extracted with 12.5 ml of *n*-hexane containing 3% 2-propanol in a reciprocating shaker for 5 min. After centrifugation (1500 *g* for 5 min), the organic layer was carefully transferred into another extraction tube and evaporated to dryness under reduced pressure. The aqueous layer was re-extracted with 12 5 ml of *n*-hexane containing 3% 2-propanol. The organic layer was transferred into the same extraction tube as the previous organic phase. Then 5 ml of 0.1 *M* hydrochloric acid were added to this tube, which was shaken for 5 min (back-extraction). The organic layer was discarded after centrifugation (1500 *g*, 5 min) The residual aqueous phase was adjusted to pH 7.0 with 0 5 ml of 1 0 *M* sodium hydroxide and 5 ml of 0.5 *M* phosphate buffer (pH 7.0)

Samples were then re-extracted twice with 12.5 ml of *n*-hexane containing 3% 2-propanol The combined organic layer was transferred to another tube and evaporated to dryness under reduced pressure. The residue was completely dissolved with 1 ml of methanol, which was evaporated again for the purpose of concentration. A 0.1-ml volume of methanol was added to the residue twice, and the resultant aliquot was then transferred to a 300-μl microvial After evaporation, the residue was finally dissolved in 20 μl of methanol, and a 1-μl aliquot was injected into the chromatograph.

Chromatographic conditions

GC–MS was carried out on a JMS DX-303 system, consisting of a GCG-06 gas chromatograph and DA5000 data system (JEOL, Tokyo, Japan). The GC column was a fused-silica DB-1 capillary (5 m × 0 53 mm I D., J & W Scientific, Rancho Cordova, CA, U.S A.). Helium was used as carrier gas of a flow-rate of 20 ml/min, and isobutane at a pressure of ca $1 \cdot 10^{-5}$ Torr was used for the chemical ionization (CI) reagent gas. The temperatures of the column oven,

injector port, separator and ion source were 155, 150, 300 and 200 °C, respectively. The ionization energy was 200 eV

Selected-ion monitoring (SIM)

Fig. 2 shows the CI mass spectra of I and the internal standard In both instances, the base peak ($MH^+ - 71$) was chosen for SIM (*m/z* 175 and 203). The peak-area ratios of I to the internal standard were used for further calculations.

Human studies

The described procedure was used in the quantitative assay of I in the serum of the healthy volunteers after oral administration of I at a dose of 100 mg (two

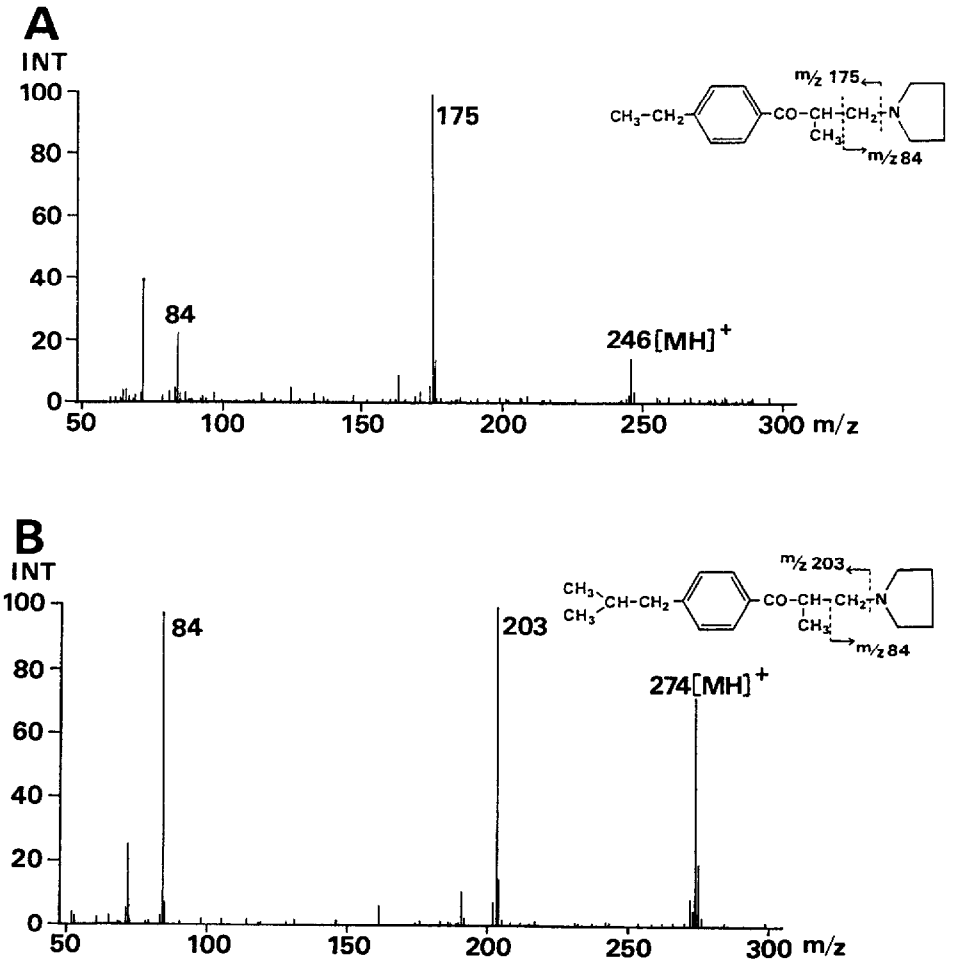


Fig 2 CI mass spectra of (A) I and (B) the internal standard

50-mg tablets). Blood samples were collected before and 0.5, 1, 2, 3, 4, 6 and 8 h after dosing. Serum samples were immediately frozen until analysis.

RESULTS AND DISCUSSION

Extraction and recovery

Because I in aqueous solution decomposes readily above pH 8.0 [4], it was extracted at pH 7 0 from serum samples The addition of 3% 2-propanol in the hexane prevented emulsion formation. The extraction recovery of I by *n*-hexane containing 3% 2-propanol from serum samples was ca. 70%, so serum samples were extracted twice to improve the recovery. Neutral interfering components were eliminated by back-extraction with 0 1 *M* hydrochloric acid. After two further extractions from 0.1 *M* hydrochloric acid solution to improve the overall recovery and the sensitivity, the extracts were concentrated by three steps and the residue was dissolved in 20 μl of methanol. The overall recovery of the assay procedure was $73.9 \pm 2.3\%$ for I and $83.8 \pm 1.6\%$ for the internal standard.

Interference studies

We have already identified six main metabolites (M2, M3, M4, M6, M8 and M9) of I [3]. The interference of the metabolites with the analysis of I was studied, and the metabolites were shown not to interfere. That is, M3, M4, M6 and M8 had no fragment peak at *m/z* 175 and 203 (Table I). Although both M2 and M9 had a fragment peak at *m/z* 175, they were not extracted and did not elute under the same chromatographic conditions, even if a small amount of M2 and M9 was extracted.

Fig. 3 shows the SIM chromatograms of blank serum, serum spiked with 5 ng/ml I and 5 ng/ml internal standard and a typical serum sample (1 h) from a healthy volunteer given 100 mg of I. No interfering peaks from endogenous compounds were observed when blank serum was assayed. The retention times of I and the internal standard were 2 and 4 min, respectively.

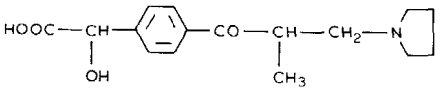
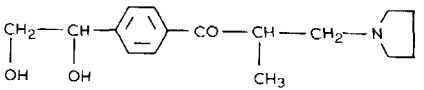
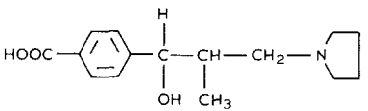
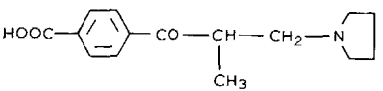
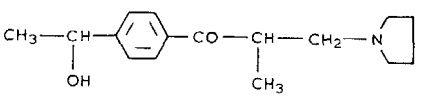
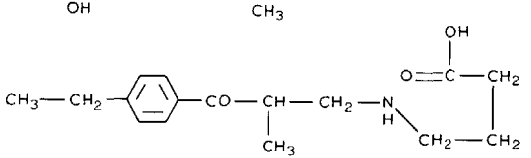
Chromatography and detection

Capillary GC using a bonded methylsilicone stationary phase was used to chromatograph I and the internal standard. Detection was done by CI mass fragmentation using isobutane as reagent gas. Selected-ion profiles were acquired for the base-peak fragments of I (*m/z* 175) and the internal standard (*m/z* 203), which originated from the pyrolysis products, 4′-ethyl-2-methylacrylophenone and 4′-isobutyl-2-methylacrylophenone, respectively

Initial experiments using conventional packed-column chromatography with a stationary phase (3% OV-225, Gasukuro Kogyo, Tokyo, Japan) were unsatisfactory owing to the thermal decomposition of I. A substantial decomposition

TABLE I

MASS SPECTRAL DATA OF METABOLITES OF I

Metabolite	Structure	MW	*m/z* of molecular and characteristic ion (relative intensity)
M2		291 35	221 (100 0), 203 (18 5), 175 (57 8), 72 (73 6)
M3		277 36	278 (MH^+, 59 6), 260 (27 6), 207 (58 4), 189 (24 5), 84 (100 0)
M4		263 34	264 (MH^+, 100 0), 84 (16 1)
M6		261 32	262 (MH^+, 11 5), 191 (100 0), 84 (10 3)
M8		261 36	262 (MH^+, 60 8), 244 (18 4), 191 (36 6), 173 (7 2), 84 (100 0)
M9		277 36	260 ($MH^+ - H_2O$, 66 9), 175 (100 0), 133 (41 3)

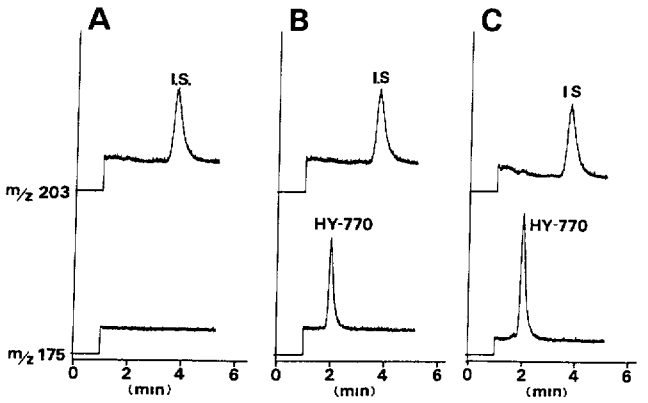


Fig 3 SIM chromatograms of serum extracts (A) drug-free serum, (B) serum to which known amounts of I (25 ng) and internal standard (25 ng) were added, (C) serum of healthy volunteer given 100 mg of I orally (subject 5, 1 h) Peaks I S = internal standard, HY-770 = compound I

TABLE II

REPRODUCIBILITY AND ACCURACY IN THE DETERMINATION OF I IN SPIKED HUMAN SERUM SAMPLES

Concentration added (ng/ml)	Concentration found (mean ± S D , $n=6$) (ng/ml)	Coefficient of variation (%)
0 2	0 191 ± 0 023	12 0
0 5	0 514 ± 0 023	4 5
2 0	2 006 ± 0 051	2 5
5 0	4 996 ± 0 169	3 4

product, 4′-ethyl-2-methylacrylophenone, was detected as an early eluting chromatographic peak when *m/z* 175 was monitored.

Sensitivity, accuracy and precision

The calibration curve was linear over the range 0.1–10 ng/ml, and the linear regression analysis yielded the equation $y=0.1161(\pm 0.0046$ S.D.$)x+0.0023$ (± 0.0087 S.D.) ($n=5$) The correlation coefficients were 0.9993–0.9999 The limit of detection was ca 0.1 ng/ml at a signal-to-noise ratio of 2, which corresponds to ca. 25 pg of I injected.

The accuracy and precision of the method were studied by analysing six identically spiked serum samples at concentrations of 0 2, 0.5, 2 and 5 ng/ml (Table II). The coefficient of variation (C.V.) was relatively high at 0 2 ng/ml, but satisfactory at other concentrations.

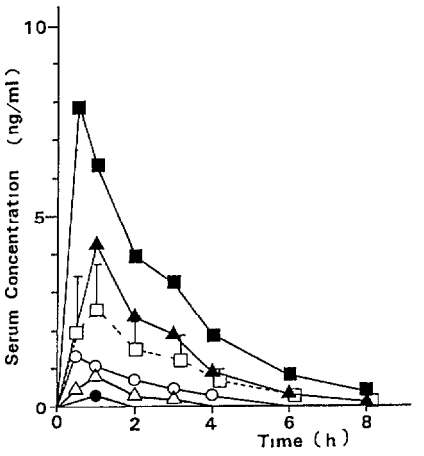


Fig 4 Time course of serum concentration in healthy volunteers receiving 100 mg of I (●) Subject 1, (○) subject 2, (▲) subject 3, (△) subject 4, (■) subject 5, (□) mean ± standard error of the mean

Human kinetics

The time course of the serum concentration of I in healthy volunteers following a single oral administration (100 mg) of I after overnight fasting is shown in Fig. 4 The average maximum serum level (ca 3 ng/ml) occurred 1 h after dosing, and the serum level declined with a half-life of ca. 1 5 h There were large inter-subject variations in the kinetic profiles, as are commonly seen with drugs that are extensively metabolized and undergo first-pass metabolism [5]. These results demonstrate that the analytical procedure described here has adequate selectivity and sensitivity for human kinetic studies.

Studies on the development of a method for the simultaneous quantitative analysis of the metabolites of I are in progress.

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