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SIMULTANEOUS DETERMINATION OF IMIPRAMINE, DESIPRAMINE AND THEIR DEUTERIUM-LABELLED ANALOGUES IN BIOLOGICAL FLUIDS BY CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A specific, sensitive and accurate method for the determination of imipramine, desipramine and their d_4 analogues in biological fluids using d_8 analogues as internal standards using capillary gas chromatography–chemical ionization mass spectrometry was developed. Drug concentrations were measured by selected-ion monitoring of the quasi-molecular ions of imipramine and the trifluoroacetyl derivative of desipramine. The coefficient of variation and relative error at a concentration of about 2 ng/ml in plasma were found less than 10% and 7%, respectively, for both drugs. No isotope effect was observed following the oral administration of an equimolar mixture of imipramine and [$^2H_4$]imipramine to a rat.

INTRODUCTION

Imipramine (IP) is a tricyclic antidepressant widely used for the treatment of depressive patients. Desipramine (DMI), an N-demethylated metabolite of IP, has approximately the same antidepressant activity as IP [1]. The structures of IP and DMI are shown in Fig. 1. The metabolic rates of tricyclic drugs vary widely, and considerable variations in their concentrations in plasma have been reported [2]. The ratio of IP to DMI at the steady state has been found to vary from 0.07 to 5.5 [3]. Therefore, IP and DMI must be determined simultaneously for the therapeutic monitoring of IP. To examine the pharmacokinetics of IP in detail, we intend in the future to determine separately DMI and [$^2H_4$]DMI derived from [$^2H_4$]IP following the co-administration of [$^2H_4$]IP and DMI to man.

Gram and Christiansen [4] reported the maximum plasma concentration of IP to be about 15–40 ng/ml and that of DMI about 10–30 ng/ml following oral

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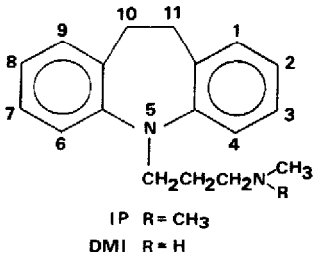

Fig. 1. Structures of IP and DMI.

administration of radioactive IP (35–50 mg) to man. A precise gas chromatographic–mass spectrometric (GC–MS) analysis is therefore required in order to detect a few ng/ml of IP and DMI in plasma. Plasma IP and DMI concentrations were determined simultaneously by GC–MS–selected-ion monitoring (SIM) using electron-impact ionization [5–9] or chemical ionization (CI) [10,11]. However, the application of these methods to our research would probably not provide satisfactory results because the accuracy and reproducibility at levels of a few ng/ml of the drug in the plasma in some instances have not been reported or the coefficient of variation at levels of a few ng/ml of the drug in plasma was about 20%.

Using conventional packed columns, Biggs et al. [7] reported that all columns and phases tested in their laboratory showed variable adsorption of IP. This adsorption is one of the reasons for the difficulty in the determination of trace amounts of IP.

In recent years, a number of capillary GC methods have been used for therapeutic drug monitoring. It might be thought that the adsorption of IP would be lower in a capillary column than in a packed column, as a capillary column is inactive. In particular, when a capillary column is inserted directly into the ion source, there are no active sites from the injection port to the ion source in this system. Although such columns should be excellent for the determination of IP or DMI by GC–MS, there are no reports to confirm this. Therefore, in this work, capillary GC–MS–SIM was used to determine IP, [$^2H_4$]IP, DMI and [$^2H_4$]DMI in biological fluids using d_8 analogues as internal standards.

This method was used to examine the isotope effects of [$^2H_4$]IP and [$^2H_4$]DMI following the oral administration of an equimolar mixture of IP and [$^2H_4$]IP to a rat.

EXPERIMENTAL

Chemicals and reagents

IP hydrochloride and DMI hydrochloride were obtained from Taiyo Yakuhin (Gifu, Japan) and Ciba-Geigy Japan (Hyogo, Japan), respectively, and recrystallized from acetone. Chlorpromazine hydrochloride was purchased from Wako (Osaka, Japan) and recrystallized from acetone. Extrelut was purchased from E. Merck (Darmstadt, F.R.G.). All other chemicals and solvents were of analytical-

reagent grade and used without further purification. [Methylene-^{14}C]-IP ([^{14}C]IP) hydrochloride ($1.78 \cdot 10^9$ Bq/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). [Methylene-^{14}C]-DMI ([^{14}C]DMI) hydrochloride ($1.64 \cdot 10^8$ Bq/mmol) was kindly supplied by Ciba-Geigy (Basle, Switzerland).

[1,3,7,9-$^2H_4$]IP hydrochloride (d_3, 3.2%; d_4, 91.2%; d_5, 5.4%; d_6, 0.2%), [1,2,3,4,6,7,8,9-$^2H_8$]IP hydrochloride (d_7, 7.5%; d_8, 88.8%; d_9, 2.8%; d_{10}, 0.8%) [12], [1,3,7,9-$^2H_4$]DMI hydrochloride (d_2, 1.0%; d_3, 2.5%; d_4, 95.8%; d_5, 0.5%; d_6, 0.1%) and [1,2,3,4,6,7,8,9-$^2H_8$]DMI hydrochloride (d_7, 3.9%; d_8, 90.6%; d_9, 5.6%; d_{10}, 0.8%) [13] were synthesized in our laboratory.

Stock solutions

Stock solutions of IP hydrochloride (6.030 mg per 250 ml), [$^2H_4$]IP hydrochloride (5.929 mg per 250 ml), [$^2H_8$]IP hydrochloride (5.412 mg per 250 ml), DMI hydrochloride (5.894 mg per 250 ml), [$^2H_4$]DMI hydrochloride (5.692 mg per 250 ml) and [$^2H_8$]DMI hydrochloride (5.675 mg per 250 ml) were prepared in ethanol. Storage of these solutions at 4°C did not result in any detectable decomposition for more than 6 months. All analyses were performed after diluting the stock solutions with ethanol.

Radio-gas chromatography

The Shimadzu (Kyoto, Japan) GC-6AM gas chromatograph equipped with a synchronized accumulating radioisotope detector having five counting tubes (tube volume 30 ml) [14] was used. GC was carried out under the following conditions: column, 1.5% OV-17 (0.5 m×3 mm I.D.); injection temperature, 270°C; column oven temperature, 220°C; carrier gas (nitrogen) and counting gas (methane) flow-rates, 50 and 250 ml/min, respectively.

Capillary gas chromatography–mass spectrometry–selected-ion monitoring

Capillary GC–MS–SIM measurements were made with a Shimadzu GCMS-QP1000 gas chromatograph–mass spectrometer equipped with a capillary column split/splitless injection system (SPL-G9, Shimadzu). The end of the capillary column was inserted directly into the ion source. SIM was recorded in the chemical ionization mode with isobutane as the reactant at a pressure of $6.65 \cdot 10^{-3}$ Pa. The ionization voltage and ionization current were 200 eV and 150 μA, respectively, and the ion source temperature was 260°C. The operating parameters for the mass spectrometer were adjusted by an autotune programme obtained from Shimadzu.

GC was performed using a 25 m×0.33 mm I.D. fused-silica capillary column with a 0.5-μm film (CBP1-S25-050, Shimadzu). The operating conditions were as follows: injection temperature, 270°C; column oven temperature programme, from 80°C at 30°C/min to 240°C, where it was maintained for 7 min; carrier gas, helium at a flow-rate of ca. 3.0 ml/min; column pressure, $4.90 \cdot 10^4$ Pa with a split vent flow-rate of 30 ml/min and a septum purge vent flow-rate of 1.0 ml/min. The helium flows through the septum and split vents were stopped prior to injection; the valves were opened 1 min after injection.

Derivatization of DMI with N-trifluoroacetylimidazole

After adding 2 μl of N-trifluoroacetylimidazole (TFAI) to 65 Bq of [^{14}C]DMI in 100 μl of *n*-hexane, each such solution was allowed to stand at 4°C, room temperature (23°C) or 40°C for 0.25, 0.5 or 1.0 h. All the solutions were evaporated to dryness under a stream of nitrogen. To each residue were added 20 μl of ethanol and 2 μl of each solution thus obtained were injected into the radio-GC system.

Sample preparation for capillary GC–MS–SIM

Frozen plasma samples were thawed rapidly in a water-bath at about 35°C and 2.0 ml of plasma were added to 19.22 ng of [$^{2}H_{8}$]IP and 20.10 ng of [$^{2}H_{8}$]DMI in a PTFE-lined screw-capped culture tube (100×16 mm O.D.). This plasma sample mixture was allowed to stand for 30 min at room temperature and, following the addition of 2 ml of distilled water and three drops of 3 *M* sodium hydroxide solution, the plasma sample was introduced into a disposable plastic syringe (10 ml; Terumo, Tokyo, Japan) packed with Extrelut (2.4 g) and eluted with 20 ml of ethyl acetate. The extract in a PTFE-lined screw-capped conical centrifuge tube (105×28 mm O.D.) was back-extracted three times with 2 ml of 0.01 *M* hydrochloric acid by vortex mixing for 1 min. After centrifugation for 5 min at 1000 *g*, the aqueous layer was pipetted into a PTFE-lined screw-capped culture tube (100×16 mm O.D.) followed by the addition of 500 μl of 1.5 *M* sodium carbonate solution and extraction twice with 2 ml of *n*-hexane by vortex-mixing for 1 min. After centrifugation for 5 min at 1000 *g*, the organic layer was pipetted into a PTFE-lined screw-capped conical centrifuge tube (105×16.5 mm O.D.). The solvent was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 100 μl of *n*-hexane and treated with 2 μl of TFAI, with which it was allowed to react for 30 min at room temperature. The reaction mixture was evaporated to dryness under a stream of nitrogen and the residue dissolved in 100 μl of 0.1 *M* sodium carbonate and extracted with *n*-hexane (2×200 μl) containing chlorpromazine (10 μg/ml) by vortex mixing for 1 min. After centrifugation for 5 min at 1000 *g*, the *n*-hexane layer was pipetted into a PTFE-lined screw-capped conical centrifuge tube (105×16.5 mm O.D.). The solvent was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 20 μl of *n*-hexane. Following centrifugation at 0°C for 5 min at 1000 *g*, a 3–4-μl aliquot of the solution was injected into the GC–MS system.

Drug measurement

A series of standard solutions, each containing 20–1000 ng of IP and [$^{2}H_{4}$]IP, 5–1000 ng of DMI and [$^{2}H_{4}$]DMI, 192.2 ng of [$^{2}H_{8}$]IP and 201.0 ng of [$^{2}H_{8}$]DMI, were all made up to 100 μl with *n*-hexane. After derivatization and subsequent extraction, the mixture was made up to 200 μl with *n*-hexane. Calibration graphs were prepared by injecting 2-μl aliquots of these solutions into a capillary GC–MS system focused on *m/z* 281, 285, 289, 363, 367 and 371. The peak-area ratios of *m/z* 281 versus *m/z* 289 (IP/[$^{2}H_{8}$]IP), *m/z* 285 versus *m/z* 289 ([$^{2}H_{4}$]IP/[$^{2}H_{8}$]IP), *m/z* 363 versus *m/z* 371 (DMI–trifluoroacetyl (TFA)/[$^{2}H_{8}$]DMI–TFA) and *m/z* 367 versus *m/z* 371 ([$^{2}H_{4}$]DMI–TFA/[$^{2}H_{8}$]DMI–TFA) were de-

TABLE I

MUTUAL CONTRIBUTIONS TO ION INTENSITY OF VARIOUS SPECIES IN THE CHANNELS MONITORED

Compound	*m/z* 281	*m/z* 285	*m/z* 289	Compound	*m/z* 363	*m/z* 367	*m/z* 371
IP	100	0.20	0.03	DMI	100	0.16	0.02
$[^2H_4]$IP	0.27	100	0.35	$[^2H_4]$DMI	0.16	100	0.22
$[^2H_8]$IP	0.09	0.43	100	$[^2H_8]$DMI	0.03	0.29	100

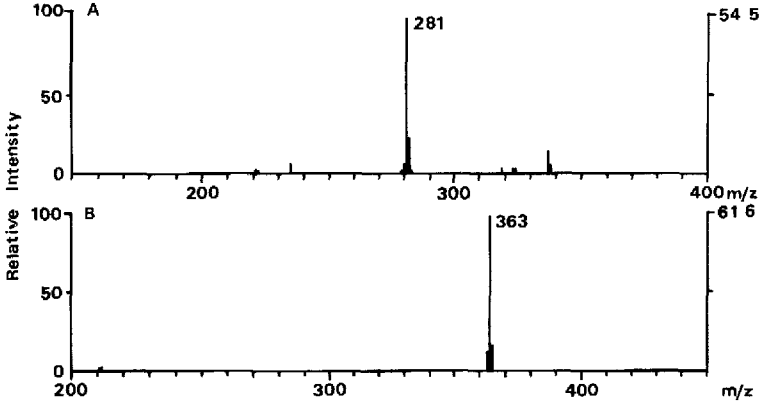

Fig. 2. Partial chemical ionization mass spectra of (A) IP and (B) DMI–TFA.

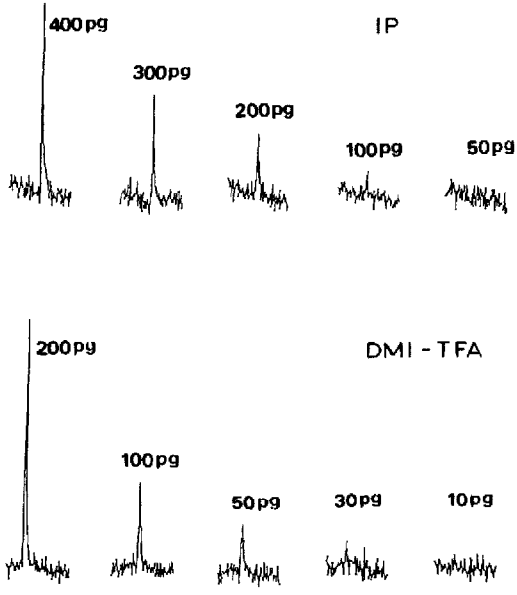

Fig. 3. Detection limits of IP and DMI–TFA.

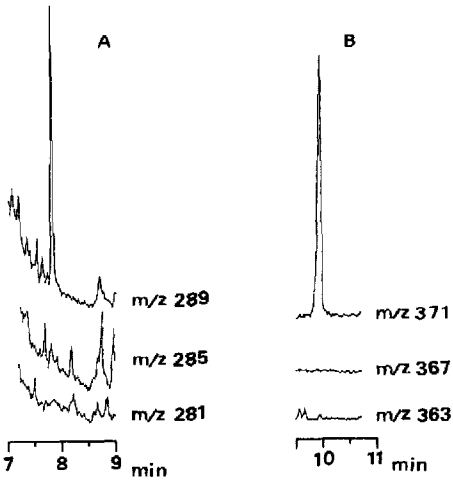

Fig. 4. Selected-ion monitoring profiles of extracts from human plasma sample (2 ml) spiked with [$^2H_8$]IP (A, 19.2 ng) and [$^2H_8$]DMI (B, 20.1 ng).

termined. The calibration graphs were obtained by weighted linear least-squares fitting of peak-area ratios versus the mixed molar ratios for each analysis of a standard mixture. Plasma concentrations were calculated by comparing peak-area ratios for unknown samples with those for the standard mixtures.

Drug administration

An equimolar mixture of IP hydrochloride and [$^2H_4$]IP hydrochloride (5 mg of each) was administered orally to a male Sprague–Dawley rat (290.0 g). Plasma was taken at 0.7, 1.1, 2.2, 3.1, 5.9, 12, 24 and 48 h following administration and 200 μl of the plasma were used for the analysis mentioned above.

RESULTS AND DISCUSSION

IP can be analysed by GC without derivatization, as it possesses no polar functional group. Although DMI can be analysed by GC without derivatization, it was derivatized to the TFA derivative so as to minimize the adsorption of DMI on the GC column. The trifluoroacetylation of DMI with TFAI in *n*-hexane was examined in detail by radio-GC using [^{14}C]DMI. GC peak yields [15] of [^{14}C]DMI–TFA exceeded 90% under the conditions described in Experimental.

This derivatization procedure caused no detectable loss of deuterium atoms from either [$^2H_8$]IP or [$^2H_8$]DMI. [$^2H_8$]IP and [$^2H_8$]DMI were labelled at all aromatic positions and [$^2H_4$]IP and [$^2H_4$]DMI at the more chemically stable 1-, 3-, 7- and 9-positions. Consequently, no deuterium elimination occurred from any of the labelled IP or DMI during derivatization.

The CI mass spectra (isobutane) of IP and DMI–TFA are shown in Fig. 2A and B, respectively. The peaks at *m/z* 281 (Fig. 2A) and *m/z* 363 (Fig. 2B) represent quasi-molecular ions of IP and DMI–TFA. These ions were chosen for SIM, in view of their high intensity. The peak at *m/z* 336 (Fig. 2A) resulted from the addition of isobutane to IP.

The mutual contributions of the monitoring ions of IP, DMI and their deuter-

TABLE II

ACCURACY OF SELECTED-ION MONITORING OF IP AND [$^2H_4$]IP IN HUMAN PLASMA

Compound	Added (ng/ml)	Found (ng/ml)				C.V. (%)	Relative error (%)
		Individual values			Mean ± S.D.		
IP	2.13	2.21,	2.31,	2.28	2.27 ± 0.05	2.3	6.6
	10.67	11.34,	10.35,	10.30	10.67 ± 0.59	5.5	0.0
	42.68	45.60,	41.77,	43.13	43.50 ± 1.94	4.5	1.9
[$^2H_4$]IP	2.10	1.90,	2.02,	1.96	1.96 ± 0.06	3.1	−6.7
	10.51	10.71,	10.43,	11.35	10.83 ± 0.47	4.4	3.0
	42.04	43.34,	43.26,	44.14	43.58 ± 0.49	1.1	3.7

TABLE III

ACCURACY OF SELECTED-ION MONITORING OF DMI AND [$^2H_4$]DMI IN HUMAN PLASMA

Compound	Added (ng/ml)	Found (ng/ml)				C.V. (%)	Relative error (%)
		Individual values			Mean ± S.D.		
DMI	2.00	2.16	1.94	2.12	2.07 ± 0.12	5.7	3.8
	9.99	9.89	9.57	9.13	9.53 ± 0.38	4.0	−4.6
	39.94	41.42	41.47	41.95	41.61 ± 0.29	0.7	4.2
[$^2H_4$]DMI	2.08	2.36	1.99	1.97	2.11 ± 0.22	10.4	1.6
	10.39	10.76	10.57	9.99	10.44 ± 0.40	3.8	0.5
	41.54	43.36	43 22	43.45	43.35 ± 0.12	0.3	4.4

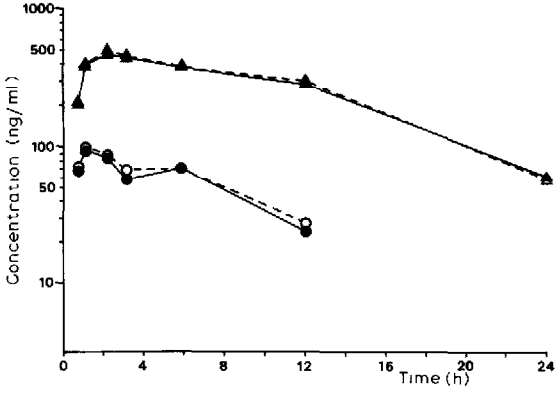

Fig. 5. Plasma concentration versus time curves for IP (●—●), [$^2H_4$]IP (○--○), DMI (▲—▲) and [$^2H_4$]DMI (△--△) following the oral administration of an equimolar mixture of IP hydrochloride and [$^2H_4$]IP hydrochloride (5 mg of each) to a male rat (290.0 g).

ated analogues are shown in Table I. The maximum contributions were from the d_8 analogues, i.e., the internal standards, to d_4 for both IP and DMI–TFA. However, when the [$^2H_8$]IP concentration was ten times that of [$^2H_4$]IP, the contribution of the d_4 species involved in [$^2H_8$]IP to the monitoring ion of [$^2H_4$]IP could be expressed within a few percent. This was also found for the contribution of [$^2H_8$]DMI to the monitoring ion of [$^2H_4$]DMI. This was the outstanding advantage of using labelled IP and DMI of high isotopic purity and separated from each other by 4 daltons.

The limit of detection of the procedure was evaluated on the basis of the signal-to-noise ratio (S/N). The lower limits of detection of GC–MS–SIM were 100 and 50 pg for IP and DMI–TFA, respectively, as shown in Fig. 3.

Although a number of organic solvents have been reported for the extraction of IP and DMI from plasma, this extraction is occasionally accompanied by emulsion formation. Recently, certain disposable clean-up methods have been developed for the separation of drugs from biological fluids. We used Extrelut to extract IP and DMI from plasma. The recoveries of IP and DMI from plasma were calculated using the ^{14}C analogues to be $60.8 \pm 6.0\%$ ($n=7$) and $64.6 \pm 9.2\%$ ($n=7$), respectively.

As amines such as IP and DMI are adsorbed on a glass surface, care must be taken to prevent this when analysing trace amounts of IP and DMI. When conducting GC–MS analyses, in some instances IP could not be detected. Adsorption could not be prevented even by silylating the glassware. No adsorption was encountered on extracting IP from plasma using [^{14}C]IP as mentioned above. Thus, chlorpromazine was added as a carrier after the derivatization and, consequently, adsorption was greatly depressed. As the adsorption of IP was not reproducible, it was difficult to determine where, when and how it was adsorbed. However, improvement of the GC–MS analysis described here was possible at least by the so-called "carrier effect" with the analogue chlorpromazine.

Fig. 4 shows the SIM for blank plasma containing 9.61 ng/ml of [$^2H_8$]IP and 10.05 ng/ml of [$^2H_8$]DMI as internal standards. The retention times of [$^2H_8$]IP and [$^2H_8$]DMI–TFA were 7.8 and 9.8 min, respectively. No interference due to contributions from other materials in the plasma appeared at the same retention times as those of IP and DMI–TFA. The retention time of chlorpromazine was 11.0 min and chlorpromazine did not interfere with IP or DMI–TFA.

Calibration graphs obtained by GC–MS–SIM showed good linearity between the peak-area ratio and molar ratio in the range 0.2–10:1 IP or [$^2H_4$]IP to [$^2H_8$]IP and 0.05–10:1 DMI or [$^2H_4$]DMI to [$^2H_8$]DMI.

The accuracy of the measurements was evaluated for IP, [$^2H_4$]IP, DMI and [$^2H_4$]DMI after their addition to 2.0-ml aliquots of blank human plasma. Plasma samples spiked with 4–80 ng of IP, [$^2H_4$]IP, DMI or [$^2H_4$]DMI were analysed by the present method. The results are presented in Tables II and III. The determined amounts of IP, [$^2H_4$]IP, DMI and [$^2H_4$]DMI are in good agreement with the actual amounts added.

In the case of co-administration using isotopically labelled compounds, it must be confirmed whether those compounds actually exert an isotope effect. An equimolar mixture of IP and [$^2H_4$]IP (5 mg of each) was co-administered orally to

a rat followed by determination of the plasma concentrations of IP, [$^2H_4$]IP, DMI and [$^2H_4$]DMI by the present procedure. Fig. 5 shows the curves of plasma concentration versus time. No appreciable differences between the plasma concentrations of d_0 and d_4 analogues were observed.

The present SIM method is a specific, sensitive and accurate means for determining IP, [$^2H_4$]IP, DMI and [$^2H_4$]DMI simultaneously. Consequently, it is possible to examine the pharmacokinetics of IP and DMI following the co-administration of [$^2H_4$]IP and DMI.

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