BIOLOGICAL MASS SPECTROMETRY, VOL. 20, 382–388 (1991)

Identification and Determination of Propafenone and its Principal Metabolites in Human Urine Using Capillary Gas Chromatography/Mass Spectrometry

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The capillary gas chromatography/mass spectrometry of trimethylsilyl-trifluoroacetyl, trifluoroacetyl and pentafluoropropionyl (PFP) derivatives of the antiarrhythmic agent propafenone (Rytmonorm®), as well as its main metabolites *N*-despropyl-propafenone and 5-hydroxy-propafenone, have been investigated. Both electron impact and positive isobutane chemical ionization mass spectrometry using the Ion Trap Detector® have been evaluated. The presence of propafenone and its co-extracted metabolites in human urine at time intervals after the oral administration of 150 mg Rytmonorm® to healthy volunteers was established, and the urinary excretion of propafenone and 5-hydroxy-propafenone was calculated using selective chemical ionization mass spectrometric detection. Only a few per cent of the dose was excreted unchanged in the urine. Large intersubject variabilities had been observed also. The large dynamic range of the Ion Trap Detector® and the high correlation coefficients (0.92–0.99) of the calibration curves were striking.

INTRODUCTION

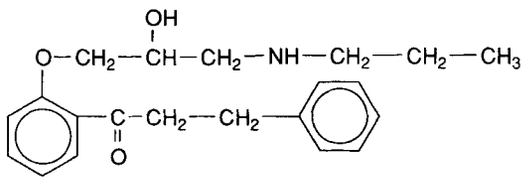
Propafenone (Rytmonorm®) is primarily a class 1 antiarrhythmic agent of group 1c, with substantial β-adrenoceptor blocking activity at therapeutical doses. Its potency as a calcium antagonist is relatively weak.

Propafenone undergoes extensive hepatic metabolism in man, via hydroxylation and conjugation. Excretion of metabolites is mainly via the faeces (53% within 48 h). Eleven metabolites are known, accounting for 90% of the administered dose, and the major ones are conjugates of *N*-despropyl-propafenone and 5-hydroxy-propafenone, the latter being pharmacologically active.[1]

The chemical structure of propafenone is common to β-adrenoceptor blocking agents (Fig. 1). Therefore, capillary gas chromatography/mass spectrometry (GC/MS) methodology as developed for the analysis of β-adrenoceptor blocking agents in biological specimens may be recommended for the identification and determination of propafenone as well as for its principal metabolites in biological specimens, e.g. urine. In order to protect the bifunctional polar groups of the aminopropanol side-chain of β-adrenoceptor blocking agents prior to GC analysis, several derivatization procedures, e.g. using *N*-methyl-*N*-trimethylsilyltrifluoroacetamide/*N*-methyl-bistrifluororacetamide (MSTFA/MBTFA), trifluoroacetic anhydride (TFAA), *n*-butylboronic acid and pentafluor(*o*)propionic anhydride (PFPA) have been described.[2–4] Therefore, we first compared and evaluated the trimethylsilyl/trifluoroacetic acid (TMS/TFA), trifluoroacetic (TFA) and pentafluoropropionic (PFP) derivatives of propafenone, 5-hydroxy-propafenone and *N*-despropyl-propafenone with respect to their GC properties and mass fragmentation using both electron impact (EI) and isobutane positive chemical ionization (CI) mass spectrometry.

Second, the presence of propafenone and its metabolites in human urine at time intervals after the oral administration of 150 mg Rytmonorm® to healthy volunteers was established. Also, the urinary excretion in man of propafenone and its main metabolites, e.g. 5-hydroxy-propafenone and *N*-despropyl-propafenone, has been investigated using selective mass spectrometric detection.

Structure of propafenone



General structure of betablockers

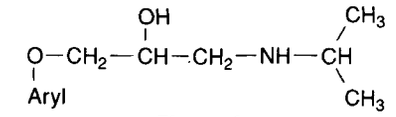


Figure 1

EXPERIMENTAL

Reagents

Methanol (Baker grade) and acetonitrile (high-performance liquid chromatography (HPLC) grade) were

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1052–9306/91/060382–07 $05.00

Received 3 April 1990
Revised 10 September 1990

purchased from J. T. Baker Chemicals BV (Deventer, The Netherlands). Ethyl acetate (Uvasol®) was purchased from Merck (Darmstadt, Germany). All other routine chemicals were of analytical grade from Merck. MSTFA and MBTFA were purchased from Macherey, Nagel & Co. (Düren, Germany), and TFAA and PFPA from Pierce Chemical Co., Rockford, Illinois, USA. Propafenone-HCl, 5-hydroxy-propafenone-HCl and *N*-despropyl-propafenone fumarate were kindly supplied by Knoll AG, Ludwigshafen, Germany. Bufuralol-HCl was kindly supplied by Hoffmann La Roche, Mijdrecht, The Netherlands. Boric acid–borax buffer (pH 9.0) was prepared by mixing 18.5 ml of 0.2 M boric acid solution with 81.5 ml of 0.05 M borax solution. Acidified methanol was prepared by mixing 300 mg of pure acetic acid with 50 ml of methanol.

Standards

Separate stock solutions of the respective compounds were prepared in methanol at a concentration of either 0.2 g l^{-1} (propafenone and bufuralol) or 0.08 g l^{-1} (5-hydroxy-propafenone and *N*-despropyl-propafenone), and stored at 4 °C for not longer than two months.

Equipment

Bond-Elut® C-2 solid-phase columns were purchased from Analytichem (Harbor City, California, USA). The vacuum manifold was purchased from J. T. Baker Chemicals BV, Deventer, The Netherlands. The derivatized samples were analysed using a Finnigan MAT Ion Trap Detector® with software revision 3.0. The AGC programme with variable ionization time was used. The ITD 800 was scanned from mass 50 u to 500 u in 1 s, using its EI mode. CI spectra were obtained using isobutane as the reaction gas and the AGC turned off. CI mass spectra were provided, scanning from mass 70 u to 650 u in 1 s. Quantitative experiments, using the CI mode from the ion trap detector were done, scanning from mass 200 u to mass 550 u in 1 s.

The gas chromatograph used was a Carlo Erba HRGC 5160 MEGA with on-column injector. The capillary column used was a J&W Durabond 1 (30 m × 0.25 mm) column with a film thickness of 25 µm. The temperature programme was as follows: 1 min at 90 °C, then a ramp of 40 °C min^{-1} to 260 °C, followed by an isothermal period of 20 min at 260 °C. The injected sample volume was 1 µl. Only disposable glassware was used.

Hydrolysis

A 10 µl volume of a β-glucuronidase–arylsulphatase mixture from *Helix pomatia* (Serva, Heidelberg, Germany) was added to 1.00 ml of urine and the respective urine samples were incubated at 37 °C overnight.

Extraction

The Bond-Elut® columns were positioned in the respective luer fittings of the vacuum manifold. A vacuum of 10–20 cmHg was applied. The columns were conditioned by eluting twice with 1 ml of methanol, once with 1 ml of distilled water and once with 1 ml of buffer (pH 9.0). The columns were prevented from running dry. An aliquot of 2 ml of urine or hydrolysed urine, containing the internal standard bufuralol, was added to each column and gently sucked through. The columns were washed twice with 1 ml of distilled water and once with 500 µl of acetonitrile. Elution of the analytes was performed with 500 µl of acidified methanol solution. The eluates were evaporated to dryness under a gentle stream of nitrogen at 55–60 °C.

Derivatization procedures

MSTFA/MBTFA. A 100 µl volume of MSTFA was added to the dried residue and vortexed for 5 s and the tube was heated at 60 °C for 5 min. Subsequently, 30 µl of MBTFA were added and the tube was again heated at 60 °C for 5 min. A 1 µl volume of the cooled mixture was injected into the gas chromatograph.

TFAA. A 50 µl volume of TFAA–ethyl acetate (2:1, v/v) was added to the dried residue and vortexed for 5 s and the tube was heated at 60 °C for 40 min. Subsequently, the mixture was evaporated to dryness under a stream of nitrogen at room temperature and the residue dissolved in 50 µl of ethyl acetate. After vortexing for 5 s, 1 µl of this mixture was injected into the gas chromatograph.

PFPA. The dried residue was dissolved in 500 µl of *n*-hexane and treated consecutively with 50 µl of PFPA and 25 µl of pyridine at 70 °C for 15 min. After washing with 1 ml of 0.1 M sodium borate solution, the mixture was evaporated to dryness under a gentle stream of nitrogen and the residue dissolved in 50 µl of *n*-hexane. After vortexing for 5 s, 1 µl of this mixture was injected into the gas chromatograph.

Precision, calibration curves and recovery

All quantitative experiments were performed using the CI mode of the ion trap detector and isobutane as the reaction gas.

The precision of relative abundance measurements in full mass range scans was demonstrated by an evaluation of the relationship between the peak areas of the respective m/z 334 (base peak) and m/z = 200–500 (full range) masses of the internal standard after consecutive GC injections by the same operator on the same day. Spectra had been taken from the apex of the gas chromatographic peak.

Calibration curves (at least five different concentrations) were prepared from the TMS/TFA derivatives of both the reference compounds, and from urinary extracts containing known amounts of propafenone, 5-hydroxy-propafenone and *N*-despropyl-propafenone. The peak areas of each compound (e.g. propafenone and 5-hydroxy-propafenone m/z = 284 u, *N*-despropyl-propafenone m/z = 240–470 u) were divided by those from the internal standard (bufuralol, m/z = 200–500 u), and plotted versus the amount of compound. Afterwards, linear regression analysis was performed. Other

calibration methods, e.g. involving analysis of peak heights, were evaluated also.

The recovery was obtained as follows: 1 ml of blank human urine was spiked with 50 µl of a stock solution containing propafenone or its principal metabolites, and extracted, derivatized and analysed subsequently. The internal standard bufuralol was added prior to the derivatization. The concentration of each compound was calculated by dividing its peak area by those from the internal standard, followed by an extrapolation from a calibration curve of the respective reference compounds. These experiments were performed in triplicate.

Human sampling

Young, healthy volunteers ($n = 5$) ingested one tablet of 150 mg Rytmonorm®. Their urine was collected at time intervals up to 48 h post-dose ($n = 2$) or up to 11–32 h post-dose ($n = 3$), and stored in the dark at −4 °C prior to analysis. Unhydrolysed or hydrolysed urine (1–2 ml) was extracted in duplicate, derivatized and analysed using capillary CI GC/MS according to the procedures described above. All samples had been derivatized using MSTFA/MBTFA, and a few samples had been derivatized using TFAA and PFPA.

In three subjects, the concentrations of propafenone (or 5-hydroxy-propafenone) were calculated by adding the areas under the curves, at $m/z = 284$, of all TMS/TFA derivatives of the drug (or its metabolite), and dividing these by those of the internal standard bufuralol, followed by an extrapolation from a calibration curve that had been calculated similarly. The correlation coefficient of the calibration curve of the added TMS/TFA derivatives of propafenone ($Y = 0.019 + 0.007X$) was 0.9848, and the correlation coefficient of the calibration curve of the added TMS/TFA derivatives of 5-hydroxy-propafenone ($Y = -0.091 + 0.004X$) was 0.9574. The relative error between duplicate determinations were 14.6 ± 13.7% for propafenone and 13.7 ± 8.8% for 5-hydroxy-propafenone (mean ± standard deviation) respectively. The renal excretion rate and the total amount excreted at time intervals up to 11–32 h post-dose was calculated using a FarmFit program.

RESULTS AND DISCUSSION

Mass spectrometry

In all mass spectra of propafenone and 5-hydroxy-propafenone (Table 1), typical stable and abundant fragments indicating the intact propyl-aminopropanol side-chain (e.g. $m/z = 284$: TMS/TFA derivative; $m/z = 308$: TFA derivative; $m/z = 408$: PFP derivative) were present. In the EI mass spectra, abundant ions at low mass represent the fragmentary degradation of this side-chain. The isobutane CI mass spectra are simple, specific and complementary to their corresponding EI mass spectra, due to the presence of characteristic fragments at high mass. These fragmentation patterns are quite similar to those reported for β-adrenergic blocking agents.[4–7]

In the isobutane CI mass spectra of the respective TMS/TFA and TFA derivative of *N*-despropyl-propafenone, the abundances of both the [M + 1] ion and the fragment which indicates the aminopropanol side-chain ($m/z = 242$ and $m/z = 266$ u) are high.

Table 1. Mass spectral data of propafenone, 5-hydroxy-propafenone and *N*-desisopropyl-propafenone

1. EI mass spectra

Compound	Derivative	EI mass spectra
Propafenone	TMS/TFA	284 (45), 270 (15), 466 (5), 223 (9), 194 (13), 103 (64), 170 (9), 147 (7), 129 (27), 91 (63), 73 (100)
	TFA	308 (100), 266 (24), 152 (14), 126 (10), 91 (25), 77 (11)
	PFP	408 (100), 366 (20), 486 (5), 244 (7), 202 (12), 176 (8), 147 (7), 103 (10), 119 (13), 91 (25), 77 (11), 57 (10)
5-Hydroxy-propafenone	TMS/TFA	284 (100), 73 (54), 129 (37), 91 (18), 168 (4), 371 (3), 209 (2), 59 (2)
N-Desisopropyl-propafenone	TMS/TFA	73 (100), 91 (52), 105 (31), 121 (18), 129 (22), 147 (7)

2. Isobutane CI mass spectra

Compound	Derivative	CI mass spectra
Propafenone	TMS/TFA	494 (1), 420 (29), 284 (100), 460 (1), 305 (1), 170 (1), 103 (2), 73 (2)
	TFA	534 (8), 420 (2), 308 (100), 196 (2)
	PFP	408 (100), 632 (4), 470 (2)
5-Hydroxy-propafenone	TMS/TFA	284 (100), 508 (27)
	TFA	308 (100), 532 (3), 196 (2)
	PFP	408 (100), 91 (12), 366 (10), 630 (3), 244 (4), 202 (7), 176 (4)
N-Desisopropyl-propafenone	TMS/TFA	242 (100), 468 (72), 540 (7), 378 (10), 129 (5)
	TFA	604 (100), 266 (36), 490 (4)

Relative abundances in parentheses.
Molecular weights of the respective derivatives: propafenone-*N*-TFA,*O*-TMS 509; propafenone-*N*-TFA,*O*-TFA 533; propafenone-*N*-PFP,*O*-PFP 633; 5-hydroxy-propafenone-*N*- TFA,bis-*O*-TMS 597; 5-hydroxy-propafenone-*N*-TFA,bis-*O*-TFA 645; 5-hydroxy-propafenone-*N*-PFP,bis-*O*-PFP 795; *N*-desisopropyl-*N*-TFA,*O*-TMS 467, *N*-despropylpropafenone-*N*-TFA-bis-*N*,*O*-TMS = 603.

The reproducibility of the EI mass spectra of the TFA and PFP derivatives of propafenone and 5-hydroxy-propafenone, as well as the TMS/TFA derivative of *N*-despropyl-propafenone, were moderate. However, especially in the case of MSTFA/MBTFA derivatization, the reproducibility of the CI mass spectra of all derivatives was good, and therefore this method was chosen for the quantification of the compounds investigated.

The TMS/TFA derivatives of 5-hydroxy-propafenone, propafenone and *N*-despropyl-propafenone were stable up to at least 3 days after their formation, on storage at −4 °C in the dark.

Identification of metabolites of propafenone in human urine

Surprisingly, the gas chromatograms of the TMS/TFA derivatives of 5-hydroxy-propafenone, propafenone and *N*-despropyl-propafenone, as either reference compounds or after the extraction from spiked human urine, were different (Fig. 2). The isobutane positive CI mass spectra indicated the presence of the respective stable *N*-TFA,*O*-TFA,(bis)*O*-TMS derivatives of 5-hydroxy-propafenone, having a shorter retention time than the corresponding *N*-TFA,(bis)*O*-TMS-derivatives (Fig. 3). We assume that the stable fragment $m/z = 369$ u in the mass spectrum of 5-hydroxy-propafenone-*N*-TFA,*O*-TFA,bis-*O*-TMS results from subsequent losses of the propyl-*N*-TFA group from the aminopropanol side-chain, the TFA group from the keto function and the TMS group from the aryl-hydroxy group.

During the elution step of the solid-phase extraction procedure used, the ionization of the enolizable keto

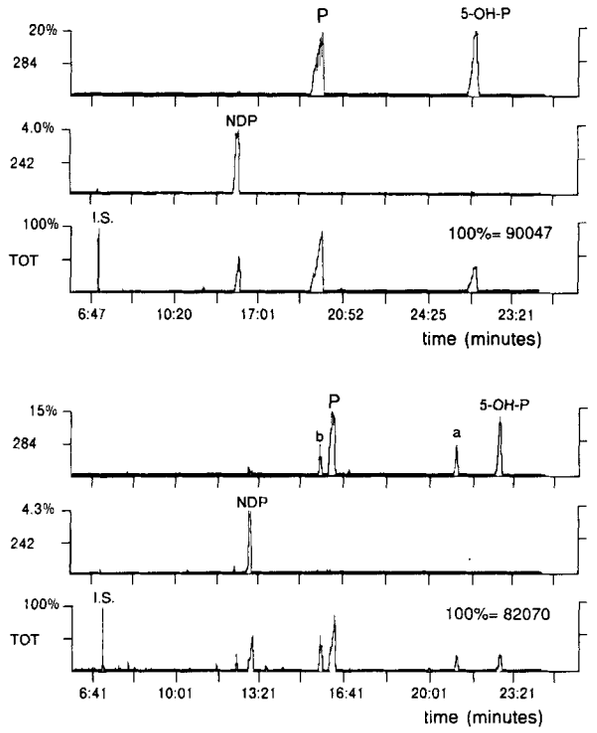

Figure 2. Gas chromatograms of propafenone (P), 5-hydroxy-propafenone (5-OH-P) and *N*-despropyl-propafenone (NDP) before (upper) and after extraction from urine (lower).

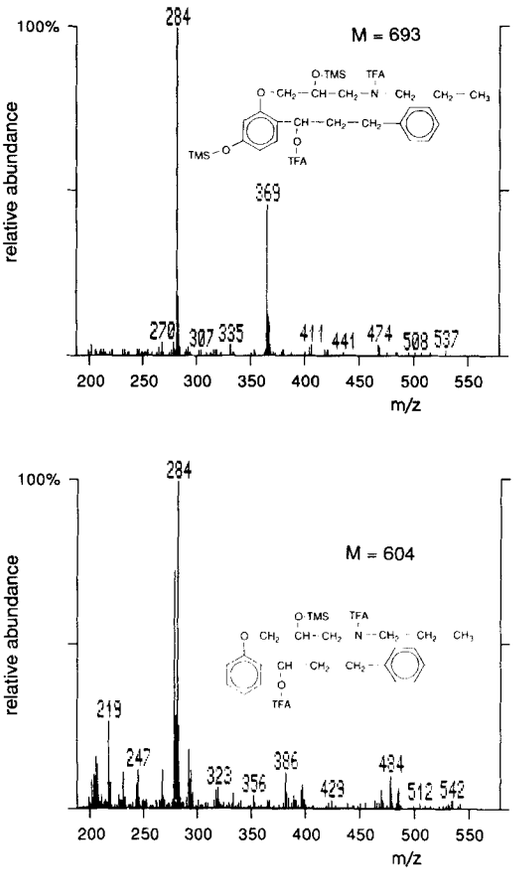

Figure 3. Isobutane positive CI mass spectra of 5-hydroxy-propafenone *N*-TFA,*O*-TFA,bis-*O*-TMS (upper) and propafenone *N*-TFA,*O*-TFA,*O*-TMS (lower).

group, which resides in the minor side-chain of (5-hydroxy)-propafenone, might be influenced by the acidified methanol, and the subsequent MBTFA derivatization of this group facilitated. Therefore, the formation of these derivatives may be considered as an unwanted artefact of the extraction procedure.

In the urinary extract of some volunteers, the occurrence of some TMS/TFA derivatives of the diol metabolite of propafenone as well as a hydroxylated *N*-despropyl-propafenone metabolite was established (Fig. 4). However, the existence of this latter metabolite of propafenone has not been reported yet,[8,9] and therefore we may not exclude that it might be some other artefact of the current procedure, e.g. from a dealkylation of the 5-hydroxy metabolite.

The gas chromatograms of the PFP and TFA derivatives of a urinary extract from a healthy volunteer 8 h after the ingestion of 150 mg Rytmonorm® are shown in Fig. 5.

In the case of the PFPA derivatization, a single peak in the chromatogram indicates the presence of the 5-hydroxy metabolite. Using TFAA as the derivatizing reagent, propafenone, 5-hydroxy-propafenone and *N*-despropyl-propafenone were positively identified in the extract. Other metabolites were absent.

Precision, calibration curves and recovery

The relative abundance of the base peak of the internal standard during 13 consecutive runs was 20.8% ±

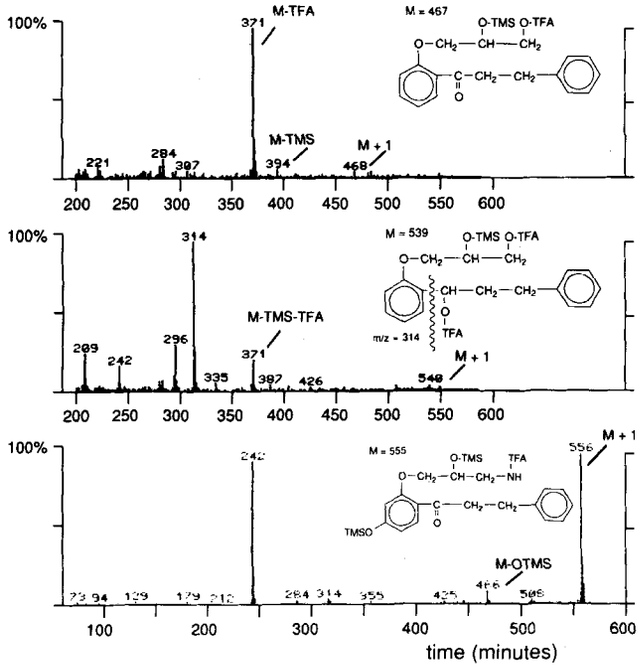

Figure 4. Isobutane positive CI mass spectra and possible structures of TMS/TFA derivatives of some metabolites of propafenone identified in human urine.

14.3% (mean ± relative standard deviation). Similar precisions have been demonstrated by others.[10] The results of the linear regression analysis of the calibration curves of the TMS/TFA derivatives of both reference compounds and urinary extracts are given in Table 2. It is evident from these results that calibration preferably occurs using peak area measurements. The excellent correlation and the high dynamic range of the ion trap detector are striking. Similar results have been reported by Lim *et al.*[11] The calibration curves of the reference

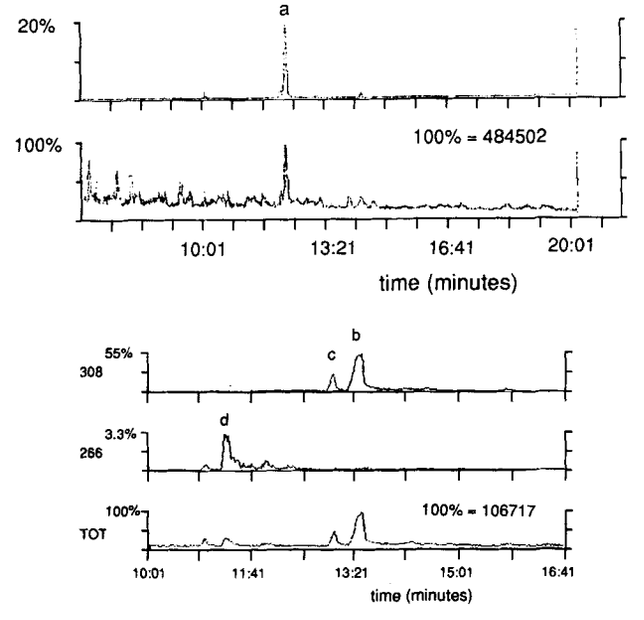

Figure 5. Gas chromatograms of urinary extracts, 8 h post-dose, (a) after PFPA and (b) TFAA derivatization. Legend: a, 5-hydroxy-propafenone *N*-PFP,bis-*O*-PFP; b, 5-hydroxy-propafenone *N*-TFA,bis-*O*-TFA; c, propafenone *N*-TFA,*O*-TFA; d, *N*-despropyl-propafenone *N*-TFA-bis-*N*,*O*-TFA.

Table 2. Calibration curves

Compound	Method	Range[e]	*a*	*b*	C.c.[a]
P (ref.)[b]	Area[c]	0–265	0.036	0.3126	0.9817
P (ref.)	Height[d]	0–265	0.0023	0.4332	0.9264
P (ur.)	Area	0–265	0.024	0.0874	0.9926
P (ur.)	Height	0–265	0.0016	0.4471	0.6095
5-OP (ref.)	Area	0–50	0.0358	−1.106	0.9234
5-OP (ref.)	Height	0–50	0.0051	−0.1217	0.9688
5-OP (ur.)	Area	0–50	0.0014	−0.2978	0.9737
5-OP (ur.)	Height	0–50	0.0029	−0.0669	0.9656
NDP (ref.)	Area	0–65	0.0075	−0.045	0.9728
NDP (ref.)	Height	0–65	0.0009	0.0021	0.9684
NDP (ur.)	Area	0–65	0.0066	−0.097	0.9548
NDP (ur.)	Height	0–65	0.0020	0.011	0.8591

Calibration curves: $y = ax + b$.
[a] C.c., correlation coefficient of the calibration curve.
[b] P, propafenone; 5-OP, 5-hydroxy-propafenone; NDP, *N*-despropyl-propafenone; ref., reference compounds; ur, urinary extract.
[c] Area, calibration method based on measurement peak areas of compounds divided by those of the internal standard, using manual mode of ITD.
[d] Height, calibration method based on measurement peak heights of compounds divided by those of the internal standard, using manual mode of ITD.
[e] Range, absolute amount injected onto the GC column in nanograms.

compounds before and after their extraction from urine are different, due to the artefact formation and/or losses during the extraction, but the correlation coefficients are sufficiently high to permit quantification. The recoveries after the extraction were 87 ± 5% for propafenone, 56 ± 6% for 5-hydroxy-propafenone and 42 ± 11% for *N*-despropyl-propafenone (mean ± standard deviation, $n = 3$). The recoveries of the metabolites are lower than those reported for β-adrenergic blocking agents,[4] due to their high polarity and/or the artefact formation (5-hydroxy-propafenone).

Urinary excretion

In hydrolysed urine, traces of 5-hydroxy-propafenone and propafenone were identified up to 24 h post-dose. In unhydrolysed urine, no parent drug and only minor amounts of the 5-hydroxy metabolite have been detected. The intersubject variability of both the metabolic pattern and the concentrations of propafenone and 5-hydroxy-propafenone in human urine was considerable (Fig. 6). Traces of propafenone were found in the urine of one subject, whereas much higher concentrations of the parent drug were determined in the urine of the two other subjects. The concentrations of other metabolites, e.g. *N*-despropyl-propafenone, were too low to permit quantification. The total amounts of propafenone and 5-hydroxy-propafenone excreted in human urine at 8 h post-dose was only a few per cent of the oral dose: 0.14 mg and 3.5 mg (subject 1), 2.4 mg and 3.7 mg (subject 2), and 2.2 mg and 2.8 mg (subject 3), respectively (Fig. 7). Such large inter-individual differences in the urinary excretion of these compounds have also been demonstrated by Hege *et al.*,[8] and it may be due to intersubject differences in the saturable metabolism of propafenone and the genetically determined oxidative status of individuals. Additionally,

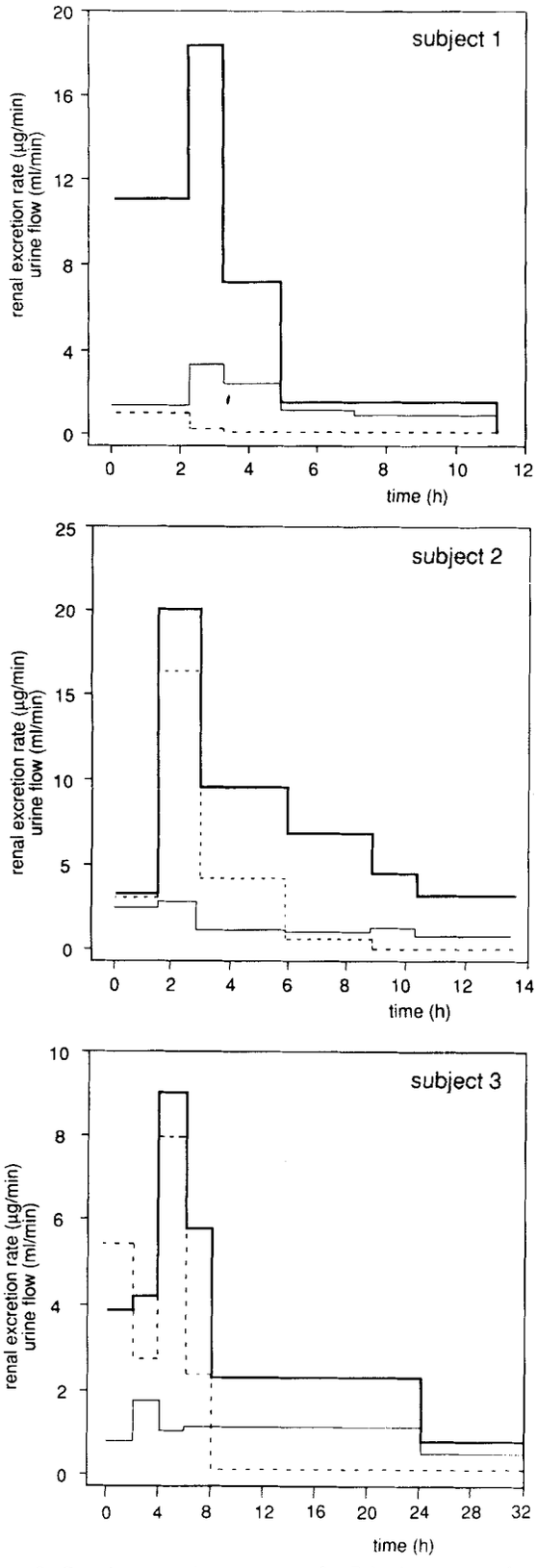

Figure 6. Renal excretion rate of 5-hydroxy-propafenone (———) and propafenone (– – – –), urine flow (———).

intersubject variations of the renal function may be of some importance as well. Therefore, differences in plasma concentrations and elimination half-lives between poor and extensive metabolizers are being observed.[1]

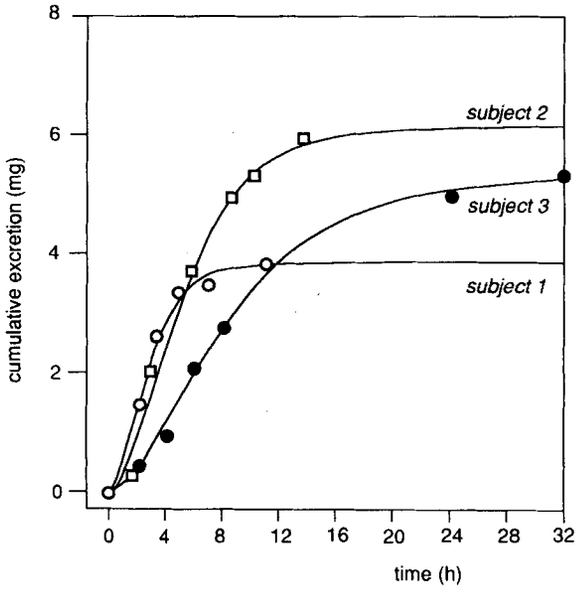

Figure 7. Cumulative excretion (mg) of 5-hydroxy-propafenone. The curves represent the sums of two exponential terms, calculated using a FarmFit program. Subject 1: T1 = 109 min, T2 = 838 min, mean residence time (MRT) = 193 min. Subject 2: T1 = 169 min, T2 = 169 min, MRT = 339 min. Subject 3: T1 = 302 min, T2 = 247 min, MRT = 550 min.

CONCLUSION

Isobutane positive CI mass spectrometry of PFP, TFA and TMS/TFA derivatives of propafenone as well as its main metabolites 5-hydroxy-propafenone and *N*-despropyl-propafenone is complementary to the EI mass spectrometry of these compounds, and their fragmentation pattern is similar to those reported for the structural related β-adrenergic blocking agents. The acceptable reproducibility of the CI mass spectra of the TMS/TFA derivatives of these compounds permits a proper quantification.

Additional TMS/TFA derivatives of 5-hydroxy-propafenone may be formed after their extraction from urine, presumably due to the influence of acidified methanol on the ionization of their enolizable keto function during the elution step of the solid-phase extraction procedure used. The formation of these side-products may be considered as an unwanted drawback of this derivatization method. However, if TFAA and PFPA are being used as the derivatizing agents, some information about trace amounts of co-extracted metabolites is lacking. The presence of such metabolites, e.g. the diol metabolite of propafenone, in the urinary extract, can still be established after MSTFA/MBTFA derivatization. Moreover, straight calibration curves having a large dynamic range and high correlation coefficients (0.92–0.99) of propafenone, 5-hydroxy-propafenone and *N*-despropyl-propafenone as reference compounds or after their extraction from urine have been obtained using the latter derivatization procedure.

Large intersubject variability in the renal excretion rate and the absolute amount of propafenone and the pharmacologically active metabolite 5-hydroxy-propafenone excreted in urine, after an oral dose of propafe-

none, was demonstrated. Only a few per cent of the dose was excreted unchanged in the urine. These inter-subject variabilities may be due to inter-individual differences in hepatic metabolism and renal function.

Acknowledgements

The authors wich to thank Miss M. R. van der Meer for her technical assistance and Dr J. G. M. de Bie for his advice using the FarmFit program.

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