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Determination of propafenone and its phase I and phase II metabolites in plasma and urine by high-performance liquid chromatography–electrospray ionization mass spectrometry

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Abstract

A sensitive method was developed to determine propafenone, 5-hydroxypropafenone, *N*-despropylpropafenone and propafenone glucuronides in human plasma and urine by HPLC–electrospray ionization mass spectrometry with the respective deuterated analogues as internal standards. The analytes were extracted by a single solid-phase extraction, collecting two fractions, one containing the glucuronides and the other propafenone and the phase I metabolites 5-hydroxypropafenone and *N*-despropylpropafenone. The mobile phases used for HPLC were: (A) 5 m*M* ammonium acetate in water and (B) 5 m*M* ammonium acetate in methanol–tetrahydrofuran (50:50, v/v). Separation of the diastereoisomeric propafenone glucuronides was achieved on a Spherisorb ODS 2 column (150×2.0 mm I.D., particle size 5 μm) at a flow-rate of 0.3 ml/min using a linear gradient from 20% B to 50% B in 15 min. For separation of propafenone, 5-hydroxypropafenone and *N*-desalkylpropafenone a linear gradient from 50% B to 80% B in 10 min was employed. The mass spectrometer was operated in the selected ion monitoring mode using the respective MH^+ ions for quantification. The limits of quantification achieved with this method were 10 pmol/ml for propafenone, 5-hydroxypropafenone, *R*- and *S*-propafenone glucuronide and 20 pmol/ml for *N*-desalkylpropafenone using 0.5 ml of plasma. Reproducibility and accuracy was below 12% for each analyte over the whole concentration range measured. The method was applied to a pharmacokinetic study assessing the influence of rifampicin on propafenone disposition.

Keywords: Propafenone

1. Introduction

Propafenone hydrochloride (PPF, Fig. 1), 2′-(2-hydroxy-3-propylamino-propoxy)-3-phenyl-propiophenone hydrochloride, is a class Ic antiarrhythmic agent with slight β-adrenergic-antagonist properties, which is effective in the treatment of supraventricular and ventricular arrhythmias [1]. PPF is administered as racemate and the enantiomers have different pharmacological activities and kinetics of disposition. PPF is extensively metabolized via phase I and phase II enzymes. 5-Hydroxylation, the major route of phase I metabolism is related to debrisoquine/sparteine polymorphism [2], and poor metabolizers of CYP2D6 form only little or no 5-hydroxy-propafenone (5-OHP), but maintain much higher propafenone concentrations than extensive metabolizers of CYP2D6 [3]. The *N*-dealkylated metabolite *N*-

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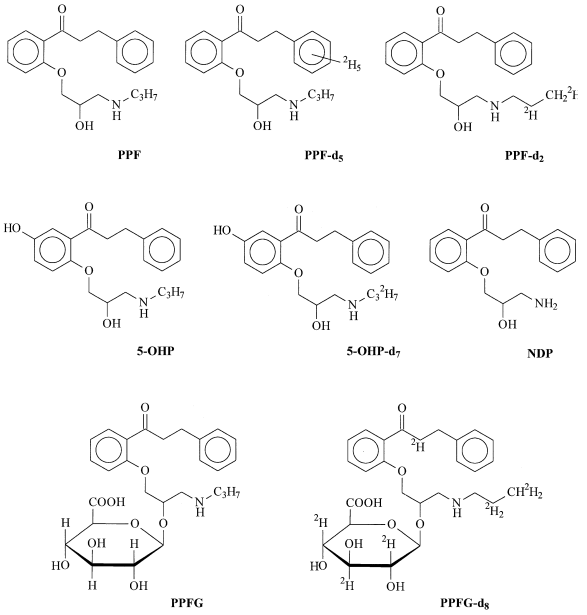

Fig. 1. Structures of PPF, PPF-d_5, 5-OHP, NDP, PPFG and the internal standards PPF-d_2, 5-OHP-d_7, PPFG-d_8.

despropylpropafenone (NDP) is formed to a minor extent [4]. A considerable proportion of the drug is glucuronidated to propafenone glucuronide (PPFG), in particular in poor metabolizers of CYP2D6. The main metabolites excreted in urine are conjugates (glucuronides and sulfates) of PPF, NDP and 5-OHP [5].

For a clinical investigation of the induction properties of rifampicin on both phase I and phase II metabolism of propafenone, an assay had to be developed for the determination of PPF, its phase I metabolites 5-OHP and NDP and the phase II metabolite PPFG in plasma and urine. We have employed a stable isotope technique which allows simultaneous and differential measuring of intravenously (i.v.) and orally (p.o.) administered drugs. Pharmacokinetics and pharmacodynamics of unlabeled propafenone administered intravenously and [$^2H_5$]propafenone (PPF-d_5) given orally were compared before and during rifampicin induction [6].

For a stable isotope approach mass spectrometric determination of PPF, PPF-d_5 and the respective metabolites is essential. One gas chromatography–mass spectrometry (GC–MS) procedure for PPF, 5-OHP and NDP is described [7], but it requires derivatization and is not applicable to the determination of the glucuronides. More recently PPF and its phase I metabolites have been determined by liquid chromatography (LC)–MS [8,9], but the conjugated metabolites including PPFG were determined after enzymatic cleavage. The present paper describes the first LC–MS assay for the direct quantification of PPFG in human plasma and urine. Direct measurement of the glucuronide instead of laborious hydrolysis procedures means a considerable saving of time, in particular as the whole extraction procedure could be performed automatically. The method was successfully applied to the determination of PPF, 5-OHP, NDP and PPFG and the respective [$^2H_5$]-labelled analogues in several pharmacokinetic studies.

2. Experimental

2.1. Materials

Solvents used were of high-performance liquid chromatography (HPLC) quality; chemicals were of analytical grade. PPF, 5-hydroxy-propafenone hydrochloride [5′-hydroxy-2′-(2-hydroxy-3-propylamino-propoxy)-3-phenyl-propiophenone hydrochloride] and *N*-despropylpropafenone fumarate [2′-(2-hydroxy-3-amino-propoxy)-3-phenyl-propiophenone fumarate] were kindly supplied by Knoll (Ludwigshafen, Germany). [$^2H_2$]-Propafenone (PPF-d_2), [$^2H_5$]-propafenone hydrochloride (PPF-d_5), [$^2H_7$]-5-hydroxy-propafenone hydrochloride (5-OHP-d_7), *R*/*S*-PPFG and *R*/*S*-[$^2H_8$]-propafenone glucuronide (PPFG-d_8) were obtained by chemical synthesis. Solid-phase extraction (SPE) cartridges, Bakerbond SPE C_{18} (500 mg) were supplied by Baker (Gross-Gerau, Germany).

2.2. Sample preparation

Samples (0.5 ml of plasma or 50 μl of urine diluted with 450 μl of water) were spiked with 50 μl of internal standard solution (8 pmol/μl PPF-d_2, 4 pmol/μl 5-OHP-d_7 and 4 pmol/μl PPFG-d_8).

Sample purification was performed automatically on an ASPEC XL system (Gilson, Abimed, Langenfeld, Germany) using C_{18} SPE cartridges. The SPE

Table 1
Solid-phase extraction scheme

SPE steps	Liquid	Volume (ml)	Dispensing flow-rate (ml/min)
Conditioning	Methanol	2	5
	Water	2	5
	Ammonium acetate (10 m*M*)	2	5
Sample loading		0.5	1
Washing	Water	0.5	3
	Ammonium acetate (10 m*M*)	4.5	3
	Methanol	0.3	1
Drying	Air	10	4
Elution fraction 1	Methanol	0.9	1
Washing	Methanol	2	10
Elution fraction 2	Acetic acid in methanol (0.1 *M*)	0.5	1

scheme is detailed in Table 1, PPF glucuronides were eluted in fraction 1, PPF and the other metabolites were eluted in fraction 2. The fractions were dried with nitrogen and the residue dissolved in 100 μl of the mobile phase, containing 20% B or 50% B for fractions 1 or 2, respectively. An aliquot of 40 μl was used for HPLC analysis.

2.3. HPLC–MS analysis

A HP Series 1100 LC–MS system (Hewlett-Packard, Waldbronn, Germany) with binary pump, degasser, autosampler and mass-selective detector equipped with an electrospray ion source was used. The mobile phases for HPLC were: (A) 5 m*M* ammonium acetate in water and (B) 5 m*M* ammonium acetate in methanol–tetrahydrofuran (50:50, v/v). Baseline separation of the diastereoisomeric propafenone glucuronides was achieved on a Spherisorb ODS 2 column (150×2.0 mm I.D., particle size 5 μm) at a flow-rate of 0.3 ml/min using a linear gradient from 20% B to 50% B in 15 min. For separation of PPF, 5-OHP and NDP a linear gradient from 50% B to 80% B in 10 min was employed.

Electrospray parameters were as follows: capillary voltage 3500 V, drying gas flow 11 l/min nitrogen, drying gas temperature 350°C, nebulizer pressure 55 p.s.i.g., fragmentor 110 V (1 p.s.i.=6894.76 Pa). The mass spectrometer was operated in the selected ion monitoring mode using the respective MH^+ ions, m/z 300 for NDP, m/z 305 for NDP-d_5, m/z 342 for PPF, m/z 344 for PPF-d_2, m/z 347 for PPF-d_5, m/z 358 for OHP, m/z 363 for 5-OHP-d_5, m/z 365 for 5-OHP-d_7, m/z 518 for *R*- and *S*-PPFG, m/z 523 for *R*- and *S*-PPFG-d_5 and m/z 526 for *R*- and *S*-PPFG-d_8.

2.4. Standardization

Calibration samples were prepared by adding increasing amounts of PPF, PPF-d_5, 5-OHP, NDP and PPFG to control plasma or urine. Standard curves in the range of 10–3200 pmol/ml for PPF, 20–3200 pmol/ml for PPF-d_5, 10–1200 pmol/ml for 5-OHP, 10–2500 pmol/ml for *R*- and *S*-PPFG and 20–1000 pmol/ml for NDP were evaluated by linear regression analysis based on internal standard calibration and were obtained by plotting peak-area ratios against the amount of the substance. Unknown concentrations were calculated with the average response factor using the respective deuterated substances (PPF-d_2, 5-OHP-d_7 and PPFG-d_8) as internal standards, except for NDP where PPF-d_2 was used. The same response factors were used for calculation of [$^2H_5$]-substituted 5-OHP, NDP and PPFG.

3. Results and discussion

3.1. Sample preparation

PPF and its phase I metabolites can be extracted by liquid–liquid extraction or SPE, whereas a liquid–liquid extraction is not possible for glucuronides due to their higher polarity. PPFG had usually been determined indirectly after enzymatic hydrolysis. Only one method for the direct determination by HPLC is described [10], PPFG is extracted from urine by SPE with a tributylammonium buffer. In order to enhance sample throughput and to minimize sample consumption we wanted to use only one extraction method for all metabolites. Therefore SPE was employed. Instead of the tributylammonium buffer, which is not suitable for electrospray ionization (ESI)-MS applications, ammonium acetate buffer was used in the wash step. Because of interfering peaks in the ion chromatograms of propafenone (m/z 342) the glucuronide fraction had to be collected separately. The propafenone glucuronides could be eluted from the C_{18} cartridges with a recovery of about 70% or higher (Table 2) with methanol. After a wash with methanol for removal of the interfering peak, PPF and the phase I metabolites have to be eluted with acidified methanol yielding a recovery of about 70%.

3.2. HPLC separation and LC–MS analysis

HPLC separation of the glucuronides is usually achieved on reversed-phase columns with ion pairing reagents [10] like tetrabutylammonium salts. Since these reagents should be avoided in ESI-MS, a substitution with ammonium acetate was performed. But with water–acetonitrile or water–methanol gradients on different C_{18} columns no separation of the glucuronides was achieved. Addition of tetrahydrofuran to the mobile phase enabled nearly baseline separation of *R*- and *S*-PPFG (Fig. 3B, $\alpha = 1.044$). The same mobile phases but with another gradient profile could be used for separation of PPF, 5-OHP and NDP (Figs. 4B and 5B). In this way all glucuronide samples and then all samples containing PPF and the phase I metabolites could be analyzed within one autosampler sequence without elongated column conditioning.

For all analytes the protonated molecular ions (MH^+) were observed as base peaks, only slight fragmentation occurred. The mass spectra of *R*/*S*-PPFG and the internal standard *R*/*S*-PPFG-d_7 are

Table 2
Extraction recoveries

Compound	Plasma		Urine	
	Concentration added (pmol/0.5 ml)	Recovery (%) (mean±SD)	Concentration added (pmol/50 μl)	Recovery (%) (mean±SD)
PPF	10	62.2±11.0	20	60.0±5.1
	400	83.0±4.1	400	73.7±1.3
PPF-d_5	20	63.2±10.4	20	77.7±6.1
	400	78.8±2.4	400	74.3±0.9
NDP	15	30.5±4.5	20	86.1±3.8
	100	58.2±2.0	100	78.9±4.8
5-OHP	10	68.1±3.8	20	63.0±4.1
	200	68.0±4.3	200	80.6±0.9
R-PPFG	10	72.5±5.5	10	79.4±1.8
	500	90.3±1.6	500	75.9±2.0
S-PPFG	10	67.9±4.7	10	83.0±2.2
	500	85.3±1.0	500	77.0±2.3

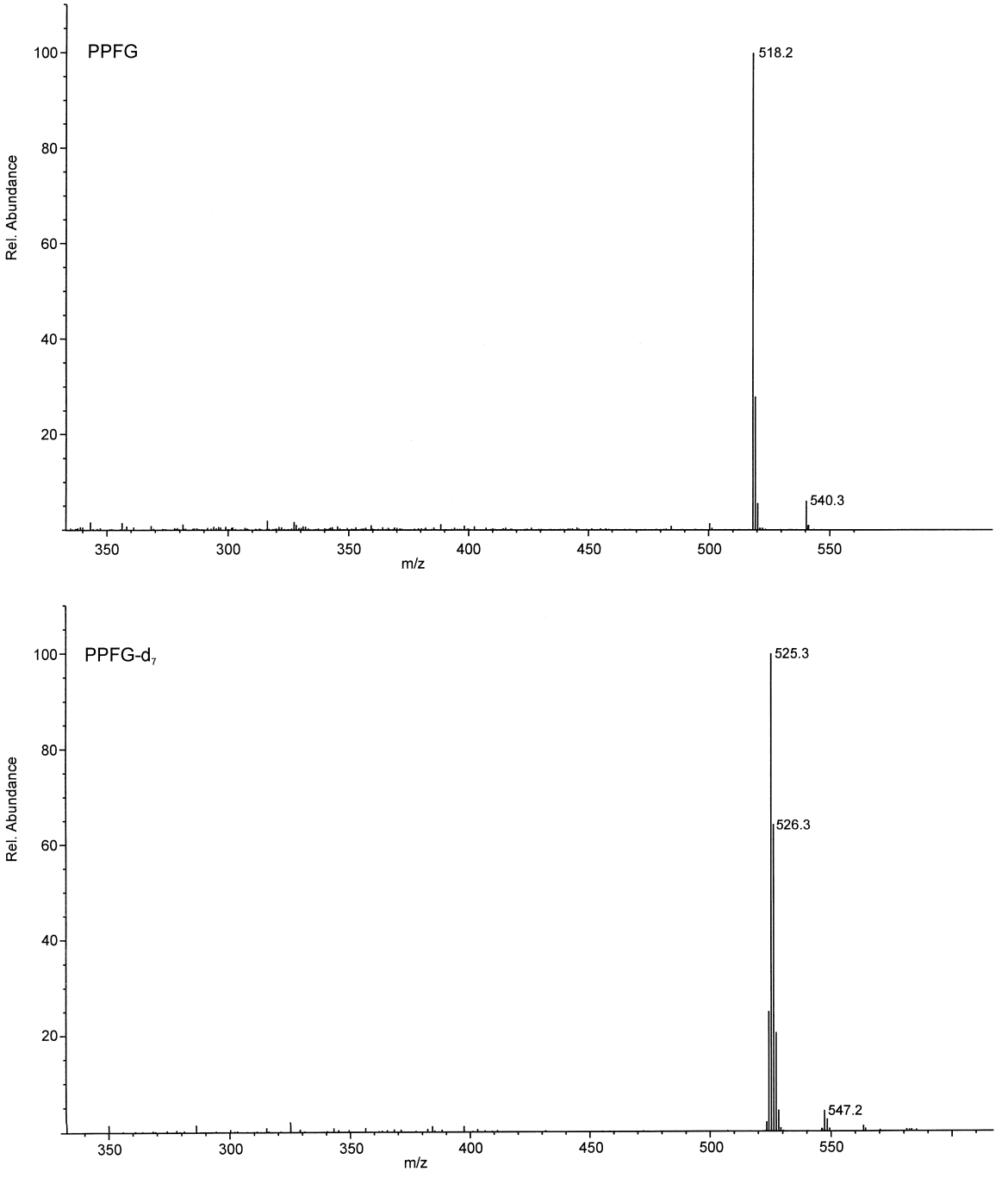

Fig. 2. Positive-ion electrospray mass spectra of *R*/*S*-PPFG and the internal standard *R*/*S*-PPFG-d_7. For quantification the ion referring to PPFG-d_8 (*m*/*z* 526) was used.

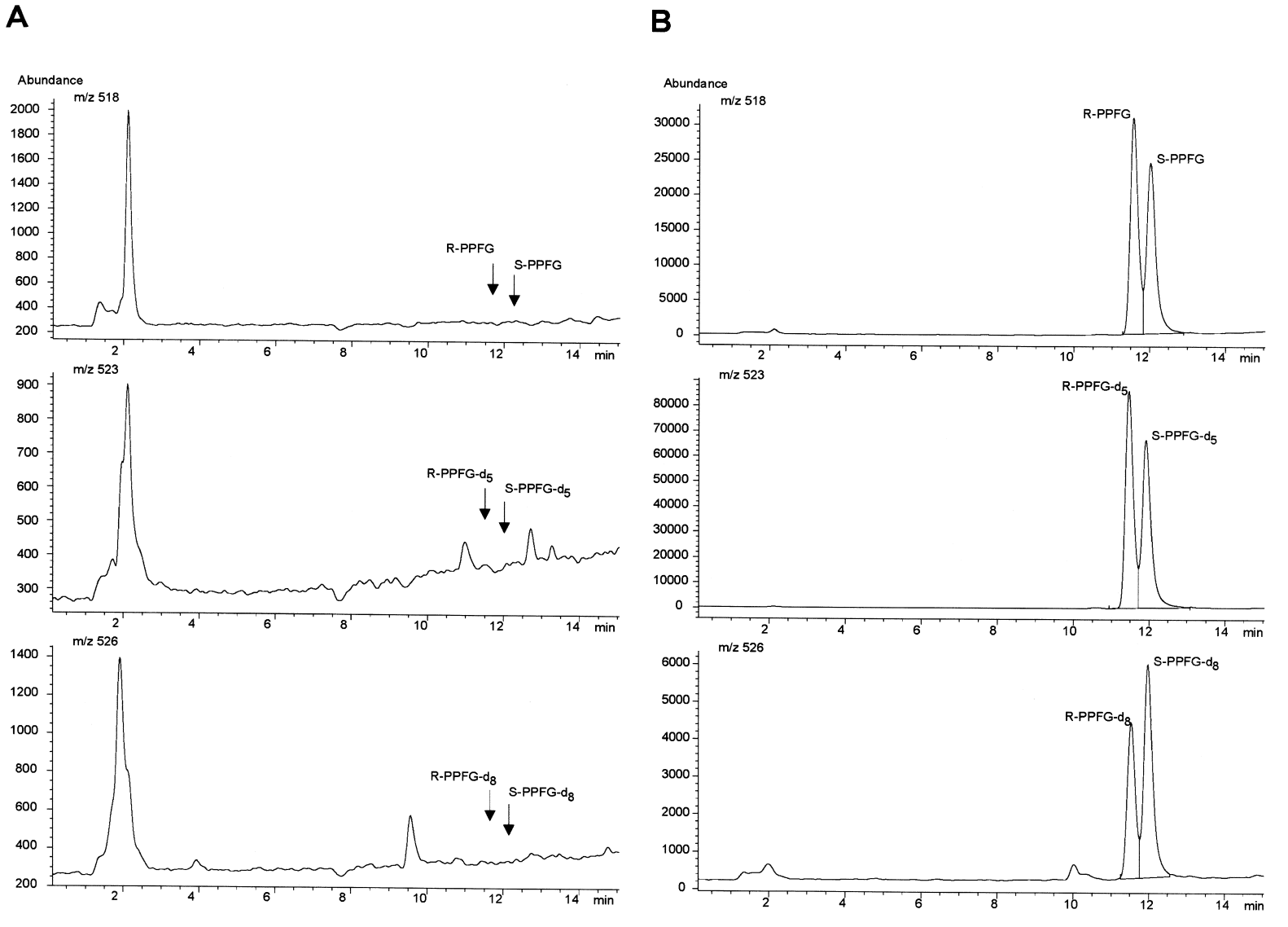

Fig. 3. Mass chromatograms of extracts from (A) blank human plasma, (B) plasma from a volunteer after single doses of 140 mg PPF i.v. ($t=0$) and 300 mg PPF-d_5 p.o. ($t=2$ h). The concentrations found were: 351 pmol/ml *R*-PPFG, 290 pmol/ml *S*-PPFG, 971 pmol/ml *R*-PPFG-d_5 and 794 pmol/ml *S*-PPFG-d_5.

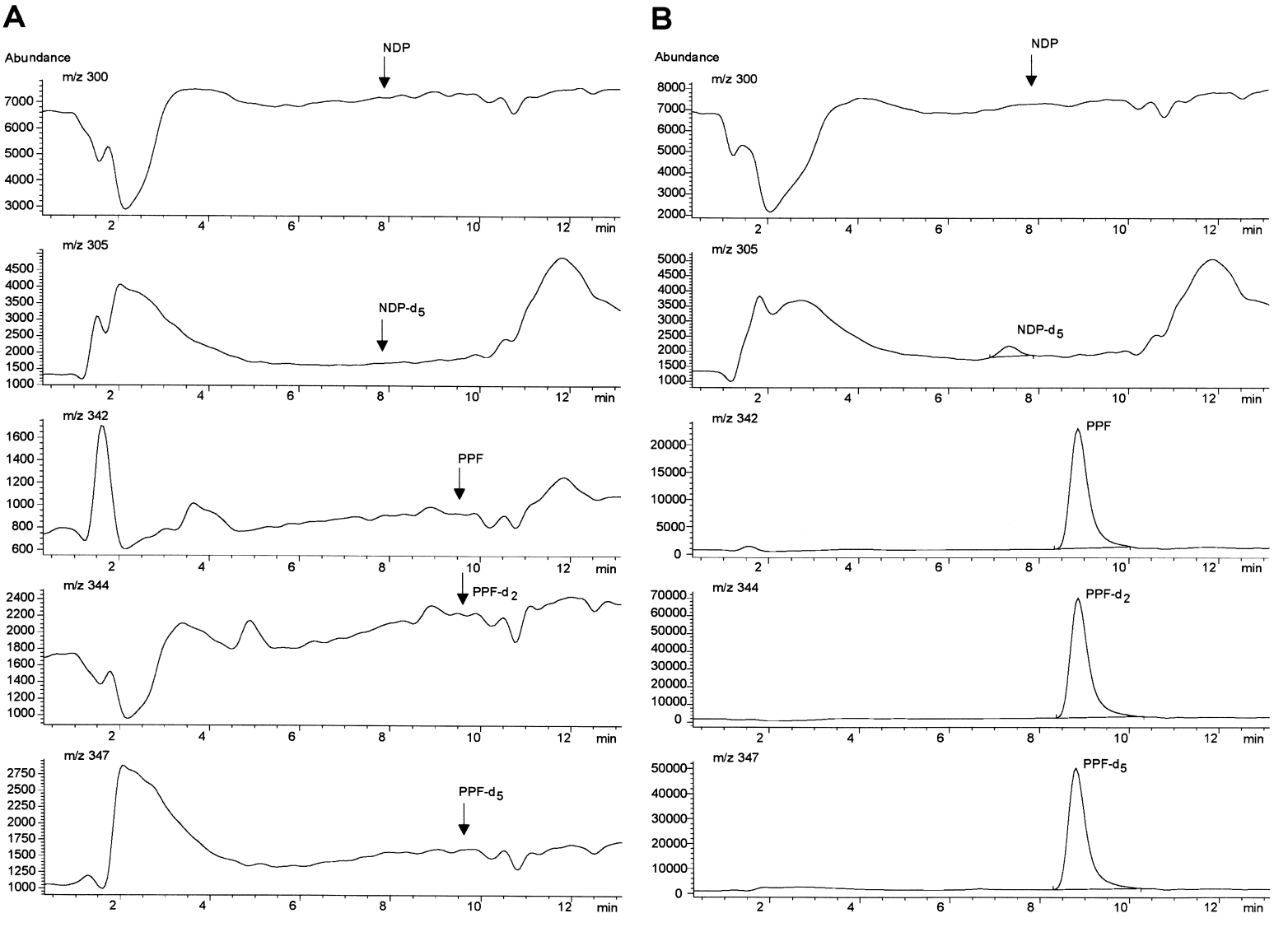

Fig. 4. Mass chromatograms of extracts from (A) blank human plasma, (B) plasma from a volunteer after single doses of 140 mg PPF i.v. ($t=0$) and 300 mg PPF-d_5 p.o. ($t=2$ h). The concentrations found were: 35.6 pmol/ml NDP-d_5, 388 pmol/ml PPF and 375 pmol/ml PPF-d_5.

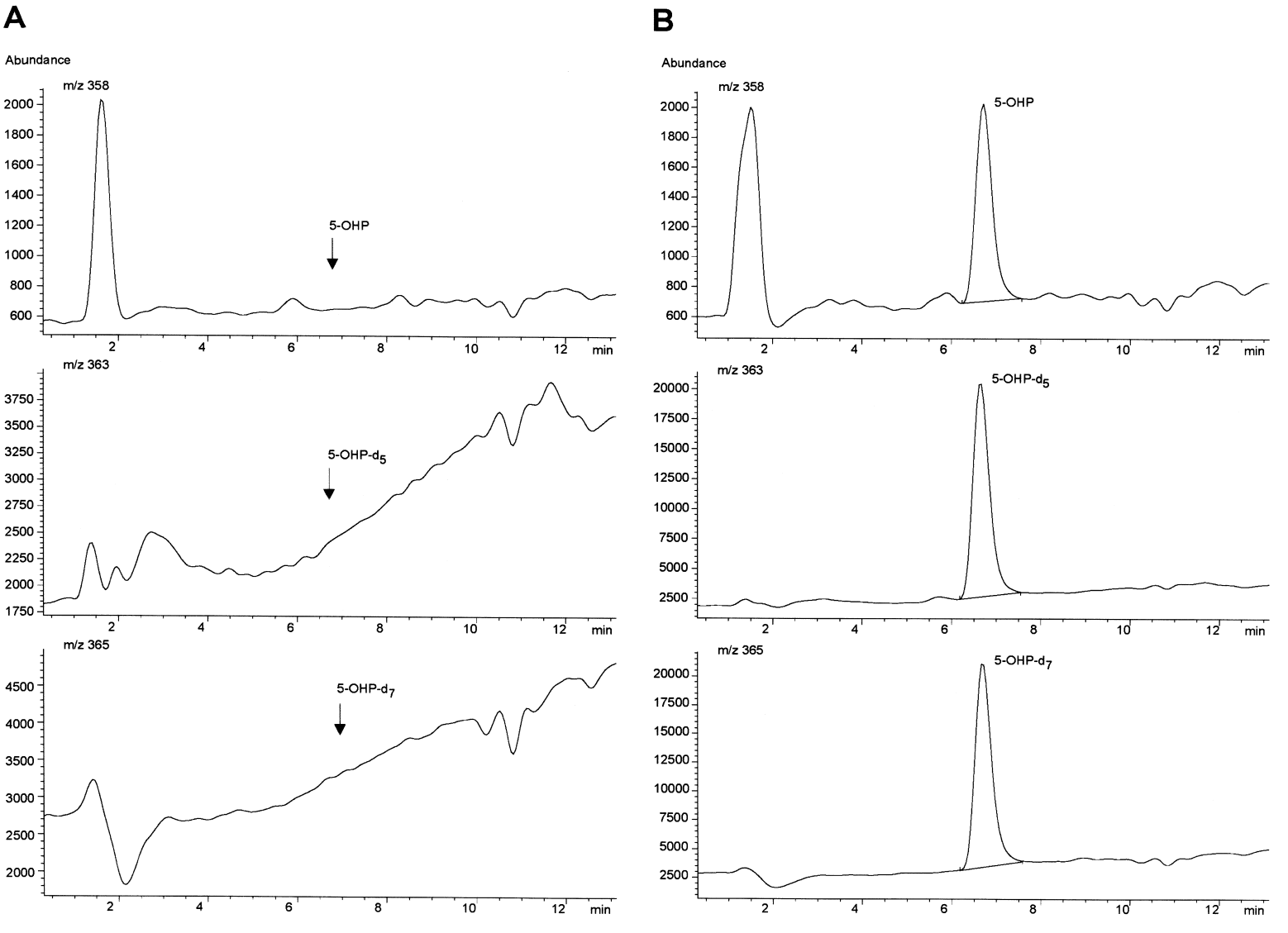

Fig. 5. Mass chromatograms of extracts from (A) blank human plasma, (B) plasma from a volunteer after single doses of 140 mg PPF i.v. ($t=0$) and 300 mg PPF-d_5 p.o. ($t=2$ h). The concentrations found were: 21.9 pmol/ml 5-OHP and 171 pmol/ml 5-OHP-d_5.

Table 3
Response factors used for calculation of unknown concentrations of PPF and its metabolites

	Response factor (mean)	RSD (%)	*n*
PPF	0.880	4.3	13
PPF-d_5	0.787	2.1	13
5-OHP	0.700	9.5	13
NDP	0.102	21.6	13
R-PPFG	3.88	3.6	13
S-PPFG	2.87	4.6	13

shown in Fig. 2. Besides the protonated molecular ions only the sodium adducts (m/z 540 for PPFG and m/z 547 for PPFG-d_7) are formed, but to a minor extent. With the electrospray parameters given in Section 2.3 no fragmentation to propafenone occurs.

Typical mass chromatograms of 0.5-ml plasma extracts are shown in Figs. 3–5. In the blank plasma no interfering peaks could be detected (Figs. 3A, 4A and 5A).

Figs. 3B, 4B and 5B show the chromatograms of plasma samples after treatment with 140 mg PPF intravenously (i.v.) (30 min) and 300 mg PPF-d_5 orally (p.o.). In extensive metabolizers of CYP2D6 the concentrations of NDP (from the i.v. dose) were below the limit of quantification (LOQ) (Fig. 4B).

3.3. Validation

The linearity of the standard curves showed to be good over the entire concentration range measured (see Section 2.4). In general, coefficients of correlation above 0.9992 were observed for PPF, *R*-PPFG and *S*-PPFG, coefficients of correlation above 0.9989 were observed for PPF-d_5 and coefficients of correlation above 0.9978 were observed for 5-OHP and NDP. Response factors used for calculation of unknown concentrations are summarized in Table 3.

Reproducibility was determined by repeatedly analyzing aliquots of plasma or urine samples with known amounts of analytes. The intra- and inter-day variabilities for plasma are given in Tables 4 and 5. Intra-day variation was below 7.5% even at the LOQ. Inter-day variation was below 11%.

In urine, not the same sensitivity is required as for plasma, as the concentrations of PPF and the metabolites are much higher (about 10-fold). The method

Table 4
Intra-day precision and accuracy for the determination of PPF and its metabolites in plasma

	Concentration added (pmol/0.5 ml)	*n*	Concentration found (pmol/0.5 ml)	Bias (%)	RSD (%)
PPF	5.0	6	5.42	8.3	5.2
	10.0	5	9.22	−7.8	1.6
	400	5	421	5.1	3.0
PPF-d_5	10.0	6	10.8	8.3	3.3
	20.0	5	19.9	−0.4	2.4
	400	5	396	−1.0	2.5
5-OHP	5.0	6	4.69	−6.2	3.8
	10.0	5	9.81	−1.9	2.1
	200	5	229	14.6	5.9
NDP	10.0	5	9.08	−9.2	6.7
	15.0	5	14.3	−4.6	7.2
	100	5	104	4.4	3.0
R-PPFG	5.0	6	5.00	0	7.4
	500	5	527	5.1	3.5
S-PPFG	5.0	6	4.85	−3.0	7.2
	500	5	519	3.7	3.9

Table 5
Inter-day precision and accuracy for the determination of PPF and its metabolites in plasma

	Concentration added (pmol/0.5 ml)	*n*	Concentration found (pmol/0.5 ml)	Bias (%)	RSD (%)
PPF	5.0	12	4.82	−3.6	9.8
	200	13	223	11.5	2.7
	1600	13	1600	0.1	4.2
PPF-d_5	10.0	13	10.7	7.4	8.2
	200	13	207	3.6	4.4
	1600	13	1530	−4.1	3.6
5-OHP	5.0	12	4.75	−5.0	10.7
	100	12	97.8	−2.2	6.2
	600	11	654	9.0	5.1
NDP	10.0	12	10.1	0.5	10.9
	100	13	90.2	−9.8	9.3
	200	11	217	8.5	8.8
R-PPFG	5.0	13	4.55	−9.0	7.7
	500	13	522	4.4	4.6
	1250	13	1330	6.6	5.2
S-PPFG	5.0	13	4.48	−10.4	8.5
	500	13	510	2.0	5.7
	1250	13	1300	3.7	5.4

Table 6
Intra-day precision and accuracy for the determination of PPF and its metabolites in urine

	Concentration added (pmol/50 μl)	*n*	Concentration found (pmol/50 μl)	Bias (%)	RSD (%)
PPF	5.0	6	4.63	−7.5	6.3
	100	6	96.9	−3.1	5.2
	1200	6	1170	−2.6	0.8
PPF-d_5	10.0	6	9.75	−2.5	4.8
	100	6	98.4	−1.6	3.4
	1200	6	1190	−0.7	0.9
5-OHP	10.0	5	11.5	14.7	1.7
	100	6	96.7	−3.4	1.7
	600	6	648	8.0	2.7
NDP	10.0	5	11.1	10.6	7.4
	100	6	106	6.0	5.5
	200	6	188	−6.2	3.7
R-PPFG	5.0	5	5.73	14.7	3.8
	50	6	46	−8.8	2.4
	375	6	370	−1.4	0.5
S-PPFG	5.0	6	5.22	4.4	4.7
	50	5	46	−8.8	4.2
	375	6	368	−1.8	1.0

optimized for plasma can as well be used for urine with the exception that only 50 μl of sample are used. Assay variabilities for urine are given in Table 6. The LOQs expressed as pmol/sample obtained in urine are nearly the same as in plasma but refer to a volume of 50 μl of urine. The variation at all concentrations was below 7.5%.

Stability of the analytes had been evaluated formerly with HPLC assays [10,11]. Samples were stable for at least 3 months, during storage at −20°C. During five cycles of freezing and thawing no deterioration could be observed. Extracted samples were stable at room temperature for at least 17 h.

3.4. Assay application

The method described enables determination of PPF, its phase I metabolites and the PPF glucuronides in human plasma or urine. Representative plasma concentration–time curves of an extensive metabolizer of CYP2D6 following single doses of 140 mg PPF i.v. and 300 mg PPF-d_5 p.o. are shown in Fig. 6.

PPF may be chosen as model drug in pharmacokinetic studies because of its complex metabolism that permits simultaneous investigation of different enzymatic phase I and phase II pathways. Studies on stereoselective metabolism of PPF may gain further insight into metabolism of racemates. Our assay may also serve as a basis to elucidate the pharmacological role of PPF glucuronides in humans.

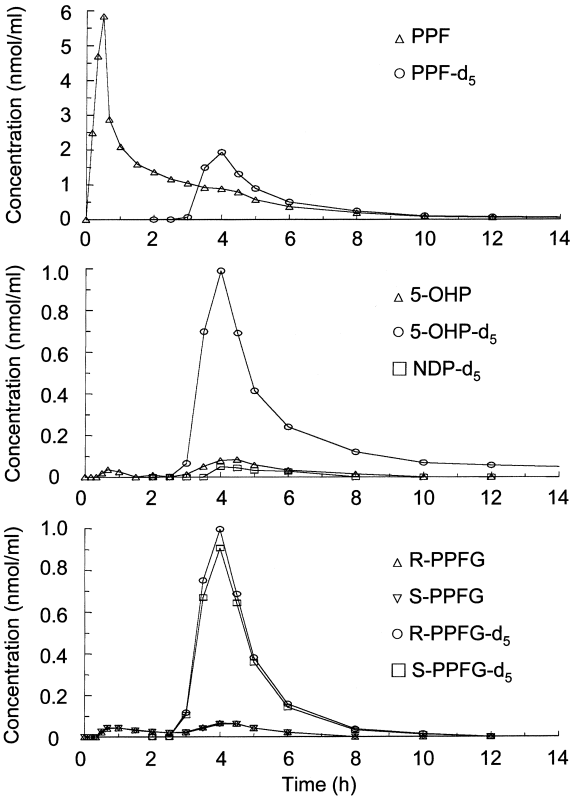

Fig. 6. Plasma–concentration profiles of PPF and its phase I and phase II metabolites in a healthy extensive metabolizer of CYP2D6 following single doses of 140 mg PPF i.v. (t=0) and 300 mg PPF-d_5 p.o. (t=2 h).

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