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High-performance liquid chromatography of imipramine and six metabolites in human plasma and urine

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ABSTRACT

A method for the simultaneous quantitation of imipramine and six metabolites (2- and 10-hydroxyimipramine, 2- and 10-hydroxydesipramine, didesmethylimipramine and desipramine) in human plasma and urine has been developed. The method is based on a three-step liquid–liquid extraction followed by isocratic, reversed-phase high-performance liquid chromatography with ultraviolet absorbance detection (detection wavelength: 220 nm). The chromatographic eluent consisted of 30% acetonitrile and 70% aqueous sodium perchlorate solution pH 2.5. Glucuronide conjugates in urine were deconjugated with β-glucuronidase/arylsulphatase prior to extraction.

INTRODUCTION

Imipramine was introduced more than 35 years ago and is still a commonly used drug for the treatment of major depression. Imipramine is eliminated almost exclusively by oxidation in the liver [1]. The major pathways are N-demethylation to the active metabolite, desipramine, and aromatic hydroxylation to 2-hydroxyimipramine and 2-hydroxydesipramine. Minor pathways are aliphatic hydroxylation to 10-hydroxyimipramine and 10-hydroxydesipramine, further N-dealkylation to didesmethylimipramine, N-oxidation to imipramine N-oxide and dealkylation of the whole side-chain to iminodibenzyl. A considerable part of the 2- and 10-hydroxy metabolites are excreted as glucuronide conjugates in the urine. About 40% of the dose is excreted in urine as unidentified polar metabolites [1].

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The therapeutic outcome of imipramine treatment depends on the plasma concentration of imipramine and desipramine [2]. Patients display 40-fold interindividual differences in imipramine plus desipramine steady-state plasma concentrations during treatment with a fixed dose of imipramine. The variability is due to variability in 2-hydroxylation, whereas differences in N-demethylation mostly contribute to the interindividual differences in the desipramine/imipramine plasma ratio [3].

The sparteine/debrisoquine oxidation polymorphism is a major determinant of interindividual differences in the 2-hydroxylation of imipramine and of desipramine [4,5], whereas N-demethylation is partially determined by mephenytoin oxidation polymorphism [6]. Imipramine has therefore become an important model drug for studying both genetic and environmental influences in drug metabolism.

Zeugin *et al.* [7] developed a high-performance liquid chromatographic (HPLC) method for the determination of imipramine and seven of its me-

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tabolites in human liver microsomes. However, the procedure for sample pretreatment was not applicable to plasma and urine samples. Therefore, we developed a sensitive and reproducible HPLC method for simultaneous quantitation of imipramine, desipramine, didesmethylimipramine, 2- and 10-hydroxyimipramine and 2- and 10-hydroxydesipramine in human plasma and urine with and without enzyme treatment in order to assess the separate pathways of imipramine metabolism in humans *in vivo*. To our knowledge, a similar method has not previously been published. The method has been applied to the analysis of samples following oral doses of imipramine and desipramine to healthy volunteers.

EXPERIMENTAL

Chemicals and reagents

Imipramine hydrochloride, desipramine hydrochloride, didesmethylimipramine hydrochloride, 2-hydroxyimipramine hydrochloride, 10-hydroxyimipramine free base, 2-hydroxydesipramine oxalate, 10-hydroxydesipramine fumarate and 2-hydroxydesmethylclomipramine free base were kindly supplied by Ciba-Geigy (Basle, Switzerland). Stock solutions were prepared in ethanol (96%) and were stored at −20°C. Chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany). β-Glucuronidase/arylsulphatase was purchased from Boehringer (Mannheim, Germany). Acetonitrile, *tert.*-butylmethyl ether and *n*-butanol were of HPLC grade and obtained from Merck. Heptane was of HPLC grade and purchased from Rathburn (Walkerburn, UK). Water was purified by osmosis and distillation. The samples were eluted with a mixture of 30% acetonitrile and 70% aqueous sodium perchlorate solution pH 2.5; the aqueous sodium perchlorate solution was prepared by addition of 14.05 g of sodium perchlorate and 1.6 ml of 60% perchloric acid to 5000 ml of water. The aqueous solution was filtered through a Millipore filter (0.45 μm) and the eluent was degassed prior to use.

Apparatus

The HPLC system consisted of Hitachi instruments (Hitachi, Tokyo, Japan): an AS-2000 autosampler with a 100-μl injector loop, a T-6300 column thermostat, an L-6200 intelligent pump and an L-4250 UV–VIS detector with variable wavelength. The system was controlled through a D-6000 HPLC interface module and a personal computer (IBM). The column was an RP-phenyl column (Nucleosil, 5 μm, 100 Å, 250 mm × 4 mm I.D., Macherey-Nagel, Düren, Germany) equipped with a guard column (Nucleosil, 7 μm, 120 Å, 20 mm × 4 mm I.D., Macherey-Nagel). The elution was carried out at a flow-rate of 1.0 ml/min and a column temperature of 30°C. The column effluent was quantified at the wavelength of 220 nm.

Sample pretreatment

Deconjugation. In order to determine total concentrations in urine, including glucuronide conjugates, the urines were enzyme-treated with β-glucuronidase/arylsulphatase prior to extraction: to 0.5 ml of urine in a 10-ml glass test tube, 470 μl of 0.2 *M* potassium dihydrogenphosphate plus 3% L-(+)-ascorbic acid and 30 μl of β-glucuronidase/arylsulphatase were added. The mixture was vortex-mixed for 2 s. Incubation was performed in capped tubes at 37°C for 16 h in a water bath. To the mixture were added 50 μl of a 2 *M* sodium hydroxide solution. The mixture was then extracted according to the following procedure.

Extraction procedure. To 1.0 ml of plasma or urine (or the enzyme-treated urine mixture) in a 10-ml glass test tube, 1 ml of a 0.6 *M* potassium carbonate solution (pH 11.3), 100 μl of a 2-hydroxydesmethylclomipramine solution in ethanol (5 and 20 μ*M* for plasma and urine samples, respectively) as an internal standard and 5 ml of heptane–*tert.*-butylmethyl ether (1:1, v/v) plus 5% *n*-butanol were added. The mixture was vortex-mixed for 1 min and centrifuged for 10 min at 1400 *g*. The test-tube was maintained at −50°C (ethanol bath) until the aqueous layer was frozen. The organic layer was transferred into a test tube containing 1 ml of a 0.02 *M* hydrochloric acid

solution. The mixture was vortex-mixed for 1 min and centrifuged for 10 min at 1400 *g* and then frozen. The organic layer was discarded, the aqueous layer was thawed and made alkaline (pH 11) after 0.5 ml of a 0.6 *M* potassium carbonate solution (pH 11.3) was added. Subsequently, 3 ml of heptane–*tert.*-butylmethyl ether (1:1, v/v) plus 5% *n*-butanol were added. The mixture was vortex-mixed for 1 min and centrifuged for 10 min at 1400 *g* and frozen. The organic layer was transferred to a conical glass test tube and evaporated to dryness at 50°C under a stream of nitrogen. The residue was dissolved in 100 μl of eluent, vortex-mixed for 5 s and centrifuged for 1 min at 1400 *g*. A 20-μl aliquot was injected onto the column.

RESULTS AND DISCUSSION

Selectivity

As shown in Fig. 1B, baseline separation of imipramine, the metabolites and the internal standard was achieved with the applied conditions. About 18 min were required for the analysis. The retention times were 5.93, 6.55, 7.37, 8.25, 10.02, 12.01, 14.32 and 16.62 min for 10-hydroxydesipramine, 10-hydroxyimipramine, 2-hydroxydesipramine, 2-hydroxyimipramine, 2-hydroxydesmethylclomipramine, didesmethylimipramine, desipramine and imipramine, respectively. No interference from impurities produced by the plasma, urine or the additives from

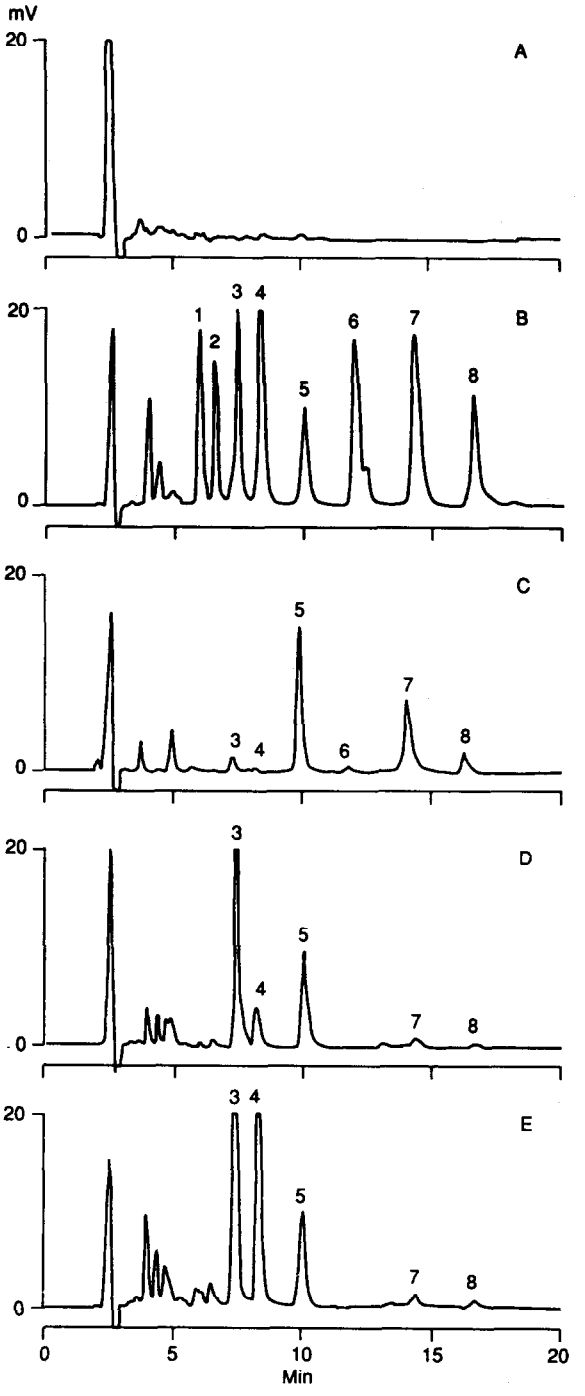

Fig. 1. (A) Chromatogram of blank, enzyme-treated urine. (B) Chromatogram of enzyme-treated urine, spiked to 10 μmol/l with imipramine and metabolites; 100 μl of 20 μmol/l **5** were added to the sample before extraction. (C) Chromatogram of a 1-ml plasma extract from a patient receiving an oral dose of 175 mg of imipramine per day containing 60 nmol/l **3**, 15 nmol/l **4**, 30 nmol/l **6**, 640 nmol/l **7** and 180 nmol/l **8**; 100 μl of 5 μmol/l **5** were added to the sample before extraction. (D) Chromatogram of a 1-ml urine extract, without enzyme treatment, from a healthy volunteer receiving an oral dose of 25 mg of imipramine containing 5.7 μmol/l **3**, 0.6 μmol/l **4**, 0.3 μmol/l **7** and 0.3 μmol/l **8**; 100 μl of 20 μmol/l **5** were added to the sample before extraction. (E) Chromatogram of a 0.5-ml enzyme-treated urine extract from the same volunteer as in D containing 14.0 μmol/l **3**, 12.3 μmol/l **4**, below 1 μmol/l **7** and **8**; 100 μl of 20 μmol/l **5** were added to the sample before extraction. Peaks: **1** = 10-hydroxydesipramine; **2** = 10-hydroxyimipramine; **3** = 2-hydroxydesipramine; **4** = 2-hydroxyimipramine; **5** = 2-hydroxydesmethylclomipramine; **6** = didesmethylimipramine; **7** = desipramine; **8** = imipramine.

the sample preparation were detected at the detection wavelength (220 nm). However, in chromatograms obtained from enzyme-treated, spiked urine there were peaks, due to artefacts, that interfered with the integration of didesmethylimipramine and 10-hydroxydesipramine peaks. These artefacts may be produced during the deconjugation procedure. Typical chromatograms for enzyme-treated, blank urine (A), enzyme-treated, spiked urine (B), patient plasma (C), urine, without enzyme treatment, from a healthy volunteer (D) and for enzyme-treated urine from the same volunteer (E) are shown in Fig. 1.

Recovery

The absolute recovery of each compound was assessed ($n = 10$) at five concentration levels by comparing the peak area after extraction with the peak area obtained from direct injection of equivalent quantities of pure standard. The five concentration levels were 0.05, 0.25, 0.50, 1.0 and 2.0 μmol/l for plasma and 0.5, 1.0, 5.0, 12.5 and 25.0 μmol/l for urine (with and without enzyme treatment). The recoveries in per cent are shown in Table I. It appears that all compounds showed some loss during the enzyme treatment procedure.

The recovery of the internal standard was 79% in plasma, 64% in enzyme-treated urine and 72% in untreated urine.

The recovery of 10-hydroxydesipramine from enzyme-treated urine was 147, 81, 63, 42 and 47% at the concentration levels 0.5, 1.0, 5.0, 12.5 and 25.0 μmol/l, respectively. Otherwise there was no trend in the relationship between recovery and concentration.

Linearity

The linearity of detector response to different concentrations of each compound was determined at plasma concentrations of 0.05, 0.25, 0.50, 1.0 and 2.0 μmol/l and at urine (with and

TABLE I

RECOVERY OF IMIPRAMINE (IP), DESIPRAMINE (DMI), 2-HYDROXYIMIPRAMINE (2-OHIP), 10-HYDROXYIMIPRAMINE (10-OHIP), 2-HYDROXYDESIPRAMINE (2-OHDMI), 10-HYDROXYDESIPRAMINE (10-OHDMI) AND DIDESMETHYLIMIPRAMINE (DDMI) IN PLASMA AND URINE WITH AND WITHOUT ENZYME TREATMENT

The analysed concentration levels were 0.05, 0.25, 0.50, 1.0 and 2.0 μmol/l in plasma and 0.5, 1.0, 5.0, 12.5 and 25.0 μmol/l in urine.

Compound		Recovery (%)		
		Plasma	Urine	
			Without enzyme treatment	With enzyme treatment
10-OHDMI	Mean	89	98	76
	Range	80–115	93–109	42–147
10-OHIP	Mean	97	89	71
	Range	92–105	79–102	49–109
2-OHDMI	Mean	75	90	74
	Range	64–81	84–98	66–84
2-OHIP	Mean	95	96	83
	Range	90–101	87–104	75–94
DDMI	Mean	68	77	53
	Range	55–78	58–96	40–65
DMI	Mean	96	89	67
	Range	89–106	83–96	57–74
IP	Mean	96	86	67
	Range	88–109	80–92	58–72

without enzyme treatment) concentrations of 0.5, 1.0, 5.0, 12.5 and 25.0 μmol/l for each compound. The standard curves for the seven compounds were linear over the investigated concentration range, when peak-area ratios (compound/internal standard), *y*-axis, were plotted against concentration, *x*-axis, and applied to a least-squares regression equation. Regression correlation data, *y*-intercept, slope and correlation coefficient for each compound are given in Table II.

Prior to analysis of unknown samples, calibration curves were prepared of three standard levels covering the expected concentration range. The linear calibration curves were fitted through the data point by linear regression.

The quantitative analysis of an unknown sample was derived with reference to the internal standard.

Repeatability

The intra-day repeatability of the method was evaluated by repeated analysis ($n = 10$) of samples of plasma and urine. Five concentrations of imipramine and the six metabolites were investigated, 0.05, 0.25, 0.50, 1.0 and 2.0 μmol/l in plasma and 0.5, 1.0, 5.0, 12.5 and 25.0 μmol/l in urine with and without enzyme treatment. In addition, the concentration levels 0.05 and 0.10 μmol/l in untreated urine were analysed. The coefficients of variation are shown in Table III. It was found

TABLE II

LINEARITY OF PEAK-AREA RATIO (COMPOUND/INTERNAL STANDARD), *y*, *VERSUS* CONCENTRATION, *x*, ASSESSED FOR IMIPRAMINE (IP), DESIPRAMINE (DMI), 2-HYDROXYIMIPRAMINE (2-OHIP), 10-HYDROXYIMIPRAMINE (10-OHIP), 2-HYDROXYDESIPRAMINE (2-OHDMI), 10-HYDROXYDESIPRAMINE (10-OHDMI) AND DIDESMETHYLIMIPRAMINE (DDMI)

The concentrations were 0.05, 0.25, 0.50, 1.0 and 2.0 μmol/l in plasma and 0.5, 1.0, 5.0, 12.5 and 25.0 μmol/l in urine (with and without enzyme treatment). The table shows *y*-intercept, slope and correlation coefficient, *r*, from the least-squares regression analysis of each compound.

Compound	Parameter	Plasma	Urine	
			With enzyme treatment	Without enzyme treatment
10-OHDMI	*y*-intercept	0.055	−0.01	0.01
	Slope	0.002	0.62	0.10
	r	0.999	0.999	0.997
10-OHIP	*y*-intercept	0.040	0.21	0.03
	Slope	0.002	0.51	0.08
	r	0.999	0.999	0.997
2-OHDMI	*y*-intercept	0.003	0.005	−0.08
	Slope	0.002	0.65	0.27
	r	0.999	0.999	0.999
2-OHIP	*y*-intercept	0.025	0.11	0.03
	Slope	0.002	0.62	0.25
	r	0.999	0.999	0.998
DDMI	*y*-intercept	−0.026	−0.31	−0.13
	Slope	0.002	0.68	0.25
	r	0.999	0.999	0.999
DMI	*y*-intercept	0.013	−0.12	−0.08
	Slope	0.002	0.71	0.31
	r	0.999	0.999	0.999
IP	*y*-intercept	0.017	−0.16	−0.06
	Slope	0.002	0.67	0.37
	r	0.999	0.999	0.999

that the coefficients of variation decreased with increasing concentration.

Reproducibility

Inter-day reproducibility was assessed for the five following days at three concentration levels: 75, 150 and 225 nmol/l in plasma and 1.0, 4.0 and 15.0 μmol/l in urine. The samples were drawn from the same pool. The coefficients of variation are shown in Table IV. The reproducibility of enzyme-treated urine was lower than that of untreated urine.

Limit of detection and determination

The limit of detection, based on a signal-to-noise ratio of 3:1, was 5 nmol/l for each compound in plasma and 10 nmol/l in urine without enzyme treatment. In enzyme-treated urine, the detection limit for the hydroxy metabolites was 100 nmol/l owing to interfering peaks, and for imipramine, desipramine and didesmethylimipramine the limit was 30 nmol/l. The limit of determination based on a signal-to-noise ratio of 10:1 was 15 nmol/l for plasma samples and 50 nmol/l for untreated urine. In enzyme-treated urine, the limit was 1.0 μmol/l for the hydroxy metabolites and 100 nmol/l for didesmethylimipramine, desipramine and imipramine.

Accuracy

Plasma and urine spiked with imipramine and the six metabolites to 75, 150 and 225 nmol/l in plasma and to 1.0, 4.0 and 15.0 μmol/l in urine were analysed once a day for five days. The mean estimate and deviation from the spiked value are shown in Table IV. The determined concentra-

TABLE III

INTRA-DAY REPEATABILITY OF IMIPRAMINE (IP), DESIPRAMINE (DMI), 2-HYDROXYIMIPRAMINE (2-OHIP), 10-HYDROXYIMIPRAMINE (10-OHIP), 2-HYDROXYDESIPRAMINE (2-OHDMI), 10-HYDROXYDESIPRAMINE (10-OHD-MI) AND DIDESMETHYLIMIPRAMINE (DDMI)

Repeatability was assessed by repeated analysis (n = 10) of the concentration levels 0.05, 0.25, 0.50, 1.0 and 2.0 μmol/l in plasma and 0.5, 1.0, 5.0, 12.5 and 25.0 μmol/l in urine with and without enzyme treatment. In addition, the levels 0.05 and 0.10 μmol/l in urine without enzyme treatment were analysed.

Compound		Coefficient of variation (%)		
		Plasma	Urine	
			With enzyme treatment	Without enzyme treatment
10-OHDMI	Mean	5.8	3.3	4.5
	Range	4.2–8.7	1.8–4.7	1.6–10.5
10-OHIP	Mean	6.9	4.3	5.8
	Range	3.0–12.0	1.6–9.5	2.0–16.7
2-OHDMI	Mean	6.0	4.1	2.3
	Range	3.4–9.5	1.1–7.2	1.0–4.0
2-OHIP	Mean	5.3	4.0	3.6
	Range	2.6–7.8	1.5–7.5	1.1–7.8
DDMI	Mean	9.0	8.2	6.2
	Range	6.3–17.9	2.0–15.3	1.0–16.1
DMI	Mean	7.4	4.3	5.5
	Range	4.7–12.4	1.9–7.0	2.2–11.8
IP	Mean	8.7	7.5	7.7
	Range	3.2–17.5	2.5–10.2	3.9–11.7

TABLE IV

REPRODUCIBILITY AND ACCURACY OF IMIPRAMINE (IP), DESIPRAMINE (DMI), 2-HYDROXYDESIPRAMINE (2-OHIP), 10-HYDROXYIMIPRAMINE (10-OHIP), 2-HYDROXYDESIPRAMINE (2-OHDMI), 10-HYDROXYDESIPRAMINE (10-OHDMI) AND DIDESMETHYLIMIPRAMINE (DDMI)

Samples from the same pool were analysed once a day for five days. The table shows mean, standard deviation (S.D.), coefficient of variation (C.V.) and deviation from spiked value.

Compound		Plasma			Urine					
		75 nmol/l	150 nmol/l	225 nmol/l	Without enzyme treatment			With enzyme treatment		
					1.0 μmol/l	4.0 μmol/l	15.0 μmol/l	1.0 μmol/l	4.0 μmol/l	15.0 μmol/l
10-OHDMI	Mean	73	155	256	1.05	4.03	14.37	1.7	5.1	16.6
	S.D.	8	10	7	0.03	0.12	0.56	0.6	0.4	1.6
	C.V. (%)	11	6	3	3	3	4	35	8	10
	Deviation (%)	3	3	14	5	1	4	63	28	13
10-OHIP	Mean	75	154	238	1.22	4.25	14.62	1.1	4.5	16.4
	S.D.	6	5	10	0.05	0.12	0.55	0.1	0.4	1.7
	C.V. (%)	8	3	4	4	3	4	14	9	10
	Deviation (%)	0	2	6	22	6	3	2	17	13
2-OHDMI	Mean	73	155	247	1.02	4.23	14.70	1.0	4.2	14.9
	S.D.	6	6	11	0.05	1.10	0.70	0.1	0.2	0.7
	C.V. (%)	8	4	5	5	2	5	13	5	5
	Deviation (%)	1	3	10	2	6	2	7	5	2
2-OHIP	Mean	72	151	226	1.13	3.84	14.15	1.2	4.3	14.8
	S.D.	7	9	10	0.06	0.12	0.29	0.1	0.2	0.5
	C.V. (%)	10	6	5	5	3	2	9	4	3
	Deviation (%)	3	1	0.4	13	4	6	18	8	0.2
DDMI	Mean	72	151	233	0.83	3.71	13.94	1.42	3.95	15.32
	S.D.	7	13	11	0.08	0.20	0.45	0.17	0.44	1.66
	C.V. (%)	10	8	5	9	5	3	12	11	11
	Deviation (%)	5	1	4	17	7	7	35	2	4
DMI	Mean	66	149	223	0.99	4.09	15.40	1.20	4.13	14.77
	S.D.	8	8	14	0.11	0.33	0.94	0.20	0.21	0.99
	C.V. (%)	12	6	6	11	8	6	17	5	7
	Deviation (%)	13	1	1	1	2	3	10	4	0.2
IP	Mean	72	158	224	0.99	4.22	14.59	1.19	4.17	14.39
	S.D.	6	12	23	0.17	0.38	1.05	0.31	0.32	1.32
	C.V. (%)	9	8	11	17	9	7	26	8	9
	Deviation (%)	4	5	1	1	6	3	10	4	3

tions of the samples of plasma and urine without enzyme treatment were in accordance with the spiked value, However, the 10-hydroxydesipramine and didesmethylimipramine were overestimated in samples of enzyme-treated urine at 1.0 μmol/l.

CONCLUSION

This study describes and isocratic, reversed-phase HPLC method developed for simultaneous quantitation of imipramine and six metabolites in plasma and urine. The three-step extraction procedure provides a good overall recovery of all compounds in plasma and urine without enzyme treatment. The lower recovery of enzyme-treated urine may thus be the result of the deconjugation procedure, though the results were reproducible. Accurate determination was performed down to 20 nmol/l in plasma, 50 nmol/l in untreated urine and 1.0 μmol/l in enzyme-treated urine, except for 10-hydroxydesipramine and didesmethylimipramine, for which it was 4.0 μmol/l. Imipramine N-oxide was not included as the metabolite was lost during sample pretreatment. This method is suitable for studies of imipramine pharmacokinetics and metabolism in humans *in vivo*.

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