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Determination of metyrapone and the enantiomers of its chiral metabolite metyrapol in human plasma and urine using coupled achiral–chiral liquid chromatography

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Abstract

A coupled achiral–chiral liquid chromatographic assay has been developed to determine the concentrations of metyrapone and the enantiomers of its chiral metabolite metyrapol in plasma and urine. The chromatographic system consisted of a silica precolumn (75 × 4.6 mm I.D.) coupled in-line to a 250 × 4.6 mm I.D. column containing cellulose tris(4-methylbenzoate) coated on silica gel (Chiralcel OJ-CSP). When plasma samples were analyzed, the mobile phase was hexane–ethanol (92:8, v/v) modified with 0.1% diethylamine and when urine samples were analyzed the mobile phase was hexane–ethanol (94:6, v/v) modified with 0.2% diethylamine. Under these chromatographic conditions the chromatographic retentions [expressed as capacity factors (k')] for metyrapone were $k' = 2.35$ (plasma) and 2.52 (urine); for (−)-metyrapol $k' = 4.22$ (plasma) and 4.62 (urine); for (+)-metyrapone $k' = 5.16$ (plasma) and 5.86 (urine); enantioselectivities (α) were 1.09 (plasma) and 1.13 (urine). The assay has been validated for use in metabolic studies. The analyses of plasma and urine samples from one subject following oral administration of 750 mg of metyrapone indicated that the enzymatic reduction of myterapone by aldo–keto reductase was enantiospecific.

1. Introduction

Aldo–keto reductases are a class of drug-metabolizing enzymes which are widely distributed among tissues. These enzymes have a broad substrate specificity including a number of therapeutically-important compounds such as anthracycline anticancer agents, warfarin and naloxone [1]. Metyrapone (MET, Fig. 1), a steroid biosynthesis inhibitor, is also an aldo–keto reductase substrate [1].

MET contains a prochiral ketone moiety and metabolism by aldo–keto reductase produces the enantiomeric metabolite metyrapol (MET-OL) (Fig. 1). The enantiospecificity of the enzymatic reduction of MET in humans has been previously investigated [2]. In this study, pooled urines from patients dosed with MET were incubated with β-glucuronidase, the MET-OL extracted, concentrated and purified. The optical rotation of the isolated MET-OL was measured and no optical activity was found. The authors concluded that the reduction of MET by aldo–keto reductase was not enantiospecific.

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As part of a study designed to determine whether MET can be used as a probe of hepatic aldo–keto reductase activity, it was decided to develop an enantioselective assay for MET-OL and to reinvestigate the enantiospecificity of this reduction. A large number of liquid chromatographic methods have been reported for the determination of MET, MET-OL and the microsomally produced mono-N-oxides of MET [3–8]. However, none of the reported assays were able to enantioselectively resolve (−)- and (+)-MET-OL.

This manuscript reports the development and validation of an enantioselective liquid chromatographic assay for the determination of MET and MET-OL in plasma and urine. The method is based upon a coupled achiral–chiral HPLC system where a precolumn containing a silica-gel stationary phase is used to separate MET and MET-OL and a column containing a chiral stationary phase based upon cellulose tris(4-methylbenzoate) coated on silica gel (Chiralcel OJ-CSP) is used to enantioselectively resolve (−)- and (+)-MET-OL. This assay has been used to analyze plasma and urine samples from a volunteer who ingested MET. The initial results indicate that in this subject the aldo–keto reductase reduction of MET was enantiospecific and favored the formation of (−)-MET-OL.

2. Experimental

2.1. Chemicals

Metyrapone (MET) and oxprenolol (internal standard) were purchased from Sigma (St. Louis, MO, USA). Analytical grade methanol, diethyl ether and dichloromethane, hexane, and ethanol were purchased from Anachemia (Montreal, Que., Canada).

Racemic metyrapol (*rac*-MET-OL) was synthesized by sodium borohydride reduction of MET. The resulting alcohol was purified by distillation at reduced pressure and characterized by IR, ^{1}H-NMR and mass spectral analyses. The enriched enantiomers of MET-OL were obtained by enantioselective chromatography on the HPLC system described below. The optical activities of the separate enantiomers were determined using a Jasco DIP-140 Digital Polarimeter (Japan Spectroscopic Company, Tokyo, Japan).

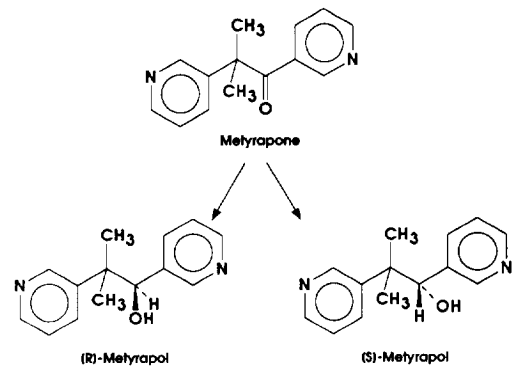

Fig. 1. The structures of metyrapone and the enantiomers of metyrapol.

2.2. Apparatus

The chromatographic system consisted of a Spectrasystem P2000 pump, a Spectrasystem AS3000 autosampler and a Spectra 100 UV detector, all from Spectra Physics (San Jose, CA, USA). The UV detector was set at $\lambda = 261$ nm.

The column configuration consisted of a 75 × 4.6 mm I.D. Ultremex 3 Silica pre-column (Phenomenex, Torrance, CA, USA) and a 250 × 4.6 mm I.D. column packed with cellulose tris(4-methylbenzoate) coated on a 10-μm silica-gel support (Chiralcel OJ-CSP, Chiral Technologies, Exton, PA, USA).

2.3. Chromatographic conditions

For analysis of the plasma samples, the mobile phase was hexane–ethanol (92:8, v/v) modified with 0.1% diethylamine. For analysis of the urine samples, the mobile phase was hexane–ethanol (94:6, v/v) with 0.2% diethylamine. A flow-rate of 1.0 ml/min and ambient temperature were used throughout the study.

2.4. Stock solutions

Stock solutions of MET (10.00 μg/ml), *rac*-MET-OL (15.40 μg/ml), and oxprenolol (249.00 μg/ml) were prepared in methanol. Serial dilutions of 8.00, 4.00, 2.00 and 1.00 μg/ml of the MET solution were prepared. Similarly, *rac*-MET-OL solutions of 7.70, 3.85, 1.92 and 0.96 μg/ml were prepared.

2.5. Sample preparation

Aliquots (100 μl) of the appropriate MET and *rac*-MET-OL stock solutions were placed in a culture tube; the tubes were placed in a water bath kept at 40°C and the solution evaporated to dryness under a stream of nitrogen. A 1-ml volume of either plasma or urine was added to the tube containing the dried analytes and the solution was vortex-mixed for 15 s.

To spiked and clinical samples were added 100 μl of 1 *M* NaOH, 250 mg of NaCl, and 100 μl of the oxprenolol solution. The resulting mixture was vortex-mixed for 30 s, 5 ml of a diethylether–dichloromethane (80:20, v/v) solution were added, the mixture vortex-mixed for 2 min and then centrifuged at 1500 *g* for 20 min. The culture tube was placed in a dry ice–acetone bath until the aqueous phase was frozen. The organic phase was decanted into a culture tube; the tube was placed in a water bath kept at 40°C and evaporated to dryness under a stream of nitrogen.

Plasma

The dried analytes were reconstituted using 500 μl of the mobile phase. A 125-μl aliquot was then placed in a 250-μl autosampler vial and a 100-μl aliquot was then injected onto the chromatographic system.

Urine

The dried analytes were reconstituted with 1 ml of distilled water, 100 μl of 1 *M* HCl were added and the resulting solution vortex-mixed for 30 s; 5 ml of a hexane–diethylether (50:50, v/v) solution were added and the resulting mixture vortex-mixed for 1 min and centrifuged at 1500 *g* for 10 min. The culture tube was placed in a dry ice–acetone bath until the aqueous phase was frozen and the organic phase was discarded. After the aqueous phase had thawed, 200 μl of 1 *M* NaOH and 250 mg of NaCl were added, the mixture vortex-mixed for 30 s and 5 ml of a diethylether–dichloromethane (80:20, v/v) solution were added. The resulting mixture was vortex-mixed for 2 min, placed in a dry ice–acetone bath until the aqueous phase was frozen, the organic phase was collected and evaporated to dryness as previously described. The dried analytes were reconstituted using 500 μl of the mobile phase and a 125-μl aliquot placed in a 250-μl autosampler vial and a 100-μl aliquot injected onto the chromatographic system.

2.6. Validation studies

Standard curve

Using the solutions of MET, spiked plasma and urine samples containing 0.10, 0.20, 0.40, 0.80 and 1.00 μg/ml were prepared. Similarly, spiked plasma and urine samples of 0.10, 0.19, 0.39, 0.77 and 1.54 μg/ml of *rac*-MET-OL or 0.05, 0.10, 0.19, 0.39 and 0.77 μg/ml of each enantiomer were prepared. Five samples were prepared for each concentration. Calibration curves plotting analyte plasma concentration as a function of the analyte/internal-standard peak-area ratios were derived for MET and each MET-OL enantiomer for both plasma and urine.

Recovery

The recovery of MET was determined using spiked plasma and urine samples at 0.10, 0.40, and 1.00 μg/ml and the recovery of MET-OL was tested at 0.048, 0.19 and 0.77 μg/ml for each enantiomer. The peak-area ratios of four extracted samples of the analyte were compared to two unextracted samples to derive a percent recovery.

Accuracy and precision

The same three concentrations which were used for the recovery experiments were used to test inter-day and intra-day variability. Five

samples at each concentration were extracted on each of four consecutive days.

The accuracy of the method was tested using blinded unknowns which were prepared by a different analyst.

2.7. Human study

A single 750-mg dose of metyrapone (CIBA, Summit, NJ, USA) was administered orally to a volunteer. Blood samples were taken at 0, 2 and 4 h after ingestion using green-top, heparinized Vacutainer collection tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ, USA). The

Table 1
Chromatographic parameters for metyrapone and the enantiomers of metyrapol

Parameter	OJ-CSP standards	Silica-OJ plasma	Silica-OJ urine
$k'_{\text{metyrapone}}$	2.35	3.02	3.48
$k'_{(-)\text{-metyrapol}}$	2.52	4.22	5.16
$k'_{(+)\text{-metyrapol}}$	2.98	4.62	5.86
$\alpha_{(+)/(-)}$	1.18	1.09	1.13
$R_{S(+)/(-)}$	1.08	1.06	1.72

Chromatographic retention expressed as capacity factor (k'), enantioselectivity ($\alpha_{(+)/(-)}$) and enantioselective resolution ($R_{S(+)/(-)}$)

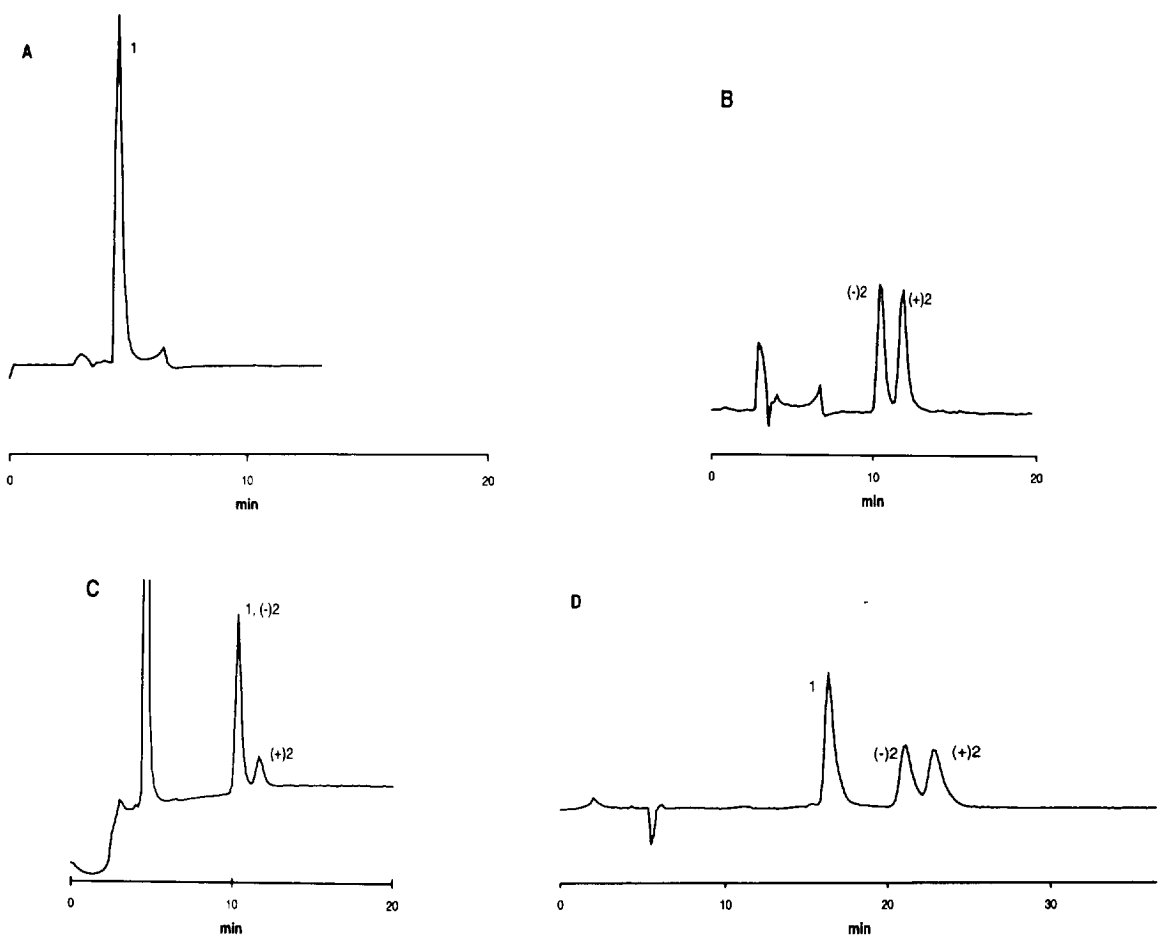

Fig. 2. Representative chromatograms from chromatography of metyrapone and the enantiomers of metyrapol on the OJ-CSP and the coupled silica/OJ-CSP liquid chromatographic systems. (A) Metyrapone on the OJ-CSP (4 ng on column); (B) *rac*-metyrapol on the OJ-CSP (2 ng of each enantiomer on column); (C) metyrapone and *rac*-metyrapol on the OJ-CSP; (D) metyrapone and *rac*-metyrapol on the coupled silica/OJ-CSP system. Peaks: 1 = metyrapone; (−)-2 = (−)-metyrapol; (+)-2 = (+)-metyrapol.

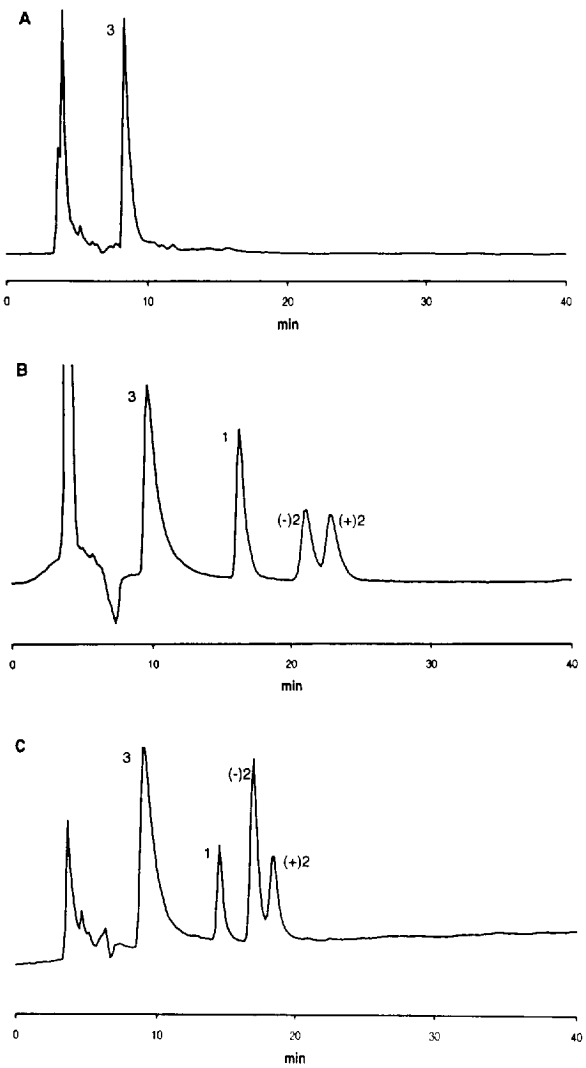

Fig. 3. Representative chromatograms of plasma samples on the coupled silica/OJ-CSP system. (A) Plasma spiked with internal standard; (B) plasma spiked with 0.40 μg/ml metyrapone and 0.39 μg/ml *rac*-metyrapol; (C) plasma sample obtained from a human volunteer 2 h after the oral administration of 750 mg metyrapone. Peaks: 1 = metyrapone; (−)-2 = (−)-metyrapol; (+)-2 = (+)-metyrapol; 3 = internal standard oxprenolol.

tubes were centrifuged at 800 *g* for 20 min, the plasma collected and stored at −20°C until analysis.

Urine samples were taken at 0, 4, 8, 12, and 24 h after ingestion. The urine was frozen at −20°C until analysis.

3. Results and discussion

3.1. Chromatographic results

Under the chromatographic conditions used in this study, MET eluted with a chromatographic retention (expressed as capacity factor, k') of 2.35 and *rac*-MET-OL was enantioselectively resolved on the OJ-CSP (Table 1, Figs. 2A,B, respectively). The enantiomeric elution order of (−)-MET-OL and (+)-MET-OL was determined by chromatographing an enantiomerically enriched sample. However, when MET and *rac*-MET-OL were chromatographed together a significant overlap occurred between MET and (−)-MET-OL making it impossible to analyze the plasma or urine sample directly on the OJ-CSP (Fig. 2C). This problem was overcome by placing a silica precolumn in front of the OJ-CSP. The resulting in-line coupled achiral–chiral system produced clean chromatograms without a significant effect on the enantioselective separation (Table 1, Fig. 2D).

3.2. Assay of plasma samples

The chromatograms resulting from the analysis of blank plasma and plasma spiked with MET

Table 2
Recovery of analytes from plasma and urine

Concentration (μg/ml) (μg/ml)	Recovery (mean ± S.D.) (%)	
	Plasma (n = 4)	Urine (n = 3)
Metyrapone		
0.1	80.6 ± 11	90.1 ± 10
0.4	77.3 ± 12	71.0 ± 1.8
1.0	100 ± 3.5	64.7 ± 1.4
(−)-Metyrapol		
0.05	96.2 ± 7.1	96.2 ± 11
0.19	86.6 ± 5.6	88.8 ± 3.3
0.77	89.4 ± 2.7	85.2 ± 1.4
(+)-Metyrapol		
0.04	73.1 ± 7.0	107 ± 2.0
0.19	84.5 ± 7.8	85.5 ± 2.0
0.77	90.0 ± 3.1	78.1 ± 1.1

Table 3
Intra- and inter-day variabilities for the assay of metyrapone and (+)- and (−)-metyrapol in plasma and urine

Concentration (μg/ml)	Coefficient of variation (%)			
	Plasma		Urine	
	Single day ($n = 5$)	Four days ($n = 20$)	Single day ($n = 5$)	Four days ($n = 20$)
Metyrapone				
0.10	11	13	13	12
0.40	7.2	7.8	12	9.5
1.00	5.0	4.8	2.7	9.3
(−)-Metyrapol				
0.05	10	12	17	18
0.19	3.8	6.8	2.8	7.9
0.77	4.4	4.0	1.4	4.1
(+)-Metyrapol				
0.05	7.2	13	15	16
0.19	3.9	6.3	5.5	8.9
0.77	4.4	3.4	1.2	3.9

and *rac*-MET-OL are presented in Fig. 3A,B, respectively.

Standard curves were derived for MET and both MET-OL enantiomers with the following regression equations and correlation coefficients (r): MET: $y = 2.603x + 0.013$, $r = 0.9984$; (−)-MET-OL: $y = 2.635x + 0.008$, $r = 0.9990$; (+)-MET-OL: $y = 2.460x + 0.013$,; $r = 0.9998$.

Two blinded samples containing unknown concentrations of all three analytes produced accuracies 82–97%. The recoveries for MET averaged 90% over the three concentrations. For (−)-MET-OL the recoveries averaged 90.5% and for (+)-MET-OL were 82.5% (Table 2). The intra-day and inter-day variabilities are presented in Table 3.

Table 4
Analyte concentrations in plasma and urine in a subject given an oral dose of 750 mg of metyrapone

Time after dose (h)	Metyrapone (μg/ml)	(−)-Metyrapone (μg/ml)	(+)-Metyrapol (μg/ml)	Ratio (−/+)
Plasma				
2	0.22	1.30	0.57	2.2
4	0.03	0.24	0.12	1.9
Urine free				
0–4	0.97	8.1	3.40	2.4
4–8	0.20	0.53	0.28	1.9
8–12	0.13	0.44	0.24	1.9
12–24	0.03	0.14	0.06	2.5
Urine total				
0–4		11.00	6.60	1.6
4–8		13.00	9.80	1.3

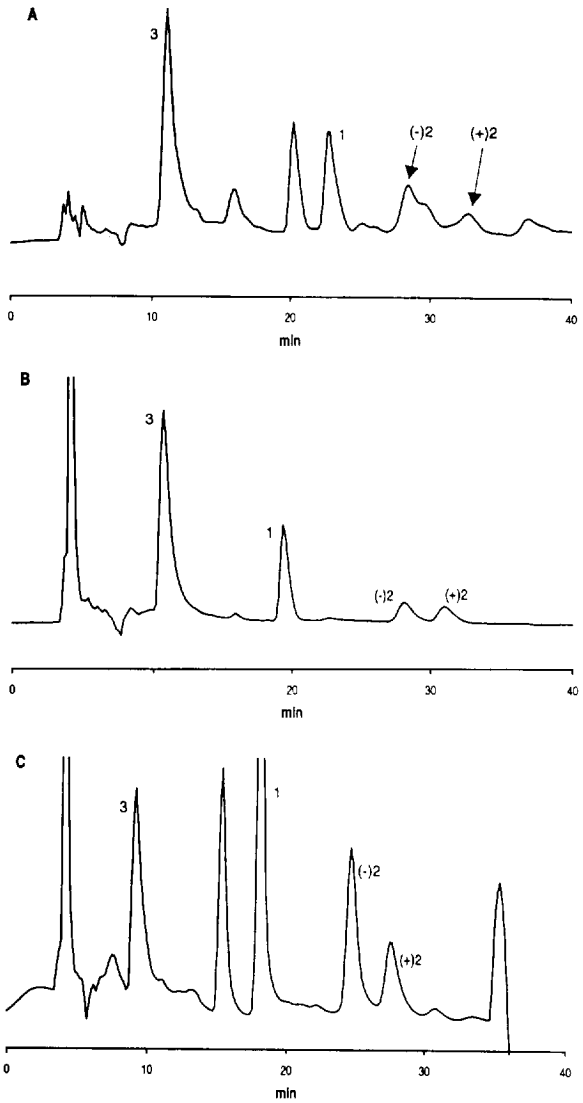

Fig. 4. Representative chromatograms of urine samples on the coupled silica/OJ-CSP system. (A) Urine spiked with 0.20 μg/ml metyrapone and 0.19 μg/ml *rac*-metyrapol after one extraction step; (B) urine spiked with 0.20 μg/ml metyrapone and 0.19 μg/ml *rac*-metyrapol after three extraction steps; (C) urine sample obtained from a human volunteer 0–4 h after the oral administration of 750 mg metyrapone. Peaks: 1 = metyrapone; (−)-2 = (−)-metyrapol; (+)-2 = (+)-metyrapol; 3 = internal standard oxprenolol.

3.3. Assay of urine samples

The achiral–chiral system developed for plasma was also used for samples extracted from urine. However, the single-extraction method used for plasma produced unusable chromatograms due to endogenous interferences (Fig. 4A). Two further extraction steps and a different mobile phase were required to produce satisfactory chromatograms (Table 1, Fig. 4B).

Standard curves were derived for MET and both MET-OL enantiomers with the following regression equations and correlation coefficients (r): MET: $y = 2.920x + 0.025$, $r = 0.9985$; (−)-MET-OL: $y = 3.373x + 0.0222$, $r = 0.9992$; (+)-MET-OL: $y = 3.438x - 0.026$,; $r = 0.9966$.

Two blinded samples produced accuracies of 84–112%. The recoveries for MET, (−)-MET-OL and (+)-MET-OL averaged 90% over the three concentrations (Table 2). The intra-day and inter-day variabilities are presented in Table 3.

3.4. Limits of detection and quantitation

The limits of detection for metyrapone, (−)-metyrapol and (+)-metyrapol in human plasma and human urine were 0.03 μg/ml for each solute. The limits of quantitation (LOQ) of metyrapone in human plasma and human urine were 0.10 μg/ml. The LOQs of (−)-metyrapol and (+)-metyrapol in human plasma and human urine were 0.05 μg/ml.

3.5. Human study

Chromatograms of a plasma and a urine sample obtained from a subject after the ingestion of MET are presented in Figs. 3C and 4C, respectively. The MET, (−)-MET-OL and (+)-MET-OL plasma concentrations 2 and 4 h after ingestion of MET and the corresponding concentrations in the urine at 4, 8, 12, and 24 h are presented in Table 4. At each time point and in both plasma and urine, the relative (−)-MET-OL/(+)-MET-OL ratios were close to 2:1. Deconjugation of the 0–4 h and 4–8 h urine samples with β-glucuronidase reduced the observed (−)-MET-OL/(+)-MET-OL ratios to 1.6:1 and 1.3:1, respectively.

The results from the human study indicate that the aldo–keto reductase mediated reduction of MET is enantiospecific, at least during the initial

metabolic clearance of the drug. However, glucuronidation appears to favor (+)-MET-OL. The consequences of these two processes on the observed enantiospecificity as well as the possibility that non-hepatic aldo–keto reductases reduce MET with different enantiospecificities is currently under investigation.

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