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ENANTIOSELECTIVE GAS CHROMATOGRAPHIC ASSAYS WITH ELECTRON-CAPTURE DETECTION FOR METHOXYPHENAMINE AND ITS THREE PRIMARY METABOLITES IN HUMAN URINE

N.R. SRINIVAS, J.W. HUBBARD, E.M. HAWES, G. McKAY and K.K. MIDHA*

College of Pharmacy, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0 (Canada)

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SUMMARY

Sensitive and enantioselective gas chromatographic assays have been developed and applied to the quantitation in human urine of the enantiomers of methoxyphenamine and its three primary oxidative metabolites, namely, N-desmethylmethoxyphenamine, O-desmethylmethoxyphenamine and 5-hydroxymethoxyphenamine. The separation of the various analytes was achieved through the combined use of high-resolution gas chromatography coupled with electron-capture detection and employing a capillary OV-225 column. The formation of diastereomeric derivatives involved the chiral acylating reagent N-heptafluorobutyryl-L-prolyl chloride. The assays for methoxyphenamine and O-desmethylmethoxyphenamine were linear over the range 0.25–2.0 μg/ml for each analytes' enantiomers, while in the case of the enantiomers for N-desmethylmethoxyphenamine and 5-hydroxymethoxyphenamine linearity was shown over the ranges 0.094–0.75 and 0.188–1.5 μg/ml, respectively. The mean coefficients of variation in all cases were less than 4%.

INTRODUCTION

Methoxyphenamine (MP), a bronchodilator, is metabolised in man and animals by routes which include three distinctly different primary oxidative pathways, namely, N-demethylation, O-demethylation and aromatic 5-hydroxylation, to form N-desmethylmethoxyphenamine (NDMP), O-desmethylmethoxyphenamine (ODMP) and 5-hydroxymethoxyphenamine (5HMP), respectively (Fig. 1A) [1–4]. The parent drug and its three primary metabolites have been quantitated by gas chromatographic (GC) methods using various detectors [5,6] in urine and plasma of man and animals dosed with MP. MP, 5HMP, NDMP and ODMP each has a chiral centre. Until now no enantioselective analytical methods have been reported for MP or any of its metabolites. The lack of sufficiently

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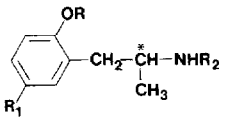

	R	R_1	R_2
MP	CH_3	H	CH_3
NDMP	CH_3	H	H
ODMP	H	H	CH_3
5HMP	CH_3	OH	CH_3

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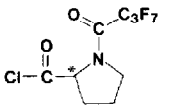

I-HPC

Fig. 1. Structures of (A) MP and its three primary metabolites (the asterisk indicates chiral center) and (B) the chiral acylating reagent, N-heptafluorobutyryl-L-prolyl chloride (L-HPC).

sensitive and selective procedures for the separation and quantitation of the individual isomers of MP and its metabolites has meant that enantioselectivity in the disposition of this drug has not been studied.

Stereoselectivity has been previously demonstrated in the disposition of certain drugs metabolised by the isozyme of cytochrome P_{450} which hydroxylates debrisoquine at the 4-position [7–11] including debrisoquine itself [12]. Given that studies on MP in different rat strains [13] and human phenotypes of debrisoquine type polymorphism [14,15] have demonstrated that O-demethylation and aromatic 5-hydroxylation, but not N-demethylation, are defective in human poor metabolisers of debrisoquine as well as their Dark Agouti rat model, it was of interest to examine the stereoselective disposition of this drug.

In this paper, enantioselective methods for the quantitation of MP and its three primary metabolites in urine samples were developed based on the use of high-resolution GC and the enhanced electron-capturing properties of the diastereomeric fluoroacylated derivatives of MP and its metabolites formed using N-heptafluorobutyryl-L-prolyl chloride (L-HPC).

EXPERIMENTAL

Materials

dl-MP·HCl was kindly donated by UpJohn Canada (Don Mills, Canada). NDMP, ODMP and 5HMP were synthesized in these laboratories [4]. Amantadine hydrochloride (AMD), *d*-ephedrine hydrochloride (*d*-E), heptafluorobutyric anhydride, L-proline, thionyl chloride and sodium tetraborate decahydrate

were purchased from Aldrich (Montreal, Canada). Glass-distilled grades of acetone, benzene, dichloromethane, ethyl acetate and *n*-pentane were purchased from BDH (Toronto, Canada). Sulfatase Type H-2 containing β-glucuronidase activity was obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals used were of analytical grade.

Stock solutions of *dl*-MP, *dl*-NDMP, *dl*-ODMP or internal standards (100 μg/ml) were prepared in distilled deionized water from their hydrochloride salts. In the case of the preparation of the stock solution of *dl*-5HMP (100 μg/ml), the free base was dissolved in a small amount of hydrochloric acid (0.1 *M*, 50 μl). All stock solutions were prepared every two months and stored at 4°C. L-HPC was prepared according to the reported procedure [16].

Instrumentation

Quantitative. A Model 5840A gas chromatograph was equipped with a ^{63}Ni electron-capture detection (ECD) system, a Model 5840A integrator (Hewlett Packard, Edmonton, Canada) and a 30 m × 0.33 mm I.D. OV-225 (0.25 μm) capillary column (Terochem Labs., Edmonton, Canada).

Qualitative. A VG-Micromass 7070HE mass spectrometric (MS) system (Manchester, U.K.) was interfaced via a single-stage glass jet separator to a Hewlett Packard 5790A gas chromatograph and was equipped with a VG11-250J data system.

Assay procedure

Extraction procedure for the analysis of the diastereomeric amide derivatives of MP and NDMP. Aliquots (1 or 2 ml) of urine samples (standards or from dosed volunteers) were dispensed into borosilicate glass tubes (16 × 125 mm) and spiked with the internal standard, AMD (1 μg/ml). Samples were mixed (Vortex Genie, Fisher Scientific, Edmonton, Canada) and 1.5 ml of saturated sodium tetraborate solution were then added (pH 9–9.5). Samples were mixed again followed by the addition of L-HPC (40 μl). The tubes were capped and the contents sequentially treated as follows: for 10 min on a rotating-type mixer set at 1600 (IKA Vibrax, VXR, Fisher Scientific), allowed to stand at 23°C for 45 min, 5 ml of *n*-pentane were added, mixed at the setting of 1400 for 10 min and centrifuged at 1720 *g* for 5 min at 4°C (TJ-6 Model centrifuge, Beckmann Instruments, Palo Alto, CA, U.S.A.). The organic layer was transferred into another clean tube and the solvent evaporated at 65°C. The residue was taken up in ethyl acetate and suitable aliquots were injected into the GC–ECD system.

Extraction procedure for the analysis of the diastereomeric amide derivatives of ODMP and 5HMP. Total concentrations of ODMP and 5HMP (non-conjugated plus conjugated) were determined by a similar procedure except that urine samples were initially treated with the sulfatase enzyme preparation. In this modified procedure, 0.2 ml of sodium acetate buffer (pH 5.2) and 40 μl of sulfatase enzyme were added to 0.5–2.0 ml of urine and incubated at 37 ± 1°C for 16 h. After incubation, *d*-E (500 ng/ml) was added as an internal standard and the pH of the mixture was adjusted to 9.5 by the addition of 100 μl of saturated sodium carbon-

ate solution and 1.0 ml of saturated sodium tetraborate buffer. Samples were mixed, L-HPC (40 μl) was added and then treated as for MP and NDMP.

Preparation of standards

Samples of N-heptafluorobutyryl-L-prolylamantadine (HFBP-AMD), N-heptafluorobutyryl-L-prolyl-*d*-ephedrine (HFBP-*d*-E), N-heptafluorobutyryl-L-prolyl-*dl*-methoxyphenamine (HFBP-MP), N-heptafluorobutyryl-L-prolyl-*dl*-N-desmethylmethoxyphenamine (HFBP-NDMP), N-heptafluorobutyryl-L-prolyl-*dl*-O-desmethylmethoxyphenamine (HFBP-ODMP) and N-heptafluorobutyryl-L-prolyl-*dl*-5-hydroxymethoxyphenamine (HFBP-5HMP) were prepared under the conditions described under *Assay procedure* except that pure samples of analytes were added to borate buffer. The residue of the derivatised analytes were taken up in ethyl acetate and suitable aliquots were examined by GC–ECD or GC–MS.

Analytical conditions

Separation of the diastereomeric amide derivatives of MP and NDMP was achieved as follows: column oven temperature, 220°C; injector temperature, 280°C; detector temperature, 300°C; 5% argon in methane (carrier gas) column flow-rate, 1 ml/min; 5% argon in methane (make up gas) flow-rate, 60 ml/min; split vent flow-rate, 32 ml/min; septum vent flow-rate, 2 ml/min; head pressure on the column, 105 kPa.

Separation of the diastereomeric amide derivatives of ODMP and 5HMP was achieved as above except that the column oven temperature was programmed as follows: initial column oven temperature 240°C, held for 4 min, thereafter increased at a rate of 10°C/min to a final temperature of 280° which was held for 10 min.

Effect of amount of L-HPC on reactivity

dl-MP (4 μg/ml), *dl*-NDMP (2 μg/ml), *dl*-ODMP (4 μg/ml) or *dl*-5HMP (2 μg/ml) were each reacted separately in duplicate with various volumes (20, 30, 40, 50 and 60 μl) of 0.02 *M* L-HPC as described under *Assay procedure*. To the residue obtained was added HFBP-AMD in ethyl acetate (50 μg/ml) as an external standard. Suitable aliquots were injected into the GC–ECD system. The enantiomeric ratio, $peak_1/peak_2$ (P_1/P_2), for each of the various derivatised analytes was calculated after the reaction with various volumes of L-HPC.

Effect of the reaction time on the formation of diastereomeric amides

dl-MP (4 μg/ml), *dl*-NDMP (2 μg/ml), *dl*-ODMP (4 μg/ml) or *dl*-5HMP (2 μg/ml) were each reacted separately in duplicate with 50 μl of L-HPC (0.02 *M*). The reaction mixtures were incubated for various times (10, 20, 30, 45 and 60 min) and then extracted with *n*-pentane. HFBP-AMD in ethyl acetate (50 μg/ml) was added to the residue as an external standard. Aliquots (1–2 μl) were injected into the GC–ECD system. The enantiomeric ratio (P_1/P_2) for each of the various derivatised analytes was calculated at each of the different time intervals.

Standard calibration curves

Calibration curves were constructed separately for both enantiomers of each compound by analysing in triplicate a series of urine samples spiked with *dl*-MP (0.25–2 μg/ml per enantiomer) and *dl*-NDMP (0.094–0.75 μg/ml per enantiomer) or *dl*-ODMP (0.25–2 μg/ml per enantiomer) and *dl*-5HMP (0.188–1.5 μg/ml per enantiomer). Derivatisation and extraction of the diastereomers were carried out as described under *Assay procedure*. Standards and unknowns were carried through this procedure at the same time.

Accuracy and precision studies

Accuracy studies of *dl*-MP and its metabolites were carried out by analysing various concentrations in replicate by the GC–ECD procedures. Such urinary determinations were made on at least three separate days in order to check the precision of the procedure.

Experimental protocol

According to a protocol approved by the local human ethics committee, three healthy male volunteers (66.67 ± 5.51 kg), attended after an overnight fast, each ingested an extemporaneously prepared capsule containing 60.3 mg of *dl*-MP·HCl (50 mg free base). Urine was collected and pooled for a period of 12 h after ingestion of the drug. After recording the volume and the pH of each sample, small aliquots (10 ml) were stored in scintillation vials at $-20\,^{\circ}$C until analysis.

RESULTS AND DISCUSSION

dl-MP and its metabolites were converted into their corresponding diastereomeric amides by reaction of the amine group with the chiral acylating agent L-HPC (Fig. 1B). Further confirmation for the formation of diastereomeric amides were obtained by GC–MS which demonstrated the formation of the N-monoacylated derivatives of the diastereomers (Table I). A simple comparison of the peak heights of the individual enantiomers extracted from plasma compared to standards indicated approximately 80% recovery for all analytes. It was not pos-

TABLE I

PRINCIPAL ELECTRON-IMPACT IONS OF CHIRAL L-HPC DERIVATIVES OF METHOXYPHENAMINE AND ITS THREE METABOLITES

Values in parentheses are relative abundances.

Compound	*m/z*
MP (*d* or *l*)	472*(1), 351(25), 294(7), 266(100), 169(8), 148(20), 121(9)
NDMP (*d* or *l*)	458*(3), 337(55), 294(93), 266(100), 169(40), 148(99), 121(55)
ODMP (*d* or *l*)	458*(1), 351(20), 294(8), 266(100), 169(10), 134(22), 107(15)
5HMP (*d* or l)	488*(8), 351(13), 294(8), 266(100), 169(12), 164(40), 137(8), 121(4), 107(8)

*Molecular ion M^+.

sible to accurately assess recovery since optically pure standards of the individual free base enantiomers were not available.

It was necessary to carry out enzymatic hydrolysis of urine samples prior to analysis of the phenolic metabolites of MP. This was found essential given the high degree of conjugation of 5HMP [15]. Consequently two separate assays were developed, one for MP and NDMP and a second for the phenolic metabolites. Each assay was optimized with respect to oven temperature conditions and selection of internal standard in order to give the best chromatographic results.

Fig. 2 shows the chromatographic separations of the diastereomeric derivatives of *dl*-MP and its primary metabolites. Note that the peaks arising from each pair of diastereomeric derivatives have been designated as P_1 and P_2 because samples of pure *d*- and *l*-enantiomers of MP and metabolites were unavailable. The peaks arising from each pair of diastereomeric derivatives showed good baseline separation and there was no interference from endogenous materials at the retention times of either internal standard or other peaks of interest. The peaks arising from the diastereomers of MP and NDMP were separated in a single isothermal run with the column oven temperature at 220°C, while on the other hand the column oven temperature was programmed (240–280°C) for the separation of the diastereomeric amides of ODMP and 5HMP. The use of temperature programming was necessary because the polar 5HMP and ODMP derivatives eluted as broad peaks at long retention times and with poor resolution under isothermal conditions. Note that satisfactory chromatography was obtained in each case despite the presence of free phenolic groups in the chiral derivatives of ODMP and 5HMP. Similar chromatographic results have been obtained with drugs such as *dl*-methylphenidate [16,17], *dl*-fenfluramine and *dl*-norfenfluramine [18] which were also separated as their diastereomeric N-monoacylated derivatives after reaction with L-HPC.

Figs. 3 and 4 demonstrate the optimisation of the present stereoselective procedure with regard to (i) the amount of L-HPC added and (ii) the time for completion of the acylation reaction. In the case of each analyte 40 μl of L-HPC and a reaction time of 45 min were shown to be adequate for the completion of the reaction. Table II shows that over the range of reaction times and amounts of reagent studied, there was no distortion in the ratio of the diastereomeric peaks (P_1/P_2) for *dl*-MP or its metabolites. The standard curves for the enantiomers of MP and its primary metabolites showed good linearity throughout the concentration ranges studied (Table III) and r^2 values of > 0.99 were obtained. Studies on inter-assay and intra-assay variability (Table IV) demonstrated that the procedures were reproducible and accurate. In each case a mean coefficient of variation of less than 10% was obtained.

The chiral procedures were then applied to examine the extent of enantioselectivity in the urinary excretions of MP and its primary metabolites in three healthy adults in 0–12 h urine samples collected after administration of MP. All three volunteers had been phenotyped previously as extensive metabolisers of MP [15]. The total amounts of the isomers of the various analytes excreted in 0–12 h urine samples are shown in Table V. Marked enantioselectivity was evident in the urinary excretions of the isomers of all the analytes except ODMP,

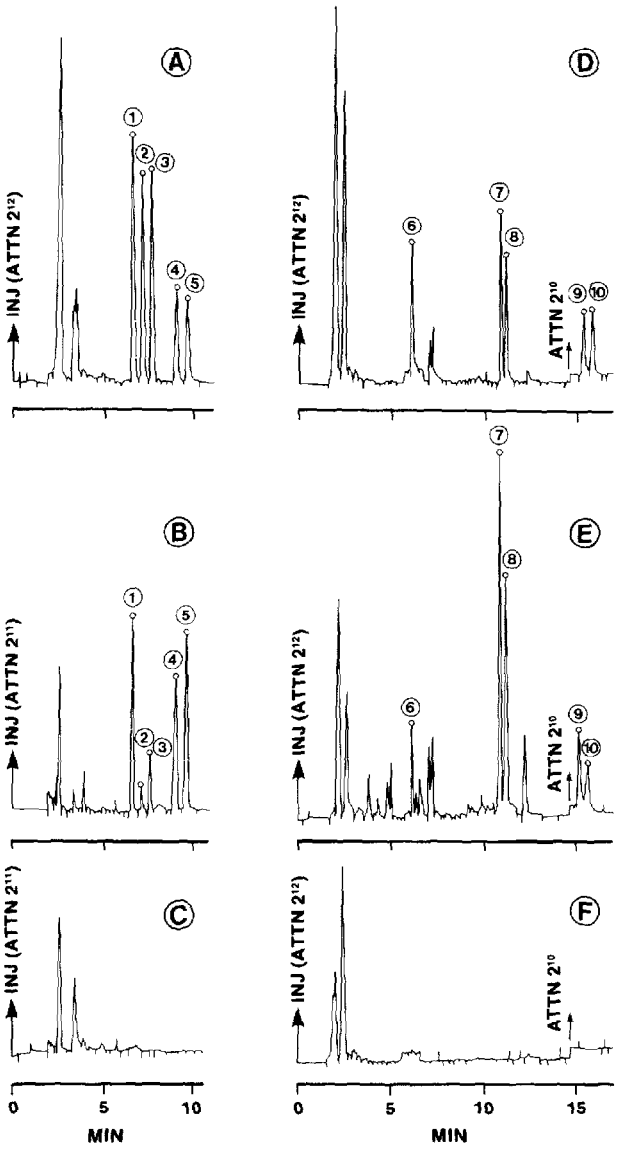

Fig. 2. GC–ECD profiles of L-HPC-derivatised extracts from (A) blank human urine spiked with AMD, MP and NDMP, (B) 0–12 h urine sample obtained from a healthy volunteer after ingestion of 60 3 mg MP·HCl, (C) blank human urine obtained from the same volunteer prior to ingestion of MP·HCl, (D) blank human urine spiked with *d*-E, ODMP and 5HMP, (E) 0–12 h urine sample obtained from a healthy volunteer after ingestion of 60.3 mg MP·HCl, (F) blank human urine obtained from the same volunteer prior to ingestion of MP·HCl. Peaks: 1 = AMD; 2 = P_1 of NDMP; 3 = P_2 of NDMP; 4 = P_1 of MP; 5 = P_2 of MP; 6 = *d*-E; 7 = P_1 of ODMP; 8 = P_2 of ODMP; 9 = P_1 of 5HMP; 10 = P_2 of 5HMP. GC conditions: A–C isothermal column oven temperature (220°C) and D–F programmed column oven temperature (240–280°C).

which was excreted in an almost equi-enantiomeric proportion. In the case of the isomers of MP and NDMP the excretion of the isomer eluting as P_2 was greater than that of the isomer eluting as P_1 by an average two- and four-fold, respec-

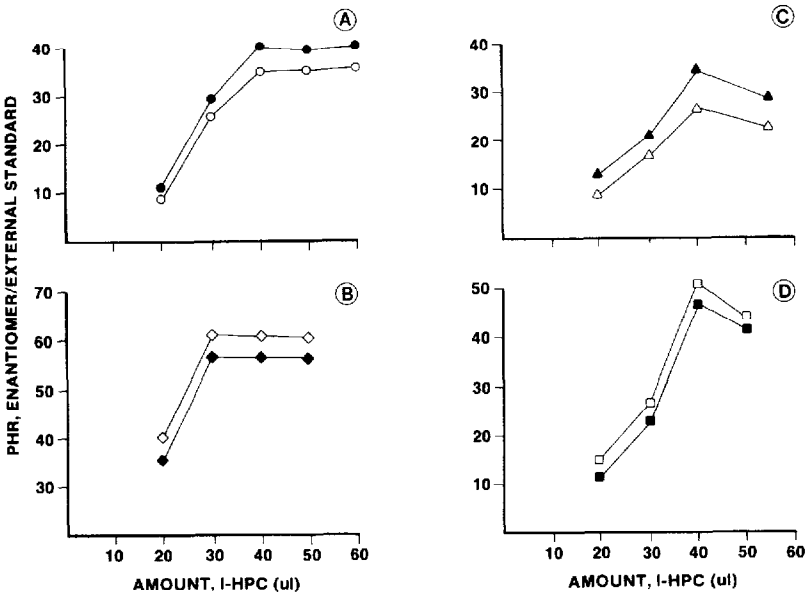

Fig. 3. Effect of the amount of L-HPC on the formation of the diastereomeric derivatives of (A) MP, (B) NDMP, (C) ODMP and (D) 5HMP. Closed symbols are P_1 peaks; open symbols are P_2 peaks; PHR is peak-height ratio.

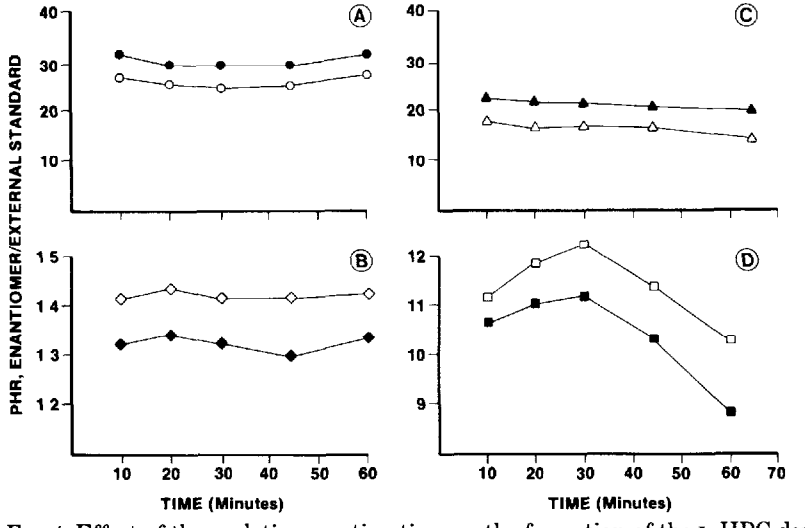

Fig. 4. Effect of the acylation reaction time on the formation of the L-HPC derivatives of (A) MP, (B) NDMP, (C) ODMP and (D) 5HMP. Closed symbols are P_1 peaks; open symbols are P_2 peaks; PHR is peak-height ratio

tively, whereas the excretion of the isomer of 5HMP represented as P_1 was about two-fold greater than that of the isomer eluting as P_2. Table VI shows the enantiomeric ratios (P_1/P_2) calculated for MP and its three primary metabolites in the 0–12 h urine samples. These enantiomeric ratios (P_1/P_2) calculated for each of the four analytes were very similar for the three extensive metabolisers, although the amounts of the individual isomers of the various analytes excreted were very

TABLE II

EFFECT OF THE AMOUNT OF L-HPC AND THE REACTION TIME ON THE ENANTIOMERIC RATIOS OF THE VARIOUS ANALYTES

Amount of L-HPC (μl)	Reaction time (min)	Enantiomeric ratio (P_1/P_2)			
		MP	NDMP	ODMP	5HMP
20	45	1.13	0.91	1.27	0.90
30	45	1.15	0.93	1.27	0.92
40	45	1.14	0.93	1.29	0.93
50	45	1.14	0.93	1.27	0.92
60	45	1.14	–	–	–
50	10	1.13	0.94	1.23	0.95
50	20	1.15	0.93	1.22	0.96
50	30	1.13	0.91	1.23	0.94
50	45	1.14	0.93	1.24	0 95
50	60	1.15	0.94	1.22	0.95

TABLE III

REGRESSION LINES FOR THE ENANTIOMERS OF MP AND ITS THREE PRIMARY METABOLITES

Analyte	Concentration range (μg/ml of each enantiomer)		Regression line ($y=mx+b$)	r^2
MP	0.25–2.0	P_1	$y=0.4375x-0.0009$	0.9987
		P_2	$y=0.2488x-0.0081$	0.9975
NDMP	0.094–0.75	P_1	$y=2.7573x+0.0258$	0.9994
		P_2	$y=2.9748x+0.0239$	0.9989
ODMP	0.25–2.0	P_1	$y=0.4815x-0.0281$	0.9986
		P_2	$y=0.3200x-0.0139$	0.9971
5HMP	0.188–1.5	P_1	$y=0.0498x-0.0032$	0.9965
		P_2	$y=0.0567x-0.0045$	0.9973

different. For example the enantiomeric ratio (P_1/P_2) calculated for NDMP in the three individuals ranged from 0.20 to 0.27, whereas the amount of the drug excreted in the urine ranged from 0.030 to 0.091 mg for the P_1 isomer and from 0.15 to 0.43 mg for the P_2 isomer.

In conclusion, enantioselective methods for the quantitation of MP and its three primary metabolites in human urine have been developed. The method involved the conversion of the enantiomers into their corresponding diastereomeric derivatives using a chiral reagent followed by chromatography on an achiral stationary phase. Sufficient sensitivity and selectivity was demonstrated to monitor the individual isomers of MP and its three primary metabolites in human urine after a single 60.3-mg oral dose of MP·HCl. This represents the first report of such procedures and provides the necessary technology to study the enantioselective disposition of this drug. In this regard MP, NDMP and 5HMP demon-

TABLE IV

ACCURACY AND REPRODUCIBILITY STUDIES FOR THE ENANTIOMERS OF MP AND ITS METABOLITES

Analyte	Spiked concentration range of each enantiomer (μg/ml)	n^*	Maximum coefficient of variation (%) observed for each enantiomer**	
			P_1	P_2
Intra-assay variability				
MP	0.25–2.0	6	4.14	3.29
NDMP	0.094–0.75	6	2.16	2.38
ODMP	0.25–2.0	6	6.25	4.64
5HMP	0.188–1.5	6	3.48	5.23
Inter-assay variability				
MP	0.25–2.0	3	7.69	8.00
NDMP	0.094–0.75	3	8.89	8.89
ODMP	0.25–2.0	4	6.16	6.82
5HMP	0.188–1.5	4	7.14	8.27

*In the case of inter-assay variability triplicate analysis was performed on the indicated "n" number of days.
**P_1 and P_2 refer to the individual enantiomers.

TABLE V

TOTAL EXCRETION OF ENANTIOMERS OF MP AND ITS THREE PRIMARY METABOLITES IN 0–12 h URINE OF THREE HUMAN EXTENSIVE METABOLISERS AFTER THE ADMINISTRATION OF 60.3 mg OF MP·HCl

Volunteer No.	Urinary excretion (mg)							
	MP isomers		NDMP isomers		ODMP isomers		5HMP isomers	
	P_1	P_2	P_1	P_2	P_1	P_2	P_1	P_2
1	2.22	4.24	0.085	0.32	2.85	2.62	0.75	0.40
2	4.03	6.11	0.091	0.43	3.28	3.02	0.60	0.29
3	1.94	3.63	0.030	0.15	2.33	2.15	1.08	0 53
Mean	2.73	4.66	0.069	0.30	2.82	2.60	0.81	0.41
S.D.	1.13	1.29	0.036	0.14	0.48	0.44	0.24	0.12

strated marked enantioselectivity while the isomers of ODMP were excreted in an almost equi-enantiomeric ratio. The implications of these findings when considered in context with the known polymorphism of MP metabolism in man [15] may play a significant role in the metabolism of this drug. The new analytical methods will enable further investigation of the enantioselectivity in the metabolism of MP and such studies are being carried out in our laboratories.

TABLE VI

ENANTIOMERIC RATIOS DETERMINED FOR MP AND ITS THREE PRIMARY METABOLITES IN 0–12 h URINE SAMPLES OF THREE HUMAN EXTENSIVE METABOLISERS WHO INGESTED 60.3 mg OF MP·HCl

Volunteer No.	Enantiomeric ratio (P_1/P_2)			
	MP	NDMP	ODMP	5HMP
1	0.52	0.27	1.09	1.90
2	0.66	0.21	1.09	2.00
3	0.53	0.20	1.08	2.03
Mean	0.57	0.23	1.09	1.98
S.D.	0.08	0.04	0.006	0.07

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