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COMPARISON BETWEEN TWO METHODS FOR THE DETERMINATION OF THE TOTAL AND FREE (*R*)- AND (*S*)-DISOPYRAMIDE IN PLASMA USING AN α_1-ACID GLYCOPROTEIN COLUMN

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SUMMARY

Two different high-performance liquid chromatographic systems for the determination of the total and free (*R*)- and (*S*)-disopyramide (DP) in plasma and urine were compared In method I a Nucleosil C_8 column was coupled in series with an α_1-acid glycoprotein column Method II consisted of two systems, a LiChrosorb Si 60 column was used for the determination of the racemic drug concentration and the *R*/*S* ratio was determined on an α_1-acid glycoprotein column The recovery of (*R*)- and (*S*)-DP from plasma was >97% in both methods The precisions of the (*R*)- and (*S*)-DP determinations in plasma are high with both methods The relative standard deviations for the determination of the free concentration do not exceed 6 5% at 1 59 μg/ml racemic DP Method II is preferred as it can also be used to determine the concentration of (*R*)- and (*S*)-monodesisopropyldisopyramide It is also easier to avoid disturbances from endogenous compounds in plasma samples with method II than with method I It was observed that DP was incorporated into urine sediment during storage A simple ultrasonic treatment of the urine samples was demonstrated to release DP from the sediment

INTRODUCTION

Disopyramide (DP) is a class I antiarrhythmic agent with complex pharmacokinetic and pharmacodynamic properties The drug is commercially available as a racemic mixture of two optical isomers, (*R*)- and (*S*)-DP As DP also possesses side-effects such as a negative inotropic effect and anticholinergic properties, its use is limited. It has recently been reported that only (*S*)-DP prolongs the QTI interval (antiarrhythmic effect) and that it has a

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smaller negative inotropic effect than the R-form [1] It has also been observed that the S-form has a more pronounced anticholinergic effect than the R-form [2].

In order to study the pharmacokinetics and pharmacodynamics of the enantiomers of DP, the concentrations of (R)- and (S)-DP in plasma and urine were determined. The binding of racemic DP to human plasma proteins is concentration-dependent at therapeutic concentrations [3] and the binding of the enantiomers of DP to plasma proteins is stereoselective [4] Therefore, the free concentrations of (R)- and (S)-DP in plasma were also determined

There are two basic approaches for the resolution and quantification of enantiomers, the indirect and the direct The indirect method involves the formation of diastereomeric derivatives which can be separated on a non-chiral column However, some compounds, including DP, lack suitable groups for derivatization, so the direct approach is employed in this study

The chiral α_1-acid glycoprotein (α_1-AGP) column was developed by Hermansson [5] This column has the advantage that it can be used for the resolution and quantification of a large number racemic drugs [6–8] However, in the determination of drugs in plasma and urine samples, metabolites or endogenous compounds can interfere with the peaks of the enantiomers This problem might be solved if the properties of the drug are altered by the preparation of enantiomeric derivatives, as was demonstrated for atenolol [9] Alternatively, a small reversed-phase column can be coupled in series with the chiral column as in the determination of (R)- and (S)-DP in plasma [10]

In this study, two different high-performance liquid chromatographic (HPLC) approaches for the determination of total and free (R)- and (S)-DP in plasma and urine are compared.

EXPERIMENTAL

Chemicals and materials

Nucleosil C_8 (5 μm) and LiChrosorb Si 60 (5 μm) were obtained from Macherey-Nagel (Duren, F R G) and E Merck (Darmstadt, F R.G), respectively An EnantioPac column (the first-generation α_1-AGP column) was kindly supplied by LKB (Bromma, Sweden) The α_1-AGP column[a] was prepared in our laboratory

Racemic DP was obtained from Sigma (St Louis, MO, U S A.) and racemic monodesisopropyldisopyramide (MND) was kindly supplied by Roussel Labs (London, U K) The structures are shown in Fig 1. The enantiomers of DP and MND oxalate were a gift from Prof Wendel L Nelson (School of Pharmacy, Department of Medical Chemistry, Seattle, WA, U S.A.) Cellulose dialysis tubing (Union Carbide, Chicago, IL, U S A) with a flat diameter of 6 mm, a wall thickness of 51 μm and a pore diameter of 24 Å was used Other chemicals used were of HPLC or analytical-reagent grade

[a]The second-generation α_1-AGP column, CHIRAL-AGP, is now available from ChromTech (Norsborg, Sweden)

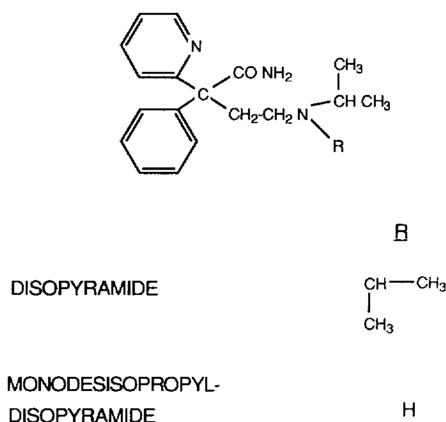

Fig 1 Structures of the compounds

Column preparation

The columns were made of precision-bore 316 stainless steel and equipped with modified Swagelock connections and Altex stainless-steel (2-μm) frits A Haskel pump operated at 300–400 bar was used for column packing

Nucleosil C_8 (30 mm×3 0 mm I D) A 0.17-g amount of Nucleosil C_8 (5 μm) was suspended in 2 ml of dichloromethane and placed in an ultrasonic bath for 10 s before being poured into a 10-ml packing column. The packing column was then filled with hexane Acetone (80 ml), methanol (100 ml) and methanol–water (1 1) (100 ml) were used as the driving and rinsing liquids, respectively

LiChrosorb Si 60 (150 mm×4 2 mm I D) A 1 5-g amount of LiChrosorb Si 60 (5 μm) was suspended in 8 ml of dichloromethane and then handled as described for the Nucleosil C_8 column. Hexane was used as driving liquid and the column was rinsed with dichloromethane.

α_1-Acid glycoprotein (100 mm×4 0 mm I D) This column was prepared as described by Hermansson et al [11]

Apparatus and chromotography

Two different HPLC systems were used and are referred to as methods I and II.

In method I, the liquid chromatographic system consisted of an LKB Model 2150 pump, a Waters U6K injector (Millipore Waters, Milford, MA, U S.A.) and a Shimadzu SPD-2A variable-wavelength UV detector operating at 261 nm A Nucleosil C_8 column (30 mm×3.0 mm I.D) was coupled in series with the first-generation α_1-acid glycoprotein column (Enantiopac, 100 mm×4 0 mm I.D.). The equipment was placed in a thermostated cabinet adjusted to

23 °C The mobile phase was a phosphate buffer (pH 5 6) (0.01 *M* phosphate) containing 5% (v/v) 2-propanol The flow-rate was 0 5 ml/min

In method II, the concentrations of racemic DP and MND were determined using a normal-phase system. The eluates containing DP and MND were collected and handled as described below under *Extraction procedures (method II)* The enantiomeric ratios for DP and MND were determined using an α_1-acid glycoprotein column (α_1-AGP, 5 μm)

The normal-phase system consisted of an LKB Model 2150 pump, a Waters U6K injector and a Waters Model 440 UV detector (254 nm) A 150 mm long LiChrosorb Si 60 column and a mobile phase composed of 9% (v/v) methanol and 0 6% (v/v) 1 *M* perchloric acid in dichloromethane were used The flow-rate was 1.0 ml/min.

The chiral HPLC system was constructed from an LKB Model 2150 pump (LKB, Bromma, Sweden), a Waters U6K injector and a Shimadzu SPD-2A variable-wavelength UV detector (261 nm) An α_1-AGP column (100 mm × 4 0 mm I.D.) was used The mobile phase (flow-rate 0 3 ml/min), a phosphate buffer (pH 7 0) with a final phosphate concentration of 0 01 *M*, contained 0 1 *M* sodium chloride[a] and 10 or 7% (v/v) 2-propanol. The system was kept at 23 °C in a thermostated cabinet. For peak integration a Nelson Analytical (Cupertino, CA, U.S.A) Series 3000 chromatography data system, version 4 0, was used

All mobile phases were degassed in an ultrasonic bath before use

A Hettich universal centrifuge and a Hettich Rotixa/KS centrifuge (temperature controllable from 10 to 40 °C) (Hettich, Tuttlinge, F R G.) were used

Ultrafiltration

Cellulose dialysis tubing (20 cm long) were soaked in water for 1 h to remove glycerin and carefully dried with Kleenex tissues A gas mixture of 6 5% carbogen in synthetic air was bubbled through the plasma sample for 10 min before starting the ultrafiltration in order to adjust the pH to 7 4 The tubes were filled with 4 ml of plasma and stuck in a centrifuge tube with a rubber stopper [12] The samples were thermostated to 37 °C in a water-bath for 30 min The tubes were then centrifuged for 5 min at 500 *g* at 37 °C, and the centrifugate (ca 50 μl) was discarded. The dialysis tubing were placed in clean centrifuge tubes and centrifuged for another 24 min at 500 *g* giving about 350 μl of plasma water

The non-specific drug binding to the dialysis tubing was studied using a phosphate buffer of pH 7 4 (μ=0.02) spiked with different concentrations of racemic DP and MND The concentration of DP was 3.79, 1.59 or 1 00 μg/ml and the concentration of MND was 1 24, 0.74 or 0 50 μg/ml Aliquots of 4 ml of the different solutions were placed in the dialysis tubing and handled as

[a]Recently, we have observed that the sodium chloride can be omitted and the flow-rate can be increased to 0 9 ml/min

described above. The centrifugate was extracted as described below The peak heights obtained were compared with those obtained from non-ultrafiltered samples

Extraction procedures (method I)

Plasma A 1-ml volume of plasma was made alkaline with 60 μl of 2 *M* sodium hydroxide and extracted for 15 min with 6 ml of water-saturated diethyl ether The tubes were centrifuged (3 min at 250 *g*) and 5 ml of the ether phase were extracted for 15 min with 200 μl of 0 001 *M* phosphoric acid The ether phase was discarded A 60-μl volume of 0 1 *M* sodium hydroxide was added to the water phase and the mixture was extracted with 6 ml of diethyl ether (15 min) and centrifuged A 5-ml volume of the ether phase was evaporated to dryness under a stream of nitrogen at 40°C.

Plasma water To 300 μl of plasma water (obtained a described above) were added 60 μl of 2 *M* sodium hydroxide and 6 ml of water-saturated diethyl ether The samples were extracted for 15 min After centrifugation for 3 min at 250 *g*, 5 ml of the ether phase were evaporated to dryness

Urine The urine samples were treated in an ultrasonic bath for 3 min To 0 5 ml of urine, 60 μl of 5 *M* sodium hydroxide were added and the mixture was extracted for 30 min with 6 ml of water-saturated diethyl ether The tubes were centrifuged for 3 min at 250 *g* and 5 ml of the ether phase were extracted for 30 min with 200 μl of 0 1 *M* phosphoric acid The ether phase was discarded A 60-μl volume of 2 *M* sodium hydroxide was added to the water phase and the mixture was extracted with 6 ml of water-saturated diethyl ether for 30 min and centrifuged A 5-ml volume of the ether phase was evaporated to dryness under a stream of nitrogen at 40°C

The residues obtained after extraction and evaporation were dissolved in 110 μl of the mobile phase and 50 μl (10–50 μl for urine) were analysed according to method I

In order to study the incorporation of DP in urine sediment, the stored urine samples were treated as follows One of the frozen urine samples was thawed and centrifuged, and two 0.5-ml samples of the supernatant were collected The tube was then vortex-mixed and two additional 0.5-ml samples containing a homogeneous mixture of sediment and urine were collected The tube was once again centrifuged and 0 5-ml of urine, including as much sediment as possible, was collected The samples were analysed as described above Another thawed urine sample was first treated in an ultrasonic bath for 3 min and then handled as described above

Extraction procedures (method II)

Plasma The extraction procedure is a modification of a method described previously [13] Both DP and MND were extracted with 1 ml of dichloromethane from a mixture of 1 ml of plasma and 60 μl of 2 *M* sodium hydroxide.

The extraction time was 10 min. The tubes were centrifuged for 2×20 min (250 *g*) with brief vortex-mixing between the two centrifugations to break the protein–dichloromethane emulsion. A 250-μl volume of the dichloromethane phase was injected directly on to the normal-phase system described under method II. The eluates containing DP and MND were collected separately and extracted with 60 μl of 2 *M* sodium hydroxide in 1 ml of water for 10 min in order to remove perchloric acid from the organic phase The dichloromethane phase was evaporated

The residue was dissolved in 30 μl of the mobile phase and 20 μl were injected on to the α_1-AGP column.

Plasma water The plasma water was extracted as described above for plasma, but only 300 μl of plasma water were used, the samples were centrifuged for 10 min and there was no need for a second centrifugation

Urine To 100 μl of urine (diluted with 400 μl of water), 60 μl of 2 *M* sodium hydroxide were added The mixture was extracted and handled as described above for plasma water.

Calibration graphs, recovery and reproducibility

Calibration graphs were prepared by adding known amounts of racemic DP and MND to plasma, urine or phosphate buffer of pH 7 4 (phosphate concentration 0 01 *M*). The standard samples were handled as described under *Extraction procedures* The calibration graphs were constructed by plotting the peak heights versus the solute concentrations At least five standard points were used for each graph To evaluate the recoveries in the two methods, blank plasma and urine were spiked with known amounts of racemic DP and MND from a stock solution (see Table IV). The samples were handled according to the above-described extraction procedures. The recovery of the enantiomers of DP and MND was calculated by comparing the peak heights obtained after extraction of spiked plasma samples with peak heights obtained by direct injections of the same amount of unextracted DP and MND Reproducibility studies for the analyses of the solutes were performed at different concentrations (see Table V) Plasma or urine were spiked with different concentrations of racemic DP and MND. The samples were ultrafiltered and/or extracted as described above in replicates of six The relative standard deviations (R.S D) presented for DP and MND in method II are calculated as

$$\{[\text{R S.D (normal-phase system)}]^2+[\text{R.S D. (chiral system)}]^2\}^{\frac{1}{2}}$$

RESULTS AND DISCUSSION

Methods I and II were used to determine the total (free+protein-bound) and free (*R*)- and (*S*)-DP concentrations in plasma and urine. In addition,

method II was also used to determine the plasma concentration of total and free (*R*)- and (*S*)-MND

Method I

(*R*)- and (*S*)-DP extracted from patients' plasma can be separated using a LiChrosorb RP-2 column in series with an α_1-AGP column as described previously [10] Method I was based on the use of a Nucleosil C_8 column (30 mm×3 0 mm I D) coupled in series with the EnantioPac column The pre-column was required to separate racemic DP from its main metabolite, MND, as (*S*)-MND and (*R*)-DP interfered when the EnantioPac column alone was used Fig 2A–D show both plasma and urine sample chromatograms obtained on the two columns coupled in series.

Fig. 2A and B demonstrate that it might be difficult to separate endogenous compounds from the enantiomers of MND and still obtain acceptable retention times for (*R*)- and (*S*)-DP To overcome these problems, method II was developed

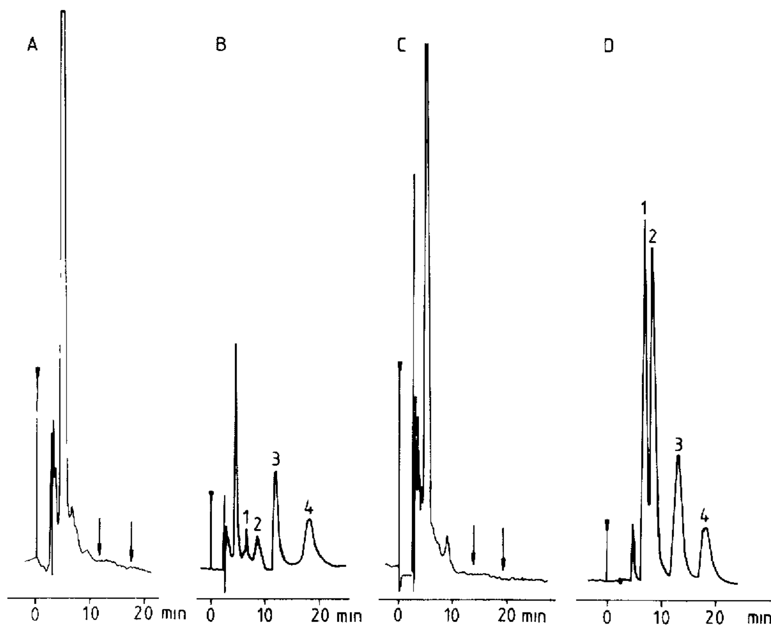

Fig 2 Separation of the enantiomers of DP and MND Column Nucleosil C_8 (30 mm×3 0 mm I D) coupled in series with a chiral EnantioPac column (100 mm×4 0 mm I D) Mobile phase phosphate buffer of pH 5 6 (0 01 *M* phosphate) containing 5% (v/v) 2-propanol Flow-rate 0 5 ml/min Peaks 1=(*R*)-MND, 2=(*S*)-MND, 3=(*R*)-DP, 4=(*S*)-DP (A) Chromatogram of ultrafiltered blank plasma The arrows indicate the retention times of (*R*)- and (*S*)-DP (0 002 a u f s) (B) Ultrafiltered plasma sample from a volunteer who had received an oral dose of racemic DP, 150 mg (0 004 a u f s) (C) Blank urine chromatogram The arrows indicate the retention times of (*R*)- and (*S*)-DP (0 002 a u f s) (D) Urine sample from a volunteer who had received an oral dose of racemic DP, 150 mg (0 064 a u f s)

Method II

Method II was based on the use of two chromatographic systems. The normal-phase system was used for the determination of the racemic concentrations of DP and MND Fig 3 presents a chromatogram of an ultrafiltered plasma sample The eluate containing DP and MND were collected separately and handled as described under Experimental The R/S ratios were determined on an α_1-AGP column. The mobile phase was a phosphate buffer of pH 7 0 containing 0.1 M sodium chloride[a] and 7 or 10% (v/v) 2-propanol. The mobile

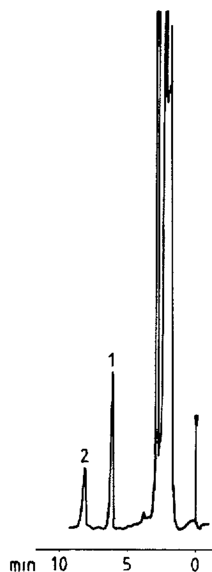

Fig 3 Separation of (R,S)-DP and (R,S)-MND (from an ultrafiltered plasma sample from a volunteer) Column LiChrosorb Si 60 (150 mm×4 2 mm I D) Mobile Phase 9% (v/v) methanol and 0 6% (v/v) 1 M perchloric acid in dichloromethane Flow-rate 1 0 ml/min Peaks 1=DP, 2=MND (0 02 a u f s)

TABLE I

CHROMATOGRAPHIC DATA OBTAINED ON THE α_1-AGP COLUMN

Column dimensions, 100 mm×4 0 mm I D Mobile phase phosphate buffer (pH 7 0) (0 01 M phosphate) containing 0 1 M sodium chloride[a] and 7 or 10% (v/v) 2-propanol Flow-rate 0 3 ml/min $\alpha = k'_S/k'_R$, $R_s = 2(t_{R_S} - t_{R_R})/(W_{bS} + W_{bR})$, where, t_{R_S} = retention time of the S-enantiomer and W_{bS} = base width of the S-enantiomer

Compound	2-propanol concentration (%)	k'_R	k'_S	α	R_s
DP	10	6 29	9 24	1 47	2 17
MND	7	6 68	9 56	1 43	1 99

[a]Recently, we have observed that the sodium chloride can be omitted and the flow-rate can be increased to 0 9 ml/min

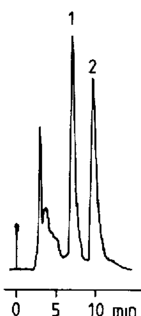

Fig 4 Separation of the enantiomers of MND Column α_1-AGP (100 mm×4 0 mm I D) Mobile phase phosphate buffer of pH 7 0 (0 01 *M* phosphate) containing 0 1 *M* sodium chloride and 7% (v/v) 2-propanol Flow-rate 0 3 ml/min Peaks 1=(*R*)-MND, 2=(*S*)-MND (0 008 a u f s)

phase with the lower concentration of 2-propanol was used for the MND metabolite and 10% 2-propanol was used for the separation of the more hydrophobic DP enantiomers. Chromatographic data obtained on the α_1-AGP column are presented in Table I. The separation factor, α, obtained for the enantiomers of DP was 1 47. Under the same chromatographic conditions the enantioselectivity for MND (the desisopropylated metabolite) was lower. Fig 4 demonstrates a separation of the enantiomers of MND, isolated from a plasma sample, on the chiral α_1-AGP column. The concentration of the enantiomers was calculated by multiplying the fraction of the total area ($R+S$) of each enantiomer with the plasma concentration of racemic DP and MND determined with the normal-phase system One advantage with method II is that it can be used to separate and determine the enantiomers of both DP and MND It is also easier to avoid disturbances from endogenous compounds in plasma by using a combination of the normal-phase system and the chiral system as in method II

Ultrafiltration

Equilibrium dialysis, ultrafiltration and ultracentrifugation are the three most often used methods for protein-binding measurements Ultrafiltration was chosen as it is simple to use and it has a high sample capacity The ultrafiltration procedure can be performed under physiological conditions regarding both temperature and pH. Protein leakage and non-specific binding of DP have been reported using commercially available Ultrafree filters, with an MW cut-off of 40 000 [14] The ultrafiltration was therefore performed in cellulose dialysis tubing (MW cut-off 12 000–14 000)

The non-specific drug binding to the dialysis tubing was examined at three different concentration levels of DP and MND as described under Experimental The concentrations of DP were 3 97, 1 59 or 1.00 μg/ml, respectively, and

of MND 1.24, 0 74 or 0.50 μg/ml The recoveries of DP and MND were >99 7% and >98 2%, respectively, at all concentrations

It has been discussed in some papers whether the temperature in protein-binding studies of DP is of any importance A small but statistically significant higher free fraction at 37°C than at 22 or 25°C has been reported for the racemate using equilibrium dialysis [3,14] In chromatographic studies it has also been observed that both the retention and the stereoselectivity of DP increase with decreasing temperature using the chiral α_1-AGP column [8] α_1-AGP is the main binding protein of DP in plasma [15] Therefore, to avoid deviations from the in vivo situation in the free fraction and enantiomeric ratio, the ultrafiltration was performed at 37°C

The influence of pH on the free fraction of DP was discussed by Hinderling et al [3] They found no change in protein binding at pH 7 4 and 8 0, but the protein binding was significantly lower at pH 6 7 Another group observed that variations within the physiological pH range (6 9–7 6) did not have any significant effect on the protein binding of DP [16] In chromatographic experiments it has been observed that the retention of (*R*)- and (*S*)-DP will decrease if the pH decreases from 7 5 to 6 0 whereas the enantioselectivity increases slightly using a chiral α_1-AGP column [5] Therefore, to obtain physiological conditions during the ultrafiltration, the plasma samples were treated with carbogen to adjust the pH to 7 4 The pH of the carbogen-treated samples varied between 7 4 and 7 7 during ultrafiltration

It has been proposed that only small fractions of ultrafiltrate (>10% of the total volume) should be produced to avoid disturbances of the protein-binding equilibrium [17,18] In these studies it was demonstrated that the free fraction increased with increasing volume of filtrate Others [19–21] demonstrated that the volume of filtrate had no influence on the free fraction obtained This ob-

TABLE II

INFLUENCE OF THE FILTERED VOLUME ON FREE CONCENTRATIONS AND ENANTIOMER RATIOS (n=5)

Compound	Total plasma concentration (μg/ml)	Peak height (mm)		*R*/*S* ratio	
		12%[a]	24%[a]	12%[a]	24%[a]
DP	1 00	9 4	9 2	2 1	2 1
	3 75	55 2	60 6	1 6	1 5
MND	0 17	2 5	2 6	1 2	1 2
	2 50	40 9	43 9	1 0	1 0

[a] Percentage ultrafiltrate of the total volume (4 ml)

servation can be expressed by the equation

$$K = \frac{[\text{protein-bound drug}]}{[\text{free drug}]\,[\text{free protein}]} \tag{1}$$

The concentration of protein-bound drug will increase as much as the concentration of free protein during ultrafiltration if the pore diameter in the ultrafiltrate membrane is small enough to retain the plasma proteins Consequently, the concentration of free drug will not be affected This was examined experimentally using a dialysis tubing with an MW cut-off of 14 000 Dialysis tubing with plasma spiked with different concentrations of DP and MND were centrifuged for 24 or 50 min The volumes of filtrate obtained were 12 and 24% of the total volume, respectively The results in Table II show that the R/S ratio of neither DP nor MND varies with the filtrate volume Further, no significant changes in the free fraction were obtained with the filtrate volume A reasonable explanation of the finding that the volume of filtrate influences the free fraction might be an overestimation of free concentration due to protein leakage into the filtrate, as large-pore membranes (MW cut-off 40 000) were used α_1-AGP has a molecular weight of 40 000 [22]

Extraction procedures

It is very important that the recovery of a solute from plasma is high, as its degree of extraction in the isolation step might be dependent on the protein concentration [23] The concentration of proteins in plasma varies between individuals Normally the concentration of α_1-AGP is 0 5–0 9 g/l, but this protein is an acute phase protein and the concentration might be strongly increased during, for example, cancer, pneumonia and rheumatiod arthiritis [22]

If a chiral compound has a high degree of protein binding and the protein binding is also stereoselective, as with DP, the concentration of protein in plasma can have a considerable effect on the degree of extraction of the enantiomers However, the effect of differing protein concentration is negligible if the degree of extraction is $>90\%$ [23] The degree of extraction of DP from plasma to diethyl ether is $>97\%$. However, the extraction yield of MND is only 57% with a phase volume ratio of $V_{org}/V_{aq}=5\ 53$ If diethyl ether is replaced with dichloromethane, the extraction yields of DP and MND are $>98\%$ with a phase volume ratio of $V_{org}/V_{aq}=0\ 92$ (see Table IV) This means that the extraction yield of the enantiomers will not be significantly affected, even if the concentration of α_1-AGP increases two- or three-fold, as it might do in plasma from patients with myocardial infarction [16]

Urine extraction

The determination of DP in authentic urine samples using method I gave low recovery and reproducibility The reason was initially not understood, as a high recovery of DP ($>96\ 4\%$) and a high reproducibility (R S D $<5\ 2\%$) were observed with spiked urine samples (see Tables IV and V) During stor-

TABLE III

EFFECTS OF URINE SAMPLE TREATMENT ON RECOVERY OF (*R*)- AND (*S*)-DP ($n=2$)

Sample treatment[a]	Peak height (mm)			
	Sample 1[b]		Sample 2[b]	
	R	*S*	*R*	*S*
A	5 40	1 80	95 6	55 6
B	32 4	11 6	99 2	53 1
C	96 3[c]	49 0[c]		

[a]A = Supernatant urine without sediment, 0 5 ml, B = homogeneous mixture of urine and sediment, 0 5 ml, C = 0 5 ml of urine including all the sediment in the tube
[b]Sample 1 = thawed urine sample, sample 2 = thawed urine sample treated in an utrasonic bath for 3 min
[c]$n=1$

TABLE IV

RECOVERY OF (*R*)- AND (*S*)-DP AND (*R*)- AND (*S*)-MND FROM URINE AND PLASMA

Method I ($n=6$)			Method II ($n=5$)			
Concentration of DP racemic (μg/ml)	Recovery (%)		Concentration of racemic DP (μg/ml)	Recovery (%)	Concentration of racemic MND (μg/ml)	Recovery (%)
	R	*S*				
Plasma						
0 41	98 4	97 5	1 00	99 9	0 17	99 8
3 10	99 0	98 1	1 90	98 2	0 97	98 2
			3 76	98 9	1 67	100
Urine						
0 80	96 4	98 7	59 8	99 8	59 5	100
30 0	100	99 0	358	99 7	357	99 9

age, urine samples produce sediment which can consist of leukocytes, erythrocytes, tubulus epithelium, squamous epithelium, urinary cylinders, amorphous salts, sperms, fungi and/or trichomonas vaginalis [24] The amount and composition can vary between individuals and within an individual depending on the food and also when the samples are taken During the extraction of DP from the urine samples, we observed that the concentration of DP increased with an increase in the amount of sediment withdrawn from the urine sample The results are summarized in Table III. The concentration of DP increased with increasing amount of sediment The most likely explanation is that DP is incorporated in the crusts of amorphous salts present in the untreated stored urine samples

Calibration graphs, recovery and reproducibility

Calibration graphs were prepared as described under Experimental. The graphs were linear in the therapeutic concentration ranges with correlation coefficients between 0 9990 and 0 9997.

The degree of extraction of the enantiomers of DP from plasma was >97% (0.40 μg/ml). The recoveries of the solutes from plasma and urine in the two methods are presented in Table IV. The reproducibility was determined from blank plasma/urine spiked with different concentrations of racemic DP and MND The samples were analysed as described earlier in replicates of six. The relative standard deviations obtained are summarized in Table V. The precision is high; even at low plasma concentrations of DP (1.00 μg/ml) and MND (0 17 μg/ml) the R S.D.s for the determination of the free concentration do not exceed 8%

TABLE V

PRECISION OF PLASMA AND URINE DETERMINATIONS ($n=6$)

Concentration of racemic solute (μg/ml)	Matrix	Relative standard deviation (%)			
		Total		Free	
		R	*S*	*R*	*S*
Method I, DP					
0 41	Plasma	4 5	4 3	–	–
2 70		–	–	4 8	5 4
3 10		4 0	3 8	–	–
0 80	Urine	2 6	2 8		
30 1		4 9	5 2		
		R and *S*		*R* and *S*	
Method II, DP					
1 00	Plasma	3 2		5 3	
1 59		3 8		6 5	
3 95		2 4		6 0	
59 8	Urine	2 0			
358		0 9			
Method II, MND					
0 17	Plasma	4 8		8 0	
0 74		5 2		7 2	
1 24		3 4		6 8	
59 5	Urine	1 8			
357		0 8			

In conclusion, method II is to be preferred as therapeutic plasma levels of the enantiomers of both DP and MND can be determined with high precision and recovery It is also easier to avoid disturbances from endogenous compound using method II

The pharmacokinetic and pharmacodynamic results have been given elsewhere [25]

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