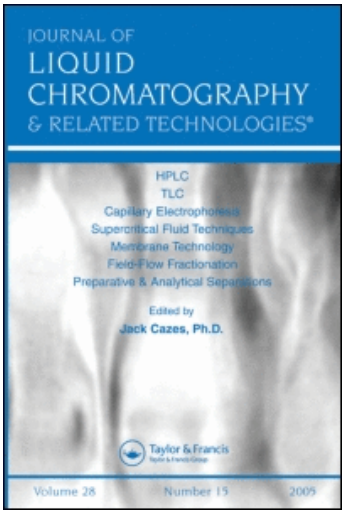


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# Drug Oxidator Phenotyping by Liquid Chromatography: A Review

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# DRUG OXIDATOR PHENOTYPING BY LIQUID CHROMATOGRAPHY: A REVIEW

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ABSTRACT

The oxidation polymorphism has been identified as one of the major factors responsible for interindividual variations in the metabolism of some drugs. The best known examples of this phenomenon are the hydroxilation of debrisoquine and mephenytoin. Poor and extensive metabolizers (oxidation phenotypes) can be separated by measuring the ratio of mother drugs to hydroxy metabolites in the biological fluid after an oral dose of the drug.

This review surveys the current knowledge of high-performance liquid chromatographic methods used in the oxidation phenotyping, most attention being paid to the debrisoquine 4-hydroxylation polymorphism.

## Introduction

Since the synthesis and operation of drug metabolizing enzymes - similarly to other proteins of the organism - is under genetic control (genotype) the "individual" manifestations of drug effects besides the environmental effects can be explained by the discrepant nature of genetic endowments. In this way it is understandbadle that the metabolism of numerous xenobiotics showing polymorphism that is a given population metabolizes the studied preparation not uniformly but polymorphically. Generally three pharmacogenetic phenotypes can be distinguished: an intensively metabolizing, a weakly metabolizing (which in extreme cases demonstrates a total lack of metabolizing capacity) and an intermediate phenotype (1).

The compounds reaching the organism are biotransformed in two phases. In the first phase the xenobiotics are subjected to one or two oxidations, reductions and hydrolysis in the second phase in the course of the so-called conjugation the either the xenobiotic compounds or the oxidative metabolites are connected with an endogenous molecule (acetic acid, glucuronic acid etc.). Both metabolizing phases are genetically determined so in both biotransformation phases such a metabolizing step can be found (that is enzyme reaction) which in the case of administering certain pharmaceuticals (that is substrates) demonsrates polymorphism in the studied population. The two most frequent and most important from a practical aspect is pharmacogenetic phenotype is the "poor/extensive" oxidator and "slow/fast" acetilator.

The oxidator type on the first phase of drug metabolism and the acetilator type gives information on the second phase with respect to its faultless or faulty nature (1).

In this paper the most important oxidator phenotype will be discussed and the analytics of debrisoquine and its 4-hydroxy metabolite will be discussed.

## The polymorphism of microsomal oxigenases of heterogenous functions

An increasing number of data reveal that the rate of one part of oxidative processes going into effect in the organism is determined by genetic factors (2, 3). More recently newer polymorphisms have been identified and their occurrence is connected with the cP-450 enzyme system.

A major percent of the studied populations is unable to carry out oxidation at the appropriate rate. These are called weak (poor) metabolizers. It has been proved that in the case of weak metabolizers what is entailed is that one isoenzyme of cP-450 is missing or is debilitated and it has changed structurally (5).

Recent investigations have verified that a genetically determined disturbance (of polymorph kind) during a monogenetic control, can be demonstrated in the oxidative metabolism of numerous drugs. The metabolism of these drugs is determined by two alleles on the same gene locus. The weak metabolizers are homozygotes in relation to an autosomal recessively inherited gene. The metabolism of debrisoquine, spartein,phenacetin, mephenytoin, nifedipin, tolbutamide, antipirine, teofillin, phenytoin was under the control of monogene cP-450 isoenzymes. The metabolism of the listed compounds goes into effect independently from each other or on deviating cP-450 subtype (3).

The listed compounds can be considered as test materials and of these debrisoquine is outstanding. Knowledge of the metabolisms given is of clinical signifi-

cance in that the rate of decomposition of drugs is closely correlated with the biotransformation speed (6).

## Polymorphism of debrisoquine and spartein metabolism

The recognition of polymorphism was the work of two research groups. Eichelbaum and coworkers (7) with respect to Spartein and Smith et al (8) confirmed in relation to debrisoquine that 6-10 % of the white population of Europe was unable to degrade the compounds at appropriate rate. In this case also two alleles on one locus determine the speed of metabolism (9). The slow metabolizing phenotypes are homozygotes with respect to mitosomal recessive alleles while the effective metabolizers can be hetero- or homozygotes when considering the dominant allele.

Inheritance is of the autosomal recessive kind. In the white population the decomposition of the two compounds occurs on the same cP-450 isoenzyme.

Major ethnic differences may be detected in the speed of hydroxylation of debrisoquine. The incidence of weak metabolizers is 1 % in Egyptians, 5-8 % in Nigerians and Europeans, while it is 30 % in Chinese (29).

The clinical importance of the metabolizer phenotype of debrisoquine is that the rate of decomposition of numerous commonly applied drugs shows a close correlation with debrisoquine hydroxylation speed. The drugs of cardiovascular effect (captopril, enkaidin, perhexilin, guanoxan) belong here, the tricyclic antidepressants (amitriptilin, desipramin, imipramin, clonipramin notriptilin) beta-receptor inhibitors (alprenolol, bufaralol, metaprolol, propranolol, timolol) and other

types of drugs (D penicillinamin, 4-methoxyemphetamic, phenacetin, phenformin) (3).

In weak metabolizers the listed drugs given in the usual dosage may cause serious side-effects.

Newer investigations have proved that in contrast with our knowledge so far, the metabolism of diphenyl-hydantoin does not depend on the debrisoquine phenotype.

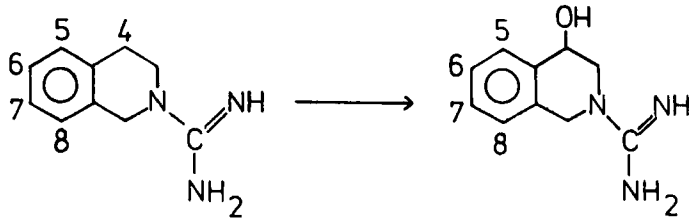
## Analytics of Debrisoquine and 4-OH metabolites from a biological medium



Among the drugs showing polymorphism in the oxidation (hydroxylation) metabolizing step, the most important is the polymorphism of the 4-hydroxylation. Following oral burdening with 10 mg debrisoquine, two phenotypes could be distinguished by determining in urine at $0\text{-}8^{h}$ interval, the eliminated debrisoquine (D) 4-hydroxydebrisoquine (HD) ratio, the so-called metabolization rate (MR). If MR $< 12.6$ we refer to extensive metabolizers (EM), if MR $> 12.6$ then we speak of poor metabolizers (PM). Thus for a quantitative description of debrisoquine 4-hydroxylation the D and HD have to be determined next to each other.

In the analysis made from the D and HD biological medium it is a problem that the original compound is a strong base (of $pK_a$ approaching 12.5) and can be extracted with solvents, if the watery medium is pH $>12$ then the highly polar HD cannot be extracted or very inadequately under the mentioned conditions (11).

This problem was solved earlier (between 1975-1977) with analytical procedures which used gas-liquid chromatography (GC) with preparation of derivates. Allen et al (11) proved with mass and NMR spectroscopy that amidino groups of D and HD is an acetylacetone



debrisoquine /D/ 4-OH-debrisoquine /HD/

Fig. 1 The main metabolization path of debrisoquine



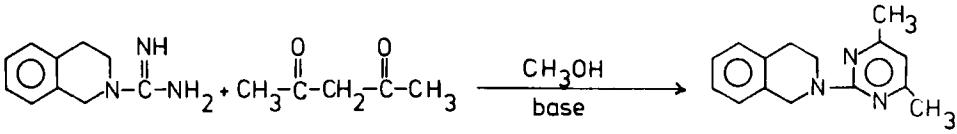



Fig. 2 Derivatization of debrisoquine with acetylacetone

alkaline methanol medium at 50 °C are converted to the 4,6-dimethyl pyrimidine ring (Fig. 2).

The 2 - (4,6-dimethyl-2-pyrimidyl) - 1,2,3,4 - tetrahydroisoquinoline recovered from urine could be well extracted with chloroform. Erdtmansky and Goehl carried out besides the D, the determination of drugs containing other monosubstituted guanido groups from a biological medium (12). They carried out ring closure of 4,6-dimethylpyrimidine at 100 °C with hexafluoro-acetylacetone in two hours. Tucker et al (13) likewise used acetylacetone for D and HD "in situ" derivatiza-

tion. The pyrimidines formed were extracted with ether and following hydrochloric acid resolution and by setting the pH to alkaline, the final extraction with carbon disulphide gave the derivates. As we mentioned they also used GC. Their method essentially was suitable for determination not only from urine but from saliva and blood. Quantification was carried out with the help of guanoxan (G) internal standard which gives the 4,6-dimethylpyridine enclosure ring with acetylacetone and the derivates behave in the same way chromatographically and chemically, as D and HD.

The most important features of the GC method are shown in Table 1.

Liquid chromatography (HPLC) the D/HD the so-called metabolization rate determination analytics made a break through in 1986. Studies of pharmacogenetic populations entailing huge numbers required methods which were quick cheap and undemanding especially with respect to preparation of samples. Westwood et al (14) filtered the urine samples on one millipore (0.22 µm) and the filtrate was injected into the liguid chromatograph. Internal standards were not used. The separations were carried out and UV detection, with reverse-phase chromatography. The limits of both materials were given in 0.1 µg/ml. The determinations of the day-to-day coefficient of variation (c.v.) was below 10 %. The conditions of the liquid chromatography are given in Table 2. Harrison et al (15) found the recovery of D and HD from urine with the use of short columns (3 ml size) of silica modified with carboxylic acid ion exchange functional groups (CBA Bond-Elut$^{TM}$). The mean recovery from urine (n=6) to be 86.9$\pm$1.7 % for D and 76.4$\pm$1.7 % for HD at a concentration of 5 µg/ml. The limits of the results were 0.2 µg/ml for D and 0.1 µg/ml for HD. The day-to-day coefficients of variation

Table 1

GC CONDITIONS FOR D AND HD ANALYSIS

| Reference | Column | Packed | Temperature (°C) | | | Detection |
|---|---|---|---|---|---|---|
| | | | injector | column | detector | |
| Allen(11) | 5 footx2 mm I.D.glass | 3% OV-225/Chromasorb W | | 180-240 | (2°C/min) | FID, GC/MS |
| Erdtmansky(12) | 1.8 m x2 mm I.D.glass | 3% OV-17/Gas Chrom Q | 200 | 160 | 200 | EC |
| Tucker(13) | 1.8 m x4 mm I.D.glass | 3% OV-225/Gas Chrom Q | | 250 | 250 | FID |
| Tucker(13) | 1.8 m x2 mm I.D.glass | 3% OV-225/Gas Chrom Q | 275 | 245 | 275 | Nitrogen selective |

Table 2.

HPLC CONDITIONS FOR D AND HD ANALYSIS

| Reference | Column | Mobile phase | Flow-rate | Detection | Derivatization | Intern. stand. |
|---|---|---|---|---|---|---|
| Westwood(14) | Brownlee RP-8, 250x4.5 mm I.D., 5 µm | 0.008 M $KH_2PO_4$ (pH 5.0)-acetonitrile (55:45, v/v) | 2 ml/min | U.V.,208 nm | - | - |
| Harrison(15) | $C_{18}$ µ Bondapak™ 250x4 mm I.D., 10 µm | acetonitrile-0.1 M $NaH_2PO_4$ (10:90, v/v) | 2 ml/min | U.V.,220 nm | - | - |
| Róna(16) | MOS-Hypersil RP-8, 200x4.6 mm I.D., 10 µm | acetonitrile-methanol--water (18.75:56.25:25, v/v) | 1 ml/min | U.V.,232 and 248 nm | + | + |
| Siest(17) | LiChrosorb CN-HIBART RT, 250x4 mm I.D, 5 µm | 8 mM phosphate Buffer(pH5)--acetonitrile (40:60, v/v) | 2 ml/min | U.V.,208 nm | - | - |
| Tillement(18) | $C_8$ in a Z module system 100x5 mm I.D., 10 µm | acetonitrile-8 mM $KH_2PO_4$ (pH 4.8) (45:55, v/v) | 2 ml/min | U.V.,200 nm | - | + |
| Chan(19) | µBondapak $C_{18}$ cartridge in a Z module system 100x8 nm I.D.,which was linked to a $C_8$ pre-column | methanol-water(pH 3.5)with orthophosphoric acid, with 10 mM 1-pentanesulphonic acid sodium salt (70:30, v/v) | 1.5ml/min | U.V.,248 nm | + | + |
| Moncrieff(20) | Spherisorb S5 nitrile (250x4.6 mm I.D.) preceded by a ODS guard column (20x4.6 mm I.D.) | acetonitrile-0.1 M $(NH_4)_2HPO_4$ (pH 2.5) (6:94, v/v) | 2 ml/min | U.V.,208 nm | - | + |

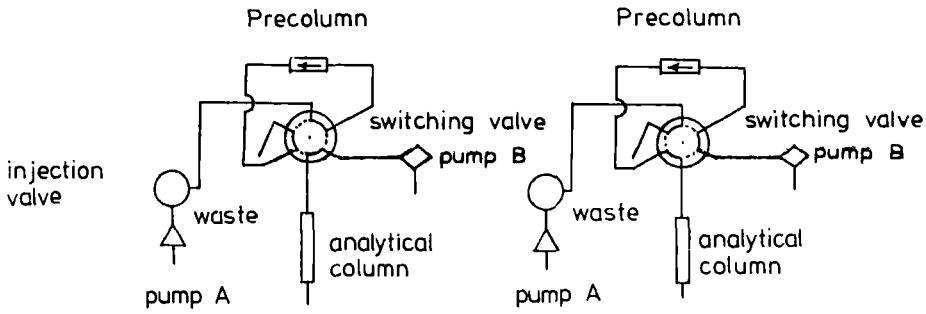



Fig. 3 Column - switching system for the analysis of D and HD in urine by Siest and coworkers



applied in reverse-phase chromatography (Table 2) of 2.0 % and 2.3 % for D and HD respectively, irrespective of whether this method did not use an internal standard, can be regarded as good. Róna et al (16) for obtaining recovery samples used in situ acetylacetone derivatization (11, 13) which has been proven suitable in GC. Also used G internal standard. Derivates from urine were obtained on Extrelut column with double diethyl ether extraction. In their reverse-phase chromatography a buffer was not used in the elution (Table 2). In the sample analyses the chromatographic peak homogenicity was checked with UV spectroscopy by using diode array detector. They found 0.1 µg D and HD/ml of urine as the limit values. Their method was the day-to-day coefficients of variation of the slope of the calibration curve which gave 6.59 % for D and 4.56 % for HD (n=5). Siest and coworkers (17) have proposed a new procedure based on direct injection of urine samples onto the HPLC system using a precolumn switching method (Fig. 3).

They injected the urine samples onto a precolumn dry-packed with Lichrosorb CN (30 x 4 mm I.D., 10 µm) and flushed at a flow rate of 0.5 ml/min with 8mM phosphate buffer (pH 5) containing 5 % v/v acetonitrile (Fig. 3 a sample clean-up). Then 3 min after injecting the valve was switched over and the sample was eluted onto the analytical column Lichrosorb CN-HIBART (250 x 4 mm I.D., 5 µm) with the same buffer containing 60 % (v/v) acetonitrile at a flow rate of 2 ml/min (Fig. 3 b sample elution step). The limit of detection of both investigated compounds was lower than 0.1 µg/ml. The C.V. of the method did not exceed 3.3 % and 3.8 % for intra-inter-assay. Considering that there is no recovery step where there is loss of material they did not use internal standards. Their method was compared on the one hand with the likewise direct sample injection of Westwood et al (14) on the other, with the GC method of Lennard et al (13). They established that in their method the C.V. was lower than that of Westwood and the metabolic status (no the measurement data but the MR = D/HD) of nineteen healthy volunteers showed a high correlation between the two methods (r=0.999).



Druche, Barre and Tillement (18) retained the previously good G internal standard but they did not use derivate preparation but obtained D, HD and G from urine samples with hexanol in the presence of 4M sodium hydroxide (see Table 2). The applied reverse-phase chromatography (see Table 2) the $67.5 \pm 3.2$ % of HD $97.3 \pm 2.3$ % of D and $94.3 \pm 3.8$ % of G were recovered. The detection limit was found to be lower than 0.2 µg/ml for D and HD. The day-to-day C.V. for the slopes of the calibration curves were 3.3 % for D and 2.1 % for HD (n=5).

Chan (19) studied the distribution of the D hydroxylation phenotypes of the Chinese population. He compared the HPLC applied by him (see Table 2) with a

3 % OV-225 (Chromosorb W stationary phase) oven maintained at 200 $^{o}$C with measured GC values. In both techniques the G internal standard and the Róna et al (16) described double ether extraction were used but not Extrelut column but in the form of liquid extraction.

The acetylacetone derivatization was carried out at 96 $^{o}$C in 2.5$^{h}$. Their method is suitable for determining D and HD from urine, plasma and saliva. The authors recommend the GC method operating with an N selective detector having ca 3 ng/ml limit for D pharmacokinetic measurements obtained from plasma and for phenotypification the HPLC method is proposed. The day-to-day coefficient of variance of their GC method for D is 7.3 %, for HD 7.9 % while in the case of HPLC 6.9 % and 8.7 %.

Moncrieff's (20) HPLC method resembles Decolin (17) pre-switching technique on-line clean-up of direct urine. Moncrieff used a standard isochratic liquid chromatograph with the modification that the Supelco 30 µm LC-18 pellicular pre-column was connected at the position of the loop. The chromatographic conditions are shown in Table 2. The urine samples after addition of puffer and practolol internal standard are injected (350 µl) onto the pre-column. This is immediately followed by the slow injection of 1 ml of 20 % methanol in 0.02 M diammonium phosphate (pH 7.6) over 30 sec to flush the very polar components to waste. After this the retained sample in the inject position is flushed onto the analytical column. After 3 min the injector valve was returned to the load position and the pre-column re-generated by flushing with 2 ml water ready for the following sample to be loaded. The inter sample percentage standard deviation was 1.84 % and 1.78 % at 5 µm/ml and 2.64 % and 4.58 % at 0.5 µg/ml for D and HD respectively (n=5).

## Enantionselectivity of 4-hydroxalation of debrisoquine

D which originally does not contain a chiral centre becomes optically active with 4-hydroxylation on the 4. carbon atom (Fig. 1) and two enantiomers are produced R-(-)-HD and S-(+)-HD. Meese et al reported on a gas chromatographic mass spectometry (GC-MS) (21) and a HPLC (22) method; used for separation of two HD enantiomers eliminated in urine and for their quantitative determination after the oral dose of D.

To describe quantitatively the 4-hydroxylation of stereo selective debrisoquine (naturally disregarding the chiral next to MR) the so-called enantiomeric excess (e.e.) concept is introduced. The % e.e. was calculated by

$$\% \text{ e.e.} = \frac{(S - (+) - HD) - (R - (-) - HD)}{(S - (+) - HD) + (R - (-) - HD)} \times 100$$

If e.e. is 0, there is no stereoselectivity and if e.e. is 100 is means that product formation is stereospecific. Meesse et al found that in the case of EM individuals e.e., $\geq$ 99 % while in PM this value ranged between 67-80 %. In other words, this means that in the case of EM the 4-hydroxylation is practically stereospecific 98-99 % of HD eliminated in the urine is S enantiomer. The HD "produce" by PM however is 10-35 % of the R-enantiomer. Extraction of samples with HPLC method (22) begins with acetylacetone derivatization in well proved alkaline medium, this time in 72 h at room temperature in the dark. The internal standard of 4-hydroxy-4-phenylpiperidine-1-carboxamidinium hemisulphate hemihydrate was synthesized by the authors. The extraction was carried out with n-hexane, the organic phase was evaporated under nitrogen at room temperature. The re-

sidues were dissolved in 100 µl (in the case of PM) or 500 µl (in the case of EM) of n-hexane and ca. 40-80 µl were injected onto a chiral "Pirkle" column (250x4.6 mm I.D.) (R) - N - 3,5-dinitrobenzoylphenylglycine covalently coupled to 5 µm amino propylsilica). The mobile phase was n-hexane-ethanol-tert. butyl-methyl ether = 98 : 1 : 1 (v/v), the flow rate was 1 ml/min. They used a fluorescence detector set at $\lambda_{ex}$ = 265 nm and $\lambda_{em}$ = 380 nm.

With the separation of the two enantiomers a separation factor of 1.03 - 1.05 ($\alpha$) and peak resolution of 0.79 - 1.15 (R) was obtained. The limit values were set at 5 nmol S- (+) - OH/ml. The R enantiomer has a shorter retention time 0.5 % of R(-)-OH/100 pmol/ml can be detected in the presence of excess S-(+)-OH.

The limit values from 1 ml of urine were determined at ca 5 pmol for D and 25 pmol in the case of both HD enantiomers.



## Hydroxylation polymorphism of mephenytoin

Mephenytoin (M) is a racemic compound. During metabolization it undergoes aromatic hydroxylation or oxidative demethylation then glucuronidation (Fig. 4).

Its metabolization is under monogenic control i.e. the S isomer is metabolized predominantly by aromatic 4-hydroxylation and the R isomer by oxidative demethylation. The disorder of metabolism shows an autosomnal recessive trait, it affects aromatic 4-hydroxylation but not N-demethylation. The S enantiomer hydroxylated rapidly and is emptied only within days or weeks. In weakly hydroxylating individuals the metabolism of both R and S mephenytoin is more prolonged which may lead to the increase into a toxic range of the blood level of the compound. It was verified in the same persons that

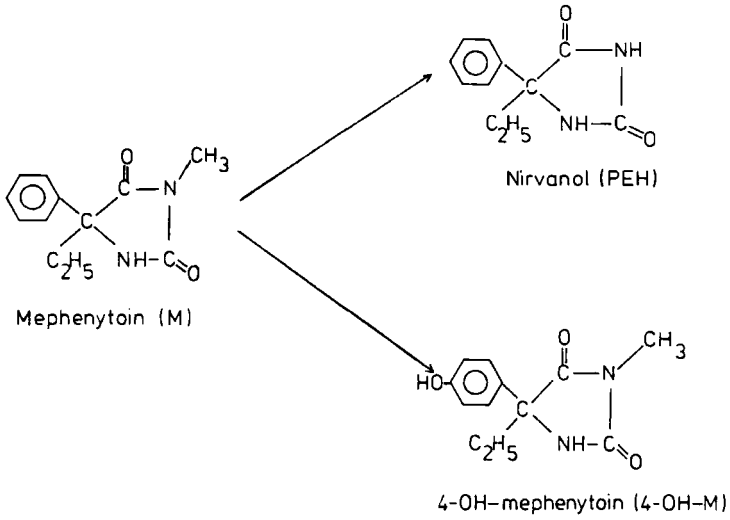



Fig. 4 The main metabolic pathways of mephenytoin



hydroxylation of mephenytoin and debrisoquine/sparteine are independent of each other. The occurrence of the weakly hydroxylating persons among the European whites is about 5 % however it is 23 % in Japanese. According to investigations made so far only mephobarbital metabolism seems to be interrelated with the mephenytoin phenotype (23, 24, 25, 26, 27, 28).

## Analysis of mephenytoin and 4-hydroxymephenytoin from a biological medium

Küpfer et al (25) who discovered of this deficiency, determined the amount of 4-OH-M excreted into the urine following decomposition of the conjugates 0-8 hours after the oral administration of 100 mg racemic M. Similar to MR used in D the concept of the so-called hydroxylation index (HI) was introduced

$$HI = \frac{\mu mol\ M\ dose\ (S\text{-}enantiomer)}{\mu mol\ 4\text{-}OH\text{-}M\ in\ urine\ 0\text{-}8^{h}}$$

S-mephenytoin figures in the numerator, because in man only the S-enantiomers of M and PEM are subject to aromatic hydroxylation (Fig. 4).

The so-called (M defect) oxidation deficiency of mephenytoin type occurring independent of the already discussed and according to our view most important (so-called S-defect) oxidation deficiency of debrisoquin / sparteine type can be defined in two ways. First by calculating HI, by the analytical determination of 4-OH-M discharged into the urine by GC (23, 24), GC-MS (28) and the HPLC (23) method to be discussed below through HI. On the other hand phenotypization can also be performed by measuring the S:R enantiomeric ratio of M in the urine. This can be made by the chiral capillary GC method (29). It should be noted that there is a HPLC method too for the separation and measurement of the two enantiomers but not from a biological medium (30). Sybilska et al (30) separated the two enantiomers on Lichrosorb RP 18 stationary phase of 30 mM cyclodextrin in 18 % ethanol acetate buffer solution of pH 5.0 (0.6 ml/min).

The urinary concentration of 4-OH-M was measured by method of Küpfer (23). Briefly, phenytoin (internal standard) and hydrochloric acid was added to urine. This mixture was then heated at 100 $^{o}$C for 2 hour, after the sample was cooled and it was extracted by dichloroethane. The dichloroethane phase was evaporated to dryness at 40 $^{o}$C under nitrogen. Hexane and KCl buffer pH 12.0 were added, the mixture was shaken and centrifugated. The aqueous phase was acidified and after was extracted by dichloroethane. The organic extract also was evaporated to dryness under nitrogen. The

residue was dissolved in mobile phase and aliquots were injected onto the μ-Bondapak C-18 (0.5 cm x 4.1 mm I.D., 10 μm) analytical column. The mobile phase was biner: 70 % 0.1 M $KH_2PO_4$ pH 6.5 and 30 % methanol. The flow-rate was 1.8 ml/min. The absorbance of the effluent was monitored at 211 nm. The calibration curve of area ratios versus investigated compound concentration were linear in the 2-100 μg/ml concentration range. The reproducibility of the assay was 7.5 %.

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