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D. de Boer, R.M.A. van de Wal, K.L. Bos, R.A.A. Maes:
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Fluoroacyl-(*S*)-prolyl derivatization for the enantiomeric profiling of 3,4-methylenedioxymethamphetamine, methylphenidate, ephedrines and their major metabolites in urine

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Introduction

The importance of enantiomeric profiling in pharmacodynamics and toxicology in general has been well established. Examples can be given for the metabolism of racemic 3,4-methylenedioxymethamphetamine (MDMA) [1] and of methylphenidate (MPH) [2]. Because of the necessity to differentiate between the enantiomers of some ephedrines and amphetamines, this type of profiling is also gaining significance in doping analysis.

MDMA, more commonly known as 'Ecstasy' or 'XTC', is an amphetamine analogue, which may be considered as a recreational drug, rather than a doping agent. In animal models, (*S*)-MDMA is a more potent psychomimetic agent than the (*R*)-enantiomer and apparently only the (*S*)-enantiomer is neurotoxic. Besides the parent compound, the major metabolites in urine, which can be found after deconjugation, are 3,4-methylenedioxyamphetamine (MDA), 4-hydroxy-3-methoxymethamphetamine (HMMA) and 4-hydroxy-3-methoxyamphetamine (HMA), respectively (Figure 1).

The ephedrines are central nervous stimulants (CNS) similar to amphetamine but their CNS effects are less marked. Their main therapeutic application is in over-the-counter cough medicines. Since ephedrine contains two asymmetric carbon atoms, four stereoisomeric representations are possible, two diastereoisomers ((*1RS,2RS*)-ephedrines[1] and (*1RS,2SR*)-ephedrines[2]) and two enantiomers per diastereoisomer. Only (*1R,2S*)-ephedrine and (*1S,2S*)-ephedrine are commonly used. The main metabolites, the norephedrines, results from *N*-

[1] Indicated generally as the "normal" ephedrines or (±)-ephedrines

[2] Indicated generally as the pseudoephedrines or (±)-ψ-ephedrines

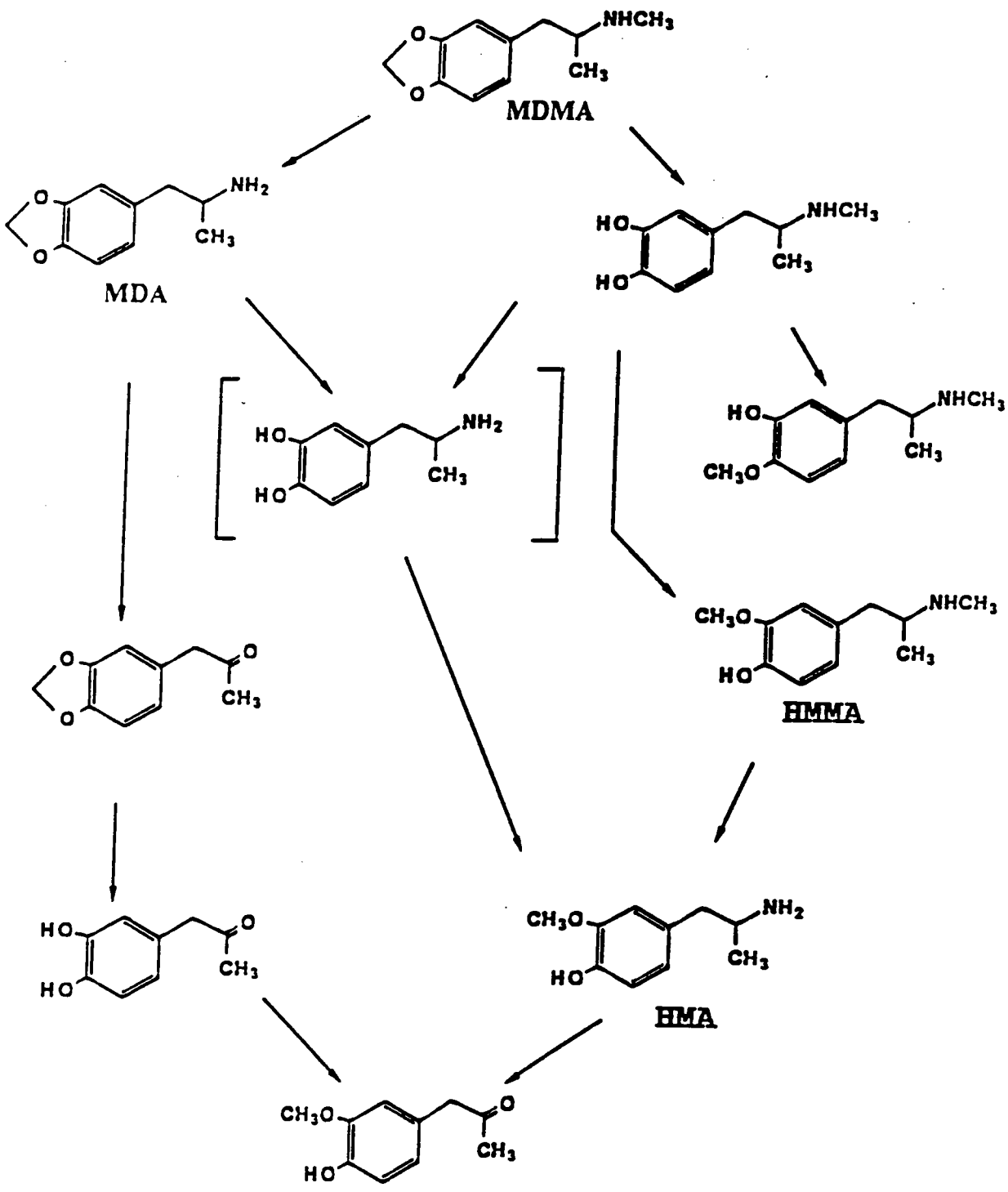

Figure 1 Main metabolic routes of 3,4-methylenedioxymethamphetamine in human

demethylation.

MPH is a mild CNS and is an important medication in the therapy of hyperkinetic syndromes in children. The pharmacological active enantiomer is (*R*)-MPH and the main metabolite is ritalinic acid. MPH is regarded as a doping agent.

We report a chiral assay for the enantiomers of the respective compounds, which is based on the formation of diastereoisomers by derivatization and detection by GC/MS. The derivatization method consists of a reaction with *N*-fluoroacyl-(*S*)-prolyl chloride combined to a consecutive reaction with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, finally resulting in *N*-(fluoroacyl-(*S*)-prolyl)-*O*-trimethylsilyl derivatives.

Experimental

Materials

Heptafluorobutyric anhydride (HFBA) and (*S*)-proline were purchased from Aldrich (Aldrich Chemie, Axel, the Netherlands). Cyclohexane was obtained from Baker (JT Baker, Deventer, the Netherlands). Pentafluoropropionic anhydride (PFPA) was purchased from Pierce (Pierce Europe BV, Oud-Beijerland, the Netherlands) and thionyl chloride from Merck (Merck Nederland BV, Amsterdam. the Netherlands). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Macherey & Nagel (Macherey & Nagel, Düren, Germany). All other chemicals were of analytical grade.

Synthesis of N-fluoroacyl-S-prolyl chloride

N-trifluoroacetyl-(*S*)-prolyl chloride (TPC) is commercially available as a 0.1 M solution in dichloromethane (Aldrich Chemie, Axel, the Netherlands). *N*-pentafluoropropionyl-(*S*)-prolyl chloride (PPC) and *N*-heptafluorobutyryl-(*S*)-prolyl chloride (HPC) were synthesized according to Lim *et al.* 1986. All chemicals were purified by distillation prior to use.

For the synthesis of PPC or HPC 1 ml of fresh distilled PFPA or HFBA was dissolved in anhydrous 6 ml of diethyl ether and added to 0.24 g of (*S*)-proline in a round-bottom flask at -78°C in a dry ice-acetone bath. After 10 min the flask was allowed to stand at room temperature for 2.5 h. The diethyl ether and unreacted HFBA were removed under reduced pressure by a rotating evaporator at room temperature. A solution of double distilled thionyl chloride (3.2 ml) in 6 ml of cyclohexane was added to the residue at 0°C and the reaction mixture was allowed to stand at room temperature for 2.5 h. The cyclohexane and unreacted

thionyl chloride were removed under reduced pressure by a rotating evaporator. The residue was dissolved in 1 ml of dichloromethane and the solvent again was removed under reduced pressure by a rotating evaporator. This process was repeated three times. The residue was finally dissolved in an appropriate volume of dichloromethane to give a concentration of 0.02 M. The purity of the reaction mixture was checked by TLC.

Isolation of metabolites from urine samples

The metabolites of MDMA and MDMA itself were isolated from urine simultaneously according to Lim and Foltz 1989 [3]. MPH and its metabolite ritalinic acid were isolated separately according to Lim *et al.* 1986 [2]. In the procedure for the metabolite, ritalinic acid was converted into MPH by esterification by the Fisher-Speier reaction and analyzed separately from MPH itself.

Derivatization

To the reaction tubes with the dry residues 1 ml of 5% carbonate buffer pH 9 were added. After cooling in an ice-water bath for 20 min, 20 µl of HPC reagent were added and the tubes were immediately capped and vortexed. The tubes were kept in the ice-water bath for 2 h and were vortexed every 30 min. The samples were extracted with 4 ml of cyclohexane by gently rocking for 20 min. Complete phase separation was achieved by centrifuging. The organic layer was transferred to another tube and evaporated to dryness under a gentle stream of nitrogen at 75°C. The dry residue was re-dissolved in 50 µl of ethyl acetate for GC/MS analysis. In the case of profiling of MDMA metabolites or the ephedrines an additional derivatization step was performed. To the residue 50 µl of MSTFA were added and the mixture was kept at 80°C for 40 min. After cooling to room temperature this mixture was ready for GC/MS analysis. The derivatization steps are illustrated in Figure 2.

GC/MS analysis

The GC/MS analysis was performed by the Varian Saturn II with a Varian 3400 Gas Chromatograph (Varian, Houten, the Netherlands) equipped with a DB-5MS column (30 m x 0.25 mm; 0.25 µm film thickness) (J&W Scientific, Folsom, CA, USA).

Results and Discussion

The *N*-fluoroacyl-(*S*)-prolyl derivatives of the enantiomers of MPH showed the best GC-resolution (Figure 3A). For the enantiomers of MDMA also complete GC-resolution was

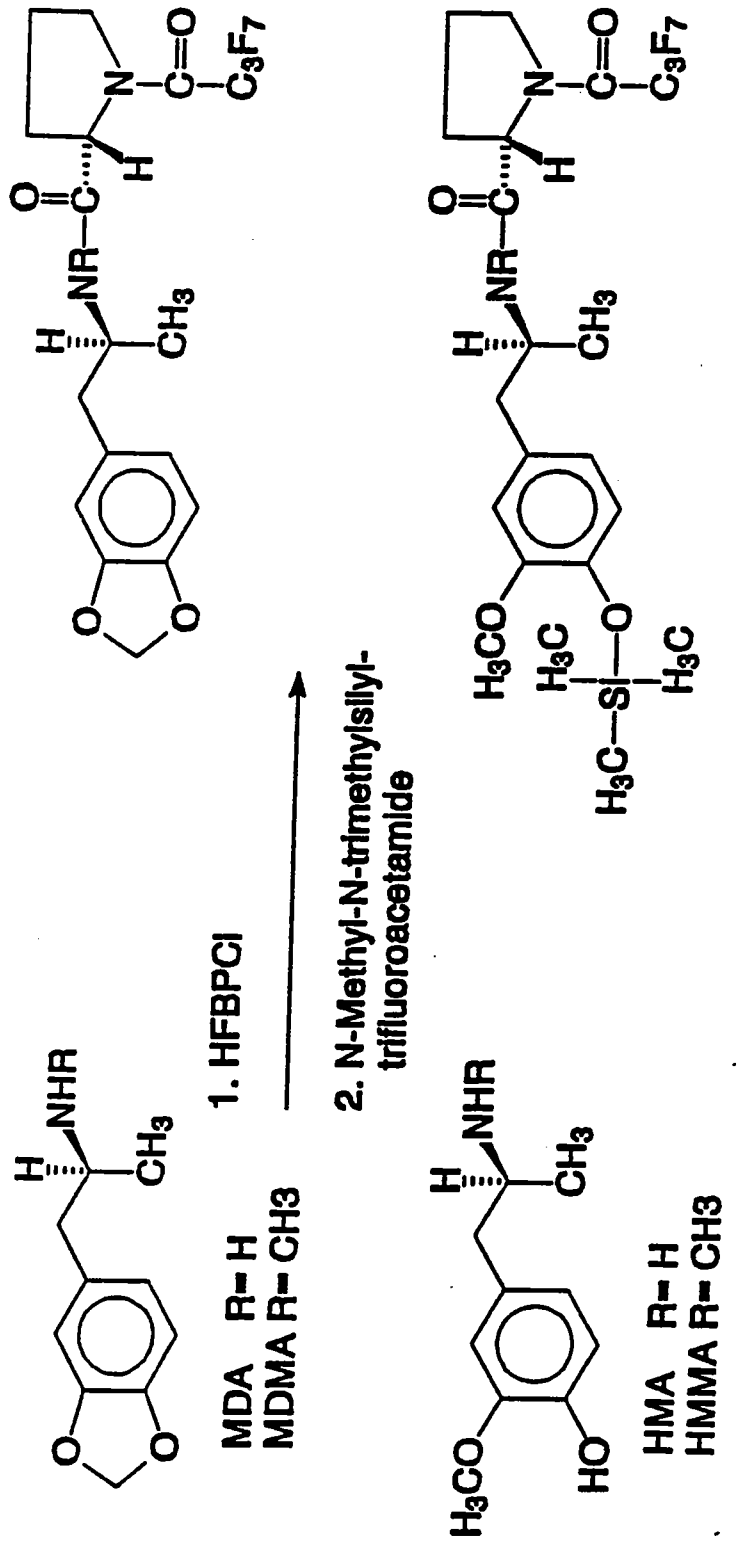

Figure 2 The *N*-(fluoroacyl-(*S*)-prolyl)-*O*-trimethylsilyl derivatization of MDMA and some of its metabolites.

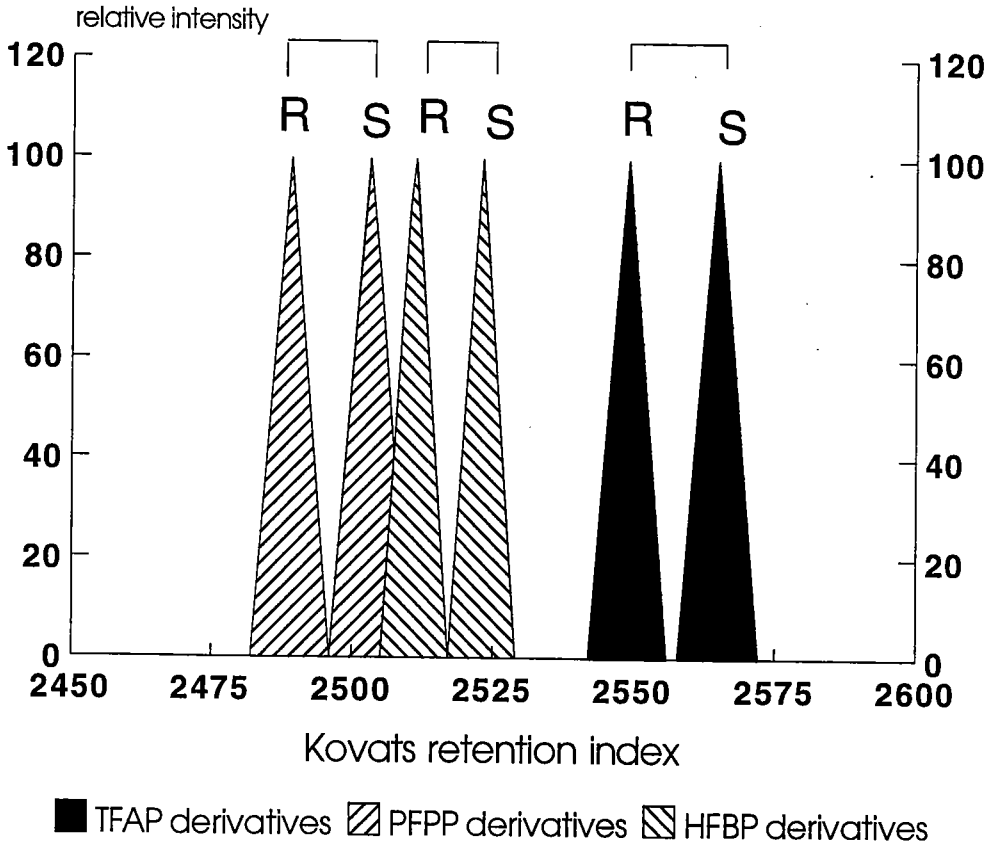

Figure 3A Kováts indices of the *N*-fluoroacyl-(*S*)-prolyl derivatives of the enantiomers of 3,4-methylenedioxymetamphetamine

obtained, although the difference between the enantiomers was smaller (Figure 3B). Because the ephedrines contained a hydroxy group, the overall derivatization was combined with a trimethylsilylation step. The diastereoisomers as well as the enantiomers of the ephedrines and the norephedrines however, were not separated (data not shown). Compared for the MS-responses, the *N*-heptafluorobutyryl-(*S*)-prolyl (HFBP) derivatives in general showed the best results (data not shown). Based on these results the HFBP derivatives were selected as the chiral derivatives of interest.

The HFBP derivatives were successfully applied for the enantiomeric profiling for the metabolism of MDMA and MPH. Because some metabolites of MDMA contained a hydroxy group, the trimethylsilylation step also had to be performed in the MDMA study. Preliminary results indicated an enantioselective metabolism in the *N*-demethylation pathway for MDMA (Figure 4). The de-esterification for MPH showed less marked enantioselectivity (data not shown).

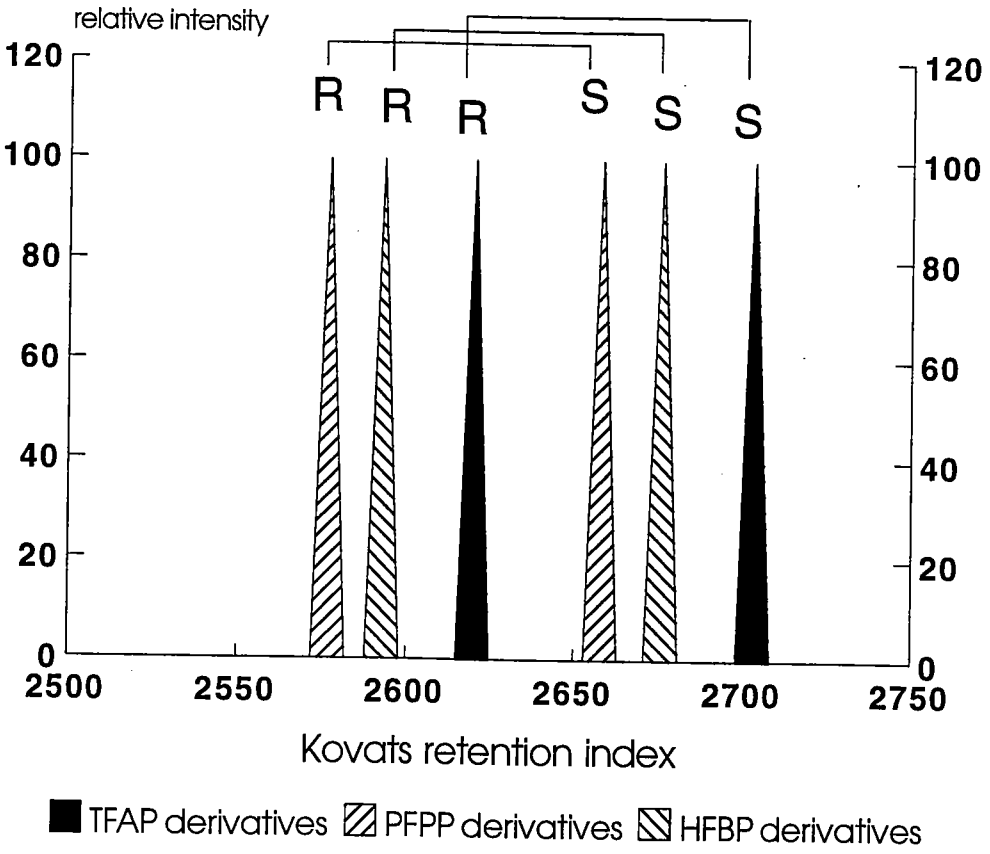

Figure 3B Kováts indeces of the *N*-fluoroacyl-*S*-prolyl derivatives of the enantiomers of methylphenidate

The usefulness of HFBP derivatives was thus demonstrated for the amphetamine anlogue MDMA. In doping analysis the enantiomeric profiling is especially relevant for the amphetamines. Although not demonstrated this type of derivatization can also be applied for the enantiomeric profiling of (*RS*)-amphetamine and (*RS*)-metamphetamine. In some cases the (*R*)-enantiomers can be detected, which are in fact not effective as doping agents. Examples of such cases are if subjects have been using selegiline or a certain Vicks® inhalers. Selegiline, which is a selective monoamine oxidase (MAO) inhibitor. Therapeutically, the (*R*)-enantiomer of deprenyl is used, because it is low-toxic, has less unwanted side-effects and is a 500 times more potent MAO inhibitor, than the (*S*)-enantiomer. The metabolites, desmethyldeprenyl, methaphetamine and amphetamine, are therefore also in the (*R*)-form [4]. The mentioned Vicks® inhalers contain the (*R*)-enantiomer of methamphetamine [5]. Without enantiomeric profiling such cases will be considered as being positive for amphetamine or metamphetamine. The distiction between the diastereoisomers of the ephedrines is also important in doping analysis.

In drugs-on-the working-place analysis the identification of the enantiomeric form is because of this required [5]. Unfortunately, for the GC/MS analysis of the ephedrines this type of derivatization was not successful under the conditions studied.

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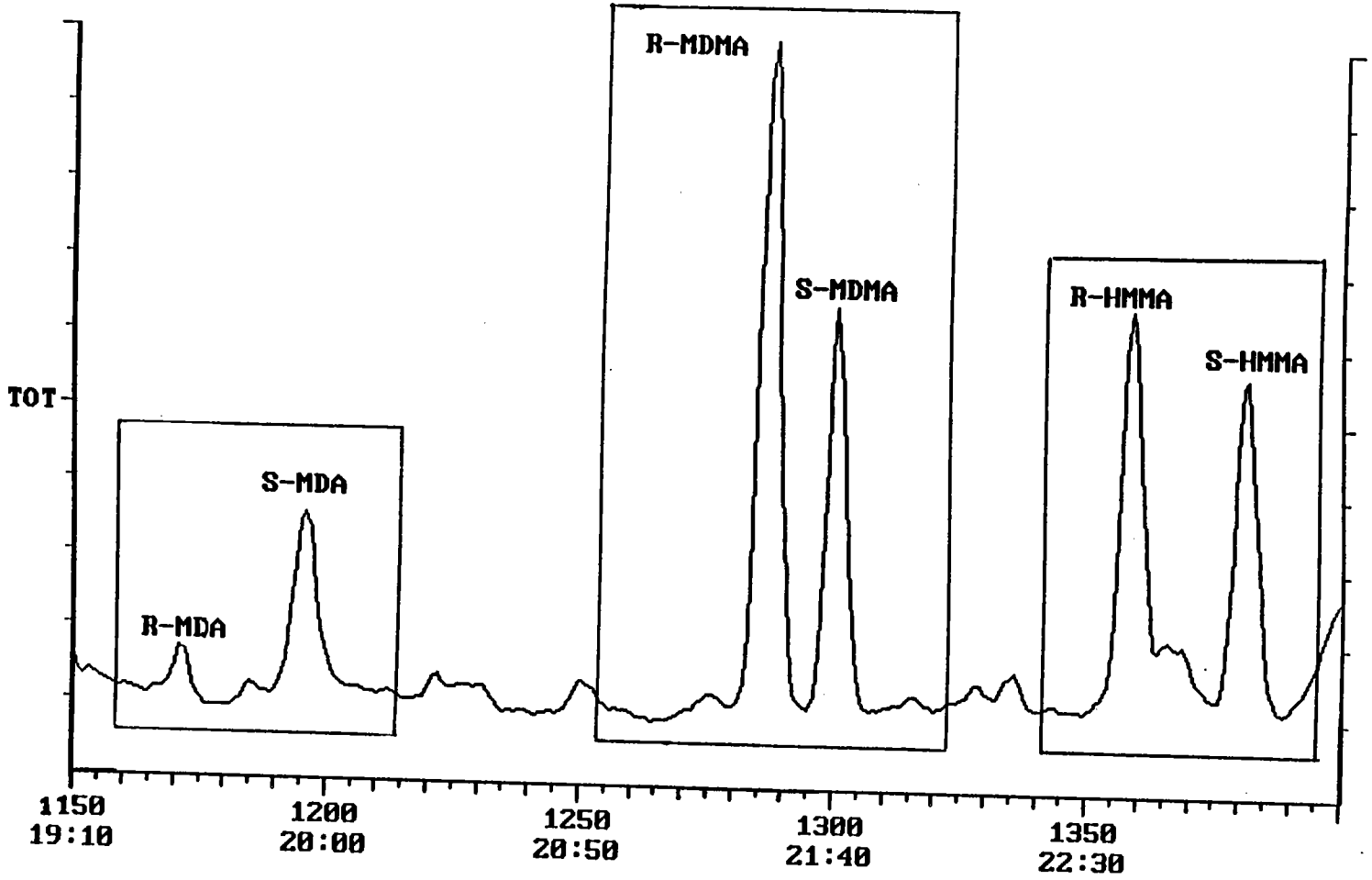

Figure 4 Total ion chromatogram of a prepared urine sample containing MDMA and some of its metabolites as obtained by GC/MS analysis of the respective HFBP derivatives