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Gas chromatography-electron capture determination of styrene-7,8-oxide enantiomers

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Abstract

The enantiomers of styrene-7,8-oxide (phenyloxirane, SO) were determined using a method based on base catalysed hydrolysis with sodium methoxide. The oxirane ring opening resulted in formation, without racemisation, of the enantiomeric pairs of the two regional isomers, 2-methoxy-1-phenylethanol and 2-methoxy-2-phenylethanol. The structure of these regional isomers was confirmed by gas chromatography–mass spectrometry (GC–MS) and proton nuclear magnetic resonance (^{1}H-NMR). To improve sensitivity of determination, the formed methoxy alcohols were subsequently derivatised with pentafluoropropionic anhydride enabling electron capture detection. This derivatization proceeded also without racemisation and the formed pentafluoropropionyl derivatives were separated on two serially coupled columns, a non-chiral AT 1705 and a chiral CP Chirasil-Dex-CB. As internal standard 2*S*,3*S*-(−)-2-methyl-3-phenyloxirane was used. The limit of quantitation of the method was 0.2 μ*M*. The repeatability of the method was assessed at two concentration levels (2.5 and 25 μ*M*) and ranged from 6 to 9% for both enantiomers. The method was applied to the determination of the rate and enantioselectivity of the cytochrome P-450 dependent oxidation of styrene to SO enantiomers in human liver microsomes.

Keywords: Enantiomer separation; Styrene; Phenyloxirane

1. Introduction

Styrene-7,8-oxide (phenyloxirane, SO) is the principal mutagenic metabolite of styrene formed by oxidation catalysed by cytochrome P-450 isoenzymes. In the biotransformation of styrene, SO is the first member of a chiral sequence of metabolites which leads to styrene glycol, SG (phenyl-1,2-ethanediol) and mandelic acid [1]. Various studies revealed a difference in the mutagenic effect in the Ames test for the two enantiomers of SO which is probably due to a difference in reactivity of the isomers towards the chiral DNA [2,3]. Since the enantiomeric nature of the styrene metabolism could have an impact on the toxicological effects, the knowledge of the enantioselectivity of the enzymes involved in the metabolism of styrene could be of ultimate importance in the risk assessment of exposure to styrene. This study was a part of a larger

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study on interindividual differences in the toxicokinetics of styrene. Within that study, the stereochemistry of the cytochrome P-450 dependent oxidation of styrene to the SO in human liver microsomes was assessed. The stereochemistry of cytochrome P-450 mediated oxidation of styrene was previously studied in rat [4,5], mouse [6,7], and rabbit liver or lung microsomes [8]. To the best of our knowledge, so far no similar study has been carried out with human liver microsomes. In an in vivo study in mice, a moderate excess of *S*-SO was recently reported by Linhart [9]. Comparable results were obtained in in vitro studies with rat liver microsomes, where enantiomeric excesses (ee) of *S*-SO of 13 and 22% were found respectively [4,5]. Contrary to these results, an enantiomeric excess of *R*-SO of 24 and 32% was found in the mouse liver and lung microsomes, respectively [7]. Preferential formation of *R*-SO (ee of 23%), was also measured in the rabbit pulmonary microsomes [8]. To support our study on cytochrome P-450 mediated oxidation of styrene in human liver microsomes, a sensitive and enantioselective method for the determination of SO enantiomers was needed. Current methods to determine the enantiomeric composition of SO involve HPLC determination of the (1) glutathione adducts of the SO [5,9], and (2) α-methoxy-α-(trifluoromethyl)phenylacetic acid diesters of *R*- and *S*-phenylethanediols formed via mEH mediated hydrolysis () [4]. Since the concentration of SO enantiomers in the microsomal incubation mixture was very low in the present study, common HPLC techniques lacked sensitivity. Currently, no GC method for the determination of the SO enantiomers in the microsomes was described in the literature. The GC determination of racemic SO has been accomplished either by direct determination using GC–FID [10] or GC–MS [11,12], or, alternatively, by indirect determination following acid catalysed hydrolysis and subsequent derivatization of the formed SG [13,14]. Due to thermal instability of SO, direct determination had to be performed using cold on-column injection [10–12]. Initially, we tried direct determination using different cyclodextrin chiral columns. Good separation of the SO enantiomers was obtained on these columns but the sensitivity needed could not be attained using either FID or MS. Gas chromatography with the more sensitive electron capture detection seemed a good alternative. In a number of studies on activity of cytochrome P-450, SO was converted to SG, using acid-catalysed hydrolysis [13,14]. To improve the sensitivity of the determination, the formed SG was subsequently derivatised with pentafluorobenzoyl chloride [14], or *n*-butylboronic acid [13] enabling electron capture detection (ECD). Applying this method for the determination of the enantiomers has, however, an important obstacle. Since the acid-catalysed hydrolysis mechanism is essentially a carbonium ion S_N1 reaction [15,16], racemisation of the enantiomers occurs, and this method is therefore not suitable for the determination of the enantiomers. Therefore to avoid racemisation, the opening of the oxirane ring should proceed via another mechanism. As an alternative to the acid-catalysed hydrolysis, the oxirane ring could be opened by a stereospecific base-catalysed S_N2 displacement reaction with sodium methoxide (NaOMe) as a strong nucleophile [15,17]. The first step in the development of the stereospecific determination of SO enantiomers was therefore to investigate the stereochemistry of this reaction. In addition, oxirane ring opening, leading to the formation of methoxy alcohols would offer a possibility to improve the sensitivity by subsequent derivatization of the hydroxy group with an acylation reagent suitable ECD. In order to optimise the determination of the enantiomers, different chiral columns were tested. The method was deployed for the determination of the SO enantiomers in human liver microsomes after in vitro incubation with prochiral styrene.

2. Experimental

2.1. Chemicals

(*R*)-phenyloxirane (98% pure, 98% ee) and (*S*)-phenyloxirane (98% pure, 97% ee), styrene and pentafluoropropionic anhydride, 98%, were purchased from Fluka (Buchs, Switzerland); *n*-Hexane, *n*-pentane and sodium methoxide (NaOMe), 2 *M* in methanol (MeOH), from Merck-Schuchardt (Darmstadt, Germany); 4-Bromophenylboric acid, 93% and (2*S*,3*S*)-(−)-2-methyl-3-phenyloxirane, 98% pure, 99% ee), from Aldrich (WI, USA); glucose-6-phos-

phate, glucose-6-phosphate dehydrogenase, G-7877 and β-nicotinamide adenine dinucleotide phosphate (NADP) from Sigma (MO, USA); Pyridine, silylation grade, from Pierce (USA); 2-(trichloromethyl) oxirane, from Boehringer, Mannheim (Germany).

2.2. Sample preparation

2.2.1. Enzymatic incubation of liver microsomes with styrene

Human liver samples were obtained from a non-commercial supplier (IIAM, USA); the microsomes were prepared according to Burke et al. [18]. Incubations with styrene were carried out with a NADPH-generating system. Microsomes were incubated at a protein concentration of 0.5 mg/ml in an incubation mixture containing 5 m*M* $MgCl_2$, 50 m*M* 1*M* K_2HPO_4 (pH 7.4), 1 m*M* NADP, 20 m*M* glucose-6-phosphate, two units of glucose-6-phosphate dehydrogenase, and 50 μ*M* 2-(trichloromethyl) oxirane as an inhibitor of microsomal epoxide hydrolase (mEH) [19,20]. The final volume was 1 ml. Heat-denaturated microsomal protein was used in control incubations to correct for background. The extent of spontaneous hydrolysis of SO in the incubation mixture was investigated in a separate study and showed to amount to less than 2% [21]. This is in agreement with the results of Bin Lin et al., reporting a half-life of SO in water at 30°C of 2 days [16], and with those of Mendrala et al. finding a conversion of SO to SG of less than 2% [12]. After incubation of the mixture for 5 min at 37°C styrene was added in the concentration range 1–1750 μ*M*. After 10 min, the incubation was stopped by adding 2.5 ml of *n*-hexane. After adding 25 μl of a 0.8 m*M* solution of 2*S*,3*S*-(−)-2-methyl-3-phenyloxirane (IS) in *n*-hexane as internal standard, the samples were vortexed for 1 min and then centrifuged for 5 min at 15 000 *g*. The *n*-hexane phase was transferred to 4 ml screw-cap vials.

2.2.2. Standard solutions and calibration curves

Stock standard solutions of SO (0.8 m*M* for both enantiomers) and IS (0.8 m*M*) were prepared in *n*-hexane. The calibration standards were prepared by adding 1.3–12.5 μl of the standard solution to the incubation mixture containing heat-denaturated microsomes (blank sample). The concentration range of the SO enantiomers in the incubation mixture was 1–10 μ*M*. For internal standardisation 25 μl of the stock solution of IS was added. The concentrations of *R*- and *S*-SO in microsomes were calculated by internal standardisation using peak height measurements. The sum of peak heights of both regional isomers of an enantiomer was used for the calculation. All samples were analysed in duplicate.

2.3. Analytical procedure

2.3.1. Oxirane ring opening of SO

The volume of the *n*-hexane extracts was reduced to approximately 100 μl under a stream of nitrogen. After addition of 0.5 ml of NaOMe/MeOH, the mixture was heated to 125°C for 30 min in hermetically closed 4 ml screw-cap glass vials. After cooling to room temperature, the resulting methoxy alcohols were extracted with 2×3 ml of *n*-pentane. These methoxy alcohols could be analysed either directly by GC–FID or, following further derivatisation, by GC–ECD.

2.3.2. Derivatization of methoxy alcohols for GC–ECD analysis

n-Pentane extracts were evaporated to dryness. To each sample 500 μl of a 0.5% pyridine solution in dichloromethane (DCM) and 10 μl of PFPA were added. The mixture was heated to 60°C for 60 min. After cooling to room temperature, the samples were evaporated under a stream of nitrogen and the residue was redissolved in 500 μl of *n*-hexane. After centrifugation for 30 s at 15 000 *g*, the clear supernatant was analysed by GC–ECD.

2.4. Chromatography

2.4.1. GC–FID analysis of methoxy alcohols

GC separation was carried out using a Carlo Erba HRGC 5300 GC equipped with a FID (Interscience, Netherlands), a Fisons AS 800 autosampler (Interscience, Netherlands) and a Turbochrom integrating system (Perkin-Elmer, Netherlands) on a CP Chirasil-Dex CB column (25 m, 0.25 mm ID, 0.25 μm film thickness; Chrompack, Netherlands). The column temperature was 105°C. Helium was used as a carrier gas and column head pressure was 100 kPa. The flow of make-up gas (nitrogen) was 30 ml

min^{-1}. The FID hydrogen flow was 30 ml min^{-1}, and the air flow was 300 ml min^{-1}. The injector and detector temperatures were set to 250°C. The sample (1 μl) was injected with a split injection technique (split ratio 1:20).

2.4.2. GC–ECD analysis of PFP derivatives of methoxy alcohols

The chromatography of the PFP derivatives of methoxy alcohols was carried out on a Hewlett-Packard 5890 GC (Hewlett-Packard, USA) equipped with a ^{63}Ni ECD, a Hewlett-Packard 7673 autosampler and a Turbochrom integrating system (Perkin-Elmer, Netherlands). For the separation, two columns connected with a press-fit connector (Chrompack, Netherlands) were used: AT 1705 (25 m, 0.25 mm ID, 0.25 μm film thickness; Alltech, Netherlands) and CP Chirasil-Dex CB (25 m, 0.25 mm ID, 0.25 μm film thickness; Chrompack, Netherlands). The initial column temperature was 103°C for 30 min; the column temperature was then increased to 200°C at 70°C min^{-1}, and held for 2 min. The column head pressure (He) was 170 kPa and the nitrogen make-up gas flow-rate was 30 ml min^{-1}. The injector temperature was 250°C and the detector temperature was 300°C. The sample (5 μl) was injected with a split injection technique (split ratio 1:20).

2.5. Identification of the reaction products after base-catalysed methanolysis

2.5.1. GC–MS analysis

GC–MS analysis was performed using a HP 6890 chromatograph (Hewlett-Packard, Amstelveen, Netherlands) equipped with a HP autoinjector, a Gerstel cooled injection system 4-plus (Gerstel GmbH, Mühlheim an der Ruhr, Germany) and a HP 5973 mass selective detector on a fused-silica CP Chirasil-Dex CB column (25 m long, 0.32 mm ID with a nominal film thickness of 0.25 μm, Chrompack, Middelburg, Netherlands). The carrier gas phase was helium at a head pressure of 51 kPa (flow of 2.5 ml min^{-1}). Injections of 1 μl were made. The following temperature programme was used: isothermal at 100°C for 20 min followed by a short ramp (50°C min^{-1}) to 170°C, temperature was kept for 2 min. The mass detector was used at electron impact mode; the quadrupole was kept at 106°C and the source at 230°C. The ionisation potential was 70 eV and the multiplier and auto tune voltage +400 eV. The data collection was in scan mode from m/e 50 to 300 at 5.56 scan s^{-1}.

2.5.2. ^{1}H-NMR analysis

In order to isolate the regional isomers of each enantiomer, semi-preparative HPLC analyses were carried out on a HP 1100 liquid chromatograph equipped with an autoinjector, a degasser and a UV detector (set at λ=254 nm). Aliquots of the reaction mixtures of each SO enantiomer with NaOMe were eluted over a 250×4.6 mm ODS Beckman Ultrasphere reversed-phase column (Fullerton, CA, USA) using a linear gradient from 0 to 20% acetonitrile in purified water over a period of 50 min, followed by a short ramp over 5 min to 100% acetonitrile kept for 2 min, then returned to 100% purified water in 3 min. The eluted fractions of multiple 75 μl injections were collected (the final volume was 1 ml) using a Gilson FC 204 fraction collector. UV spectra were obtained from all fractions collected between 40 and 50 min. The fractions containing the different isomers were pooled for each of the 4 isomers and extracted twice with an equal volume of *n*-pentane. The organic phase was evaporated under a gentle stream of nitrogen and the oily residues were taken up in a small volume of $CDCl_3$. Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) spectras were recorded on a Varian Gemini-200 spectrometer (Varian, Houten, Netherlands). $CDCl_3$ was used as the deuterated solvent and chloroform (at δ=7.25 ppm) as the internal standard.

3. Results and discussion

3.1. Oxirane ring opening

The initial step in developing the method for the determination of SO enantiomers was opening of the SO oxirane ring. Using methanolysis, the sensitivity of the determination could be improved by derivatization of the formed reaction product using an ECD suitable reagent, such as PFPA. To follow the ring opening reaction, a GC–FID determination was

used such that the time course of the reaction could be studied and the reaction conditions optimised. After 30 min, the reaction was completed and the peaks of SO and IS had completely disappeared from the chromatogram. As seen from the FID chromatogram, the reaction yielded two peaks for each SO enantiomer (Fig. 1a and b). These two peaks represent, as was confirmed by ^{1}H NMR and GC–MS, the two regional isomers: 2-methoxy-2-phenylethanol and 2-methoxy-1-phenylethanol. The formation of regional isomers is the result of a nucleophilic attack of the methoxide ion on either the α- or β-carbon (Fig. 2) [15,17]. The attack on the β-carbon leads to retention and attack on the α-carbon to inversion of configuration. In the latter case it was, therefore, important that racemisation did not occur. The extent of racemisation was measured by the analysis of the enantiopure standards of *R*-SO and *S*-SO following methanolysis and derivatization. As seen from the FID chromatograms, in the case of both regional isomers a small amount of the other enantiomer was found (Fig. 1a and b). As calculated from the corresponding calibration lines, the relative contribution of the other enantiomer was 1 and 1.5% for *R*-SO and *S*-SO, respectively. This was in agreement with the enantiomeric purity of the SO standards (ee of the *R*- and *S*-SO standards was 98 and 97%, respectively); indicating that the ring opening reaction proceeds without racemisation.

3.2. Identification of the reaction products after base-catalysed methanolysis

3.2.1. GC–MS analysis

The mass spectra of the *R*- and *S*-SO enantiomers after base-catalysed methanolysis are shown in Fig. 3. The first peak in the chromatograms of the methanolysis products of both SO enantiomers was assigned to 2-methoxy-2-phenylethanol (Fig. 1a and b). The absence of the molecular ion (m/z 152) and the fragment of m/z=121 (loss of CH2–OH) is typical for a primary alcohol (Fig. 3a). The second, larger peak was assigned to 2-methoxy-1-phenylethanol, based on the presence of the molecular ion (m/z 152) and the loss of water (m/z 134), which are indicative of a secondary alcohol (Fig. 3b). The m/z serial 107 to 79 to 77 is typical for a benzyl alcohol.

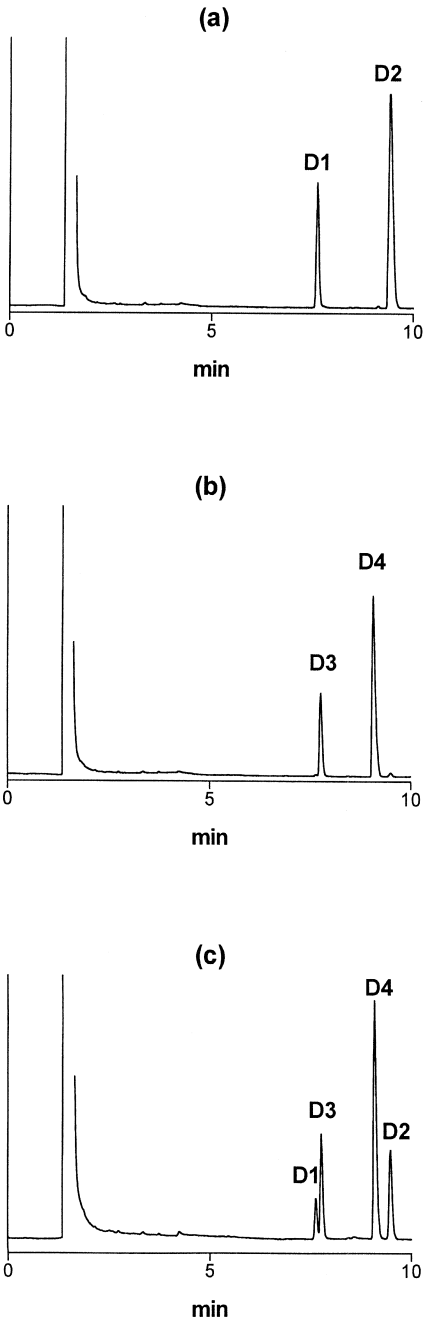

Fig. 1. GC–FID chromatogram after base catalysed methanolysis of (a) *R*-SO, (b) *S*-SO, and (c) their mixture (the concentration of *S*-SO is approximately 2.5 times higher than the concentration of *R*-SO). Chromatographic conditions as described in Section 2.4.1. D1 (2-methoxy-2-phenylethanol obtained from *R*-SO). D2 (2-methoxy-1-phenylethanol obtained from *R*-SO). D3 (2-methoxy-2-phenylethanol obtained from *S*-SO). D4 (2-methoxy-1-phenylethanol obtained from *S*-SO).

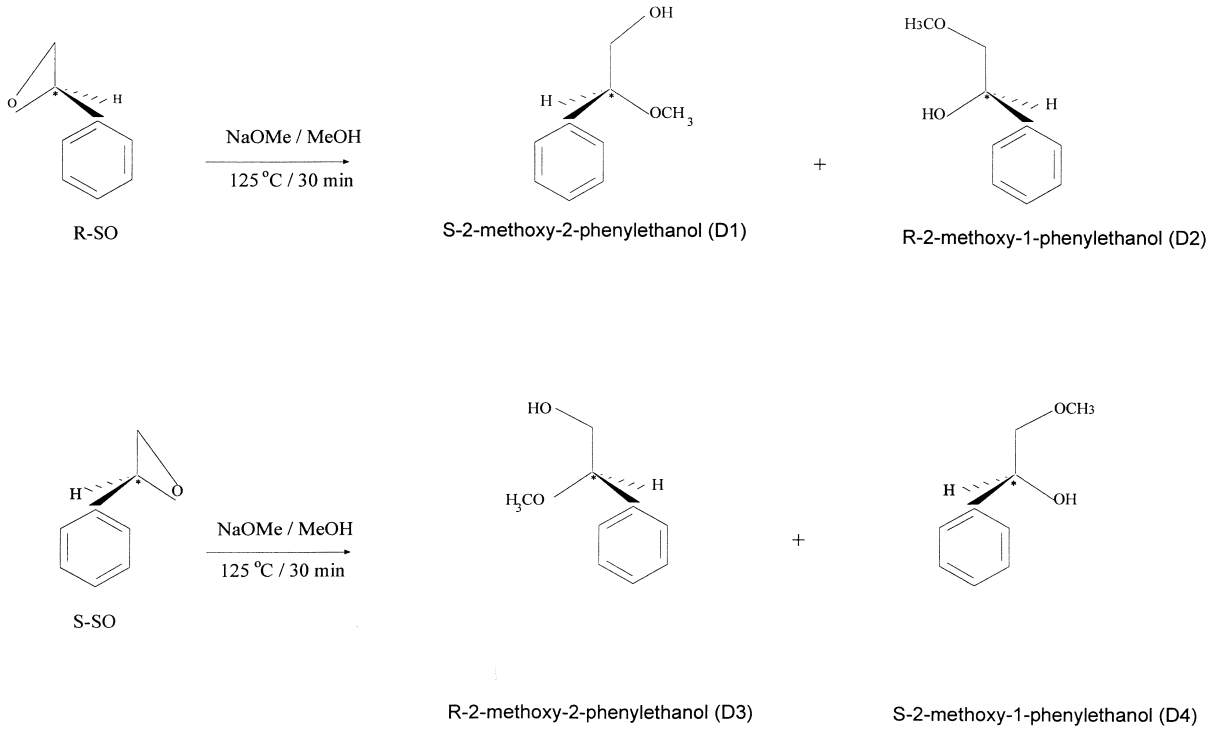

Fig. 2. Base-catalysed methanolysis of *R*-SO and *S*-SO. The structure of the two regional isomers that were formed from each of the SO enantiomers was confirmed by GC–MS and ^{1}H-NMR. The assignment of the stereochemical configuration of the 2-methoxyphenylethanols is based on the assumption that a nucleophilic attack (S_N2) on the chiral α-carbon of SO leads to inversion of configuration, while the configuration is retained following attack on the achiral β-carbon.

3.2.2. ^{1}H–NMR analysis

In order to perform ^{1}H-NMR analysis, the regional isomers of the *R*- and *S*-SO enantiomer products after base-catalysed methanolysis were separated and isolated using semi-preparative HPLC. Similar to the FID chromatograms, two peaks appeared in the HPLC chromatogram for each enantiomer (chromatograms not shown). The first peak in the chromatogram was for both enantiomers larger than the second one. The first peak was assigned to 2-methoxy-1-phenylethanol, and the second peak to 2-methoxy-2-phenylethanol. This assignment was confirmed by ^{1}H-NMR, the first peak showed δ (ppm) 3.3 (2H, φ-CHOH–C**H**$_2$OCH$_3$), 3.5 (3H, φ-CHOH–CH$_2$OC**H**$_3$) and 4.9 (1H, φ-C**H**OH–CH$_2$OCH$_3$) and the second peak showed δ 3.3 (3H, φ-CHOC**H**$_3$–CH$_2$OH), 3.6 (2H, φ-CHOCH$_3$–C**H**$_2$OH), and 4.3 (1H, φ-C**H**OCH$_3$–CH$_2$OH), while both peaks showed δ 7.4 (5H, the phenyl protons).

3.3. Derivatization and ECD determination of the methoxy alcohols

As seen from the ECD chromatograms of the PFP derivatives of the methoxy alcohols, the derivatisation proceeded without racemisation (Fig. 4a and b). Both enantiomers gave two peaks, which correspond to the PFP derivatives of the regional isomers 2-methoxy-1-phenylethanol and 2-methoxy-2-phenylethanol. Although the derivatives of SO were well separated on the CP Chirasil-Dex CB column, they were not well separated from the derivative of the internal standard, which is structurally similar to SO. To achieve better separation, different serially connected columns were used. The best separation was achieved by using a non-chiral AT1705 column and a chiral CP-Chirasil Dex CB (resolution factor, R_s was 1.4). Somewhat lower resolution was achieved by using two chiral columns: CP Cyclodex B 236 *M* and CP-Chirasil Dex CB connected in series (Rf 1.2;

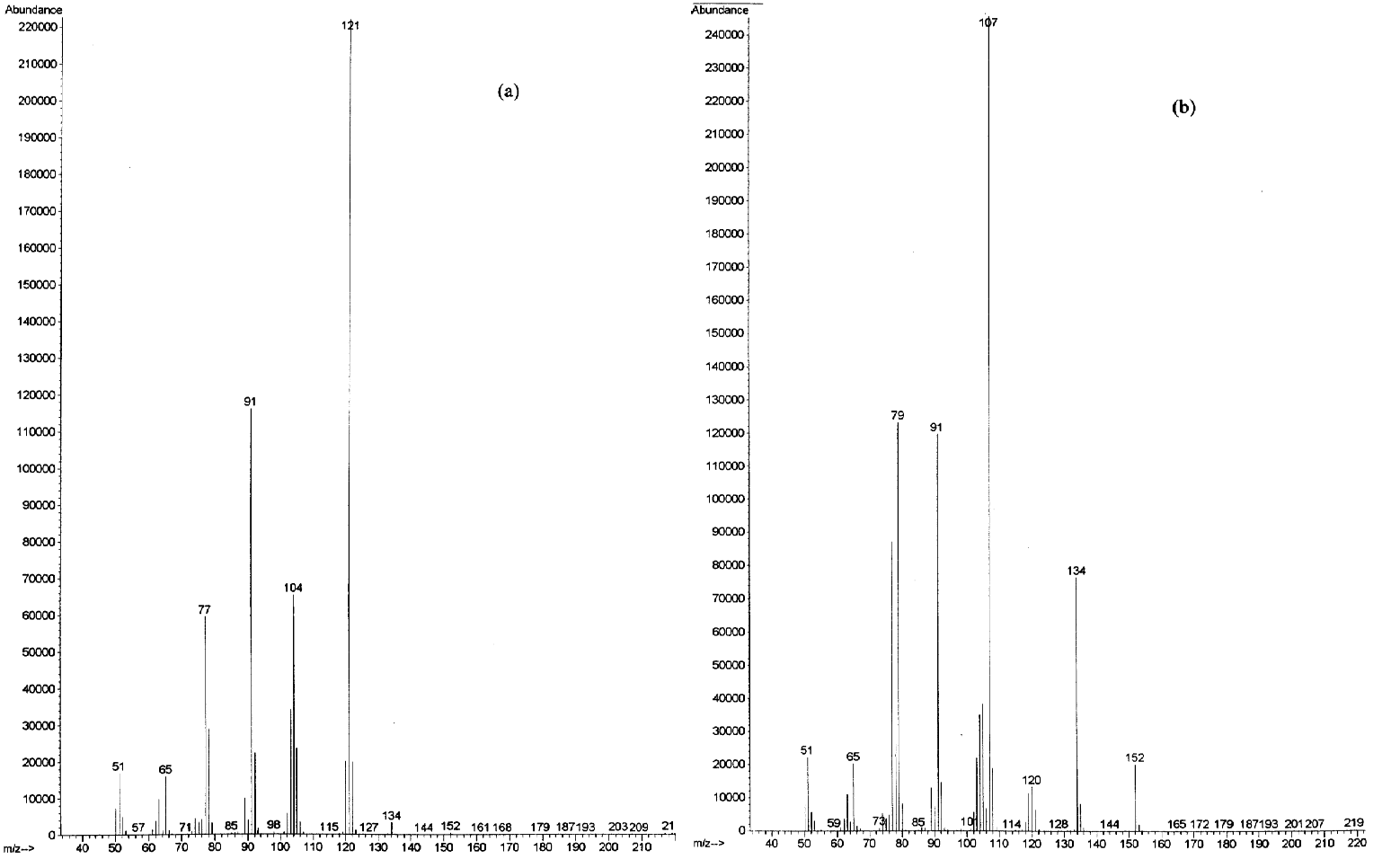

Fig. 3. Mass spectrum of the regio-isomers of 2-methoxyphenylethanols formed after base catalysed methanolysis of *R*-SO and *S*-SO (FID chromatogram is shown in Fig. 1): (a) the first smaller peaks of both enantiomers, D1 and D3; and (b) larger second peaks, D2 and D4. Chromatographic and instrumental conditions as described in Section 2.5.

chromatograms not shown). Although longer retention times were obtained in this way, SO derivatives were well separated from IS derivatives. Good separation of PFP derivatives of SO enantiomers and IS was also achieved in the chromatography of the samples from the microsomal incubations, with no interference from background. In Fig. 5 chromatograms obtained from the liver microsomes incubated with styrene are shown (sample from control incubation with heat-inactivated microsomes, the control sample spiked with standards of SO and IS, and the sample after incubation with 1.1 m*M* styrene). As seen from the chromatogram from the microsomes after incubation with styrene, the amount of *R*-enantiomer was higher than that of *S*-enantiomer indicating product enantioselectivity of the cytochrome P450 enzyme.

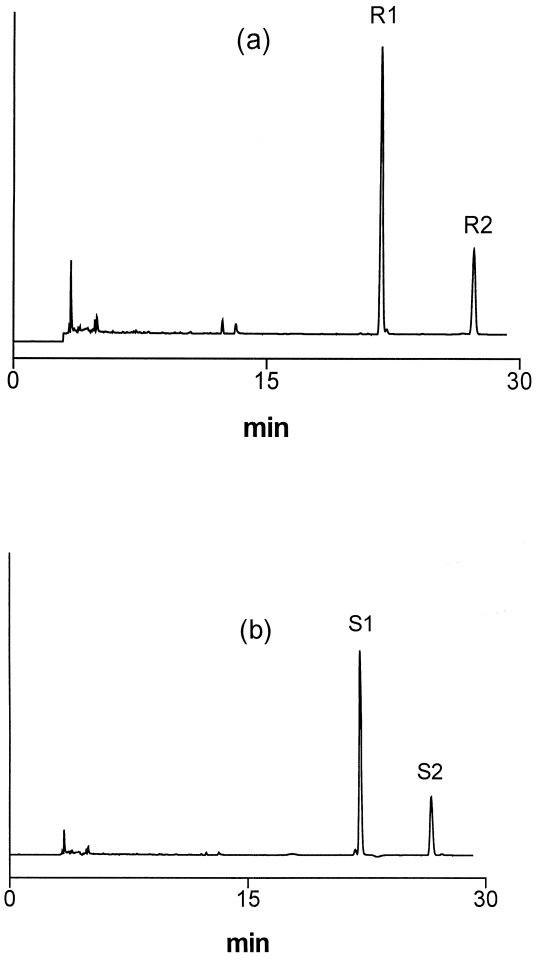

Fig. 4. GC–ECD chromatogram of derivatives of *R*-SO (a) and *S*-SO (b) after base catalysed methanolysis and derivatisation with PFPA. R1, R2 and S1, S2=PFP derivatives of the regional isomers of 2-methoxyphenylethanols obtained from *R*-SO and *S*-SO, respectively. Chromatographic conditions as described in Section 2.4.2.

3.4. Reproducibility and limit of detection

The limit of quantitation (LOD) was calculated using the method described by Miller and Miller [22]. For that purpose a calibration line in the low concentration range was made by addition of SO to blank microsomes. The SO standard concentrations ranged from 0.2 to 3 μ*M* and five determinations were performed at each concentration. The determined LOD for both *R*-SO and *S*-SO enantiomers amounted to 0.2 μ*M*.

The repeatability (within-day precision) of the method was determined at two concentration levels (2.5 and 25 μ*M*) by analysis of ten aliquots of a microsome sample. The relative standard deviation of the mean, amounted to 9.1 (*R*-SO) and 8% (*R*-SO) at the concentration level of 2.5 μ*M*, and to 6.0 (*R*-SO) and 6.1% (*S*-SO), at the concentration level of 25 μ*M*.

3.5. Samples

The method was applied to determine the rate and product enentioselectivity of the cytochrome P-450 dependent oxidation of styrene to SO enantiomers. So far, the stereochemistry of styrene oxidation has only been studied in experimental animals. To the best of our knowledge, this is the first study performed in human liver microsomes. The concentration of SO enantiomers was measured in microsomal samples of 20 human livers after incubation with styrene in the concentration range of 1 to 1750 μ*M* for 10 min. Since the enzymatic hydration of oxiranes via mEH represents an enantioselective process, the correct determination of product stereoselectivity in enantioselective epoxidation required complete inhibition of mEH [20]. In the present study, inhibition of mEH was achieved by preincubation of the microsomes with 2-(trichloro-

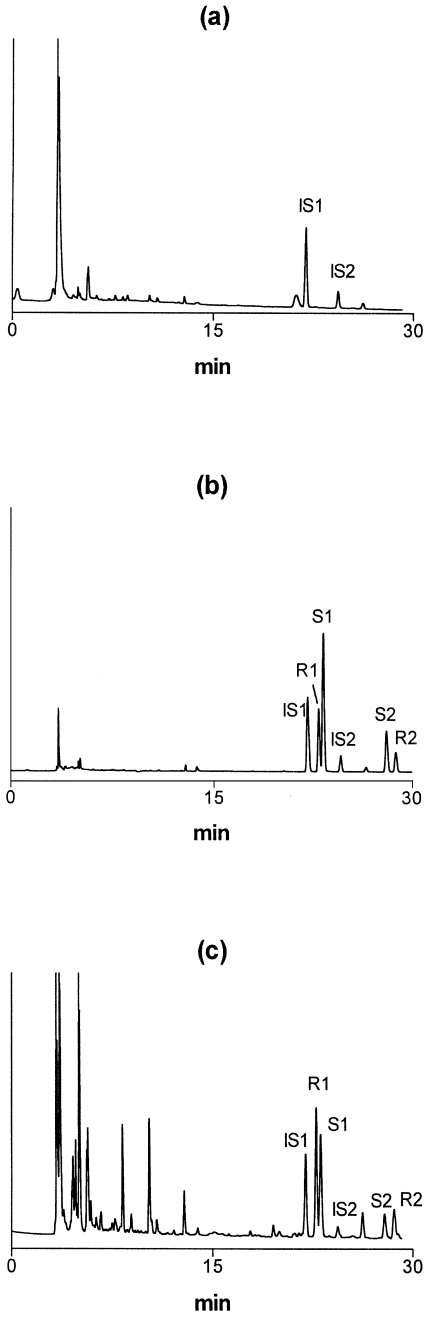

Fig. 5. GC–ECD chromatogram after base catalysed methanolysis and derivatisation with PFPA of (a) blank microsome sample, (b) blank microsome sample spiked with standards of *R*-SO, *S*-SO and IS, and (c) microsome sample incubated with 0.5 m*M* styrene for 10 min. R1, R2 and S1, S2 = PFP derivatives of the regional isomers of 2-methoxyphenylethanols obtained from *R*-SO and *S*-SO, respectively. Chromatographic conditions as described in Section 2.4.2.

methyl) oxirane. The SO concentration was in all samples above the limit of quantitation and ranged from 0.25 to 10 μ*M*. Cytochrome P450 showed a moderate stereoselectivity, with an excess of *S*-SO (15%) at low styrene concentration (16 μ*M*) and an excess of *R*-SO (7%) at high styrene concentration (1100 μ*M*). This dependence of the enantiomeric excess of SO on the substrate concentrations, suggests involvement of different P450 isoforms [20,23]. The results of that study will be published elsewhere [24]. As an example, in Fig. 6, the concentration time course of the enzymatic oxidation of styrene to *R*- and *S*-SO in one human liver sample is shown.

To summarise, in the presented study an enantioselective and sensitive method for the determination of the SO enantiomers in human microsomes is developed. The determination is based on base-catalysed methanolysis. In contrast to the generally used method involving acid-catalysed reaction, the opening of the oxirane ring by base-catalysed methanolysis proceeded without racemisation. The oxirane ring opening led to the formation of two regional isomers for each enantiomer, 2-methoxy-1-phenylethanol and 2-methoxy-2-phenylethanol. The presence of a hydroxyl group in these products offered the possibility of improving the sensitivity of GC analysis by subsequent derivatization of SO enantiomer products using PFPA. This allowed the use of the very sensitive and specific electron capture

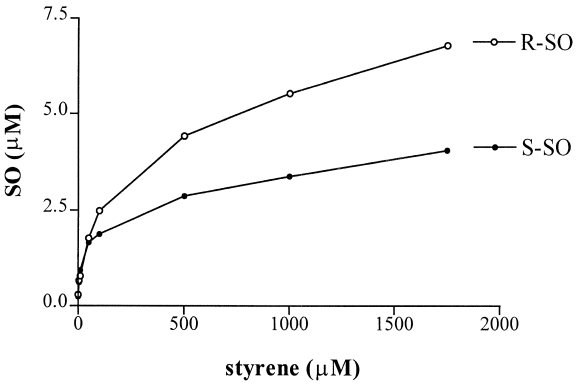

Fig. 6. The concentration-time course of the SO enantiomers after the incubation of human liver microsomes with different concentration of styrene for 10 min.

detection. This derivatization proceeded also without racemisation, and the derivatives of the SO enantiomers and the IS were successfully separated using a non-chiral AT 1705 column and a chiral CP Chirasil-Dex CB column connected in series. The method was shown to be suitable for the determination of the SO enantiomers in the human liver microsomes after incubation with styrene. The presented method could also be applied to the determination of the SO enantiomers in blood of persons occupationally exposed to styrene. There are very few studies related to the stereochemistry of styrene metabolism in persons occupationally exposed to styrene. In none of these studies, however, SO enantiomers have been measured and most of them report the enantiomeric ratio of mandelic acid (MA) in urine [25–27]. The enantiomeric ratio of the MA, however, depends on the enantioselectivity of all enzymes involved in the styrene metabolic path. This is particularly true for the main detoxification step in humans, e.g. hydrolysis of the SO via mEH, which is a strongly substrate enantioselective process [20]. In the study of Maestri et al. [28], the internal exposure to SO was determined indirectly by measuring the mercapturic acids of SO enantiomers in urine using HPLC. However, the used method, although specific and sensitive, was not recommended for the routine analysis being rather complicated and time consuming [28].

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