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Chiral high-performance liquid chromatography and gas chromatography of the stereoisomers of hexyl 2,5-dichlorophenyl phosphoramidate

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ABSTRACT

O-Hexyl O-2,5-dichlorophenyl phosphoramidate (HDCP) is a chiral organophosphorus compound that undergoes enzymatic hydrolysis in the rat and hen. Studies of the stereospecificity of its biodegradation are necessary to establish HDCP toxicity. To this effect, methods have been developed for the analysis of the HDCP stereoisomers by gas chromatography (GC) and high-performance liquid chromatography (HPLC). The best resolution and analysis were obtained by HPLC with UV detection, a OA-4100 Techocel chiral column and the mobile phase: hexane–1,2-dichloroethane–ethanol (92:5:3, v/v/v). The detection limit was 25 μM for HDCP and 5 μM for one of its hydrolytic products: 2,5-dichlorophenol (DCP). The method was reproducible *intra o inter die*. Moreover, a method is described for the liquid extraction of HDCP and DCP with 1,2-dichloroethane in biological samples, with a yield of $(80.3 \pm 9.7)\%$ ($n = 10$, S.D.) for HDCP and $(84.1 \pm 10.0)\%$ ($n = 10$, S.D.) for DCP. The method is compared with the solid-phase extraction technique with C_{18} sorbent. The hydrolysis of HDCP by hen plasma is studied.

INTRODUCTION

The great differences observed in the biological activities of enantiomeric organophosphates (OP) [1] dictate the utilization of highly efficient analytical methods to separate and detect these compounds. The introduction of chiral stationary phases has led to important progress in both liquid and gas chromatographic techniques.

At present, OP are widely used in agriculture due to their easy degradation. In this sense, several enzymes – fundamentally belonging to mammalian species – are known to hydrolyse OP [2]. Some of these hydrolysing systems are stereospecific, like the soman (1,2,2-trimethylpropylmethylphosphonofluoridate) hydrolysing activity [3].

O-Hexyl O-2,5-dichlorophenyl phosphoramidate (HDCP) is a chiral phosphoramidate compound (Fig. 1) that has been used to study the toxicological properties of the insecticide methamidophos. It is known that HDCP possesses a number of different *in vitro* and *in vivo* inhibiting effects on both acetyl cholinesterase (AChE) and neurotoxic target esterase (NTE) [4,5]. Based on these studies, it has been hypothesized that such discrepancies might be attributable to the *in vivo* transformation of one of the stereoisomers of HDCP [5]. Earlier studies demonstrated the existence of an enzyme activity capable of hydrolysing HDCP in rat and hen tissues [6].

The purpose of the present study was to develop a chromatographic method for the anal-

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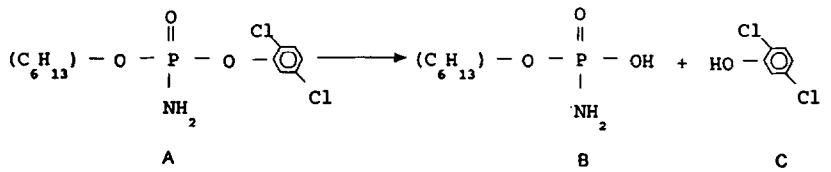

Fig. 1. Structure of HDCP and its metabolites.(A)O-hexyl O-2,5-dichlorophenyl phosphoramidate, (B) O-hexyl phosphoramidic acid, and (C) 2,5-dichlorophenol.

ysis and quantitation of the different HDCP stereoisomers, along with the metabolite resulting from its enzymatic hydrolysis: 2,5-dichlorophenol (DCP). Several methods were evaluated for the detection of HDCP stereoisomers based on high-performance liquid chromatography (HPLC) and gas chromatography (GC). An HPLC method with UV detection is proposed, using a chiral column to identify the different HDCP and DCP stereoisomers. Finally, a method was developed for the extraction from aqueous samples of HDCP treated with biological samples. These methods have made it possible to study the stereospecific biotransformation of HDCP.

EXPERIMENTAL

Chemicals

O-Hexyl O-2,5-dichlorophenyl phosphoramidate (HDCP) was supplied by Dr. Naumann (Bayer Chemical Co., Leverkusen, Germany). Purity was *ca.* 95%. HPLC-grade *n*-hexane (95%), 1,2-dichloroethane, absolute ethanol, ethyl acetate, acetone and isopropyl alcohol (from Scharlau, Barcelona, Spain) were passed through a 0.45-μm, 47-mm diameter nylon filter (Scharlau). Other chemicals were of analytical grade from Panreac (Barcelona, Spain) and Merck (Darmstadt, Germany). Ethanol was previously dried with 0.3-nm molecular sieve beads through *ca.* 2 mm, followed by filtration as for the other solvents.

Gas chromatography

A Hewlett-Packard 5890 series GC equipped with an autosampler 7671A, split/splitless injector and NPD detector were employed. Data acquisition was with an integrator 3392A from Hewlett-Packard (Barcelona, Spain). Two chiral columns were assayed: the Cyclodex-B column (30 m × 0.32 mm I.D., CDX-B 0.25 μm)(J and W Scientific, Altech, Sugelabor, Madrid, Spain) and the Chirasil-Vall column (25 m × 0.32 mm I.D.)(Heliflex, Altech).

High-performance liquid chromatography

HPLC was performed using a Hewlett-Packard 1050 series equipped with a variable wavelength UV detector operating at 230 nm, a quaternary pump and an autosampler with a 21-vial tray. Data acquisition was by peak height from a Hewlett-Packard HPLC ChemStation.

The columns tested were: Supelcosil LC-(R) Phenyl urea (250 × 4.6 mm I.D.) from Supelco (Barcelona, Spain), Chiracel OD (600 × 0.32 mm I.D.)(microcolumn), Chiracel OC (250 × 4.6 mm I.D.), Chiracel OF (250 × 4.6 mm I.D.), Techocel OA 4900 (5 μm, 250 × 4.0 mm I.D.), Techocel OA 4700 (5 μm, 250 × 4.0 mm I.D.), Techocel OA 4500 (5 μm, 250 × 4.0 mm I.D.), and Techocel OA 4100 (5 μm, 250 × 4.6 mm I.D.). The Chiracel columns were from Daicel Chemical Industries (Tokyo, Japan), and the Techocel columns were from HPLC Technology (Macclesfield, UK). In the study with tissue treated and extracted samples, a Hypersil precolumn (Silica) was used (5 μm, 20 × 4.0 mm I.D., Hewlett-Packard, USA), which was frequently changed. The Chiracel OD microcolumns were tested by Dr. J.P. Langenberg and Mr. Hans de Vette, using an HPLC equipment for microcolumns.

Source of plasma

Adult Rhode Island Red hens (*Gallus domesticus*) at least 9 months old and weighing 1.5–2 kg were used. The hens were sacrificed and blood collected in heparinized tubes. Plasma was separated by centrifugation at 2000 *g* for 10 min, and stored at − 30°C.

Biodegradation assay

The assay medium (2 ml) contained 0.95 ml of Tris/HCl buffer (10 m*M* pH 7.4) and 1 ml of plasma solution (plasma diluted in buffer 1:9, v/v). The reaction was started by adding a volume of 50 μl of 4 m*M* HDCP in dry acetone to yield a final concentration in the assay medium of 100 μM HDCP. The mixture was incubated at 37°C for 0 min for the development of the extraction procedure and for 2, 15, 30, 60, 120 and 240 min for the study of the hydrolysis of HDCP. The reaction was stopped by adding PCA solution (0.35 *M* HCl/0.36 *M* sodium acetate) or HCl solutions or SDS-AAP [sodium dodecyl sulphate (2% w/v) in Tris/EDTA pH 8 containing 0.25 mg 4-aminoantipyrine/ml] depending on the further treatment. Details are described later.

Control samples were incubated at the same conditions without plasma solution and used as controls for non-enzymatic hydrolysis. The latter was not observed. Other controls were run under the same conditions with or without plasma but stopping the reaction immediately after adding the HDCP. These were used as controls for zero reaction time and for the concentration of HDCP without hydrolysis but with all the treatments used for the samples. The treatment with PCA or HCl to stop the reaction did not affect the stability of HDCP.

Colorimetry

The reaction was stopped by adding 1.5 ml of SDS-AAP followed by 1 ml of buffer. Finally, 0.75 ml of 0.4% potassium ferricyanide (FCN) was added for development of the colour reaction. The DCP liberated forms a complex with AAP which reacts with FCN to give a coloured product that can be monitored with a spectrophotometer at 505 nm. The complex is stable for 1 hour. The molar extinction coefficient is $\epsilon = 27\,158 \pm 884\ M^{-1}\ cm^{-1}$. This procedure is described by Sogorb *et al.* [7].

Solid-phase extraction

The biological samples (2 ml) were treated with 1 ml of PCA solution to stop the enzyme reaction and precipitate the proteins. Centrifugation at 2000 *g* for 10 min was followed by supernatant extraction. The C_{18}, C_8, C_2, CH and PH cartridges from Bond Elut (Analytichem International, CA, USA) were used after preconditioning by flushing with 3 ml of 1,2-dichloroethane, 3 ml of methanol and 6 ml of sample medium (Tris/HCl buffer–PCA solution, 3:1, v/v). Elution was carried out with 3 ml of 1,2-dichloroethane. The eluate was collected in a tube with a conical tip, and concentrated to 0.4 ml at room temperature under a nitrogen stream. Aliquots of 5 μl were injected into the Techocel OA-4100 column.

Liquid–liquid extraction

The biological samples (2 ml) were treated, in a glass screw-cap test tube, with 75 μl of 0.2 *M* HCl to stop the enzyme reaction and denature the proteins. Four ml of 2,5-dichloroethane were added and mixed in a tube mixer (ABT-2 from SBS Instruments, Barcelona, Spain) for one hour, and centrifuged (2540 *g* for 15 min, 7°C) in a G-S-6 centrifuge with a GH-3.7 horizontal rotor (Beckman, Madrid, Spain). After centrifugation, 3 ml of the 1,2-dichloroethane layer were transferred to a tube with a conical tip and concentrated to 0.4 ml under a nitrogen flow. Five μl were injected into the Techocel OA-4100 column.

RESULTS AND DISCUSSION

Gas chromatography

The Cyclodex-B column yielded a low resolution of the stereoisomers while the Chirasil-Vall column gave a satisfactory separation. However a low sensitivity for HDCP was observed. This was attributed to injection problems and adsorption of a portion of the sample on the column. These drawbacks, along with the instability of the column and a very short half life, render gas chromatography less than ideal for the study of the HDCP stereoisomers.

Liquid chromatography

Table I indicates the different columns used to resolve the HDCP stereoisomers. Peak assignment is unknown and we refer to them as HDCP 1, the first stereoisomer, and HDCP 2, the second one. The different retention times (t_R)

TABLE I

PARAMETERS DETERMINING THE PERFORMANCES OF THE DIFFERENT COLUMNS TESTED

The mobile phase for each column was: hexane–1,2-dichloroethane–ethanol (100:20:1, v/v/v) for Techocel OA-4100, Techocel OA-4700 and Techocel OA-4500; hexane–ethyl acetate (85:15, v/v) for Supelcosil LC-R; hexane–isopropanol (80:20, v/v) for MicroChiracel OD; hexane–2-propanol–diethylamine (95:5:0.1, v/v/v) for Chiracel OC and Chiracel OF. t_R is the peak retention time (in min); N is the number of column plates; L is the lengh of the column; w is the peak width at 10% of the baseline (in min); R is the resolution achieved between the two stereoisomers.

	HDCP 1		R	HDCP 2	
	t_R	N/L		t_R	N/L
Techocel OA-4100	14.43	12.57	2.00	17.12	9.86
Techocel OA-4700	17.63	6.70	1.70	20.95	6.56
Techocel OA-4500	12.55	9.50	0.86	13.52	7.99
Supelcosil LC-R	20.64	16.89	0.53	21.38	12.65
Chiracel OC	22.40	13.90	1.23	24.50	10.64
Chiracel OF	40.40	7.05	1.30	48.45	2.10
MicroChiracel OD	9.38	4.30	1.67	10.7	4.28

are indicated, along with the number of plates/column length per column; the resolution between the two stereoisomers obtained in each column is expressed for Gaussian peaks, where $R = 2\Delta t/(w_A + w_B)$, $N = 16(t_R/w)$, and w is the peak width at one-tenth of the height of the peak from the baseline. The most adequate column for the resolution of the HDCP stereoisomers is the OA-4100 column (HPLC Technology) because it gives the highest value for the resolution and a good N/L ratio (plate height). Furthermore, it is able to detect simultaneously HDCP and its hydrolytic metabolite, DCP. This column was used in all subsequent experiments. The resolution achieved with this column was comparable to that reported by others for the enantiomers of methamidophos [8].

The optimum elution conditions initially employed were: hexane–1,2-dichloroethane–ethanol (78:17:5, v/v/v)(Fig. 2A). The flow-rate was 1 ml/min. During column utilization, the retention time of DCP was seen to decrease and approach that of the stereoisomers; the elution times of the latter were not modified. Eventually, after performing *ca.* 144 chromatograms over a period of three months, the DCP peak interfered with the peaks of the stereoisomers. This phenomenon may be attributed to the existence of polar groups in the column material that gradually become blocked with use of the column, resulting in a smaller retention time for DCP. Consequently, the elution conditions had to be modified to: hexane–1,2-dichloroethane–ethanol (92:5:3, v/v/v) (Fig. 2B). Under these new conditions DCP was found to elute before the HDCP stereoisomers, and the column response remained stable for a period of at least 8 months, corresponding to approximately 1265 chromatograms. The number of plates of the column during this period was 13 809 ± 1216 and 13 915 ± 946 for each stereoisomer and the res-

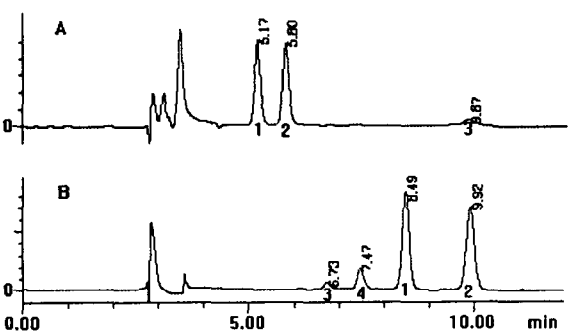

Fig. 2. Chiral separation of HDCP by HPLC. The column employed was OA-4100 (5 μm, 250 × 4.6 mm I.D.), UV detection at 230 nm; 5 μl of racemic mixture of 500 μ*M* HDCP were injected. Peaks 1 and 2 are the HDCP enantiomers; peak 3 corresponds to DCP (represented as an impurity of the HDCP analysis); and peak 4 is an impurity of 1,2-dichloroethane. (A) eluent: hexane–1,2-dichloroethane–ethanol (78:17:5, v/v/v); (B) eluent: hexane–1,2-dichloroethane–ethanol (92:5:3, v/v/v).

olution was higher (3.75 ± 0.1) than under the initial conditions.

The response was seen to be linear over a concentration range of 25–800 μM and 5–500 μM for HDCP and DCP, respectively (correlation coefficient 0.999 and 0.998, respectively). The detection limit for HDCP was 25 μM (12.5 μM for each stereoisomer), *versus* 5 μM for DCP (in the solution injected in the column). Finally, the reproducibility was tested with an external standard. As an example, in one experiment, 21 samples were chromatographed over an 8-h period. A control external standard was injected every 5 samples to check the stability of the measurements. The standard deviation of the mean peak heights corresponding to the 5 standards was 0.7–0.9% for the HDCP stereoisomers, and 2.3% for DCP. The standard deviation of 5 experiments performed on different days was 0.9–1.1% for the HDCP stereoisomers, and 4.2% for DCP. This *intra o inter die* reproducibility is considered acceptable for HDCP biodegradation studies, and is comparable to that obtained with other methods used to determine organophosphates via HPLC [9].

Using this column, *ca.* 1400 chromatograms were performed over 11 months. After having established the final optimum conditions, column response remained constant, and thus may be regarded to be relatively stable. This stability contrasts with the instability reported for other HPLC chiral columns [10].

Extraction procedure

In studying the extraction of HDCP stereoisomers from biological samples, we have employed liquid chromatography as the analytical method. Samples containing HDCP and plasma solution not subjected to hydrolysis (0 min) were extracted as described in the Experimental section. Quantitative assay of the extracted samples was carried out with an external standard containing HDCP at a nominal concentration of 500 μM (250 μM for each stereoisomer). This corresponds to the final concentration of HDCP in the samples extracted and concentrated with a theoretical 100% recovery.

Non-polar cartridges were tested (C_{18}, C_8, C_2, CH), in the hope to achieve greater HDCP retention. A polar cartridge was also evaluated (PH). Table II shows the percentage extraction achieved with the different cartridges. C_{18} was found to exhibit the greatest HDCP retention, and was studied in detail. 1,2-Dichloroethane was chosen as the extraction eluent, because it provided higher yields than the other eluents tested: acetone, acetonitrile, tetrahydrofurane, and the chromatographic mobile phase (hexane–1,2-dichloroethane–ethanol, 87:17:5, v/v/v). The minimum elution volume required for optimum extraction was 3 ml. Increasing of the cartridge size and passing the sample twice through the same cartridge failed to improve the extraction performance. Each cartridge could be reused three times without loss of performance. The extraction yield with C_{18} was 64.9 ± 7.4% ($n = 10$, S.D.). However, in the absence of tissue, extraction with C_{18} was 75%, indicating that treatment with PCA and elimination of proteins through precipitation caused a systematic 10% loss.

TABLE II

EXTRACTION YIELDS (%) FOR EACH HDCP STEREOISOMER USING THE CARTRIDGES INDICATED

Cartridge	Extraction yield (%)	n
C_{18}	64.9 ± 7.4	10
C_8	54.6 ± 11.1	5
C_2	49.1 ± 9.0	6
CH	47.6 ± 15.6	7
PH	41.7 ± 1.2	4

In performing liquid–liquid extraction, enzyme activity was stopped by adding 0.2 *M* HCl instead of PCA. This avoided HDCP losses resulting from protein precipitation with PCA. The extraction yield was 80.3 ± 9.7% ($n = 10$, S.D.) for HDCP and 84.12 ± 10.02% ($n = 10$, S.D.) for DCP. Moreover extraction with C_{18} could be reproducible and efficient enough to determine the levels in water or in other samples. Solid-phase extraction may be more selective than liquid–liquid extraction. However, for the extraction of HDCP stereoisomers, liquid–liquid extraction was chosen because it gave

higher yields, and the chromatograms did not give peaks that could interfere with the HDCP stereoisomers or DCP.

Application to the hydrolysis of HDCP by hen plasma

Plasma solutions were incubated with 100 μM HDCP for 0, 2, 15, 30, 60, 120 and 240 min. After stopping the reaction with HCl, the residual HDCP and the DCP formed were extracted by liquid–liquid extraction with 1,2-dichloroethane and concentrated prior to injection. Each time was sampled in triplicate. The quantitative analysis of the samples was performed with a calibration curve ranging from 25 μM to 600 μM of a racemic mixture of HDCP. An external standard of 500 μM HDCP was injected every 5 samples to check the stability. This standard was prepared as follows: 50 μl of 4 mM HDCP in dry acetone was diluted in 3 ml of 1,2-dichloroethane and concentrated to 0.4 ml to yield a concentration of 250 μM for each stereoisomer. The injection volume was always 5 μl. Fig. 3 shows an example of the hydrolysis of HDCP *versus* time. Peaks 1 and 2 are the HDCP residual stereoisomers; peak 3 is the DCP formed and peak 4 is an impurity of 1,2-dichloroethane resulting from the concentration of the 3-ml 1,2-dichloroethane extract to 0.4 ml for injection. This figure shows that it is possible to quantitate the hydrolysis of each stereoisomers *versus* time by HPLC. With increasing time, the height of the peaks of the HDCP stereoisomers decreases and the height of the DCP peak increases while the impurity peak remained unchanged. In Fig. 4, the disappearance of each stereoisomer *versus* time is shown. It is possible to quantitate each stereoisomer at every time. The second stereoisomer is hydrolysed slightly faster than the first one. However this difference is not large enough to explain the differences in *in vivo* and *in vitro* behaviour of this compound.

In earlier studies, quantification of HDCP hydrolysis was performed with a colorimetric method based on the measurement of the DCP liberated during the hydrolysis of HDCP [7]. In order to correlate those results with the measurement by HPLC, the hydrolysis of HDCP was accomplished identically in both studies. Paired

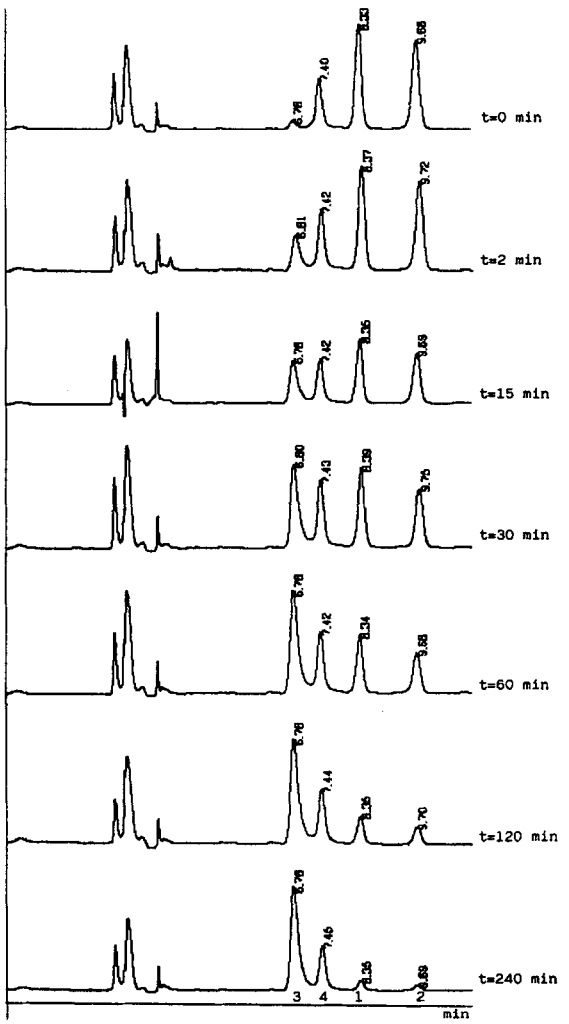

Fig. 3. Example of chromatograms corresponding to an HDCP sample treated with biological tissue for different times, extracted and concentrated prior to injection. The remaining HDCP stereoisomers and the DCP formed were extracted with 4 ml of 1,2-dichloroethane from a biological sample (1.95 ml Tris/HCl 10 mM pH 7.4 buffer, and 1 ml plasma diluted 1:9 in buffer) after 0, 2, 15, 30, 60, 120 and 240 min of incubation at 37°C. Peaks 1 and 2 are the HDCP residual stereoisomers; peak 3 corresponds to DCP formed; and peak 4 is an impurity of 1,2-dichloroethane.

aliquots of HDCP solution were incubated simultaneously with aliquots of plasma solution for 0, 2, 15, 30, 60, 120, 240 and 360 min at 37°C. At the end of the incubation time, samples were used for measuring either the DCP liberated and the remaining stereoisomers by HPLC,

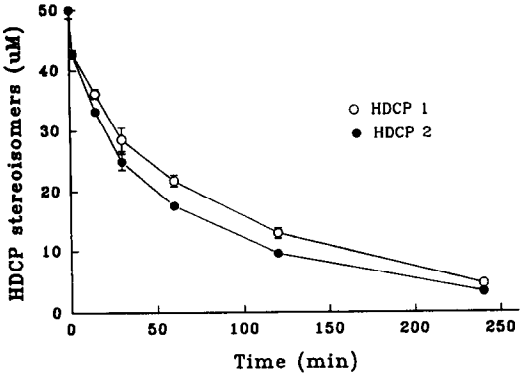

Fig. 4. Disappearance of both HDCP stereoisomers resulting from incubation with hen plasma. HDCP solution (100 μM) was incubated with hen plasma for 0, 2, 15, 30, 60 and 120 min at 37°C. The samples were extracted and concentrated. The concentration of the remaining HDCP stereoisomers is plotted *versus* time. The data shown are the results of one experiment performed in triplicate.

or the DCP liberated colorimetrically. In Fig. 5, the DCP measured colorimetrically and the DCP measured by HPLC and the HDCP hydrolysed measured by HPLC are represented. The latter was calculated from the difference between the initial HDCP and the remaining HDCP, *i.e.* the sum of the remaining concentrations of the two

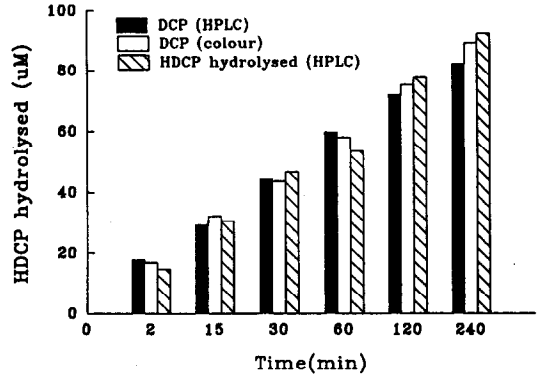

Fig. 5. Comparison of the amount of hydrolysed HDCP as determined by HPLC and by a colorimetric method. Paired aliquots of HDCP solution (100 μM) were incubated simultaneously with hen plasma for 0, 2, 15, 30, 60, 120 and 240 min at 37°C. The figure shows the amount of HDCP hydrolysed and the amount of DCP liberated, both measured by HPLC, together with the amount of DCP measured colorimetrically. The data shown are the results of one experiment performed in triplicate.

stereoisomers. No discrepancy in the measurements by both methods was observed.

CONCLUSIONS

A method has been described for analysing the HDCP stereoisomers by HPLC. The OA-4100 Techocel chiral column was chosen in view of its good performances. After a period of stabilization, the column response was constant and reproducible over more than 1400 chromatograms performed in a period of 11 months. Liquid–liquid extraction of the HDCP stereoisomers was carried out on biological samples. The method described was found to be valid for the direct quantification of the HDCP stereoisomers and DCP as hydrolytic product. The concentration of the remaining stereoisomers correlated well with the DCP measured by HPLC or colorimetrically. The rate of disappearance of both HDCP stereoisomers was almost the same. We concluded that the hydrolysis of HDCP by hen plasma was not stereospecific. So, other tissues may be involved in the stereospecific hydrolysis of HDCP. However, this methods could be applied for the detailed study of the hydrolysis of HDCP by hen plasma and other tissues such as liver or brain, even with other chiral organophosphates. Further investigations for a micropreparative isolation of the pure stereoisomers may be required to study the specific toxicity of each HDCP stereoisomer.

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