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High-performance liquid chromatographic method for determination of DDT and its degradation products in rat plasma, liver and brain: validation and application to a pharmacokinetic study

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Abstract

A sensitive and reliable high-performance liquid chromatographic (HPLC) method, using a solid-phase extraction (SPE), was established and validated for determination of *p,p′*-DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane] and its metabolite *p,p′*-DDE [1,1-(2,2-dichloroethanylidene)-bis(4-chlorobenzene)] in rat plasma, liver and brain. After being diluted with water, plasma, liver and brain samples were applied to a solid-phase extraction C_{18} cartridge. The extraction containing *p,p′*-DDT and *p,p′*-DDE from the cartridge were cleaned-up using a Florisil Sep-Pak cartridge. The samples were analyzed by HPLC using UV detection at 238 nm. The limit of detection for *p,p′*-DDT and *p,p′*-DDE was 0.1 mg kg^{-1} liver or brain and 0.1 mg l^{-1} plasma. For six replicate samples at 40, 4 and 0.2 mg kg^{-1}, intra-day precision values were within 4.9% for plasma, 6.4% for liver, and 9.7% for brain. Inter-day precision values at 4 mg kg^{-1} were within 8.2% for plasma and tissues. The method performances were shown to be selective for *p,p′*-DDT and *p,p′*-DDE, and linear over the range 0.04–12 mg kg^{-1} (mg l^{-1} for plasma). The absolute recoveries of *p,p′*-DDT and *p,p′*-DDE in rat plasma and tissues were over 92%. The method was proved to be applicable to the pharmacokinetic study of DDT in rats after a single oral administration.

Keywords: DDT

1. Introduction

Endocrine disrupters are exogenous compounds that can disturb endocrine system of animals by interfering hormonal activity. Some of these compounds may have been accumulated in the environment and cause serious problems to wildlife and humans. *p,p′*-DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane] has been detected at high concentrations on the Pacific Coast and Eastern Finland [1,2]. A concentration of 3 mg kg^{-1} of total DDT has also been found in rail eggs [3]. *o,p′*-DDT has estrogenic activity in rats [4], and recently, Robison et al. have confirmed the binding of *o,p′*-DDT to the estrogen receptor [5]. According to the report of Keice et al., *p,p′*-DDE [1,1-(2,2-dichloroethanylidene)-bis(4-chlorobenzene)], a major de-

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composition product of DDT, also has strong anti-androgenic properties in male rats [6].

To evaluate the adverse effects of DDT and DDE on wildlife or humans, it is important to accurately determine the concentrations and behavior of these compounds in the environment, wildlife or animals by using a simple, rapid and sensitive method. The reported analytical methods for DDT and DDE include gas chromatography–electron capture detection (GC–ECD) for rat, dog and water samples [7–17], and high-performance liquid chromatography–ultraviolet detection (HPLC–UV), gas chromatography–flame ionization detection (GC–FID) and gas chromatography–mass spectrometry (GC–MS) for water and soil samples [18–20].

It is thought that, among these methods, HPLC is the most suitable for toxicokinetic studies of DDT, due to its stable sensitivity.

In this study, we developed a HPLC–UV method for quantification of *p,p′*-DDT and *p,p′*-DDE in the rat plasma, liver and brain. Then this method was applied to a subchronic toxicokinetic study.

2. Experimental

2.1. Reagent and materials

p,p′-DDT (DDT, 98% in purity) and *p,p′*-DDE (DDE, 99.0% in purity) were obtained from GL Science (Tokyo, Japan) and Riedel-de Haen (Germany), respectively. HPLC-grade methanol, acetonitrile and distilled water, and pesticide-grade acetone and hexane were obtained from Kanto Chemical (Tokyo, Japan). Bond elute C_{18} cartridges (1 g/6 ml) were obtained from Varian (Harbor, CA, USA) and Sep-Pak Florisil (690 mg) from Waters (Milford, MA, USA). Polytron (Kinematica LITTAU, Switzerland) was used as a homogenizer for tissue. Blood, livers and brains were collected from F-155 male rats (Charles River Japan Inc.) at 1, 2, 4, 6, 8, 12, 18, 24, 48, 72, 96 and 192 h after administration of a single oral dose of 106 mg DDT kg^{-1} body weight. A 5 ml blood sample from each rat was treated with 100 μl of heparin and centrifuged at 1870 *g* at 4°C for 10 min to obtain a plasma fraction. Then the plasma was pooled. The plasma, liver and brain samples were stored at −20°C before being analyzed.

2.2. Chromatography

Chromatography was performed on a HPLC system consisting of a HP 1100 series (Hewlett-Packard, Waldbronn, Germany) equipped with pump system, auto-sampler (G1329A, ALS), UV–Vis diode array detector (G1315A) and computer (Vectra, XM, Series 4) with a HP Chem Station. The analytical column was an L-column ODS 5 μl, 250×4.6 mm I.D. reversed-phase column (Chemical Inspection and Testing Institute, Tokyo, Japan). The column oven temperature was set at 40°C. The mobile phase was methanol–water (95:5, v/v), which was deaerated by sonication prior to use, and the flow-rate was set at 1.0 ml min^{-1}. The eluate was monitored at 238 nm with a UV–Vis detector.

2.3. Extraction procedure

2.3.1. Plasma

An aliquot (0.4 ml) of plasma was gently mixed with 5 ml of water and 0.5 ml of acetone in a 20-ml test tube on a Vortex mixer (Model G-560, Vortex-GENIE.2, NY, USA) for 1 min. After the mixture was passed through a C_{18} cartridge, which previously had been activated with 5 ml of methanol and conditioned with 5 ml of distilled water, the column was rinsed with 2 ml of water–acetone (90:10, v/v) and this was followed by a rinse with 5 ml of water–acetone (40:60, v/v). Then DDT and DDE were eluted with 3 ml of hexane. The eluate was evaporated to dryness by a rotary evaporator at 40°C and finally under a nitrogen stream. The obtained residue was dissolved in 5 ml of hexane and transferred to the top of a Florisil cartridge that was preconditioned with 10 ml of hexane; the same procedure was repeated three times. DDT and DDE were eluted with a total of 15 ml of hexane. The entire eluate was evaporated to dryness in the same manner as before. The residue was finally dissolved in 2 ml of acetonitrile and a 50 μl aliquot was injected into the HPLC system.

2.3.2. Liver

The livers were homogenized in water (five times the liver weight). After a 2-g aliquot was added to a water (5 ml)–acetone (0.5 ml) solution, it was

processed for HPLC analysis according to the same procedures for plasma.

2.3.3. Brain

The brains were also homogenized in water (five times the brain weight). A 1-g aliquot was added to a water (5 ml)–acetone (0.5 ml) solution and processed according to the same procedures for plasma; the only exception was the amount (1 ml) of acetonitrile used for reconstitution of the residue.

2.4. Preparation of analytical standards and calibration standards

From analytical stock solutions of DDT and DDE (1000 mg l^{-1} acetonitrile), working standards (20, 2 and 0.2 mg DDT or DDE per litre of acetonitrile) were prepared. A mixture of an appropriate volume of the working solutions and blank plasma (0.4 ml), liver (0.4 g) or brain (0.2 g) samples were diluted with water, and the mixture was extracted as described in Section 2.3. The calibration standards were obtained with final concentrations of 0.04, 0.2, 0.8, 2.0, 4.0, 8.0 and 12 mg l^{-1} in acetonitrile.

2.5. Method validation

2.5.1. Specificity

Blank plasma, liver and brain samples ($n=6$) were analyzed by using the described method. The chromatograms were visually inspected for the presence of substances which might interfere with the peaks of DDT and DDE.

2.5.2. Absolute recovery, accuracy and precision

Intra- and inter-day precision and accuracy of the developed method were evaluated in plasma, liver and brain samples spiked with DDT and DDE. For intra-day accuracy and precision, the samples ($n=6$) spiked at concentration of 0.2, 4, 40 and 300 mg kg^{-1} were assayed.

The inter-day accuracy and precision were evaluated in six replicates of the samples spiked at a concentration of 4 mg kg^{-1} and taken at three different days in a week. For evaluation of accuracy, the relative error percentage was determined from the formula [(mean of detected concentration − added concentration)/added concentration] × 100, while the precision was evaluated as the coefficient of variation (C.V.). The absolute recovery was calculated from standard calibration curves: namely the peak heights of DDT and DDE in plasma, liver and brain samples were compared with those in standard solutions.

2.5.3. Limit of detection and limit of quantification

The limit of detection (LOD) was defined as the lowest concentration of DDT and DDE ($S/N=3$). The quantification limit was set at the lowest standard concentration on the calibration curve.

2.5.4. Linearity of calibration curve

Calibration standards containing both DDT and DDE at concentrations of 0.04–12 mg l^{-1} (seven concentrations) were prepared from working solutions of plasma, liver or brain. Calibration curves were prepared by peak height vs. DDT or DDE amount.

2.5.5. Influence of dilution procedure

A 300 mg kg^{-1} concentration sample was used after being diluted 10-fold with acetonitrile.

2.5.6. Stability study

For the freeze–thaw stability of DDT and DDE, six replicate samples spiked at a concentration of 4 mg kg^{-1} were analyzed after repetition of freezing and thawing (freeze at −20°C, thaw at 50°C and freeze with dry-ice in acetone solution). For the stability of DDT and DDE in plasma, liver and brain during freezing storage, samples were analyzed at 7 and 14 days of storage at −20°C for plasma samples and at 50 and 150 days for liver and brain samples. The stability was evaluated as [mean of detected concentration/spiked concentration] × 100.

2.6. Application to pharmacokinetic study

In order to apply our analytical methods to pharmacokinetic studies, 5-week-old male F344 rats were orally given DDT dissolved in corn oil at a dose of 106 mg kg^{-1} body weight. This dose is approximately half of the oral LD_{50} value. Plasma, liver or brain samples were collected from three rats

at 1, 2, 4, 6, 8, 23, 28, 48, 72, 96 and 192 h after DDT administration.

3. Results and discussion

3.1. Analytical methods

The described HPLC method proved to be efficient for simultaneous separation and quantification of DDT and DDE when combined with cleaning procedures with solid-phase extraction columns such as C_{18} and Florisil. Solid-phase extraction was highly efficient when compared to liquid–liquid extraction because it requires less time and consumes less solvent. As given in Table 1, absolute recoveries of DDT and DDE from plasma and liver were over 99%. In the brain samples, the recoveries were raised from 66% to 92% when half the sample amount (0.2 g) was used. This might be due to an enhancement of extractability of DDT that would interact with fat in the brain.

3.2. Method validation of plasma, liver and brain

Chromatographic separations of DDT and DDE are shown in Fig. 1. The retention times were 5.6 min and 6.4 min for DDT and DDE, respectively. Both chemicals were completely separated and no apparent interfering or coeluting peaks with similar retention times were found on the chromatograms of blank plasma, liver and brain samples. The LOD ($S/N = 3$) of both chemicals was 0.10 mg kg^{-1} for the plasma and tissues tested. Linear calibration curves were obtained for DDT and DDE over the concentration range 0.04–12 mg l^{-1} (Table 2). The correlation coefficients (R) were greater than 0.9997.

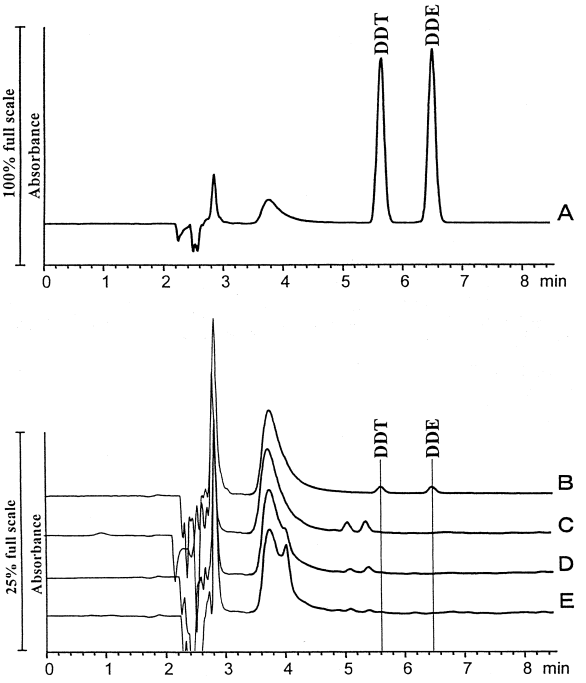

Fig. 1. Typical chromatograms of standard solution 2 mg l^{-1} (A) and 0.02 mg l^{-1} (B), and of blank plasma (C), liver (D) and brain (E).

Intra-day and inter-day accuracy and precision were determined to evaluate reliability of the analytical method. The results are listed in Table 3. Intra-day accuracy for DDT and DDE in plasma at concentrations of 0.2, 4 and 40 mg kg^{-1} was between −4.8 and +0.5, with C.V. being 4.9% or less. Similar results were also obtained for liver and brain. Inter-day accuracy in plasma, liver and brain ranged

Table 1
Absolute recovery of DDT and DDE in rat plasma and tissues

Sample	Sample volume	Concentration added (mg kg^{-1})	DDT		DDE	
			Mean conc.[a] ±SD	Absolute recovery (%)	Mean conc. ±S.D	Absolute recovery (%)
Plasma	0.4 ml	4	4.03±0.05	101	4.00±0.02	100
Liver	0.4 g	4	3.72±0.16	93	3.75±0.18	94
Brain	0.4 g	4	2.64±0.57	66	2.60±0.49	66
Brain	0.2 g	4	3.67±0.05	92	3.67±0.02	92

[a] Mean conc., mean concentration (mg kg^{-1}) [$n = 3$].

Table 2
Regression parameters calculated from calibration curves

	n	Slope	Intercept	γ[a]
DDT				
Plasma	6	16.8388±0.0996	−0.0088±0.0521	$0.9999\pm1.87\times10^{-5}$
Liver	5	16.8164±0.3904	−0.0061±0.0211	$0.9997\pm2.23\times10^{-4}$
Brain	6	16.5834±0.3160	+0.0114±0.0086	$0.9998\pm3.13\times10^{-4}$
DDE				
Plasma	6	17.6595±0.1698	+0.0055±0.0206	$0.9999\pm1.95\times10^{-5}$
Liver	5	17.6106±0.3982	+0.0015±0.0273	$0.9999\pm8.16\times10^{-5}$
Brain	6	17.2946±0.4114	+0.0129±0.0102	$0.9998\pm2.43\times10^{-4}$

[a] Coefficient of the linear regression analysis.

from −3.5% to +1.5% for DDT and from −6.0% to −0.3% for DDE. Inter-day precisions of DDT and DDE in the same tissues were less than 8.2% and 8.1%, respectively. These results indicate that intra- and inter-day assays are not affected by the sample matrix. Furthermore, intra- and inter-day assays of plasma sample spiked at 4 mg kg^{-1} of DDT or DDE were also determined by using calibration curves prepared with standards in acetonitrile. They gave an accuracy of 96% (−4.0) and 97% (−2.8) for DDT and DDE, respectively, for intra-day assay, and 99% (−1.5) and 100% (0.5) for DDT and DDE, respec-

Table 3
Intra-day and inter-day assay of DDT and DDE in rat plasma and tissues

Sample	Concentration	DDT			DDE		
	added (mg kg^{-1})	Concentration determined (mg kg^{-1})	Accuracy[a]	Precision[b]	Concentration determined (mg kg^{-1})	Accuracy	Precision
Plasma							
Intra-day[c]	300	288	−4.0	1.3	289	−3.7	1.2
	40	40.2	+0.5	1.1	39.9	−0.3	0.2
	4	3.81	−4.8	1.1	3.87	−3.3	1.3
	0.2	0.20	0	2.8	0.20	0	4.9
Inter-day[d]	4	3.93	−1.8	2.7	3.99	−0.3	2.6
Liver							
Intra-day	300	307	+2.3	3.9	307	+2.3	3.7
	40	39.3	−1.8	5.2	38.1	−4.8	5.4
	4	388	−3.0	6.4	3.77	−5.8	5.3
	0.2	0.19	−5.0	6.1	0.19	−5.0	6.1
Inter-day	4	3.86	−3.5	4.0	3.76	−6.0	4.0
Brain							
Intra-day	300	315	+5.0	4.7	314	+4.7	3.7
	40	41.6	+4.0	5.9	40.7	+1.8	5.8
	4	3.94	−1.5	9.7	3.92	−2.0	8.7
	0.2	0.21	+5.0	3.6	0.20	0	5.0
Inter-day	4	4.06	+1.5	8.2	3.99	−0.3	8.1

[a] Relative error (%).
[b] C.V. (%).
[c] $n=6$ for each concentration for intra-day assay.
[d] $n=18$ for six replicate for 3 days in a week for inter-day assay.

tively, for inter-day assay. Thus, an acceptable assay was achieved without an internal standard.

Table 3 indicates also the results of a dilution procedure at a concentration of 300 mg kg^{-1}. The accuracy in plasma, liver and brain was −4.0 to +5% for DDT and −3.7 to +4.7% for DDE. Thus, the upper limit of determination can be raised to 300 mg kg^{-1} by dilution.

Freeze–thaw cycle stability test in rat plasma revealed no appreciable degradation of DDT and DDE (Table 4). When rat plasma was stored at −20°C for 14 days, both chemicals were detected at 98% or more of the initial concentrations. Similar results were also obtained for the liver samples stored at −20°C for 150 days. On the other hand, the remaining percentages of DDT and DDE in the brain were 82% and 75%, respectively, under the same condition as used for the liver. It is not certain at present if this is due to a degradation or an interaction with fat.

3.3. Application to pharmacokinetic study

The described method was applied to a pharmacokinetic study in rats after administration of DDT at a single oral dose of 106 mg kg^{-1} body weight. Convulsions were first seen at 8 h after administration with mild severity and became severe in all treated rats at 12 h. These results were in accordance with those of Dale et al. [21]. Fig. 2 shows mean concentration–time profiles of DDT. The maximum concentrations (C_{max}) were 69.02 mg kg^{-1} liver, 21.39 mg kg^{-1} brain and 6.33 mg l^{-1} plasma. The maximum concentration of DDT in plasma was detected at 4–8 h after administration, while the maximum concentrations were obtained at 8 h in the liver and brain. After reaching the maximum level, the DDT concentrations in each tissue decreased slowly. These phenomena were also in agreement with the results of Woolley and Rurmells [10] and Krechniak, which were obtained at 8 h in the liver and brain. After reached the maximum level, DDT concentrations in each tissue decreased slowly. These phenomena were also in agreement with the results of Woolley and Rurmells [10] and Krechniak [22]. With decreasing DDT concentrations, the severity of the neurological symptoms decreased and completely disappeared by 18 h.

The concentration–time profiles of DDE are illustrated in Fig. 3. The magnitude of the detected DDE concentrations was high in liver, brain and plasma in that order with the maximum values of 2.02, 0.59

Table 4
Freeze–thaw and storage stability of DDT and DDE in rat plasma and tissues[a]

Sample	*n*	DDT		DDE	
		Mean conc.[b] ±SD	Remaining percentage (%)[c]	Mean conc. ±SD	Remaining percentage (%)
Plasma					
Freeze–thaw	6	3.98±0.10	100	4.03±0.07	101
Storage days 17	3	3.82±0.02	96	3.88±0.03	97
Storage days 14	3	3.91±0.02	98	3.97±0.05	99
Liver					
Freeze–thaw	6	4.08±0.17	102	4.04±0.15	101
Storage days 50	3	3.89±0.06	97	3.83±0.04	97
Storage days 150	3	3.63±0.01	91	3.88±0.09	99
Brain					
Freeze–thaw	6	4.11±0.28	103	4.11±0.28	103
Storage days 50	3	3.64±0.15	91	3.65±0.06	91
Storage days 150	3	3.27±0.25	82	3.02±0.17	75

[a] Concentration added, 4 mg kg^{-1}.
[b] Mean conc., mean concentration (mg kg^{-1}).
[c] Remaining percentage (%), (Mean concentration)/(Concentration added)×100.

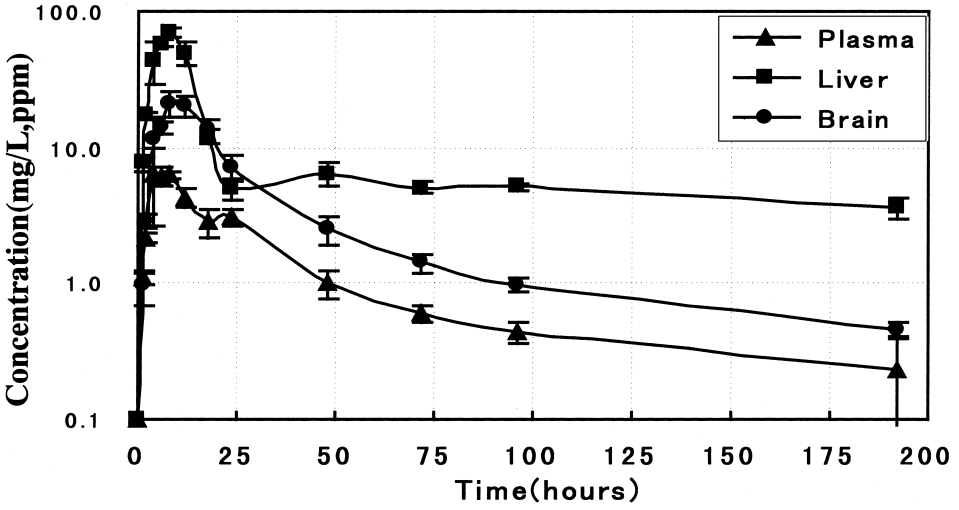

Fig. 2. Concentrations of DDT in rat plasma, liver and brain after single oral administration of DDT at a dose of 106 mg kg^{-1} body weight.

and 0.41 mg kg^{-1}. DDE rapidly disappeared from plasma and was below the limit of detection at 48 h. However, DDE remained long in the liver and was still detected at 0.17 mg kg^{-1} at 192 h.

The maximum concentrations of DDT or DDE from liver, brain and plasma to the amounts of DDT given to the rats were calculated to be 2.12, 0.30 and 0.19% for DDT, respectively, and 0.07, 0.01 and 0.0 1% for DDE (converted to DDT), respectively.

4. Conclusion

A sensitive and repeatable HPLC method with UV detection was developed for simultaneous determination of DDT and DDE in rat plasma, liver and brain using C_{18} and Florisil cartridges for clean-up. This method did not require a tedious procedure and eliminated the interfering materials. Validation experiments showed very good precision and accuracy

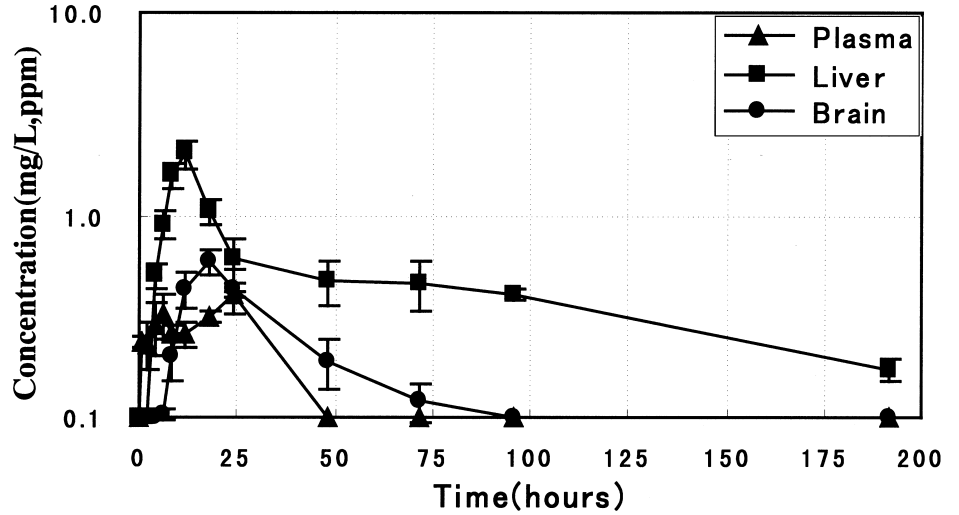

Fig. 3. Concentrations of DDE in rat plasma, liver and brain after single oral administration of DDT at a dose of 106 mg kg^{-1} body weight.

of the method with coefficients of variation and relative errors of less than 10%.

This method was also shown to be suitable for rat pharmacokinetic studies of DDT.

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