Journal of Chromatography, 232 (1982) 119—128
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1349

SIMULTANEOUS DETERMINATION OF TOLMETIN AND ITS METABOLITE IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received February 3rd, 1982; revised manuscript received April 29th, 1982)

SUMMARY

A highly sensitive and selective high-performance liquid chromatographic assay has been developed for the separation and quantitation of tolmetin and its major metabolite in human biological fluids, viz. plasma, urine and synovial fluid. Analysis of plasma and synovial fluid required only 0.5 ml of the sample. The sample was washed with diethyl ether and extracted with diethyl ether—chloroform (2:1). The extracted compounds were injected onto a reversed-phase column (RP-2) and absorbance was measured at 313 nm. The standard curves in plasma were found to be linear for both tolmetin and the metabolite at concentrations from 0.04 to 10.0 μg/ml. Urine samples (0.5 ml) were diluted (1:1) with methanol containing the internal standard and were directly injected onto the reversed-phase (RP-2) column. Standard curves of tolmetin and metabolite in urine were linear in the range 5—300 μg/ml. Serum and synovial fluid concentrations of tolmetin and its metabolite in patients receiving multiple doses of tolmetin sodium were determined using the assay procedure.

INTRODUCTION

Tolmetin (1-methyl-5-(4-methylbenzoyl)-1H-pyrrole-2-acetic acid) is a non-steroidal anti-inflammatory agent [1, 2] effective in the treatment of rheumatoid arthritis and osteoarthritis. Tolmetin is rapidly adsorbed after oral administration, both in healthy subjects and in arthritic patients [3, 4]. It is eliminated by metabolism to 1-methyl-5-(4-carboxybenzoyl)-1H-pyrrole-2-acetic acid (MCPA) [5], and both tolmetin and MCPA are excreted via kidneys.

Several methods involving spectrophotometric [6], gas chromatographic (GC) [7, 8] and high-performance liquid chromatographic (HPLC) procedures [9] have been developed for the determination of tolmetin in plasma and urine. GC procedures involved derivatization prior to analysis, while the spectrophotometric assays required large volumes of plasma for analysis. Ayres

and co-workers [9, 10] developed an HPLC assay procedure for the analysis of tolmetin and its metabolite in plasma and urine but the lower limit of quantitation was 0.250 μg/ml. Samples of plasma or urine containing less than 0.250 μg/ml of drug were assayed by utilizing 2.5 ml of plasma or urine.

Since the above procedures either did not quantitate the major metabolite or were too time consuming and laborious, an HPLC assay that could accurately and rapidly determine the levels of tolmetin and its metabolite in plasma, urine and synovial fluid was developed.

The distinct advantages of this method over previous methods are: (a) a lower limit of quantitation (0.04 μg/ml) resulting in greater sensitivity, thus allowing smaller volumes of plasma to be used; (b) accurate determination of the major metabolite, MCPA; and (c) applicability of the assay for both tolmetin and MCPA in synovial fluid.

EXPERIMENTAL

Chromatographic conditions

The HPLC system was equipped with the following components: Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph, Model ALC/GPC 204, equipped with a U6K injector, a M-6000A solvent delivery system and a UV detector (Model 440) with 313-nm wavelength filter. A 25 cm × 4.6 mm I.D. column (E. Merck, Darmstadt, G.F.R.) packed with LiChrosorb® RP-2 (10 μm) was used.

The mobile phase was methanol—water (1:1) with 0.005 *M* tetrabutylammonium hydrogen sulfate (TBA) and 0.0016 *M* dibasic sodium phosphate. The individual components of the mobile phase were filtered through a 0.45-μm Millipore® filter and the mobile phase was prepared fresh daily. The LiChrosorb RP-2 column was conditioned with at least 30 ml of the mobile phase prior to use at a flow-rate of 2.0 ml/min which was then maintained constant for sample analysis. The retention times for MCPA, the internal standard and tolmetin were 3.3, 4.6 and 6.5 min, respectively.

Chemicals and reagents

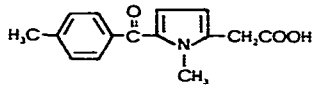
Sulfuric acid (analytical grade), anhydrous diethyl ether (analytical grade), methanol (nanograde), and chloroform (reagent grade) were all purchased from Mallinckrodt (St. Louis, MO, U.S.A.). Tetrabutylammonium hydrogen sulfate was purchased from Aldrich (Milwaukee, WI, U.S.A.). Both monobasic potassium phosphate (KH_2PO_4) and dibasic sodium phosphate (Na_2HPO_4) were certified ACS grade (Fisher Scientific, Pittsburgh, PA, U.S.A.). Citrated human plasma was purchased from Interstate Blood Bank (Philadelphia, PA, U.S.A.).

Fig. 1 illustrates the structural formulae of tolmetin, MCPA and the internal standard, McN-2512 (McNeil Pharmaceutical, Spring House, PA, U.S.A.) used in the assay.

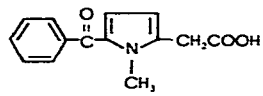
Glassware treatment

Prior to use, all glassware was soaked in detergent (Micro, International Products, Trenton, NJ, U.S.A.) for 2 h, rinsed thoroughly with distilled water

A. Tolmetin: 1-methyl-5-(4-methylbenzoyl)-1H-pyrrole-2-acetic acid



C. McN-2512: 1-methyl-5-benzoyl-1H-pyrrole-2-acetic acid



B. MCPA: 1-methyl-5-(4-carboxybenzoyl)-1H-pyrrole-2-acetic acid

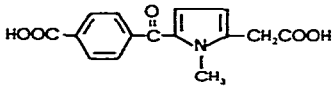


Fig. 1. Chemical structures of tolmetin, MCPA and the internal standard, McN-2512.

and heat treated for 3 h at 270°C. Polyethylene-lined screw-caps were soaked in *n*-heptane for at least 2 h and dried in an oven until all the solvent had evaporated.

Standard curves in plasma

The solutions used in the construction of the standard curve (0.02—5.0 μg/ml) were prepared by serial dilution of a stock solution containing 1 mg/ml each of tolmetin and MCPA with distilled water and were prepared daily. The stock solution was prepared by dissolving sodium salts of tolmetin and MCPA in distilled water.

Stock solution of the internal standard was prepared in methanol (1 mg/ml). A 1-ml volume of this solution was diluted to 500 ml with distilled water (2 μg/ml) and utilized for analysis.

Extraction procedure

Citrated human plasma (0.5 ml) and 1.0 ml of solutions containing 0.02, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 3.0 and 5.0 μg/ml of tolmetin and MCPA were added to 15-ml disposable screw-cap bottles each containing 1.0 ml of phosphate buffer, pH 7.4, 0.5 ml of internal standard solution (2 μg/ml) and 10.0 ml of diethyl ether. Duplicate sets were prepared at each concentration. The samples were shaken on a table-top shaker for 15 min and centrifuged for 10 min at 800 *g*. The diethyl ether (upper) layer was aspirated and discarded. A 0.5-ml volume of 6 *N* sulfuric acid and 9.0 ml of the diethyl ether—chloroform (2:1) mixture were added to the samples which were then shaken for 15 min and centrifuged for 10 min. An 8-ml volume of the organic (upper) layer was transferred to a 12-ml centrifuge tube and evaporated to dryness under nitrogen. The sample extract was reconstituted with 30 μl of methanol, vortexed and 20 μl of this solution was injected into the liquid chromatograph using the stated chromatographic conditions at attenuations between 0.05 and 0.2 a.u.f.s. depending on the concentration of tolmetin and MCPA in the sample.

Extended standard curve

In order to analyze plasma samples containing more than 10 μg/ml of tol-

metin and/or MCPA, four plasma pools containing tolmetin and MCPA, each in concentrations of 20, 30, 40 or 50 μg/ml, were prepared. These pools were analyzed in exactly the same manner as described before, except that only 0.1 ml of plasma sample was taken for analysis and the volume was made up to 0.5 ml with 0.4 ml of blank plasma. Following analysis, the peak height ratios were multiplied by five in order to account for the dilution of the two compounds in plasma.

Synovial fluid assay

Concentrations of both tolmetin and MCPA in synovial fluid were quantitated using the assay procedure described for plasma. Plasma standards were used for the daily calibration curve. Synovial fluid samples were assayed starting at step one of the extraction procedure for plasma and there were no changes in the assay procedure.

Standard curves in urine

The solutions used in the construction of the standard curve were prepared by diluting a stock solution containing 1 mg/ml each of tolmetin and MCPA in distilled water with urine and were prepared daily. Solutions of the following concentrations were prepared in urine: 5, 10, 25, 50, 100, 200 and 300 μg/ml. Internal standard solution (0.2 mg/ml) was prepared in methanol.

Procedure

A 0.5-ml aliquot of each of the above solutions was added to 12-ml tapered centrifuge tubes. Internal standard solution (0.5 ml, 0.2 mg/ml) was added to each tube, the samples were vortexed for 30 sec and 20-μl aliquots were injected into the liquid chromatograph.

Analysis of patient samples

Five patients with rheumatoid arthritis were placed on a regimen of 400 mg of tolmetin every 6 h for seven days. On day 8, following the administration of a single dose of 400 mg of tolmetin, blood, synovial fluid and urine samples were obtained from these patients at selected time intervals. The blood samples were processed for serum. The serum, synovial fluid and urine samples were analyzed for tolmetin and MCPA by the procedure described here.

RESULTS AND DISCUSSION

Typical chromatograms of blank plasma and plasma spiked with tolmetin, MCPA and the internal standard are shown in Fig. 2A and B, respectively. Good correlation was obtained between the peak height ratios and tolmetin plasma concentrations. The standard curve data were obtained by analyzing duplicate samples at each of the concentrations, each day for three days (n=6, at each concentration). Linear regression analysis using 1/variance weighting of the data gave a correlation coefficient (r) of 0.9998, Student's t of 433.0, a slope of 0.363 ± 0.00084 and a y-intercept of −0.0036 ± 0.00043. The standard curve was reproducible from day-to-day. Coefficients of variation (C.V.) in the peak height ratios of tolmetin plasma concentrations be-

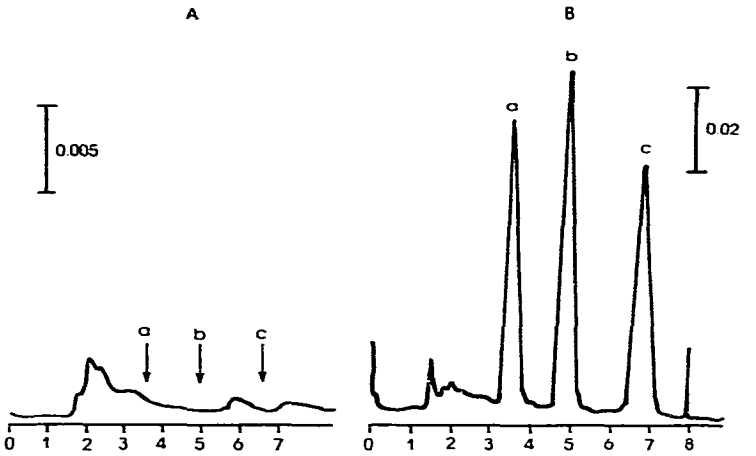


Fig. 2. HPLC assay of tolmetin and MCPA in human plasma. Chromatograms of: A, blank plasma (a.u.f.s. = 0.05); B, plasma spiked with 2 μg/ml each of MCPA (a) internal standard, McN-2512 (b) and tolmetin (c); a.u.f.s. = 0.2.

tween 0.2 and 10 μg/ml were less than 2% and less than 11% for 0.1 and 0.04 μg/ml.

Good correlation was also obtained between the peak height ratios and MCPA plasma concentrations. Linear regression analysis using 1/variance weighting of the data gave a slope of 0.435 ± 0.0012, *y*-intercept of 0.0016 ± 0.0005, r=0.9998, t=376.0. The standard curve was reproducible from day-to-day. C.V. values for MCPA plasma concentrations between 0.1 and 10.0 μg/ml were less than 3% and 20% at 0.04 μg/ml.

Regression analysis of the plasma standard curve data using 1/variance gave the best statistical evaluation, i.e., Student's t value, r, and accuracy of the back calculated concentrations. The back calculated tolmetin and MCPA concentration values were within 2% of the theoretical concentrations in the range 0.1—10.0 μg/ml. At 0.04 μg/ml, the percent deviation was within 12.5% for both compounds. A summary of both tolmetin and MCPA standard curve data in plasma is presented in Table I.

The standard curves for both tolmetin and MCPA were found to be non-linear above 10.0 μg/ml using 0.5 ml of plasma. Regression analysis of the data, obtained by analyzing 0.1-ml aliquots of plasma samples containing tolmetin and MCPA in concentrations greater than 10.0 μg/ml (i.e. 20, 30, 40 and 50 μg/ml), using 1/variance weighting factor again gave excellent back calculated concentration values within ±4% of the theoretical values. The percent deviation of the slopes of the two regression lines, i.e. 0.04—10.0 μg/ml versus 20—50 μg/ml, was 3.7% from the mean for tolmetin and 0.7% for MCPA. Thus, samples containing tolmetin and MCPA in excess of 10 μg/ml can be analyzed by using a 0.1-ml aliquot of the samples, calculating the concentrations from the regular standard curve, i.e. 0.04—10.0 μg/ml, and then multiplying the concentrations by five to give the actual sample concentrations.

The extraction efficiencies for both tolmetin and MCPA were determined

TABLE I

SUMMARY STATISTICS FOR TOLMETIN AND MCPA STANDARD CURVES IN PLASMA

Concentration in plasma (μg/ml)	Tolmetin (n = 6)				MCPA (n = 6)			
	Mean peak height ratio	Standard deviation	Coefficient of variation (%)	Percent deviation from theoretical concentration	Mean peak height ratio	Standard deviation	Coefficient of variation (%)	Percent deviation from theoretical concentration
0.04	0.012	0.0013	10.6	10.0	0.017	0.0033	19.7	12.5
0.1	0.034	0.0028	8.2	0	0.045	0.0013	2.9	0
0.2	0.069	0.0012	1.7	0	0.089	0.0023	2.6	0
0.4	0.140	0.0014	1.0	1.0	0.176	0.0036	2.1	0
0.8	0.284	0.0055	1.9	1.3	0.351	0.0056	1.6	0
1.2	0.433	0.0036	0.8	0	0.524	0.0038	0.7	0
1.6	0.578	0.0065	1.1	0	0.699	0.0210	2.9	0
2.0	0.721	0.0100	1.4	0	0.866	0.0190	2.2	0.5
6.0	2.205	0.0176	0.8	1.7	2.608	0.067	2.6	0
10.0	3.610	0.0350	1.0	0.5	4.292	0.093	2.2	1.3
Slope	0.363 ± 0.0008				0.435 ± 0.001			
Intercept	−0.0036 ± 0.00043				0.0016 ± 0.0005			
Correlation coefficient	0.9998				0.9998			
Student's t	433.0				376.0			
Weighting procedure	1/variance				1/variance			

at 0.8 and 3.0 μg injected and that for McN-2512, at 1.0 μg injected. Results are shown in Table II and show a reduction in extraction efficiency at lower concentrations although the differences were marginal.

TABLE II

EXTRACTION EFFICIENCIES OF TOLMETIN, MCPA AND INTERNAL STANDARD FROM PLASMA

Compound	Amount injected (μg)	Extraction efficiency from plasma* (%)
Tolmetin	3.0	75.3
	0.8	66.9
MCPA	3.0	75.2
	0.8	70.6
Internal standard	1.0	72.8

*(Mean peak height of six extracted samples)/(mean peak height of six directly injected samples).

The synovial fluid samples for analysis were obtained from patients who were already on a multiple dosing regimen of tolmetin. Hence, pre-dose samples also contained tolmetin and MCPA. Therefore, it is not known whether any endogenous substances in synovial fluid would interfere with tolmetin and MCPA peaks. However, from the characteristics of the peaks in the chromatograms (i.e. peak sharpness, lack of front or back tailing), it would seem to indicate that no endogenous material in synovial fluid was eluting from the column at the same time as tolmetin and/or MCPA.

Tolmetin and MCPA were identified in synovial fluid samples by spiking a sample with 1.0 μg each of tolmetin and MCPA and comparing the peak shape and retention times of compounds in the spiked sample to those in the unspiked sample. The retention times of tolmetin and MCPA in the spiked sample were the same as for the peaks seen in the unspiked sample. Fig. 3A and B show the chromatograms of a synovial fluid sample from a rheumatoid arthritic patient receiving tolmetin sodium and the same sample spiked with 1.0 μg each of tolmetin and MCPA, respectively. A 1-μg sample of McN-2512 was used as the internal standard in both samples.

Typical chromatograms of blank urine and urine spiked with tolmetin, MCPA and the internal standard are shown in Fig. 4A and B, respectively. Good correlation was obtained between the peak height ratios and tolmetin urine concentrations. Linear regression analysis using 1/variance weighting of the data gave a correlation coefficient of 0.995, a slope of 0.0074 ± 0.00011 and a y-intercept of −0.0053 ± 0.0014. The back calculated tolmetin concentrations were within 5% of the theoretical concentrations (5—300 μg/ml). C.V. values for tolmetin peak height ratios were less than 11% at all concentrations.

Good correlation was also obtained between the peak height ratios and

TABLE III

SUMMARY STATISTICS FOR TOLMETIN AND MCPA STANDARD CURVES IN URINE

Concentration in urine (μg/ml)	Tolmetin (n = 6)				MCPA (n = 6)			
	Mean peak height ratio	Standard deviation	Coefficient of variation (%)	Percent deviation from theoretical concentration	Mean peak height ratio	Standard deviation	Coefficient of variation (%)	Percent deviation from theoretical concentration
5.0	0.031	0.003	10.4	2.0	0.040	0.005	12.4	4.0
10.0	0.073	0.008	11.0	5.0	0.090	0.009	10.4	4.0
25.0	0.187	0.018	9.6	3.2	0.230	0.019	8.3	3.2
50.0	0.372	0.034	9.1	1.4	0.461	0.025	5.5	0
100.0	0.728	0.076	10.4	1.4	0.907	0.078	8.6	2.2
200.0	1.470	0.142	9.7	0.9	1.800	0.163	8.8	0.6
300.0	2.190	0.130	6.0	1.6	2.700	0.151	5.4	0.5
Slope	0.0074 ± 0.00011				0.0093 ± 0.00012			
Intercept	−0.00533 ± 0.00143				−0.0054 ± 0.002			
Correlation coefficient	0.9949				0.9965			
Student's t	62.943				75.646			
Weighting procedure	1/variance				1/variance			

MCPA urine concentrations. Linear regression analysis using 1/variance weighting of the data gave a correlation coefficient of 0.997, a slope of 0.0093 ± 0.00012 and a *y*-intercept of −0.0054 ± 0.002. The back calculated MCPA concentrations were within 4% of the theoretical concentrations (5—300 μg/

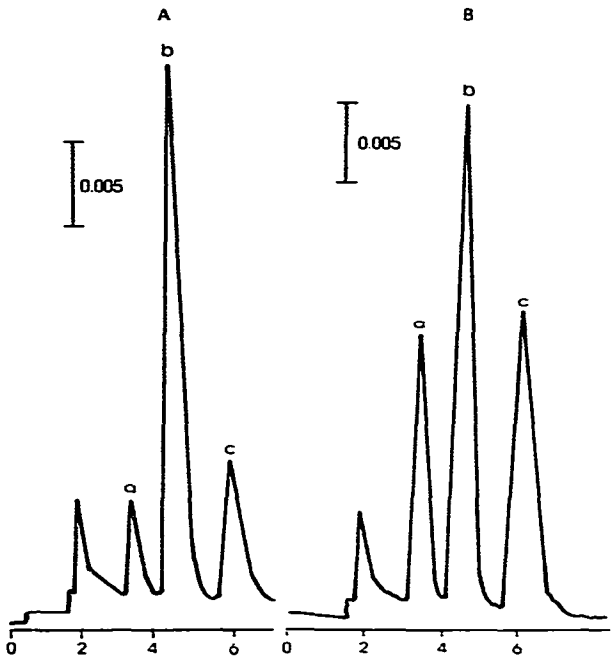


Fig. 3. HPLC assay of tolmetin and MCPA in human synovial fluid. Patient No. 1, A, 8-h synovial fluid sample with 1 μg internal standard (a.u.f.s. = 0.05); B, 8-h synovial fluid sample spiked with 1 μg each of tolmetin (c), MCPA (a) and internal standard (b) (a.u.f.s. = 0.05).

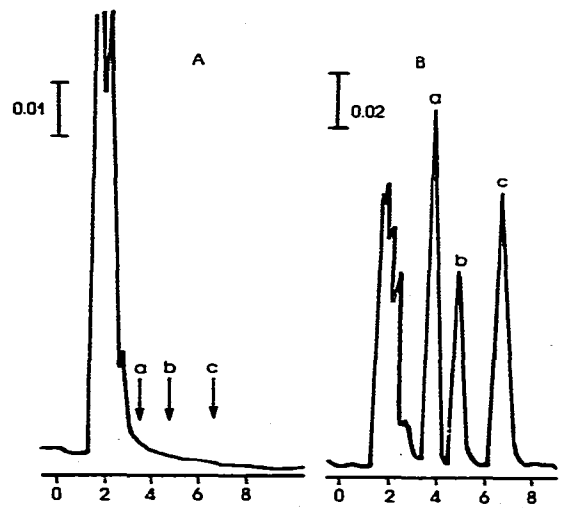


Fig. 4. HPLC assay of tolmetin and MCPA in human urine. Chromatograms of: A, blank urine sample (a.u.f.s. = 0.1); B, MCPA (a), internal standard (b), and tolmetin (c) each in concentrations of 200 μg/ml in urine (a.u.f.s. = 0.2).

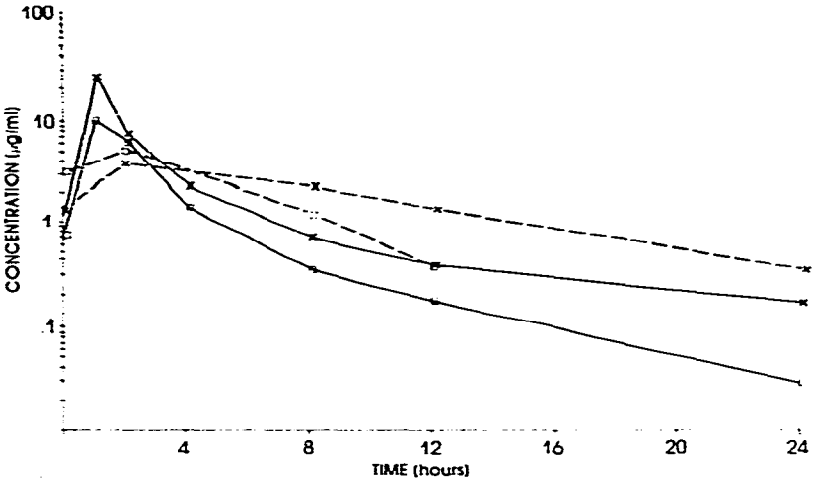


Fig. 5. Serum (——) and synovial fluid (- - - -) concentration profiles of tolmetin (×) and MCPA (□) in Patient No. 1 following a single oral dose of 400 mg of Tolectin®, tolmetin sodium on day 8 (on day 1 through 7, the patient was receiving 400 mg of Tolectin®, tolmetin sodium four times a day).

ml). C.V. values for MCPA peak height ratios were 12.4% or less at all concentrations. A summary of tolmetin and MCPA standard curve data in urine are presented in Table III. No interference peaks due to endogenous material in urine were observed during the analysis of samples from normal volunteers and from patients with rheumatoid arthritis.

To show the applicability of the technique, the results of analysis of serum and synovial fluid samples obtained from one patient are presented here. Fig. 5 illustrates the semi-logarithmic plot of serum and synovial fluid concentration—time profiles of tolmetin and MCPA in a rheumatoid arthritic patient. The serum samples obtained at 1 and 2 h were analyzed using 0.1 and 0.5 ml aliquots since historical data suggested tolmetin concentrations above 10 µg/ml at these time points. The synovial fluid concentration of MCPA in this patient at 24 h was below the quantitation limit of the assay.

This technique has been successfully utilized in the analysis of samples from arthritic patients receiving tolmetin sodium. The pharmacokinetic results will be presented in a subsequent publication.

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