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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE DETERMINATION OF *p*-(3,3-DIMETHYL-1-TRIAZENO)BENZOIC ACID IN MOUSE PLASMA

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SUMMARY

A reproducible and sensitive method is described for assaying *p*-(3,3-dimethyl-1-triazeno)-benzoic acid (pCOOH-DMT) and for identifying the N-desmethyl metabolite, *p*-(3-methyl-1-triazeno)benzoic acid (pCOOH-MMT) using high-performance liquid chromatography. The method measures concentrations as low as 1.25 nmol/ml of plasma. Extraction efficiency of internal standard or of added triazenes averages 88% and the coefficient of variation of the method is less than 10%. pCOOH-DMT is stable at room temperature at pH 7.4, whereas pCOOH-MMT undergoes rapid decomposition (half-life 6 min). pCOOH-MMT is more stable in an albumin-containing solution or in plasma, but not in boiled 9000 *g* mouse liver. After 80 min incubation with a 9000 *g* mouse liver fraction and reduced nicotinamide-adenine dinucleotide phosphate, only 24% pCOOH-DMT was metabolized. Plasma pharmacokinetic studies in mice treated with 200 mg/kg intraperitoneally showed that the potassium salt of pCOOH-DMT has a half-life of 67 min.

INTRODUCTION

Among various aryldimethyltriazenes synthesized and tested for antineoplastic activity in animals [1—3], *p*-(3,3-dimethyl-1-triazeno)benzoic acid potassium salt (pCOOK-DMT) [4] appears of particular interest. This compound has shown marked antimetastatic activity in Lewis lung carcinoma

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of the mouse [5], significant antitumoural activity against L1210 and P388 leukaemias and antitumour and antimetastic activity on the M5076 (M5) ovarian reticular cell sarcoma of the mouse [6], three murine tumour models currently used to select new anticancer agents. In some of these murine experimental tumours, pCOOK-DMT appears more effective and less toxic than imidazole-4-carboxamide-5-(3,3-dimethyl-1-triazeno) (DTIC), a drug currently used for the therapy of some human tumours [7–9].

An analytical method for pCOOK-DMT determination in biological samples is needed for further studies on its mode of action, metabolism and pharmacokinetics. In this paper, we describe a high-performance liquid chromatographic (HPLC) method for the determination of *p*-(3,3-dimethyl-1-triazeno)benzoic acid (pCOOH-DMT) and its probable metabolite *p*-(3-methyl-1-triazeno)-benzoic acid (pCOOH-MMT). The practical application of this method is illustrated by presenting the first results on the in vitro metabolism of pCOOK-DMT and its disappearance from plasma of mice treated with the drug.

EXPERIMENTAL

Standards and reagents

Triazenes were synthesized by published methods [4] and used as standards. Stock solutions were prepared in methanol every two weeks and kept at −20°C. Bovine albumin fraction V and tetrabutylammonium hydroxide (TBAH) were purchased from Sigma (St. Louis, MO, U.S.A.), and methanol, acetone and acetonitrile (HPLC grade) from Merck (Darmstadt, F.R.G.).

High-performance liquid chromatography

Separation by HPLC was performed on a Waters Model 440 instrument equipped with a 340-nm absorbance detector. An isocratic solvent system of 0.005 *M* TBAH in double-distilled water (pH 7.6, adjusted with phosphoric acid) and acetonitrile (82:18, v/v) was delivered at the rate of 1.5 ml/min using an Erbasil C_{18} column (10 μm particle size, 250 mm × 4.6 mm I.D.) from Carlo Erba (Milan, Italy).

Extraction of triazene from biological samples

p-(3,3-Dimethyl-1-triazeno)carboxamide ($pCONH_2$-DMT) as internal standard was added to 0.1 ml of mouse plasma and to 1.0 ml of homogenized liver fractions in the quantities of 2 and 5 μg, respectively. Samples were diluted with two equivalent volumes of ice-cold methanol and mixed vigorously on a vortex mixer for 30 s. Samples were cooled to −60°C with acetone and dry ice for 2 min, then centrifuged at 600 *g* for 3 min to precipitate proteins, and the clear methanolic solution was injected directly into the HPLC column.

Calibration curve and recovery

Various amounts of pCOOK-DMT (1, 2, 4 and 8 μg) and internal standard were added to 0.1 ml of mouse plasma or 1.0 ml of homogenate mixture. Samples were mixed, then extracted for pCOOH-DMT as described. Experiments were run in triplicate for each concentration and replicated four times. After chromatographic analysis, the peak-area ratios of pCOOH-DMT to

internal standard (i.e. $pCONH_2$-DMT) were plotted for linear regression analysis against the theoretical concentration in the samples. In order to calculate the recovery, the pCOOH-DMT to $pCONH_2$-DMT (added as external standard) peak-area values were compared with those obtained by injecting external standards at the same concentrations.

In vitro stability

The stability of pCOOK-DMT and of *p*-(3-methyl-1-triazeno)benzoic acid potassium salt (pCOOK-MMT) was determined at 37°C in 0.15 *M* potassium chloride buffer solution (pH 7.4) and in a boiled 9000 *g* liver fraction. The stability of pCOOH-MMT was also determined in 4 or 8% albumin solution (pH 7.4, adjusted with 0.5 *M* sodium hydroxide) and mouse plasma. The concentration of a freshly prepared standard solution of pCOOH-MMT was 14 μM (3 μg/ml) and its chemical degradation was investigated as a function of incubation time. Tubes were not protected from light. At different intervals (0, 10, 20, 40 and 80 min), 0.1-ml aliquots of the incubation mixtures were diluted (1:2) with a methanol solution of 5 μM $pCONH_2$-DMT as standard. Samples were then treated as reported above (extraction procedure) and injected into the HPLC system.

The concentration of pCOOH-DMT or pCOOH-MMT at time 0 was assumed to be equal to the standard solution. Each experiment was run in triplicate and the results were expressed in terms of percentage changes in pCOOH-DMT and pCOOH-MMT concentration with time.

In vitro metabolism

C57Bl/6J Female mice (20 ± 2 g body weight) were killed and their livers were immediately removed, weighed and homogenized in a Potter-Bellco homogenizer with ice-cold 0.15 *M* potassium chloride buffer solution (pH 7.4) in a ratio of 1:5 (w/v). The homogenates were centrifuged at 9000 *g* for 20 min. Incubations were started by adding triazene dissolved in 1.0 ml 0.15 *M* potassium chloride buffer solution to the supernatant fraction (1 ml) with reduced nicotinamide-adenine dinucleotide phosphate (NADPH) as cofactor (2 mg). The pCOOK-DMT concentration was 43 μM. Incubation was at 37°C in a shaking water bath in the presence of atmospheric oxygen.

In vivo studies

For the determination of plasma kinetics of pCOOK-DMT, C57Bl/6J male mice (20 ± 2 g body weight) were used. The drug was dissolved in saline (9 mg/ml sodium chloride) and injected at the dose of 200 mg/kg intraperitoneally. At various times after the injection (5, 15, 30, 60, 120, 240 and 360 min), three animals per point were killed and plasma was taken and frozen at −20°C until analysed.

Semi-preparative identification by thin-layer chromatography and gas chromatography–mass spectrometry

In order to obtain sufficient samples for mass spectral pCOOH-DMT and metabolite identification, the concentrated methanolic solution from deproteinized pools of homogenized mouse liver fractions extracted for pCOOH-

DMT determination as described was submitted to thin-layer chromatography (TLC) on silica gel 60 F-254 plates (Merck). Co-chromatography with authentic samples of triazene was also done. For TLC, acetone—ethyl acetate—water (4:1:1.5, v/v/v) was used as eluent. The plate was sprayed with N-[naphthyl-(1)]-ethylenediammonium dichloride, a specific reagent for detection of triazenes [10].

The compounds isolated were scraped off the TLC plate, dissolved in acetone—water (9:1, v/v) and injected into the HPLC system or analysed by mass spectrometry after drying under a nitrogen stream. An LKB 2091 mass spectrometer with an LKB 2130 computer was used for gas chromatographic—mass spectrometric (GC—MS) analysis of pCOOH-DMT. The GC conditions were: column OV-1 3% on Gas-Chrom Q 80—100 mesh, 2 m × 2 mm I.D.; oven temperature 260°C, electron-impact energy 70 eV; helium carrier-gas flow-rate 25 ml/min, pCOOH-DMT was identified as the trimethylsilyl derivative, obtained with bis(trimethylsilyl)trifluoroacetamide in pyridine in the presence of 1—2% trimethylchlorosilane as catalyst. All silylating agents were purchased from Pierce (Rockford, IL, U.S.A.).

RESULTS

The resolution of a standard mixture using the C_{18} reversed-phase system is illustrated in Fig. 1a. Fig. 1b shows the chromatogram of mouse plasma to

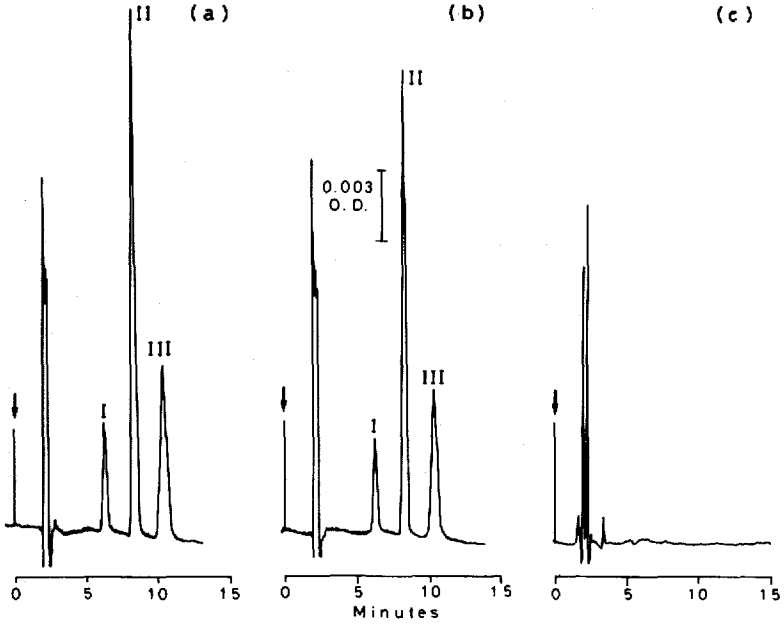


Fig. 1. (a) Chromatogram of a standard mixture of 50 ng each of N-desmethyltriazene pCOOH-MMT (I), $pCONH_2$-DMT (II) and pCOOH-DMT (III). (b) Chromatogram of mouse plasma containing the standard compounds: pCOOH-MMT (I), $pCONH_2$-DMT (II) and pCOOH-DMT (III). The equivalents of 50 ng of each standard were injected at 0.02 a.u.f.s. (c) Chromatogram of mouse plasma extract before addition of triazenes. The equivalent of 10 μl of plasma was injected at 0.02 a.u.f.s. Chromatographic conditions and extraction procedure are described in the text.

TABLE I

REPRODUCIBILITY OF THE pCOOH-DMT ASSAY

The calibration line was linear ($r = 0.99$; $P < 0.05$) and had a slope of 0.12 and an intercept of 0.005, not significantly different from zero. $n = 12$.

Plasma concentration (μg/ml)	Peak-area ratio (mean ± S.D.)	Coefficient of variation (%)
10	0.123 ± 0.0076	6.2
20	0.249 ± 0.0216	8.7
40	0.467 ± 0.0272	5.7
80	0.987 ± 0.0445	4.5

which 1.5 μg of each of the standard compounds were added. The limit of detection was 25 ng per injection. No HPLC interfering peaks from mouse plasma were observed before addition of standards (Fig. 1c). The same chromatographic profile was observed after extraction from a 9000 *g* liver fraction used as a control (data not shown). The extraction efficiency of added triazenes was 87% for pCOOH-MMT and 89% for pCOOH-DMT. The reproducibility of the pCOOH-DMT is reported in Table I; the calibration curve was linear ($r = 0.99$) and the maximum coefficient of variation was 8.7%. No chemical degradation of pCOOH-DMT was observed in 0.15 *M* potassium chloride buffer solution (pH 7.4) after 24 h at room temperature.

Fig. 2 shows the stability of pCOOH-MMT in different experimental conditions. At 37°C, from potassium chloride buffer solution, 50% of the incubated amount of pCOOH-MMT was degraded in about 6 min; after 20 min incubation, pCOOH-MMT was almost completely broken down to *p*-aminobenzoic acid (pNH_2-COOH) (values not reported in Fig. 2). The HPLC peak of pNH_2-COOH is not present in the chromatogram because this compound is not detectable at 340 nm. No pCOOH-MMT degradation was observed in

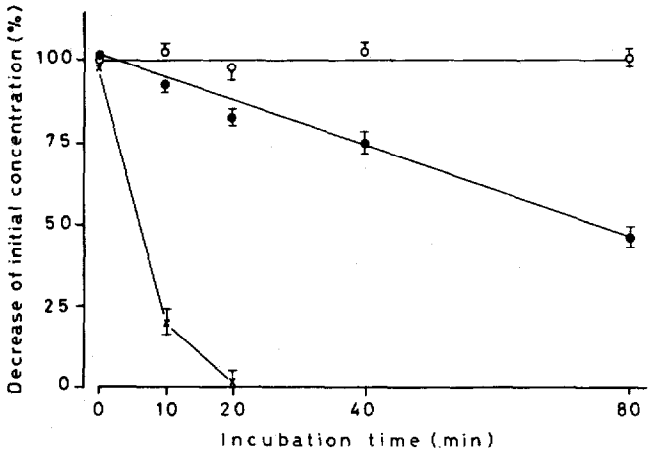
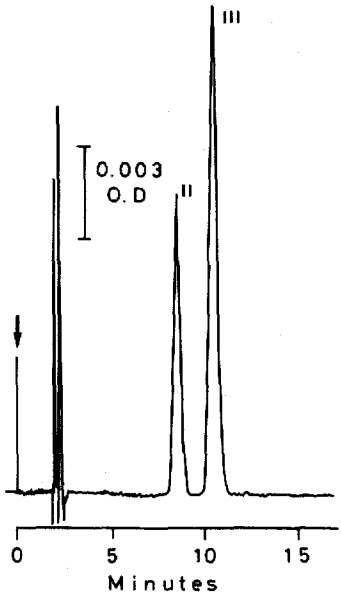


Fig. 2. In vitro stability of pCOOH-MMT from solutions of 0.15 *M* potassium chloride buffer (×), 4% albumin (•) and mouse plasma (○) as a function of incubation time at 37°C; details in the text.



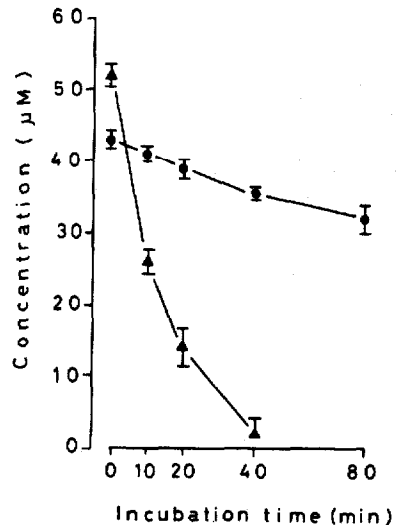


Fig. 3. Chromatogram of pCOOH-DMT in a 9000 *g* liver fraction after 40 min incubation. The final calculation indicates a concentration of 6.9 μg of pCOOH-DMT per ml of incubation mixture.

Fig. 4. Metabolism of pCOOK-DMT (•) and $pCOCH_3$-DMT (▲) by 9000 *g* mouse liver fractions. The initial substrate concentrations were 43 *μM* pCOOK-DMT and 52 *μM* $pCOCH_3$-DMT (mean of three values ± S.E.).

mouse plasma or in 8% bovine albumin solution, whereas about 50% of the incubated amount of pCOOH-MMT was degraded from 4% bovine albumin solution after 80 min incubation.

Fig. 3 shows the chromatogram of pCOOH-DMT in a 9000 *g* mouse liver fraction 40 min after the start of incubation; its metabolic disappearance is reported in Fig. 4 and compared to 1-(4-acetylphenyl)-3,3-dimethyltriazene ($pCOCH_3$-DMT), a control substrate for 9000 *g* N-demethylation activity [11]. After 80 min incubation of pCOOK-DMT in a boiled 9000 *g* liver fraction at 37°C, no chemical degradation was observed. Quantitative determination of pNH_2-COOH (chemical degradation product of pCOOH-MMT possibly formed) from biological samples was not possible because of background interference at 254 nm (i.e. the wavelength at which pNH_2-COOH has maximal absorbance). Since we failed to find any pCOOH-MMT after incubation of pCOOH-DMT with a 9000 *g* liver fraction, we investigated its stability in the same conditions. Only 5 min after the beginning of incubation of pCOOH-MMT in boiled liver extract, no peak corresponding to this compound was observed.

Fig. 5a shows the trimethylsilyl derivative mass spectrum of the compound corresponding to peak III in Fig. 3. This peak has the same HPLC retention time as authentic pCOOH-DMT. Fig. 5b shows the mass spectrum of the trimethylsilyl derivative of authentic pCOOH-DMT, which is virtually identical to that in Fig. 5a. The ion at *m/z* 265 is the molecular ion and loss of a methyl radical leads to the peak at *m/z* 250. The elimination of 44 u ($Me_2N^{\cdot}$) gives

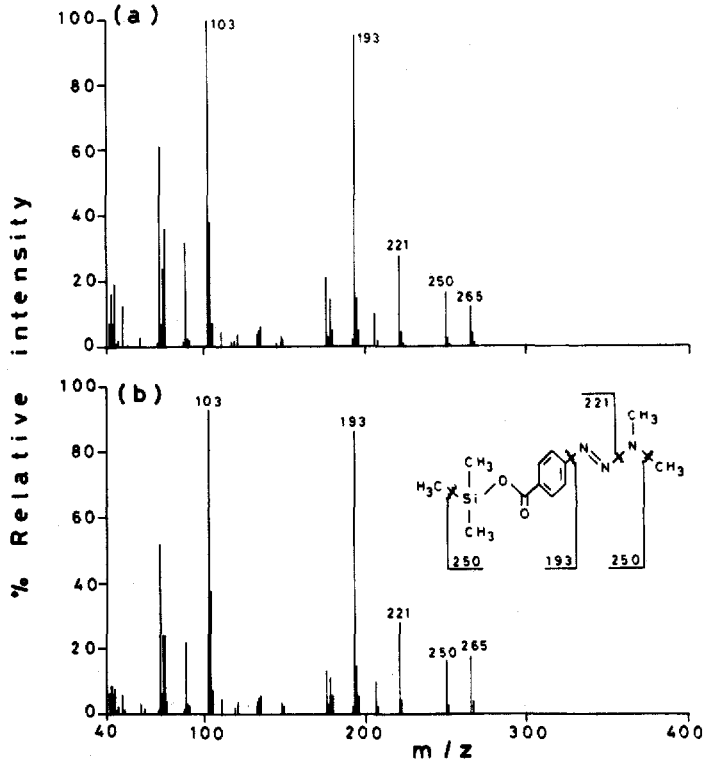


Fig. 5. Electron-impact mass spectrum of (a) component III (see Fig. 3) as its trimethylsilyl derivative after TLC isolation and (b) pCOOH-DMT standard as its trimethylsilyl derivative.

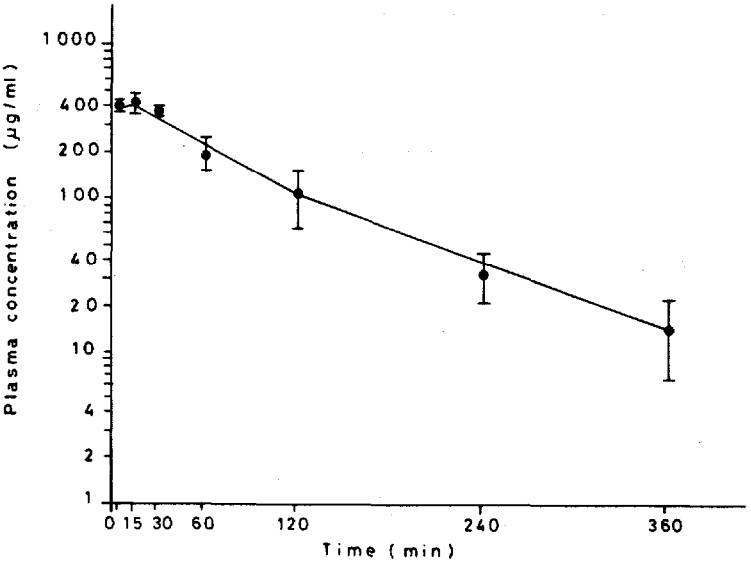


Fig. 6. Plasma concentration–time curve from mice after an intraperitoneal dose of 200 mg/kg pCOOK-DMT (mean of three values ± S.D.).

the ion at *m/z* 221. The peak at *m/z* 193 is probably due to the loss of the triazene chain. These fragmentations are in analogy with those of other 3,3-dimethyltriazenes [12, 13]. Losses of the triazene chain and of trimethylsilanol give the ion at *m/z* 103. Peaks at *m/z* 77 and 73 are due to $C_6H_5^+$ and $MeSi^+$, respectively. Arylmonomethyltriazenes rapidly decompose by hydrolysis in aqueous solution to the corresponding aniline, nitrogen and carbon cation derivative [14] so that the added pCOOH-MMT HPLC peak in plasma was identified as *p*-(3-methyl-1-triazeno)benzoic acid only by comparison with the authentic pCOOH-MMT HPLC retention time, not by MS.

Fig. 6 depicts pCOOH-DMT levels in plasma of mice treated with 200 mg/kg intraperitoneally. The pCOOH-DMT plasma peak level was achieved 15 min after injection, then the drug concentrations declined with a half-life of 67 min.

DISCUSSION

The HPLC method described in the present study appears specific, reproducible and sufficiently sensitive for assaying pCOOH-DMT and for identifying its N-desmethyl metabolite. As can be seen in Table I, the calibration curve of pCOOH-DMT was linear (r = 0.99) and the coefficient of variation (C.V.) was les than 10%. No interference from endogenous compounds was observed. Extraction efficiency of added triazenes averaged 88% and their limit of detection was about 25 ng per injection. The sensitivity appears sufficient to study the pharmacokinetics of pCOOH-DMT in animals treated with therapeutic doses.

The 1-aryl-3,3-dimethyltriazenes have shown antitumour efficacy in experimental tumours in animals but their mechanism of antineoplastic activity is still not understood. They probably require metabolic activation in the liver [1, 2] through a pathway involving the formation of 1-aryl-3-methyltriazene [15], which is known to methylate biologically significant nucleophiles [16]. The HPLC method described is suitable for studying the metabolism of this class of compounds. We found that pCOOH-DMT was only weakly metabolized in vitro. After 80 min incubation with a 9000 *g* mouse liver fraction and NADPH, only 24% of pCOOH-DMT was metabolized compared to 79% of $pCOCH_3$-DMT, known to be largely N-demethylated [11]. These data are in agreement with those of Sava et al. [17] who reported in vitro oxidative N-demethylation of pCOOK-DMT expressed as the amount of formaldehyde product during in vitro incubation. The pCOOH-MMT was very unstable in the incubation conditions, its half-life in a boiled 9000 *g* liver fraction being less than 5 min (data not shown). This explains why the low rate of formation of pCOOH-MMT could not be detected under our conditions.

The low rate of N-demethylation of pCOOK-DMT is interesting since the antitumour activity of dimethyltriazenes is believed to be related to the formation of the monomethyl derivatives, which have been proposed as the active species [15]. The low N-demethylation rate of pCOOK-DMT and the instability of pCOOH-MMT argue against the hypothesis that pCOOH-MMT is really responsible for the antitumour activity of pCOOK-DMT. On the other hand, pCOOH-MMT appears stable in plasma, thus suggesting that once formed in the liver it may reach the tumour in sufficient amounts before being

degraded. Studies in mice failed to give us a definitive answer to this point. In fact, the chromatograms of plasma of mice treated 5 min previously with pCOOK-DMT showed a peak with the same retention time as pCOOH-MMT. However, it was very small, its height was approximately 2% of the pCOOH-DMT peak, it was no longer observable later on and it requires further characterization. In addition, in the chromatograms of plasma of mice treated with pCOOK-DMT there was a new major peak corresponding to an unknown metabolite. It is a triazene as assessed by TLC separation and colorimetric identification [10], it is water-soluble, but its chemical structure has still to be established.

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