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TRACE-LEVEL DETERMINATION OF 3′-AZIDO-3′-DEOXYTHYMIDINE IN HUMAN PLASMA BY PRECONCENTRATION ON A SILVER(I)–THIOL STATIONARY PHASE WITH ON-LINE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

H. IRTH*, R. TOCKLU, K. WELTEN, G.J. DE JONG, U.A.Th. BRINKMAN and R.W. FREI[a]

Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam (The Netherlands)

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SUMMARY

A method was developed for the determination of 3′-azido-3′-deoxythymidine (AZT) in plasma. The method is based on the trace enrichment of AZT on a pre-column packed with a silver-loaded thiol stationary phase at pH 11.6. On-line desorption to the reversed-phase liquid chromatographic system is performed by injecting a plug of 50 μl of 1 *M* perchloric acid on the silver(I)–thiol pre-column. Two different sample pretreatment methods – protein precipitation with perchloric acid and on-line clean-up via a polymeric PRP-1 pre-column – were applied for the determination of AZT in human plasma. The latter method allows the direct injection of plasma samples into the analytical system and can therefore easily be automated. With both methods detection limits in the order of 10^{-8} *M* AZT were obtained after preconcentration of 1.0 ml of plasma, using UV detection at 267 nm.

INTRODUCTION

Since it has become known that AIDS (acquired immunodeficiency syndrome) is caused by the human immunodeficiency virus (HIV), much effort has been devoted to develop drugs capable of attacking this virus. AZT (3′-azido-3′-deoxythymidine; Fig. 1) is considered to be one of the most promising substances for the treatment of AIDS [1]. The action of AZT is based on its conversion into AZT triphosphate, which is incorporated into DNA by the HIV DNA polymerase. The azido group at the 3′-position blocks the further pro-

[a]Author deceased.

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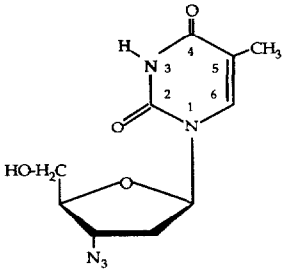

Fig. 1. Structure of AZT.

duction of DNA, because it replaces the hydroxyl group that is needed for the chemical bond to the next DNA link [1].

In order to monitor plasma concentrations of AZT and to learn more about its toxic and antiviral action, sensitive and selective analytical methods are required. Recently, analytical methods for the determination of AZT in plasma and urine were reported, which were based on the reversed-phase liquid chromatographic (LC) separation of AZT from interfering plasma components and UV detection at 267 nm [2–5]. Three methods [2–4] require off-line clean-up steps (liquid–liquid or liquid–solid extraction, evaporation, centrifugation) which are time-consuming and may lead to losses. An alternative approach to liquid–liquid extraction is the application of pre-column techniques [6] which can simultaneously be used for the on-line clean-up of, e.g., biological samples and the enrichment of trace amounts of analytes. Daoud and co-workers [7,8] and Nielen et al. [9] described pre-column techniques for the direct injection of untreated blood plasma using, e.g., 10- or 40-μm C_8- or C_{18}-bonded silica as pre-column packing materials. Van der Horst and Martens [5] used a C_{18} pre-column for the clean-up of plasma samples containing AZT. Since AZT is a rather polar compound and has a small breakthrough volume on, e.g., C_{18}-bonded silica, only 50 μl of plasma could be injected. For the trace enrichment of higher sample volumes (of up to 1 ml) and for a higher selectivity, other pre-column packing materials are required. We recently reported the use of a silver-loaded sorbent for the on-line trace enrichment of nucleobases (uracil, cytosine, thymine), nucleosides (uridine) and other uracil derivatives (5-fluorouracil, bromacil) [10]. Retention of these compounds occurs via the formation of a coordination bond between Ag^I and the nitrogen at the 3-position of the pyrimidine ring (see Fig. 1). Owing to this specific interaction, the selectivity of the analytical system is considerably enhanced. Since AZT also is an uracil derivative, we applied the same method to the determination of AZT in human plasma.

EXPERIMENTAL

Apparatus

The LC system (Fig. 2) consisted of three laboratory-made six-port Valco-type injection valves, a 1.0-ml sample loop, a 50-μl loop to contain the perchloric acid (1 *M*) needed for the desorption of the analytes, two 10 mm×4 mm I.D. laboratory-made stainless-steel pre-columns and holders (Chrompack, Middelburg, The Netherlands) and a 200 mm×4.6 mm I.D. 5-μm Hypersil ODS (Shandon Southern, Runcorn, Cheshire, U.K.) analytical column. The carrier solution for preconcentration was delivered by a Kontron (Zürich, Switzerland) 414 LC pump and the LC eluent by a Pye Unicam PU 4015 pump (Philips, Eindhoven, The Netherlands). Each pump was provided with a pulse damper. The preconcentration pump was connected to a Rheodyne (Berkeley, CA, U.S.A.) solvent-select valve. The LC eluent was acetonitrile–aqueous 10 m*M* acetate buffer (pH 5.0) (11:89, v/v). A Kratos (Ramsey, NJ, U.S.A.) Spectroflow 757 absorbance detector was used for detection at 267 nm. The analytical system was operated under ambient conditions.

Chemicals and reagents

AZT was a gift from the Academic Hospital of the Free University (Amsterdam, The Netherlands). All chemicals and organic solvents used were an-

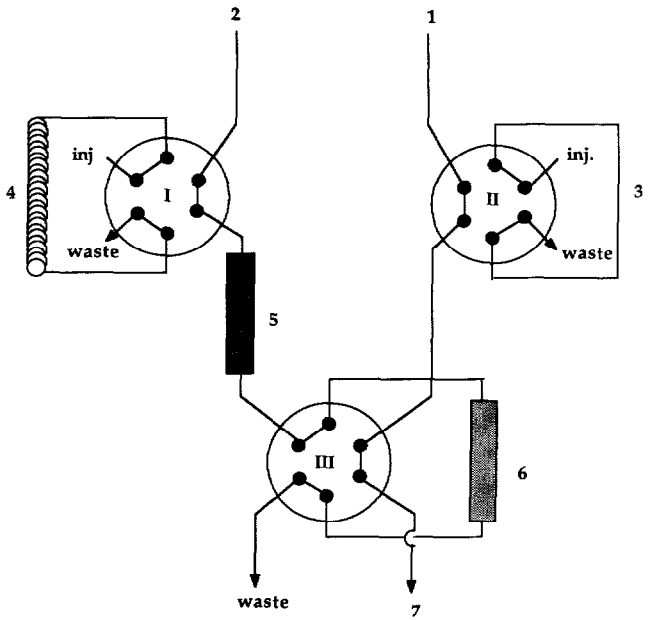

Fig. 2. Scheme of the analytical system: 1 = LC pump; 2 = preconcentration pump; 3 = injection loop (perchloric acid); 4 = sample injection loop; 5 = PRP-1 clean-up pre-column; 6 = Ag^{I}–thiol pre-column; 7 = to analytical column and detector.

alytical-grade reagents (Baker, Deventer, The Netherlands). Fresh human plasma was obtained from the Blood Transfusion Service (Academic Hospital of the Free University).

Procedure for trace enrichment

The polystyrene–divinylbenzene copolymer PRP-1 (10 μm; Hamilton, Bonaduz, Switzerland) and the thiol-containing methacrylate polymer Spheron Thiol 1000 (40–63 μm; Lachema, Brno, Czechoslovakia) were used as stationary phase for clean-up (No. 5 in Fig. 2) and trace enrichment (No. 6 in Fig. 2). They were slurry-packed into the stainless-steel pre-columns via a 5-ml syringe; the packed thiol pre-column was loaded off-line with 5 ml of 10 m*M* silver nitrate and flushed with 5 ml of deionized, distilled water and 5 ml of HPLC-grade methanol before use.

The clean-up and trace enrichment procedure is summarized in Table I. In the first step the sample is injected via a 1.0-ml loop into the carrier solution – 10 m*M* acetate buffer (pH 5.0) – and flushed through the PRP-1 clean-up pre-column at a carrier flow-rate of 2.0 ml/min, while the Ag^I–thiol pre-column is switched off-line. The column is washed with 4.0 ml of 10 m*M* acetate buffer (pH 5.0), and then the system is flushed with 1 ml of aqueous potassium hydroxide (pH 11.6). The analyte is transferred to the Ag^I–thiol pre-column by flushing the PRP-1 pre-column with 3.0 ml of potassium hydroxide solution (pH 11.6)–methanol (80:20, v/v) at a flow-rate of 0.5 ml/min. On-line desorption of AZT to the analytical column was carried out by injecting a plug of 50 μl of 1 *M* perchloric acid into the LC eluent stream, and switching to the pre-column to desorb the preconcentrated analyte and transferring it to the analytical column. A 3-s delay time was introduced between the injection and switching step.

TABLE I

PROCEDURE FOR THE CLEAN-UP AND PRECONCENTRATION OF AZT

For desorption of AZT to the analytical column, see Experimental; (+) PRP-1 and Ag^I–thiol pre-column are switched on-line; (–) PRP-1 and Ag^I–thiol pre-column are switched off-line.

Carrier solution	Volume (ml)	Flow-rate (ml/min)	Ag^I–thiol	Event
1 *M* $HClO_4$	2.0	2.0	+	Regeneration of Ag^I–thiol
Acetate (pH 5)	4.0	2.0	–	Loading and washing of PRP-1 pre-column
KOH (pH 11.6)	1.0	2.0	–	Increase of pH value
KOH (pH 11.6)–methanol (80:20)	3.0	0.5	+	Transfer of AZT to Ag^I–thiol pre-column[a]

[a]Step shown in Fig. 2.

Sample pretreatment

Deproteination of plasma with perchloric acid. Plasma samples were deproteinized by adding 0.5 ml of perchloric acid (70–72%) to 4.5 ml of plasma. The mixture was vortexed for 1 min and centrifuged for 10 min (1000 *g*). Solid potassium hydroxide was then added to the supernatant to adjust the pH to 11.6, and the solution was centrifuged again. The supernatant was directly injected onto the Ag^I–thiol pre-column using a 1-ml loop.

Protein removal via a PRP-1 pre-column. A 0.5-ml volume of 1 *M* acetate buffer (pH 5.0) was added to 4.5 ml of plasma. The solution was filtered through a 2-μm membrane filter and injected onto the PRP-1 pre-column using a 1-ml loop.

RESULTS AND DISCUSSION

Trace enrichment of AZT on Ag^I–thiol

Whereas apolar compounds can easily be retained on hydrophobic stationary phases such as C_{18}-bonded silica or PRP-1, alternative pre-column materials have to be chosen if polar or ionic compounds are to be preconcentrated. We recently showed that a thiol stationary phase loaded with Ag^I can be used for the trace enrichment of uracil nucleobases [10], and that the retention of these compounds is mainly determined by the pH of the sample solution and by the flow-rate at which it is pumped through the pre-column. By investigating different model compounds, such as substituted uracil derivatives and barbiturates, we found that the presence of a single NH–C=O group in the pyrimidine ring is sufficient to retain this type of compound on Ag^I–thiol. Since AZT also contains a non-substituted NH–C=O group, we investigated its trace enrichment on Ag^I–thiol under the optimum pH conditions earlier obtained for the pyrimidine nucleobases (sample pH 11.6). We found that AZT was indeed retained on Ag^I–thiol under these conditions. The dependence of the AZT recovery on the sampling flow-rate is shown in Fig. 3. The curve is similar to the one obtained for uracil or thymine [10] and shows a recovery of $97 \pm 3\%$ ($n = 10$)

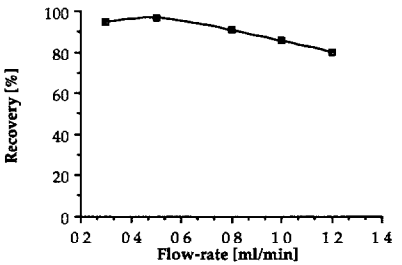

Fig. 3. Percentage recovery of AZT at pH 11.6 as a function of the carrier flow-rate. Direct preconcentration of 1-ml samples containing 10^{-7} *M* AZT; for other conditions, see Experimental.

at a flow-rate of 0.5 ml/min. The breakthrough volume obtained was higher than 9 ml. All further experiments were therefore carried out with a sample pH of 11.6 and at a preconcentration flow-rate of 0.5 ml/min.

Somewhat surprisingly, the recoveries for AZT were distinctly better than would be expected on the basis of earlier data obtained for uridine [10]. The lower recoveries obtained for uridine may be explained by steric hindrance caused by the substitution of N-1 by a ribose ring. Therefore, further experiments with other N-1-substituted uracil derivatives, e.g. thymidine or 5-fluorouridine, should be carried out in order to find out the reason for the different complexation behaviour.

Desorption of AZT to the analytical system

The on-line desorption of AZT to the analytical system was performed (cf. ref. 9) by lowering the pH value. The N-3 position of the pyrimidine ring is then protonated, which breaks the coordination binding to Ag^I. In practice, desorption of AZT was performed by injecting small plugs (50 μl) of a strongly acidic solution onto the Ag^I–thiol pre-column. Fig. 4 shows the dependence of the AZT recovery (based on peak area) and peak height on the desorption pH. While the recovery remains constant at $98 \pm 2\%$ ($n=4$) for all pH values investigated, the peak height decreases with increasing pH, which indicates that the desorption kinetics are strongly dependent on the pH. Larger desorption volumes of solutions of a higher pH did not result in a smaller peak width. For example, desorption with 50 μl of 1 *M* perchloric acid resulted in an additional peak broadening of $\sigma=1.9$ s, compared with a 10-μl direct injection ($\sigma=4.2$ s); the value for a desorption with 500 μl of 0.1 *M* perchloric acid was 2.8 s.

Desorption via analyte protonation leaves Ag^I bound to the thiol ligand, and the Ag^I–thiol phase can be re-used for at least 25 injections without further regeneration steps. No negative effects on the lifetime of the analytical column have been observed due to the repeated injection of 50-μl plugs of a strongly acidic solution.

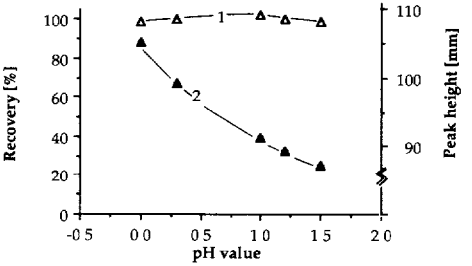

Fig. 4. Percentage recovery of AZT based on peak area (1) and peak height (2) as a function of the desorption pH value. Direct preconcentration of 1-ml samples containing 10^{-7} *M* AZT; sample pH, 11.6; for other conditions, see Experimental.

Analytical data

With the present method a detection limit of $4 \cdot 10^{-9}$ *M* (trace enrichment from 1.0-ml sample; signal-to-noise ratio 3:1) was obtained. The repeatability ($n=7$) was 2.0% for 10^{-7} *M* AZT and 5.6% for $6 \cdot 10^{-9}$ *M* AZT. A linear response ($r=0.9993$) of AZT was obtained over three orders of magnitude in the concentration range of interest ($5 \cdot 10^{-9}$–$5 \cdot 10^{-6}$ *M*). The column efficiency, measured in terms of plate number, showed only a slight decrease: 11 260 for preconcentration versus 12 660 for direct 10-μl injections.

Determination of AZT in plasma

Early experiments showed that the direct injection of untreated plasma samples on the Ag^I–thiol pre-column results in the occurrence of several large peaks (see Fig. 5), which interfere with the determination of early-eluting, polar analytes, such as 5-fluorouracil [10]. These interferences may be attributed to proteins, peptides or amino acids that contain a sulfhydryl group, which forms strong complexes with Ag^I. Removal of these plasma constituents is therefore a necessary sample pretreatment step if Ag^I–thiol is used as pre-column stationary phase. We investigated the suitability of two different deproteination techniques for the determination of trace levels of AZT (10^{-7}–10^{-8} *M*) in plasma samples, viz. precipitation with perchloric acid or clean-up over a PRP-1 pre-column.

Deproteination with perchloric acid. Plasma proteins were precipitated by

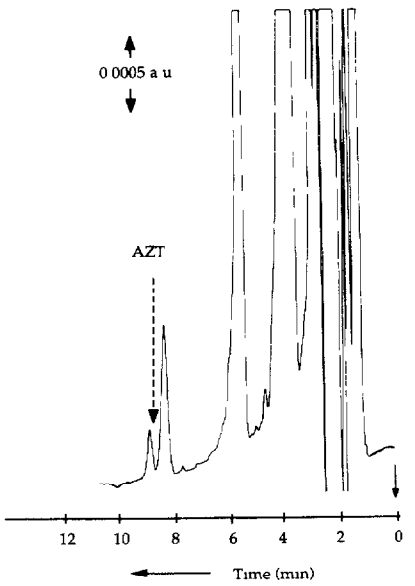

Fig. 5. Direct injection of 1.0 ml of blank plasma (pH 11.6) onto the Ag^I–thiol pre-column. The stippled line indicates the retention time of AZT. Eluent, acetonitrile–10 m*M* acetate buffer (pH 5.0) (12.5:87.5, v/v); for other conditions, see Experimental.

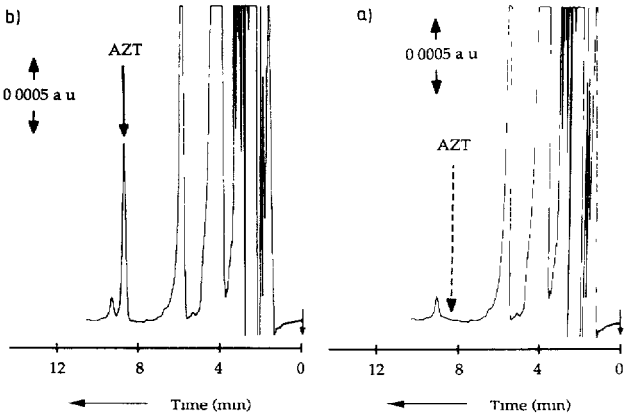

Fig. 6. Direct injection of 1.0 ml of plasma onto the Ag^{I}–thiol pre-column after deproteination with perchloric acid. (a) Blank plasma (pH 11.6); (b) plasma (pH 11.6) spiked with $1.5 \cdot 10^{-7}$ *M* AZT. Eluent, acetonitrile–10 m*M* acetate buffer (pH 5.0) (12.5.87.5, v/v); for other conditions, see Experimental.

the addition of 1 part of perchloric acid to 9 parts of plasma sample (final perchloric acid concentration, 7%). The solution was vortexed, and solid potassium hydroxide was added to increase the pH to 11.6 without further dilution of the sample. After centrifugation and filtration over a 2-μm membrane filter, the sample was directly injected onto the Ag^{I}–thiol column via a 1.0-ml loop. Fig. 6 shows the chromatograms of a plasma blank (a) and of plasma spiked with $1.5 \cdot 10^{-7}$ *M* AZT (b). The chromatogram of blank, deproteinized plasma does not show any interfering peaks at the retention time of AZT. The recovery of AZT is 98% with a relative standard deviation (R.S.D.) of 6% ($n=4$). For the trace enrichment of 1-ml plasma samples a detection limit (signal-to-noise ratio 3:1) of 5 ng of AZT was obtained.

On-line clean-up over a PRP-1 pre-column. Since the precipitation technique includes several off-line pretreatment steps that are difficult to automate, pre-separation of proteins via a clean-up pre-column packed with C_{18}-bonded silica [7,8] or PRP-1 was investigated. Nielen et al. [11] used a hydrophobic sorbent prior to a metal-loaded phase in order to remove interfering urine constituents (dual pre-column system). The analyte was retained on the hydrophobic material, while ionic compounds were removed with the aqueous flushing solution. The analytes were then transferred to the metal-loaded phase with methanol–water (70:30).

We used the same technique to separate AZT from ionogenic plasma constituents. After filtration over a 2-μm membrane, the plasma was adjusted to pH 5.0 and flushed through a PRP-1 pre-column (No. 5 in Fig. 2). Owing to the rather high polarity of AZT, the PRP-1 pre-column can only be flushed with 4 ml of acetate buffer (pH 5.0), otherwise breakthrough of AZT will occur.

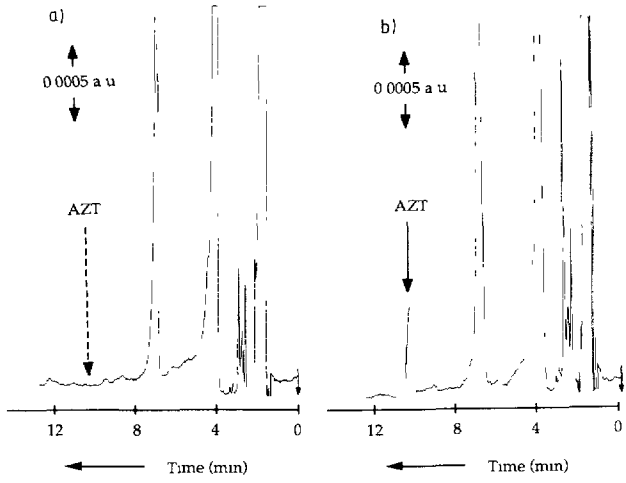

Fig. 7. Direct injection of 1.0 ml of plasma onto a PRP-1 pre-column. (a) Blank plasma (pH 5.0); (b) plasma (pH 5.0) spiked with $7 \cdot 10^{-8}$ *M* AZT. Eluent, acetonitrile–10 m*M* acetate buffer (pH 5.0) (11:89, v/v); for other conditions, see Experimental.

During the washing process the Ag^I–thiol pre-column is switched off-line. The pH is then increased by flushing the system with 1 ml of aqueous potassium hydroxide (pH 11.6). After switching the Ag^I–thiol pre-column on-line, AZT is transferred with 3.0 ml of potassium hydroxide solution (pH 11.6)–methanol (80:20, v/v) at a flow-rate of 0.5 ml/min. The whole procedure is summarized in Table I.

With this technique, plasma samples can be injected directly into the analytical system without significant dilution or pretreatment. Fig. 7 shows chromatograms of a plasma blank (a) and plasma spiked with $7 \cdot 10^{-8}$ *M* AZT (b). The recovery of AZT was 93% with an R.S.D. of 4% ($n=4$). The detection limit was 10^{-8} *M* for the trace enrichment of 1 ml of plasma. As with the perchloric acid procedure, no interfering peaks occur at the retention time of AZT; the clean-up actually is better in the present case.

The main disadvantage of the technique is that the PRP-1 material is irreversibly loaded with apolar plasma constituents, which results in recoveries of less than 10% if the pre-column is used for a second time. Attempts to regenerate the PRP-1 pre-column with methanol or 0.01 *M* perchloric acid were not successful, therefore the clean-up pre-column had to be repacked after each run. The Ag^I–thiol pre-column could be used for at least 25 plasma injections. In order to remove plasma components with a high affinity for Ag^I, the Ag^I–thiol pre-column was flushed with 2 ml of 1 *M* perchloric acid after each run.

CONCLUSIONS

An Ag^I-loaded thiol stationary phase can be used for the trace enrichment of AZT from plasma samples. After on-line desorption to a reversed-phase LC

system, detection limits of $4 \cdot 10^{-9}$ *M* for standard solutions and 10^{-8} *M* for plasma samples are obtained with UV detection. Two deproteination techniques – precipitation by perchloric acid and direct plasma injection onto a PRP-1 pre-column – have been applied, which both successfully remove interfering plasma components. Precipitation with perchloric acid includes several off-line pretreatment steps and is therefore less suitable if the method has to be automated. The direct injection of plasma samples via a PRP-1 column requires virtually no sample treatment and can therefore easily be automated. Since the PRP-1 pre-column has to be replaced after each run, an automated cartridge exchanger [12] should be available.

Future work will deal with the determination of active (phosphorylated) AZT metabolites, such as AZT triphosphate, which are responsible for the antiviral action [1]. These compounds possess a negative charge and are therefore difficult to extract from plasma or tissue samples with organic solvents. Neither can they easily be preconcentrated on hydrophobic stationary phases. Since the N-3 position of the pyrimidine ring remains unaffected during metabolism, the complexing properties of the phosphorylated AZT metabolites should be similar to those of AZT itself. Trace enrichment on an Ag^{I}-loaded stationary phase may therefore be a promising approach to determining the compounds in plasma or cell tissues, thus providing more knowledge on the antiviral action of AZT and similar compounds.

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