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Rapid determination of theophylline in serum by selective extraction using a heated molecularly imprinted polymer micro-column with differential pulsed elution

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Abstract

Molecular imprinting of theophylline in poly(methacrylic acid–ethylene dimethacrylate) form binding sites with complementary size, shape and chemical functionalities to theophylline. This molecularly imprinted polymer (MIP) can be packed into a micro-column for selective solid phase extraction (SPE) of theophylline from 20 μl of sample solution. Its chemical inertness and thermal stability allow the use of various organic solvents and elevated column temperatures for effective binding of theophylline. Non-specific adsorption of interfering drugs on the MIP surface is eliminated by an intermediate wash with 20 μl of acetonitrile, prior to quantitative desorption of the bound theophylline by 20 μl of methanol for in-line UV spectrophotometric determination. In this differential pulsed elution (DPE) technique, both the column temperature and solvent flow rate can be optimized to enhance selectivity. Application of this micro-analytical method, molecularly imprinted solid phase extraction–DPE (MISPE-DPE), is demonstrated for accurate determination of theophylline in human blood serum. The method is validated over a linear range from 2 μg/ml to at least 20 μg/ml. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Molecularly imprinted polymer; Theophylline; Solid phase extraction; Serum; Pulse elution; Analysis

1. Introduction

Asthma is an inflammatory disease characterized by bronchial hyper-responsiveness that can proceed to life-threatening airway obstruction. Theophylline is a bronchodilator that is widely used in the treatment of asthma and bronchospasm in adults. It is one of the most commonly prescribed pharmaceuticals [1] and was one of the three drugs most frequently monitored by pharmacokinetics services in the US Veterans Affairs medical centers [2]. A developmental study found that theophylline caused clastogenic but not genotoxic effects in human lymphocytes after long-term exposure [3]. As a phosphodiesterase inhibitor, theophylline synergized with chloram-

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bucil in inducing apoptosis of β-chronic lymphocytic leukemia cells, which suggested that the combination might have therapeutic value [4]. Since its concentration at the active sites strongly depended on characteristic parameters of the patient's response, an individualization of dosage regimen was argued for [5]. Theophylline was demonstrated to inhibit the repair of potentially lethal DNA lesions [6]. Its pharmacokinetics has recently seen a resurgence of interest in the development of new oral macrolide and fluoroquinolone antibiotics [7].

Biological samples such as urine [8] plasma or serum [9,10], and tissue [11] have been used in theophylline doping control investigations, clinical pharmacokinetic experiments [12], and human liver metabolism studies [13]. The ubiquitous nature of methylxanthines and the complexity of biological sample matrices demand a highly selective technique for theophylline determination. Analytical and pharmaceutical chemists traditionally employed high performance liquid chromatography (HPLC) to assay theophylline and other methylxanthines [7,14]. However, HPLC methods were generally disadvantaged in terms of analysis time and sample handling requirements. An international health control survey showed that commercial immunoassay kits were comparable in performance to reverse-phase HPLC (coefficient of variation = 7.5%, accuracy = 3.2%) when measuring theophylline in serum [15]. One shortcoming of immunoassays is the availability and relatively short working life of antibodies. A significant cross-reactivity of 6.7% was also found when theophylline was measured by fluorescence polarization immunoassay (FPIA) in the presence of caffeine [16]. Such cross-reactivity with caffeine may have important consequences in the monitoring of premature newborns, in which levels of both analytes can be comparable due to biotransformation of theophylline to caffeine.

Species of RNA that bound with 10 000-fold greater affinity and specificity to theophylline than to caffeine were earlier identified by selection from an oligonucleotide library [17]. However, molecular imprinted polymers (MIP) were later demonstrated to provide selective affinity for theophylline and various other drugs [18–25]. The technique is based on creating cavities, in a highly cross-linked polymer matrix, which correspond to the size and shape of the target drug molecule. Intermolecular interactions such as hydrogen bonding between the MIP cavities and the drug molecules in a sample solution can drive the selective molecular recognition process that causes selective binding of the drug molecules. The design of MIPs and their extensive applications to numerous areas of scientific research (including chromatography, antibody and receptor binding mimics, artificial enzymes, and biosensors) have been reviewed [26–32]. Specifically, they show significant promise for use as MIP columns in solid-phase extraction (SPE) methods [33–37]. These columns are resistant to mechanical stress, heat, acids, bases, water and organic solvents. Their storage life is very long (years) at ambient temperatures, with no apparent reduction in molecular recognition performance.

Recently, Mullett and Lai successfully developed an on-line method that utilized a MIP column for the selective SPE of theophylline, followed by pulsed elution (PE) of the bound theophylline with several 20 μl aliquots of a protic polar solvent for direct UV detection [38]. In comparison with conventional HPLC and immunoassay methods of theophylline determination in serum, this novel molecularly imprinted solid phase extraction–pulsed elution (MISPE-PE) technique offered a very competitive detection limit, greatly reduced analysis time when compared to HPLC [39], no antibody preparation, and a lower cost for each sample analysis. It also allowed for analyte preconcentration through injection of a relatively large volume of dilute sample solution, thereby improving the detection limit. More recently, the MISPE-PE technique was further developed for faster determination of theophylline. Since the theophylline MIP material had a high capacity for theophylline binding, a micro-column could be employed for MISPE of theophylline [40]. The small amount of MIP in the micro-column permitted more effective desorption of the bound theophylline by a single PE. Also, the narrow column diameter reduced peak broadening due to radial diffusion of the injected sample, resulting in greater analytical sensitivity.

Non-specifically bound interferences could easily be removed from the micro-column using a single 20 μl wash with an aprotic polar solvent, followed by a single PE to desorb the bound theophylline. This differential pulsed elution (DPE) technique alleviated the sample cleanup requirement for accurate theophylline determination. In the present work, micro-column heating and flow rate optimization are combined with DPE to demonstrate the most effective binding of theophylline and complete removal of non-specifically bound interferences. This new technique is applied for the rapid determination of theophylline in human blood serum.

2. Materials and methods

2.1. Chemicals

Theophylline and caffeine were purchased from Sigma (St. Louis, MO). Acetaminophen, and hydrochlorothiazide were supplied by Health Canada (Ottawa, ON). All solvents (chloroform, acetonitrile and methanol) were HPLC grade from Caledon (Georgetown, ON).

2.2. Preparation of columns

The theophylline MIP was prepared using methacrylic acid (MAA) as the functional monomer, ethyleneglycol dimethyacrylate as the cross-linker, theophylline as the print molecule and AIBN as the initiator according to a method previously reported by Mosbach and co-workers [41]. The product polymer was ground into fine particles using a mortar and pestle. Removal of the theophylline print molecule from the MIP particles was accomplished through a Soxhlet extraction with methanol–acetic acid (9:1 v/v). The MIP particles were sieved, and the ≤ 63 μm size fraction was used for column packing. A small volume of methanol was added to the MIP particles, and the slurry was dispensed into a 1/16″ stainless steel tubing (1.0 mm i.d., 80 mm long). The fully-packed column was capped at both ends by a 1/16″ zero-volume union fitted with a 2 μm screen (Chromatographic Specialties, Brockville, ON). Hence, the size distribution of MIP particles used in the column was from 2 to 63 μm.

2.3. MISPE-PE and -DPE

MISPE of theophylline was performed on the MIP micro-column using chloroform as the solvent. The micro-column was either sitting at room temperature or submerged in a Haake Instruments variable-temperature circulating water bath (Paramus, NJ) set at 40 or 60°C. An Eldex 9600 solvent delivery system (San Carlos, CA) was operated at a flow rate of 0.05–1.00 ml/min. A Rheodyne 7125 switching valve (Cotati, CA) containing a 20 μl sample loop was used for sample injection, PE and DPE. The absorbance of the micro-column eluate was monitored with a Gilson 115 UV detector (Middleton, WI) set at 270 nm, and the output signal was recorded by a Dionex 4270 integrator (Sunnyvale, CA) for retention time and peak area measurements. Various standard solutions of theophylline, caffeine, acetaminophen and hydrochlorothiazide in chloroform were analyzed by MISPE-PE. After 1–2 min of waiting time was allowed for the detection of any break-through peak, a 20 μl pulse of methanol was injected through the Rheodyne valve to cause PE of any bound theophylline or non-specifically bound drug compounds. For DPE, after detection of the break-through peak, up to three 20 μl pulses of acetonitrile were used to wash off any non-specifically bound drug remaining on the micro-column. A 20 μl pulse of methanol was next injected to determine if the acetonitrile pulses had removed the drug quantitatively.

2.4. Serum analysis

A 1 ml sample of human serum was extracted with an equal volume of chloroform, vortexed for 10 s and centrifuged at 4000 rpm for 5 min. The chloroform layer was removed and spiked with theophylline (in chloroform) to provide a series of working standard solutions over the concentration range 0–20 μg/ml. A calibration curve was constructed by triplicate 20 μl injections of these standard solutions onto the micro-column for analysis by MISPE-DPE at 60°C.

2.4.1. Safety considerations

Human serum samples are a potential biohazard. Unused serum samples should be treated with Javex before disposal as hazardous waste.

3. Results and discussions

3.1. Interference removal

The effect of solvent polarity, as discussed elsewhere [40], represented a convenient parameter for controlling the binding of theophylline versus drug interferences on the micro-column using chloroform as the mobile phase. By adjusting the polarity of the PE solvent, any non-specific binding of drugs could be selectively desorbed by an intermediate wash with 20 μl pulses of a polar solvent, such as acetonitrile. This differential pulse technique (DPE) technique was tested on several drugs that covered a wide range of pK_a values and concentrations. It was understood that a higher pK_a value for a drug compound would provide a stronger interaction between its basic nitrogen(s) and the carboxylic acid moieties of the MIP binding sites [40]. As a first example, a 100 μg/ml standard solution of hydrochlorothiazide (p$K_a = 4.35$) was injected onto the MIP micro-column. Rapid elution of a break-through peak was first detected. Three 20 μl pulses of acetonitrile were next injected successively to wash off any hydrochlorothiazide remaining on the micro-column due to non-specific adsorption. To confirm if the wash removed the hydrochlorothiazide quantitatively, three 20 μl pulses of methanol were injected. The overall results indicated that acetonitrile was successful in removing 95% of the hydrochlorothiazide, while the other 5% was eventually eluted by methanol. As a second example, a 50 μg/ml standard solution of acetaminophen (p$K_a = 16.07$) was injected onto the micro-column. Three 20 μl pulses of acetonitrile were able to successfully desorb the non-specifically bound acetaminophen. To confirm if the acetonitrile pulses desorbed the acetaminophen quantitatively, three 20 μl pulses of methanol were then carried out. At room temperature, the overall results indicated that acetonitrile was successful in desorbing 98% of the acetaminophen, while the remaining 2% was eventually desorbed by methanol. For comparison purposes a 100 μg/ml sample of theophylline was tested by DPE, and three acetonitrile pulses desorbed only 43% of the bound theophylline. This percentage might be interpreted as all those theophylline molecules that were non-specifically bound to the MIP surface sites. Non-specific binding was likely occurring at the surface sites of the MIP, where incomplete polymerization around the print molecules had formed partial theophylline-selective cavities. The DPE technique allowed the determination of theophylline after interference removal, even though the MISPE-DPE sensitivity was sacrificed by a significant loss of theophylline in the acetonitrile pulses. Consequently, the detection limit for theophylline was degraded from 1.0 to 2.3 μg/ml for a chloroform flow rate of 0.5 ml/min and a micro-column temperature of 20°C. This detection limit fortunately would still satisfy the therapeutic range of 10–20 μg/ml for theophylline.

3.2. Flow rate dependence at 20°C

The selective binding of theophylline to the micro-column had to be further optimized in order to circumvent a decreased sensitivity of the MISPE-DPE technique. For enhanced theophylline selectivity in the MISPE binding process, accessibility to the binding sites inside the porous MIP particles by molecular diffusion was essential. This problem of accessibility was exacerbated by the small diameter of the micro-column which generated a high solvent flow velocity, thereby kinetically limiting the access of theophylline molecules to the inner selective cavities [42,43]. Consequently, the kinetic selectivity of the MIP suffered when compared to its thermodynamic selectivity under batch binding conditions, as recently reported for the slow kinetics of equilibrium sorption in packed-bed experiments [44]. Since the solvent flow velocity dictated the time available for diffusion, analyte selectivity of MISPE was evaluated by varying the solvent flow rate over a range of 0.05–1.00 ml/min. Standard solutions of 50 μg/ml theophylline and acetaminophen (prepared in chloroform) were sepa-

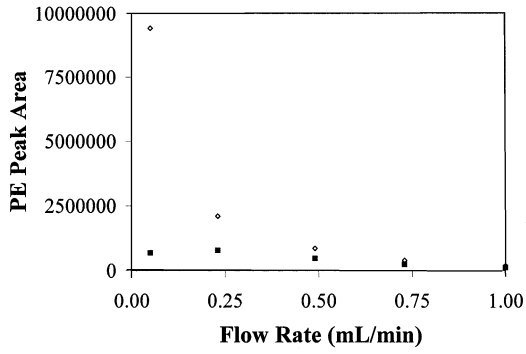

Fig. 1. PE peak areas for theophylline (◇) and acetaminophen (□) at various flow rates. Column temperature = 20°C; mobile phase = 100% chloroform; sample volume = 20 μl; methanol pulse volume = 20 μl; detection $\lambda = 270$ nm.

rately injected onto the micro-column, followed by desorption of the bound analyte by PE with methanol. This PE peak area was used to determine the amount of theophylline or acetaminophen bound to the MIP at each flow rate, as illustrated in Fig. 1. Theophylline displayed a trend of increased binding efficiency with decreasing flow rate, which provided more time for the diffusion of theophylline molecules to the strong and selective binding sites inside the MIP particles. This confirmed the critical role of diffusion in the binding process. Such molecular recognition was not shared by acetaminophen, as evidenced in its fairly constant amount bound over the evaluated flow rates. A ratio of PE peak areas, theophylline to acetaminophen, now provides in Table 1 a theophylline binding selectivity factor for the micro-column at each flow rate after the UV molar absorptivities of theophylline and acetaminophen at 270 nm are factored in. It highlights the trend of increasing theophylline binding selectivity factor with decreased flow rates, thereby implicating the predominance of molecular recognition. On the contrary, non-specific binding was stronger for acetaminophen than theophylline as indicated by binding selectivity factor values of less than 1.00 at the high flow rates. This was merely the consequence of a higher pK_a value for acetaminophen than theophylline, which provided a stronger interaction between the basic nitrogen of acetaminophen and the acidic carboxylic moieties in the MIP matrix. Although a reduction in mobile phase flow rate could alleviate the interference problem, it would obviously compromise the analysis time. Even at the lowest flow rate of 0.05 ml/min, which was most favorable to the selective binding of theophylline, non-specific binding of acetaminophen and other interferences still occurred and their elimination was essential before theophylline could be determined accurately.

Table 1
Theophylline binding selectivity factor for the micro-column at different flow rates

Flow rate (ml/min)	Binding selectivity factor[a]
1.00	0.490
0.73	0.516
0.49	0.569
0.23	0.832
0.05	4.235

[a] Calculated from the ratio of PE peak areas, theophylline to acetaminophen.

3.3. Temperature dependence

An alternative solution would be temperature elevation by submerging the micro-column into a thermostatic water bath. Temperature was an optimization parameter that had often been overlooked in SPE studies, despite its well-known advantages of increased sample solubility, improved column efficiency and reduced column pressure drop [45]. The theophylline MIP had good thermal stability that permitted the use of elevated column temperatures up to 60°C for better theophylline accessibility to the binding sites inside the porous MIP by molecular diffusion. As mentioned above and suggested in a previous report [46], solvent polarity was critical in the MISPE selectivity. The highest selectivity was obtained with solvents of low polarity, where electrostatic forces dominated the binding process. Over the temperature range of 20–60°C, the dielectric constant of chloroform was confirmed by refractive index measurements to decrease with increasing temperature. Hence the solvent polarity was decreased in the chloroform mobile phase for

Table 2
Percentage of injected drug interferences desorbed from theophylline MIP micro-column at 60°C by one 20 μl pulse of acetonitrile

Analyte	Concentration (μg/ml)	% Removed by acetonitrile
Acetaminophen	50	100
Dyphylline	50	100
Phosphatidyl-choline	10 000	100
Theobromine	50	100
Theophylline	50	10

MISPE, providing a reduced elution power and greatly improved molecular recognition of theophylline.

DPE experiments were next performed on the micro-column heated to 40 and 60°C. Better results were accomplished at 60°C, where all tested interferences were completely removed as summarized in Table 2. In contrast, removal of theophylline from the micro-column *decreased* to a low value of 10%. The elevated temperature increased the diffusion rate of theophylline (and interferences) and hence its mass transfer to the strong and selective binding sites inside the porous MIP particles. Due to the high selectivity of these inner binding sites, however, only theophylline were strongly bound. Interferences would be engaged only in non-specific binding, which was easily overcome by the subsequent wash with a 20 μl pulse of acetonitrile. The improved molecular recognition at 60°C was also essential to the success of quantitative removal of interferences with 90% retention of theophylline in the present DPE technique. It attained maximal detection sensitivity, while maintaining the high flow rate of 0.5 ml/min and the short analysis time of 3 min. To the best of the authors' knowledge, this is the first report that has utilized temperature to enhance MIP selectivity based on mass transfer and solvent polarity effects.

3.4. Flow rate dependence at 60°C

When a temperature of 60°C was used for MISPE-PE at various chloroform flow rates, much reduced efficacy of PE was observed at 0.05 ml/min. One plausible explanation is that such a low flow rate allowed plenty of time for the methanol to approach 60°C, which decreased the polarity of methanol and hence its elution strength. At 0.25 and 0.50 ml/min, the efficacy of PE was re-established probably due to insufficient time for the methanol to reach 60°C. The combined effect of theophylline accessibility and PE efficacy is illustrated in Fig. 2, where blank-subtracted PE peak areas are plotted against theophylline concentrations for the two flow rates at 60°C. As Table 3 indicates, while a linear response was generated in both cases, a greater analytical sensitivity was attained by 0.25 ml/min. This flow rate also yielded a better detection limit of 0.7 μg/ml, as compared with 1.1 μg/ml for 0.50 ml/min. However, both detection limits would satisfy the therapeutic drug monitoring (TDM) range of 10–20 μg/ml for theophylline [47,48].

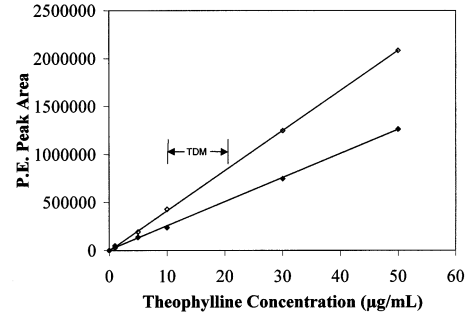

Fig. 2. MISPE-PE standard calibration curve for theophylline, using mobile phase flow rates of 0.25 (◇) and 0.50 (◆) ml/min. Column temperature = 60°C; mobile phase = 100% chloroform; sample volume = 20 μl; methanol pulse volume = 20 μl; detection $\lambda = 270$ nm.

Table 3
MISPE-PE standard curve statistics for two flow rates

Flow rate (ml/min)	Slope	Correlation coefficient
0.25	41 800	0.9993
0.50	25 098	0.9999

3.5. Serum analysis by MISPE-DPE

In the traditional use of HPLC and GC methods for therapeutic drug analysis in such human biological fluids as blood serum, extensive sample cleanup is necessary to remove interferences and extended chromatographic time is required. Conventional SPE for sample cleanup can involve much effort and time, as the sorbent must be pre-conditioned, followed by analyte elution with a large volume of solvent (>0.5 ml) under a vacuum manifold. In addition, there are several chemical limitations (including pH sensitivity in the case of silica-based sorbents) and the awkward requirement of a wetted sorbent that must not dry out.

Method validation of the MISPE-DPE technique for the rapid determination of theophylline in blood serum was achieved by utilizing a micro-column temperature of 60 ± 1°C and a mobile phase flow rate of 0.5 ml/min. The use of a simple isocratic mobile phase (100% chloroform, non-buffered) and pulsed elution solvents (acetonitrile and methanol) provides robustness to the technique, as it eliminates the requirement for the preparation of more complex gradient mobile phases that are commonly used in the HPLC analysis of theophylline [15,49–51]. A further merit is that the reproducibility of packing MIP columns for MISPE-DPE analysis is not critical because the analyte peak is always identified by its late appearance due to the selective molecular recognition, not by retention time as in HPLC methods. The method is robust when there is no need to adjust experimental parameters to compensate for changes in retention time even if column performance (such as flow rate) changes over time. A main limitation of the MISPE-DPE procedure is that only biological samples dissolved in chloroform can be analyzed. This may seem to involve a rather laborious and time-consuming liquid–liquid extraction step. However, the method is indeed faster when compared with a typical SPE-HPLC procedure for drug determination in plasma, since peculiar substances in complex samples may require extensive HPLC elution times. Blood serum samples were first extracted with chloroform, which served to isolate

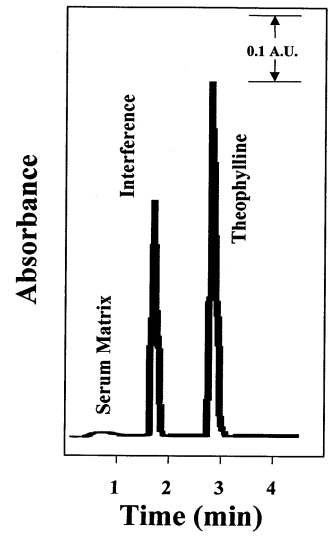

Fig. 3. MISPE-DPE of spiked chloroform extract from serum containing 10.0 μg/ml of theophylline using a theophylline MIP micro-column. Column temperature = 60°C; mobile phase = 100% chloroform; flow rate = 0.5 ml/min; sample volume = 20 μl; acetonitrile pulse volume = 20 μl; methanol pulse volume = 20 μl; detection $\lambda = 270$ nm.

theophylline while simultaneously removing interferences such as proteins in the serum. Since theophylline is weakly bound to serum proteins [52] such as albumin [53], and the presence of an organic solvent like chloroform has been shown to displace protein-bound drugs in plasma [54], it is expected that the total theophylline concentration is determined with this MISPE-DPE technique. After the chloroform extract was injected onto the micro-column, some interferences would pass through rapidly in approximately 1 min. DPE of non-specifically bound interferences was next performed, ending with quantitative desorption of theophylline.

A MISPE-DPE standard calibration curve for theophylline serum analysis at 60°C was constructed, with excellent linearity ($R^2 = 0.997$) in the concentration range up to 20 μg/ml and a detection limit of 1 μg/ml. Note that this detection limit was better than the 2.5 μg/ml obtained at room temperature, and that the highly selective determination of theophylline was accomplished in less than 3 min as shown in Fig. 3. Instrument

precision was studied by multiple analysis (more than ten injections) of the serum sample, to exhibit a RSD of $< 3\%$. Method repeatability was evaluated by independent preparation and analysis of different serum samples, to produce an intra-assay precision of $< 5\%$. Two analysts in our laboratory confirmed the ruggedness of the technique over a span of 30 weeks, to yield an intermediate precision of $< 6\%$. Effectively the selective MISPE-DPE technique with UV detection has accomplished all of the sample cleanup, interference removal and analyte determination for serum analysis, with excellent column reusability [38].

4. Conclusions

A deeper understanding has been gained about the fundamental molecular recognition process that is responsible for the selective binding of theophylline molecules with the MIP particles. The developed MISPE-DPE method applies 20 μl acetonitrile and methanol pulses to achieve complete removal of chemical interferences from a heated micro-column 60°C for the selective, direct UV determination of theophylline. Application of this technique has fully demonstrated the high capability of the MIP micro-column to selectively isolate theophylline from other serum matrix components for fast, accurate determination over a linear dynamic range of 2–20 μg/ml theophylline. The whole procedure has also been properly validated in terms of linearity, repeatability, reproducibility, selectivity, robustness, ruggedness and interferences. Fast analysis time, simple interference removal, high analyte recovery, low detection limit, minimal consumption of solvents (1.5 ml per analysis) and excellent column reusability (even after drying out) all made this new MISPE-DPE technique particularly attractive in comparison to established methods of serum theophylline determination. The MISPE-DPE technique can potentially be extended to a wide variety of MIPs for simple, rapid and selective drug screenings in biomedical research, such as nicotine in tobacco extract [55], gum and patches.

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