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Journal of Chromatography B, 742 (2000) 247–254

JOURNAL OF CHROMATOGRAPHY B

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Determination of moxifloxacin (BAY 12-8039) in plasma and lung tissue by high-performance liquid chromatography with ultraviolet detection using a fully automated extraction method with a new polymeric cartridge

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Received 5 November 1999; received in revised form 13 January 2000; accepted 1 March 2000

Abstract

The aim of this study was to develop a high-performance liquid chromatographic (HPLC) assay for the determination of moxifloxacin in human plasma and lung tissue. The assay was based on HPLC with a Supelcosil ABZ+ column and ultraviolet detection set at a wavelength of 296 nm. The extraction procedure was characterized by a fully automated liquid–solid extraction using an OASIS column for the solid phase. The assay has been found to be linear and validated over the concentration range 3.2 to 0.025 μg/ml for moxifloxacin in plasma and from 16 to 0.25 μg/g for moxifloxacin in lung tissue. In future, the assay will support the pharmacokinetic study of the penetration of moxifloxacin in human lung tissue.
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Keywords: Moxifloxacin

1. Introduction

Moxifloxacin {1-cyclopropyl-7-(2,8-diazobicyclo-[4.3.0]nonane)-6-fluoro-8-methoxy-1,4-dihydro-4-oxo-3-quinolone carboxylic acid} is a new methoxyquinolone that combines enhanced in vitro activity against Gram-positive bacteria with maintenance of activity against Gram-negative organisms [1–4]. It has activity against all community-acquired respiratory pathogens, including those commonly recognised such as *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* and the less common (*S. aureus* and *K. pneumoniae*) as well as atypical respiratory pathogens (*Mycoplasma*, *Chlamydia* and *Legionella*) [5–7]. These broad-range activity pathogens, based on in vitro data on minimum inhibitory concentration (MIC), time-kill kinetics on post-antibiotic effect, suggest that moxifloxacin may be a suitable candidate for the treatment of range respiratory tractus infections (RTIs), including pneumonia. Based on recent guidelines the reference therapy is injectable a

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PII: S0378-4347(00)00166-3

β-lactam antibiotic, such as amoxicillin, cephalosporin or a macrolide [8,9]. However, the emergence of penicillin-resistant strains of *S. pneumonia*, 30% of cases, presents a therapeutic challenge. Traditionally the plasma concentrations for an antibiotic and its relationship to the MIC (MIC_{90} for likely pathogens), have been used to predict its likely efficacy for a variety of infections. This approach is justifiable in the case of bacteraemia, but is less attractive where the infection is predominantly within an extravascular site such as lung tissue. The rationale for measuring the concentrations of antimicrobial agents at potential sites of infections other than serum is that pathogens may be confined to sites which are separated from the blood by significant barriers to antimicrobial agent movement. Lung tissue comprises several potential sites of pulmonary infection including those involved in airway infections such as sputum and bronchial mucosa, and those in bronchioalveolar infections such as the epithelium lining fluid and alveolar macrophage. In addition whole lung tissues contain lymphatics and elements of both the bronchial and pulmonary circulation. The lung is usually homogenized, resulting in a antibiotic concentration which is an average of that present in the various compartments in this tissue, including all of the sites listed above. In order to assess human lung pharmacokinetics of moxifloxacin, a sensitive and specific assay was required to quantify moxifloxacin concentration both in plasma and in lung tissue. Microbiological assays, capillary electrophoresis with laser-induced fluorescence and high-performance liquid chromatography (HPLC) with fluorescence detection were described in the literature for moxifloxacin measurements [10,11]. The aim of this study is to propose a new assay to quantify moxifloxacin in human lung. This assay is based on HPLC with UV detection using an extraction method with a new polymeric cartridge (OASIS). For ethical considerations we chose to validate this new HPLC assay on porcine biological lung matrix. However for different moxifloxacin concentrations, we developed quality controls on human lung matrices to make correlations between the two lung matrices.

This new method will be applied to determine plasma and lung concentrations of moxifloxacin, study human lung pharmacokinetic profiles and evaluate the ratio of concentrations (lung/plasma).

2. Experimental

2.1. Chemicals

Moxifloxacin and enrofloxacin, the internal standard, shown in Fig. 1 were obtained from Bayer (Puteaux, France). Potassium dihydrogenphosphate, concentrated trifluoroacetic acid and concentrated orthophosphoric acid, all Normapur quality, and methanol were from Prolabo (Nogent sur Marne, France). PBS (phosphate-buffered saline) DULBECCO'S came from Gibco BRL (Cergy Pontoise, France). Acetonitrile, Chromar HPLC quality, was purchased from Carlo-Erba (Val de Reuil, France). Drug-free plasma was purchased from Gibco BRL.

2.2. Solid-phase extraction (SPE) and HPLC instrumentation

An automated SPE method on 30 mg (1 ml) OASIS HLB extraction cartridges (Waters, MA, USA) was performed using an ASPEC Xli system (Gilson Medical Electronics France, Villiers le Bel, France) equipped with a Rheodyne valve with a 50-μl loop. The Kontron HPLC system (Milan, Italy) consisted of a Model 525 pump, a Model 360 autosampler and a Model 332 UV detector and data acquisition was performed on a D450 software for signal treatment. The crushing phase was effected by an Ultra Terrax from Bio Block (Strasbourg, France),

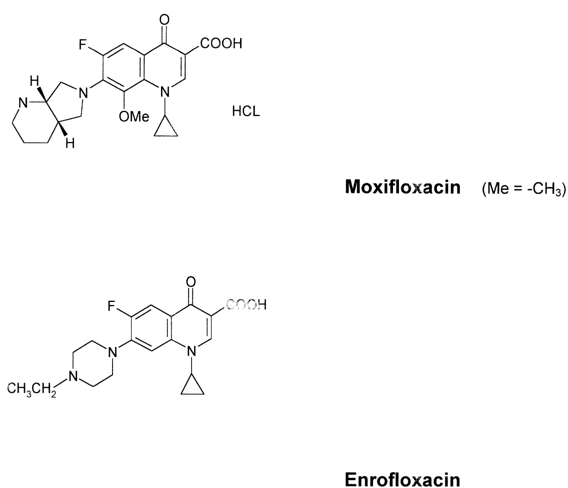


Fig. 1. Molecular structures of moxifloxacin and enrofloxacin.

and the centrifugation by a GR422 from Jouan (Paris, France).

2.3. Sample extraction procedure

An automatic sample processor was used for the extraction (ASPEC Xli; Gilson). During the first step, the automate mixed 1200 μl of sample with 300 μl of internal standard solution (10 μg/ml enrofloxacin in water). Conditioning of the OASIS cartridge (1 ml of methanol, 1 ml of water) was followed by loading with 1.25 ml of sample prepared previously. The extractive columns were washed with 1 ml water. The cartridges were dried by air and the compounds were eluted with 1 ml of methanol–trifluoroacetic acid (99.9:0.1, v/v). The solvent was evaporated at 50°C under a stream of nitrogen. The residue was reconstituted in 0.5 ml of the mobile phase and 50 μl of this solution was injected into the chromatographic system.

2.4. Chromatography

The mobile phase consisted of a mixture of acetonitrile–10 m*M* potassium dihydrogenphosphate buffer (18:82, v/v). The pH was adjusted to 4 with concentrated orthophosphoric acid and the mobile phase was filtered through a 0.45-μm filter from Millipore (Saint Quentin en Yveline, France). The elution conditions were isocratic, and the mobile phase flow-rate was set at 1.25 ml/min. The analytical column was a Supelcosil ABZ+ (5 μm, 150×4.6 mm) from Supelco (Saint Quentin Fallavier, France). The sample injection volume was 50 μl. UV absorbance detection at 296 nm was carried out and the range of the detector was set at 0.05 AUFS (absorbance unit full scale) for plasma and 0.02 AUFS for lung samples. The chromatographic run time was 13 min.

2.5. Preparation of standards and quality controls

2.5.1. Plasma calibration and quality controls

Stock solutions of moxifloxacin and enrofloxacin were prepared by dissolving the appropriate amount of moxifloxacin and enrofloxacin, accurately weighted, in distilled water to yield a final drug concentration of 3200 μg/ml for moxifloxacin and 10 μg/ml for enrofloxacin. Working stock solutions of 320, 240, 160, 80, 40, 20, 10, 5, 2.5 μg/ml were prepared by appropriate aqueous dilution of the 3200 μg/ml standard solution.Then, each working stock solutions were diluted 100-fold into free plasma to obtain a concentration range from 3.2 to 0.025 μg/ml for moxifloxacin. Aliquots of those solutions were stored at −20°C. This range was based on human moxifloxacin concentrations found in previous pharmacokinetic studies, when the drug was administered at 400 mg once a day [12]. Quality control concentrations representing 0.03, 0.7, 2.5 μg/ml of moxifloxacin in plasma were prepared by specific dilution of the 320 μg/ml stock solution in drug-free plasma.

2.5.2. Lung calibration and quality controls

Stock solutions of moxifloxacin and enrofloxacin are the same for plasma and lung samples. Working stock solutions of 160, 120, 80, 40, 20, 10, 5, 2.5 μg/ml were prepared by appropriate dilution in PBS. A 250-mg amount of lung tissue was accurately weighted and spiked with 25 μl of each working stock solution. To each tube, we added 2.475 ml of PBS before tissue homogenization. The resulting standards ranged in concentration from 16 to 0.25 μg/g. Quality control concentrations representing 0.4, 6, 15 μg/g of moxifloxacin were prepared by the same procedure as the calibration sample. Then the samples were crushed and centrifuged at 3080 *g* during 15 min.

2.6. Sample treatments

2.6.1. Plasma samples

Blood samples were collected in heparinized tubes and centrifuged (10 min, 1850 *g*, 20°C) as soon as possible before storing at −80°C.

2.6.2. Lung tissue samples

Prior treatment of lung samples reduced interference in the assay due to external blood contamination. This treatment involved wiping off external traces of blood. No washing was performed in order to avoid transferring the antibiotic to the washing liquid. After this first treatment, a fragment of lung parenchyma of approximately 250 mg was removed and weighed precisely. It was then ground in 2.5 ml

of PBS. The tissue suspension obtained was homogenized, stirred gently to allow diffusion of the moxifloxacin, and crushed. Finally the sample was centrifuged at 3080 *g* during 15 min.

2.7. Calibration and calculation procedures

Daily calibration curves were constructed using the ratios of the observed peak areas of moxifloxacin and the internal standard. Unknown concentrations were computed from the unweighted linear regression equation of the peak area ratio against concentration for the calibration curve.

2.8. Accuracy, precision and recovery

The intra-day accuracy and precision of the method were estimated from the back-calculated standard concentrations. The overall mean precision was defined by the relative standard deviation (RSD) with relative errors from six standards analyzed on the same day. Inter-day variability was estimated from the sixplicate analysis of three samples on six separate days.

2.9. Specificity and selectivity

Specificity was assessed in the presence of clavulanic acid, tazobactam, sulbactam, amoxicillin, ampicillin, cefixime, cefotaxime, cloxacillin, imipenem, cefalotin, mezlocillin, latamoxef, penicillin G, piperacillin, ticarcillin, fusidic acid, ciprofloxacin, ofloxacin, pefloxacin, fosfomycin, vancomycin, erythromycin, rifampicin, amikacin, tobramycin, netilmicin, gentamicin, itraconazole, amphothericin B.

2.10. Limit of detection and limit of quantitation

The limit of detection (LOD) in plasma was defined by the concentration with a signal-to-noise ratio of 3. The limit of quantitation (LOQ) is the minimum injected amount that gives precise measurements (accuracy and RSD both less than 15%). If the required precision of the method at the LOQ has been specified, the Eurachem approach can be used [13]. A number of samples with decreasing amounts of the analytes are injected six times. The calculated RSD of precision is plotted against the analyte amount. The amount that corresponds to the previously defined required precision (15%) is equal to the LOQ. The minimum quantifiable concentration (MQC) measuring the lowest concentration at which there is satisfactory a priori measures of accuracy and precision using the appropriate biological matrix was calculated.

3. Results

3.1. Chromatographic characteristics

Fig. 2 shows chromatograms of an extracted blank sample, an extracted plasma sample containing 2.5 μg/ml of moxifloxacin, an extracted lung sample containing 15 μg/g of moxifloxacin and an extracted human lung containing 15 and 0.4 μg/g of moxifloxacin. The mean retention time of moxifloxacin was 6.09 min.

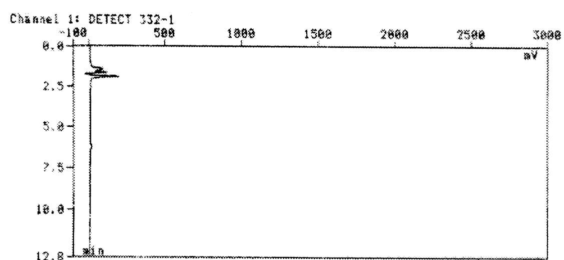
3.2. Calibration curve

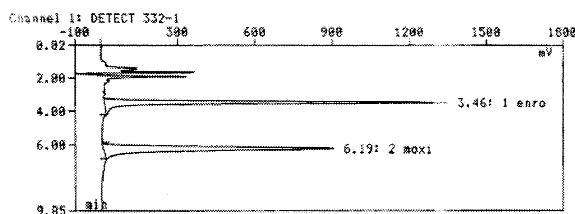
The analysis of moxifloxacin in plasma and lung tissue exhibited excellent linearity through the coefficient of correlation r^2: $r^2=0.9999$ and $r^2=0.9985$, respectively, for plasma and lung tissue samples. Regression intercepts for the calibration curves were generally very small and were not statistically significant compared to zero. These daily calibration curves were used for calibration and calculation purposes. The results indicated that the assay of moxifloxacin had acceptable precision ($<10\%$ for plasma and $<15\%$ for lung tissue), and accuracy (relative error $<5\%$ for plasma and $<10\%$ for lung tissue); lower LOQ was 0.03 μg/ml and 0.4 μg/g, respectively, for plasma and lung tissue.

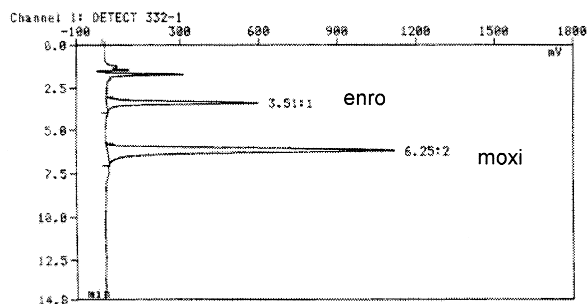
3.3. Accuracy, precision and recovery

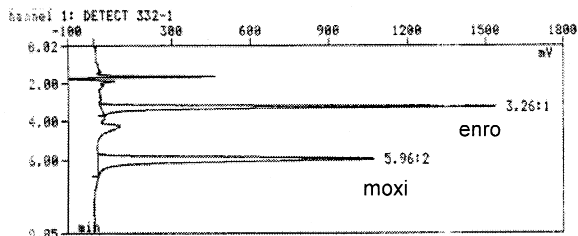
The intra-day accuracy and precision of the method were estimated from the back-calculated standards. The overall mean precision as defined by the RSD, ranged from 1.51 to 6.78% and from 1.82 to 8.77%, respectively, for plasma and lung tissue from six series standards analyzed within the same day (Table 1).

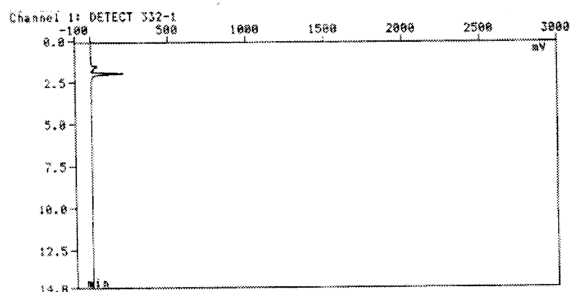
Inter-day variability, as estimated from the sixpli-











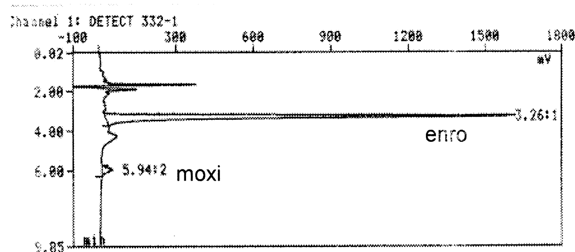


Fig. 2. Chromatograms of blank human plasma sample (a), human plasma sample spiked with 2.5 μg/ml of moxifloxacin and 10 μg/ml of enrofloxacin (b), blank of porcine lung tissue sample (c), porcine lung tissue sample spiked with 15 μg/g of moxifloxaxin and 10 μg/g of enrofloxacine and two human lung tissue samples spiked, respectively, with 15 μg/g (e), and 0.4 μg/g (f) of moxifloxacin.

cate analysis of three samples on six separate days (Table 2), was low, with RSDs ranging from 1.12 to 9.11% and from 3.44 to 14.23%, respectively, for plasma and lung tissue samples and with relative error ranging from 0.19 to 1.80% and from 3.30 to 5.96%, respectively, for plasma and lung tissue samples. The extraction mean recovery of moxifloxacin from quality control samples are 97.40% for plasma and 94.57% for lung tissue (Table 3).

3.4. Specificity and selectivity

Blank plasma showed no interfering endogenous substances in the analysis of moxifloxacin. Poten-

Table 1
Intra-day accuracy and precision from the determination of moxifloxacin in plasma and lung samples

Theoretical concentration (μg/ml)	Concentration found (mean±SD) (μg/ml)	Accuracy (%)	RSD (%)	Relative Error (%)	*n*
Plasma					
2.5	2.486±0.038	99.45	1.51	0.55	6
0.7	0.704±0.014	100.55	1.95	0.55	6
0.03	0.030±0.002	98.93	6.78	1.066	6
Lung[a]					
15	14.776±0.272	98.50	1.82	1.49	6
6	5.835±0.107	97.25	1.83	2.75	6
0.4	0.376±0.033	94.00	8.77	6.00	6

[a] Theoretical concentration and concentration found in μg/g for lung samples.

Table 2
Inter-day accuracy and precision from the determination of moxifloxacin in plasma and lung samples

Theoretical concentration (μg/ml)	Concentration found (mean±SD) (μg/ml)	Accuracy (%)	RSD (%)	Relative Error (%)	*n*
Plasma					
2.5	2.506±0.028	100.22	1.12	0.22	36
0.7	0.687±0.011	98.19	1.57	1.80	36
0.03	0.030±0.003	99.81	9.11	0.19	36
Lung[a]					
15	15.504±0.499	96.69	3.44	3.30	36
6	5.642±0.291	94.03	5.16	5.96	36
0.4	0.384±0.054	96.00	14.23	4.00	36

[a] Theoretical concentration and concentration found in μg/g for lung samples.

tially coadministered drugs tested had retention times that were different from moxifloxacin or were not detected.

Table 3
Plasma and lung sample recovery

Theoretical concentration	Mean recovery (%)
Plasma (μg/ml)	
2.5	97.4
0.7	100.6
0.03	94.2
Lung (μg/g)	
15	95.4
6	90.0
0.4	98.4

3.5. Limit of detection, limit of quantification and minimal quantifiable concentration.

The LODs were 0.00652 μg/ml and 0.05 μg/g, respectively, for plasma and lung tissue.

At 0.03 μg/ml for plasma and 0.4 μg/g for lung tissue, the percent deviation from the respective nominal concentrations and the RSD were both less than 15%. Thus, 0.03 μg/ml for plasma and 0.4 μg/g for lung tissue were defined as the LOQs. The minimal quantifiable concentrations were 0.03 μg/ml for plasma and 0.4 μg/g for lung tissue.

3.6. Application in human lung matrix

This presented assay has been validated in porcine lung matrix. In order to determine if there is a matrix

Table 4
Comparison between porcine and human lung moxifloxacin concentrations

Theoretical concentration (μg/g)	Porcine concentration found (mean±SD) (μg/g)	Human concentration found (mean±SD) (μg/g)	*n*
15	14.959±0.230	15.345±0.728	6
6	6.027±0.166	5.836±0.364	6
0.4	0.396±0.057	0.401±0.032	6

effect on lung moxifloxacin concentrations we decided to measure moxifloxacin lung concentrations from human lung samples spiked. We used the quality control concentrations (0.4, 6, 15 μg/g) to evaluate the correlation between porcine and human moxifloxacin lung concentrations. Results are shown in Table 4 and statistical analysis supported by a slope test demonstrated that porcine and human moxifloxacin concentrations were well correlated ($r^2=0.9931$) and that the slope of the linear regression between the two concentrations was not significantly different from 1 ($t=33.84$, $\nu=16$ df and $\alpha<0.1\%$). We concluded that there is no matrix effect and this assay will be used to assess the moxifloxacin pharmacokinetic profiles in human lung.

4. Discussion and conclusion

We developed a specific HPLC assay to determine moxifloxacin concentrations in both plasma and lung tissue. The chromatographic parameters were UV detection ($\lambda=296$ nm), LOD and LOQ were, respectively, 0.00652 μg/ml and 0.03 μg/ml for plasma samples, and 0.05 μg/g and 0.4 μg/g for lung tissue samples. The MCQ was estimated to be 0.03 μg/ml in plasma samples. Previous assays were described in the literature and concerned the measurement of moxifloxacin using fluorescence detection.

Stass and Dalhoff [10] described a reserved-phase HPLC method with fluorescence detection allowing the sensitive and specific quantification of moxifloxacin in biological fluids (blood, urine, saliva). Concentrations down to a LOQ of 0.0025 μg/ml can be quantified in plasma, saliva and urine. Precision and accuracy of the bioanalytical results were below 10% of theory in the whole working range of the method. The LOQ was adequate to enable a full description of the human pharmacokinetics phase I studies during clinical studies. The comparison between the two assays showed that the assays were similar for precision and accuracy, but our assay showed higher LOD, LOQ. These results were justifiable by the fact that the objectives of the assays were different. Stass and Dalhoff measured very low moxifloxacin concentrations in different biological fluid matrices to follow its total elimination during phase I clinical studies.

Our objective was to propose a sensitive, specific and robust assay to quantify moxifloxacin in tissue matrices (e.g., lung tissue) in a range of concentrations adapted to previous pharmacokinetic results. One important point to underline was the sample preparation presented in our assay. The quality of elution and chromatographic results depended on the quality of sample preparation. The sample preparation presented in Stass and Dalhoff's assay was a precipitation using acetonitrile and aqueous phosphoric acid. This preparation was a very simple procedure but there were some inconveniences. The major inconvenience was, that to quantify low concentrations the preparation needed large sample volumes. The important proportion of acetonitrile (60% in sample) gave rise to some alterations of chromatographic parameters, and showed band broadening, shifting retention times and interfering peaks. The technique of on-column focusing was interesting to minimize these influences [14,15]. We used a liquid–solid fully automated extraction procedure for the preparation of samples which permitted one to increase the selectivity of the assay. The major advantage was that the liquid–solid extraction was more ''chromatographically compatible'' and permitted one by evaporation to concentrate the sample as we wanted. We chose to concentrate the

samples two-fold but we could concentrate 10-fold and still measure lower LOD and LOQ. An another important thing to underline was the choice of solid phase for liquid–solid extraction and the chromatographic column. We used a polymeric cartridge for SPE. The OASIS HLB cartridge exhibited a large specific area (800 m^2/g) which provided a good recovery of drugs [16]. Another advantage of polymeric sorbents was that typically only one predominant mode of interaction occurred, that was, reversed-phase mechanism. Silica sorbents used for SPE had strong secondary silanol interactions which complicated the retention and the elution of analytes. For the chromatographic column, the use of the Supelcosil ABZ+ column provided all the benefits of silica-based reversed-phase HPLC columns; a polar group incorporated in the Supelcosil ABZ+ phase gave a high level of silanol deactivation and selectivity, markedly different from that of conventional or deactivated C_{18} reversed-phase columns [17]. Supelcosil ABZ+ columns provided good peak shape and efficiency for polar compounds such as fluoroquinolones.

In conclusion all the factors discussed suggest that the HPLC assay with UV detection presented here allows fast analysis (run time of 13 min) of moxifloxacin in human plasma and lung tissue. It provides data with pharmacokinetic analysis of the moxifloxacin penetration in human lung tissue.

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