Journal of Chromatography, 530 (1990) 397–406
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5377

Determination of aniracetam and its main metabolite, N-anisoyl-GABA, in human plasma by high-performance liquid chromatography

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(First received January 23rd, 1990; revised manuscript received April 21st, 1990)

ABSTRACT

Two different reversed-phase high-performance liquid chromatographic methods for the determination of aniracetam (I) and its metabolite N-anisoyl-GABA (II) in human plasma are described. The procedure for I involves direct injection of plasma samples spiked with the internal standard on a clean-up column followed by reversed-phase chromatography on a C_{18} column. The limit of quantification was 5 ng/ml, using a 200-μl specimen of plasma. The mean inter-assay precision of the method up to 800 ng/ml was 3%. The procedure for II involved liquid–liquid extraction of II and the internal standard from plasma with ethyl acetate, and reversed-phase chromatography on a C_{18} column. The limit of quantification was 50 ng/ml using a 0.5-ml plasma specimen. The mean inter-assay precision up to 50 μg/ml was 6%. The applicability and accuracy of the methods were demonstrated by the analysis of over 1000 plasma samples from two bioavailability studies in healthy volunteers.

INTRODUCTION

Aniracetam [Ro 13-5057, 1-(*p*-methoxybenzoyl)-2-pyrrolidinone, I, Fig. 1] is a

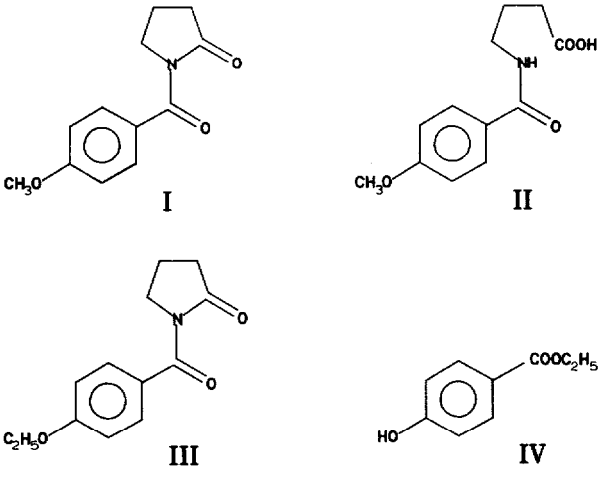

Fig. 1. Structures of aniracetam (I) and N-anisoyl-GABA (II), and the internal standards III and IV.

0378-4347/90/$03.50

new 2-pyrrolidinone derivative currently in the registration phase as a cognitive performance enhancer. Following oral administration of labelled I to rats, dogs and humans, the substance was very rapidly absorbed from the gastrointestinal tract, although the bioavailability of the parent drug was extremely low. I is extensively metabolized in the body according to two species-specific pathways [1]. In humans, I is mainly (*ca.* 70%) biotransformed into N-anisoyl-GABA (Ro 13-6680, II), and the pathway leading to anisic acid and 2-pyrrolidinone accounts for the remaining 30% of the dose. Owing to the rapid metabolic degradation of I its peak plasma concentrations are very low even following high oral doses, and decline rapidly. Therefore plasma concentrations of both I and II, its main active metabolite [2], have to be determined for pharmacokinetic studies in humans. The chemical properties of I and II hinder the possibility of using a single method for their determination; furthermore the existing methods for I (gas chromatography with selected-ion monitoring mass spectometry [3] and high-performance liquid chromatography with ultraviolet detection (HPLC–UV) [4]) and II (HPLC–UV [2]) require either instrumentation not available in all laboratories, or conditions that are time-consuming or not easily reproducible.

This report describes two different, reversed-phase HPLC methods, one for the determination of I in plasma by means of a column-switching technique and the other for the determination of II in plasma after liquid–liquid extraction.

EXPERIMENTAL

Reagents and solvents

Methanol (Rückstandsanalyse and LiChrosolv), ethyl acetate (Rückstandsanalyse), potassium dihydrogenphosphate (p.a.) and orthophosphoric acid (85%, p.a.) were obtained from E. Merck (Darmstadt, F.R.G.), acetonitrile (RS for HPLC) and triethylamine (RPE) from Carlo Erba (Milan, Italy), N,N-dimethylacetamide (puriss.) from Fluka (Buchs, Switzerland) and Aquasil from Pierce (Rockford, IL, U.S.A.). Water was delivered from a Milli-Q 4 system (Millipore, Milford, MA, U.S.A.), fed with deionized water. Aniracetam, anisoyl-GABA and the internal standards 1-(*p*-ethoxybenzoyl)-2-pyrrolidinone (Ro 13-8606, III) for the determination of I and ethyl *p*-hydroxybenzoate (IV) for the determination of II were all of pharmaceutical grade from F. Hoffmann-La Roche (Basle, Switzerland). All compounds were used as received.

Fresh-frozen plasma from CPAD human blood (AVIS, Milan, Italy) was used for the preparation of quality control (QC) and calibration plasma samples.

Internal standard solutions. A solution of III was prepared in water (concentration 5 μg/ml) from a stock solution obtained by dissolving 5 mg of III in 1 ml of N,N-dimethylacetamide and diluting to 10 ml with water. Both solutions were prepared weekly and stored at 4°C.

A solution of IV was prepared in methanol (concentration 40 μg/ml) by diluting the methanolic (1 mg/ml) stock solution of IV. Both solutions could be stored at 4°C for at least three months.

Plasma calibration and quality control samples

Plasma calibration samples (for I) and QC samples (for both I and II) were prepared by spiking blank plasma with 1% of the corresponding working solutions. They were divided into aliquots and stored deep-frozen (−20°C) until required for analysis. Plasma calibration samples for II were prepared daily by spiking 0.5-ml blank plasma specimens with 25 μl of the corresponding methanolic working solution. Working solutions of II could be stored at 4°C for at least three months.

To obtain optimum control of the assay, working solutions and QC samples were prepared by different analysts using different stock solutions.

Instrumentation

Determination of I. A schematic representation of the column-switching system is given in Fig. 2. A single-piston pump (Model 410, equipped with a Model 812 pulse-damper; both from Kontron, Zurich, Switzerland) was used as pump P1 delivering water as purge solvent at a flow-rate of 2 ml/min. Aliquots of 200 μl of plasma were injected by an automatic sample injector (W; Model WISP 712 equipped with a cooling unit that maintained the sample compartment at 4°C; Waters, Milford, MA, U.S.A.) onto the clean-up column (C1; 20 mm × 2.1 mm I.D.; Hewlett-Packard, Palo Alto, CA, U.S.A.), dry-packed with Hypersil ODS (particle size 30 μm; Shandon, Astmoor, U.K.). A UV detector (Model 4020; Pye-Unicam, Cambridge, U.K.) operating at 282 nm was used as D1. The pump P3 (Model T-414, equipped with a Model 812 pulse-damper; both from Kontron)

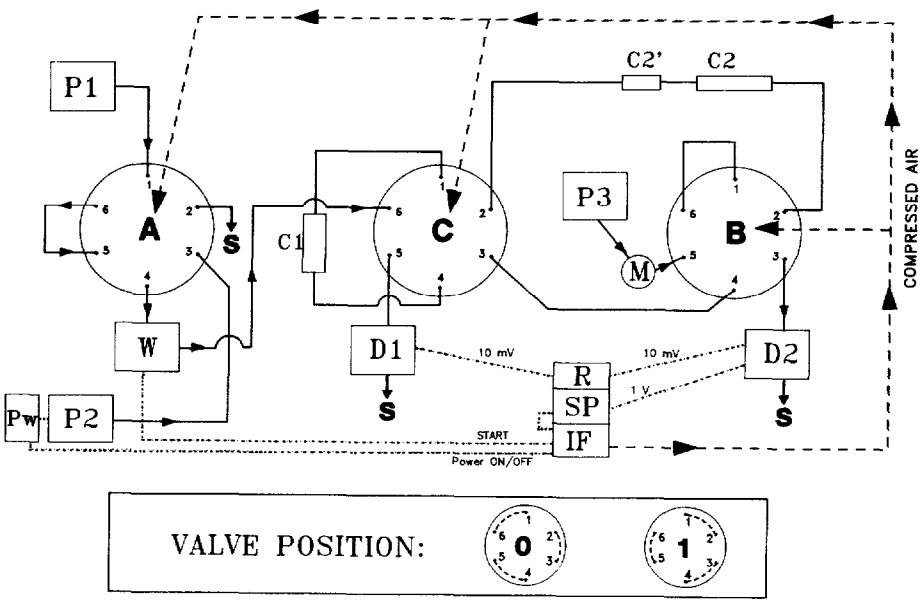

Fig. 2. Schematic representation of the column-switching system for the analysis of I in human plasma.

delivered the mobile phase [water–methanol–acetonitrile (70:30:10, v/v)] at a flow-rate of 1 ml/min to the analytical column (C2; Hypersil ODS, 60 mm × 4.0 mm I.D., particle size 3 μm; Shandon) protected by a guard column (C2′; RP-2, 30 mm × 4.6 mm I.D., particle size 10 μm; Brownlee Labs., Santa Clara, CA, U.S.A.). A manual injector (M; Model 7125; Rheodyne, Cotati, CA, U.S.A.) was used for injection of the control solutions directly onto the analytical column. A UV detector set at 282 nm (Spectromonitor 3100; Milton Roy, Riviera Beach, FL, U.S.A.) was used as D2. A third single-piston pump P2 (Model 410, Kontron), connected to a relay switch (Pw), delivered methanol at a flow-rate of 1 ml/min. The three air-actuated switching valves (A, B, C; Model 7000A; Rheodyne), assembled with three solenoid valves (Model 7163; Rheodyne), were controlled by the external time events of a computing integrator (SP; Model SP 4200; Spectra Physics, San José, CA, U.S.A.) by means of laboratory-made interface.

Acquisition, reduction and documentation of the data were performed by means of SP working with a modified version of a BASIC program developed for the Model SP 4100 computing integrator [5]; the integrator was connected to the 1-V unattenuated output of the detector D2, and a dual-pen strip-chart recorder (R; Model 8252; Pye-Unicam) was connected to the 10-mV attenuated outputs of D1 (0.04 a.u.f.s.) and D2 (0.001 a.u.f.s.).

The elution times were *ca.* 8.3 and 11.8 min for I and III, respectively, corresponding to retention times of *ca.* 4.3 and 7.8 min, respectively, on the analytical part of the system (C2′–C2). The analysis time was *ca.* 26 min.

Determination of II. The HPLC system consisted of a Model 420 two-piston pump (Kontron) delivering the mobile phase [methanol–triethylamine–0.1 *M* phosphate buffer pH 3.0 (530:0.05:470, v/v)] at a flow-rate of 1 ml/min, an automatic sample injector (WISP 710B, Waters), a guard column (RP-8 Spheri, 30 mm × 4.6 mm I.D., particle size 5 μm; Brownlee Labs.), an analytical column (μBondapak C_{18}, 300 mm × 3.9 mm I.D., particle size 10 μm; Waters), a diode-array UV detector (LC 235; Perkin Elmer, Norwalk, CT, U.S.A.). and a strip-chart recorder (GP-100, Perkin Elmer).

Acquisition, reduction and documentation of the data were performed by means of the custom-modified [6] Nelson X-tra Chrom II software (Nelson Analytical, Cupertino, CA, U.S.A.) on a Hewlett-Packard 9000 Series 300 minicomputer; the computer interface was connected to the 2-V unattenuated output of the diode-array detector (acquisition wavelength 255 nm).

The retention times of II and IV were 5.3 and 10.1 min, respectively. The analysis time was *ca.* 14 min.

Extraction procedure for the determination of I

A 0.5-ml aliquot of plasma sample (calibration standard, QC or unknown) was mixed thoroughly with 25 μl of internal standard solution III in a glass tube. About 250 μl of the sample were transferred to the glass microinsert of the autosampler vial.

The six column-switching steps were as follows.

Step 1 (A = 0, B = 0, C = 0; 4 min). A 200-μl volume of plasma sample was injected onto the clean-up column C1. Polar plasma components were washed out with water to the waste S, while less polar components, including I and III, were retained on C1. The analytical column C2 was switched to the normal-flow direction in preparation for the analysis, while pump P2 was idle.

Step 2 (A = 0, B = 0, C = 1; 1 min). Enriched material was transferred from C1 onto C2 in the backflush mode by means of the mobile phase delivered by pump P3. The chromatographic separation of I and III, detected by D2, started. Pump P1 washed the autosampler circuit to S.

Step 3 (A = 0, B = 0, C = 0; 0.2 min). The flow through the enrichment part of the system was separated from that through the analytical part.

Step 4 (A = 1, B = 0, C = 0; 8.8 min). The clean-up column C1 was washed with methanol, delivered from P2, to waste S. The analytical seperation on C2 continued.

Step 5 (A = 1, B = 1, C = 0; 2 min). Methanol purging of C1 continued, while backflushing of C2 started in order to remove strongly retained substances.

Step 6 (A = 0, B = 1, C = 0; 10 min). While backflushing of C2 continued, C1 was re-equilibrated with water, and P2 stopped delivering methanol.

Extraction procedure for the determiniation of II

A 0.5-ml aliquot of the plasma sample was mixed with 0.5 ml of 1 *M* orthophosphoric acid in a conical tube. After thorough mixing, 25 μl of internal standard solution IV were added, followed by 6 ml of ethyl acetate. A piece of aluminium foil was placed over the mouth of the tube, which was then stoppered. Extraction was performed on a rotating (head-over-head) shaker for 15 min at 30 rpm, followed by centrifugation at 1500 *g* and 4°C for 5 min. A 5-ml volume of the organic phase was transferred to a 7-ml conical tube, which had previously been silanized with Aquasil and rinsed with methanol in an ultrasonic bath; the extract was evaporated to dryness at 20°C under a gentle stream of dry nitrogen. The residue was dissolved in 200 μl of mobile phase by vortex-mixing and sonication. After centrifugation at 3000 *g* for 1 min, *ca.* 100 μl of the clear supernatant were transferred to the plastic microinsert of the autosampler, and 20 μl were injected.

Calibration and calculations

Five and six calibration samples for the determination of I and II, respectively, covering the expected concentration range, were processed daily together with QC and unknown samples, as described above. In both cases, the calibration curve was obtained by weighted linear least-squares regression (weighting factor $1/y^2$) of measured peak-height ratios I/III (or II/IV) (y) versus the concentrations of I (or II) added to plasma (x). The curve was then used to interpolate concentration of I (or II) in QC and unknown samples from the measured peak-height ratios I/III (or II/IV).

RESULTS AND DISCUSSION

Validation

The recovery of I, II, III and IV was almost quantitative.

A linear correlation between the peak-height ratio of I to III (or II to IV) and the concentration of I (or II) was found in the ranges 5–800 ng/ml and 0.05–50 μg/ml in plasma for I and II, respectively. The coefficient of determination (r^2) was generally better than 0.9980, and the mean deviation from the calibration graph was better than 3% for both methods. The quantification limit definition adopted was that of the lowest concentration that could be determined during the inter-assay validation with either precision or accuracy of less than or equal to 20% and 15%, respectively: no reliance was placed onto the detection limit (signal-to-noise ratio 3:1), which was 2 ng/ml for I and 5 ng/ml for II, respectively.

The inter-assay accuracy and precision of both methods were evaluated by replicate analysis of spiked QC samples on five days over a period of two weeks. Results are shown in Table I.

Selectivity from endogenous plasma components as well as other metabolites of I (2-pyrrolidinone, anisic acid, 4-methoxyhippuric acid) was also demonstrated for both methods.

TABLE I

INTER-ASSAY PRECISION AND ACCURACY FOR THE DETERMINATION OF I AND II

Compound	Concentration added (A) (ng/ml)	Concentration found (B) (ng/ml)	n	Precision (R.S.D.%)	Accuracy $(B-A)100/A$
I	5.00	5.62	5	3.5	+12.5
	7.50	7.48	10	6.9	−0.3
	37.5	39.0	10	1.8	+4.0
	75.0	76.9	10	2.0	+2.5
	600	599	10	1.0	−0.2
II	50	48.3	5	15.5	−3.4
	200	200	10	5.3	0
	15000	15233	10	6.7	+1.6

Determination of I

Initial attempts were aimed at re-validating, in this laboratory, the sample preparation associated with the original HPLC method [4], involving liquid–liquid extraction of buffered plasma; however, it proved impossible to reproduce either the chromatogram in the regions of interest or the sensitivity reported, owing to interferences. Different buffers and/or organic solvents were then tried, as well as solid-phase extraction on Extrelut (E. Merck) columns but attemps

were unsuccessful although some improvements were observed in the chromatogram. On-line sample enrichment [7], which avoids lengthy sample preparation and generally increases laboratory productivity, was then explored. Water was chosen as clean-up solvent, because (1) both I and III are uncharged molecules, (2) both substances are completely retained on the clean-up column and (3) any problem associated with the use of organic modifiers and/or buffers (clogging of frits or tubing caused by precipitation of plasma proteins) is avoided.

Since the efficiency of automated HPLC procedures is much improved by the removal of late-running, strongly retained compounds from both the analytical and the clean-up columns, the analytical column was cleaned by reversing the flow of the mobile phase delivered by pump P3 (Fig. 2), while the clean-up cartridge was purged with methanol. Although the column lifetime is reportedly reduced by continual reversing of the flow, it was found that the analytical column performance remained almost unaltered, even after *ca.* 1000 plasma injections. The clean-up column was changed daily during routine work (50 injections) or when the back-pressure at P1 exceeded *ca.* 50 bar.

Compound I is stable in frozen plasma for at least three months [1]. At room temperature, however plasma concentrations of I halve over 24 h owing to enzymic hydrolysis to 2-pyrrolidinone and anisic acid; the internal standard III is even less stable in plasma. In order to stabilize the two substances in plasma, addition of different cholinesterase inhibitors and cooling at 4°C were studied. The results of these experiments, shown in Table II, indicate that both dichlorvos and sodium fluoride slow the degradation of I and III, although to different extents, but that their effect is still unsatisfactory; furthermore, high concentrations of dichlorvos introduce additional, late-eluting peaks in the chromatogram. The solution to the degradation problem is to cool plasma samples at 4°C, a temperature that still allows convenient handling of this biological fluid: although hydrolysis of I and III is not stopped completely, it is reduced to an acceptable level (1% for I and 8% for III after 24 h). It is therefore necessary to conduct all

TABLE II

DEGRADATION OF I AND III IN PLASMA AFTER 24 h UNDER DIFFERENT CONDITIONS

Condition	Degradation (%)	
	I	III
RT[a], no additives	57	79
RT, + 40μg/ml dichlorvos	42	67
RT, + 200 μg/ml dichlorvos	29	23
RT, + 25 mg/ml NaF	26	61
4°C, no additives	1.3	8.0

[a] RT = room temperature.

operations involving plasma (centrifuging, dosing, transferring) at 4°C, and, if replicate determinations of different days are required, to freeze the samples immediately after use.

Determination of II

The original procedure [2] involved a basic hydrolysis step in order to free anisic acid from its glucuronide conjugate, a neutralization step and two extractions with ethyl acetate. Since the extraction procedure was rather time-consuming, and the recovery of II and IV was variable, direct extraction of acidified plasma with ethyl acetate was attempted. Results were optimal, since recovery of both II and IV was almost quantitative and constant, and no interference of other metabolites or endogenous plasma components was observed. It was found that the recovery of the internal standard IV was affected by the evaporation temperature of the organic extract, since IV tended to be evaporated/sublimed. The problems were solved by carrying out the evaporation of the extract at 20°C in silanized tubes.

Application of the methods to biological samples

The two methods have been applied successfully to the analysis of over 1000 plasma samples from two bioequivalence studies in humans, using several oral dosage forms. Figs. 3 and 4 show representative chromatograms from these studies.

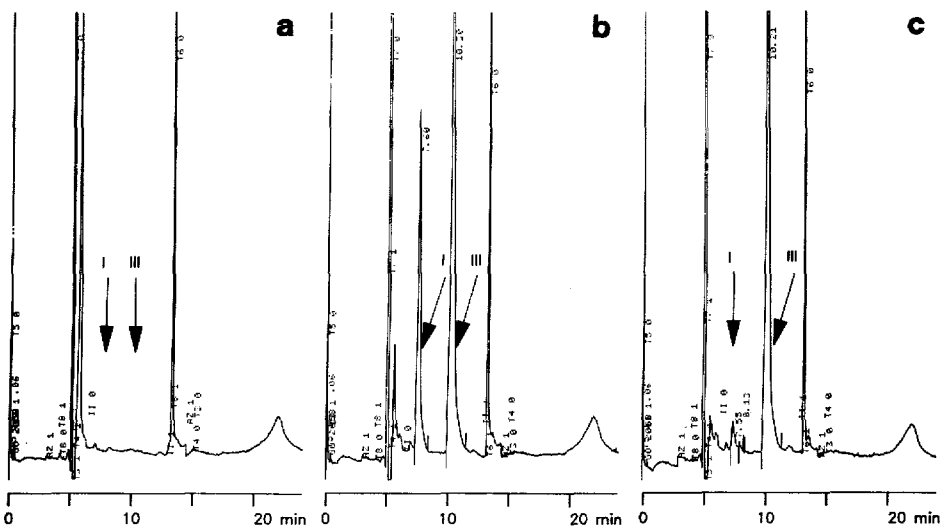

Fig. 3. HPLC of plasma from volunteer G.An. after oral administration of a single, 1500-mg dose of I (determination of I). (a) Blank (predose) plasma; (b) plasma 5 min after administration: calculated concentration 94.9 ng/ml I; (c) plasma 20 min after administration: calculated concentration 6.62 ng/ml I.

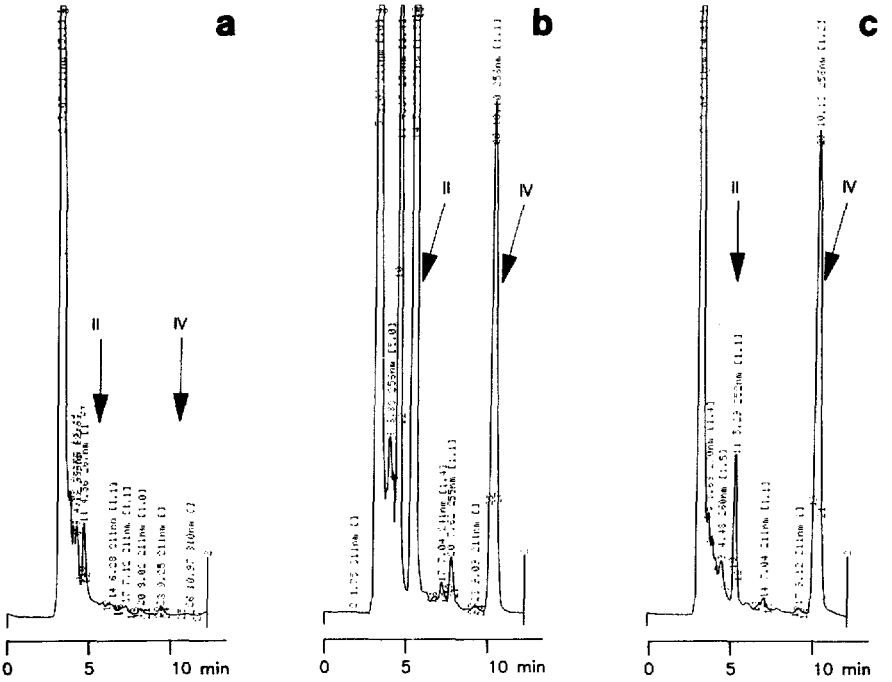

Fig. 4. HPLC of plasma from volunteer T.M. after oral administration of a single, 1500-mg dose of I (determination of II). (a) Blank (predose) plasma extract; (b) plasma extract 45 min after administration: calculated concentration 11 287 ng/ml II; (c) plasma extract 360 min after administration: calculated concentration 450 ng/ml II.

The method for the determination of II allows the assessment of its pharmacokinetic parameters from plasma concentration–time data after a single oral dose corresponding to the envisaged therapeutic regimen for I (1500 mg daily). The method for the determination of I, on the other hand, rarely allows the measurement of plasma levels after oral administration of the drug owing to extensive first-pass metabolism, which is the result of an extremely high body clearance (*ca.* 10 l/min); kinetic parameters could be determined easily, however, after bolus intravenous administration of the drug.

ACKNOWLEDGEMENTS

The authors thank Drs. D. Dell and G. Wendt, PF/MET, F. Hoffmann-La Roche (Basle, Switzerland), for helpful criticism and for correcting the manuscript, and Dr. U. Timm, PF/MET, F. Hoffmann-La Roche, for making available an interface for column-switching.

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