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Resolution of Salbutamol Enantiomers in Human Urine by Reversed-Phase High Performance Liquid Chromatography after Derivatization with 2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl Isothiocyanate

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A stereospecific HPLC method has been developed for the resolution of the enantiomers of salbutamol in human urine. After solid-phase extraction and derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate, the diastereomeric derivatives were resolved (Rs=1.83) on 5 μm octadecylsilan column using 35% acetonitrile in 0.05M ammonium acetate buffer (pH=6) as a mobile phase with electrochemical detection. The diastereomeric derivatives were formed within 30 min. The detection limit of each enantiomer was 20 ng/ml (S/N=3).

Key words : Chiral separation, HPLC, Chiral derivatizing reagent, Salbutamol, Enantiomer, 2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate

INTRODUCTION

Salbutamol, 2-(tert-butylamino)-1-(4-hydroxy-3-hydroxymethylphenyl)-ethanol, also known as albuterol, is a relatively selective β_2-adrenergic bronchodilator, which has been used to relieve bronchospasm in patients with reversible obstructive airway diseases (Cullen *et al.*, 1969). It is used clinically as a recemic mixture of two optical isomers, R-(-)- and S-(+)- salbutamol. Even though both enantiomers show high selectivity for β-adrenoceptors in bronchial muscle compared to cardiac muscle, the drug's agonistic activity resides mainly in the R-(-) enantiomer (Brittain *et al.*, 1973; Hartley *et al.*, 1971; Hawkins *et al.*, 1973)

Stereoselective disposition of enantiomers can result in different pharmacological profiles owing to different rate of absorption or stereoselective presystemic metabolism, distribution or clearance. Owing to increasing regulatory interest, the analysis of the enantiomeric forms of chiral molecules has received much attention in recent years(Allenmark, 1991). A few enantioselective assays for salbutamol have been reported. Tan *et al.* (1987) resolved salbutamol enantiomers in human urine using α_1-AGP column at 0°C. But the resolution was not satisfactory (Rs=1.06) and detection limit was high (259 ng/ml) because of the low sample capacity of the α_1-AGP column. Adams *et al.* (1993) and Boulton *et al.* (1995) determined salbutamol enantiomers in human blood using Pirkle-type chiral stationary phase with fluorescence detection. Though the assays were sensitive enough for the analysis of salbutamol enantiomers in human biological samples, prelimenary equilibrium between the nonpolar mobile phase and Pirkle-type chiral stationary phase required long time.

Seo *et al.* (1994) determined salbutamol enantiomers in human plasma using off-line achiral-chiral coupled chromatogrphy. Salbutamol racemate extracted from human plasma was first separated on the achiral silica column and fractionated. Secondly the salbutamol racemate was resolved into each enantiomer and quantitized on the Pirkle-type chiral stationary phase with fluorescence detection. The assay was sensitive enough but the procedure took long time.

As an indirect chiral separation method, 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl isothiocyanate (TAGIT) was used as a chiral derivatizing reagent to determine the enantiomers of salbutamol in human serum with fluorescence detection by HPLC (He *et al.*, 1992). We tried to resolve the enantiomers of salbutamol by HPLC using another general chiral derivatizing reagent, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC). The daistereomers were formed easily and resolved by RP-HPLC but the fluorescence property of the salbutamol disappeared.

This paper describes a simple method that involves

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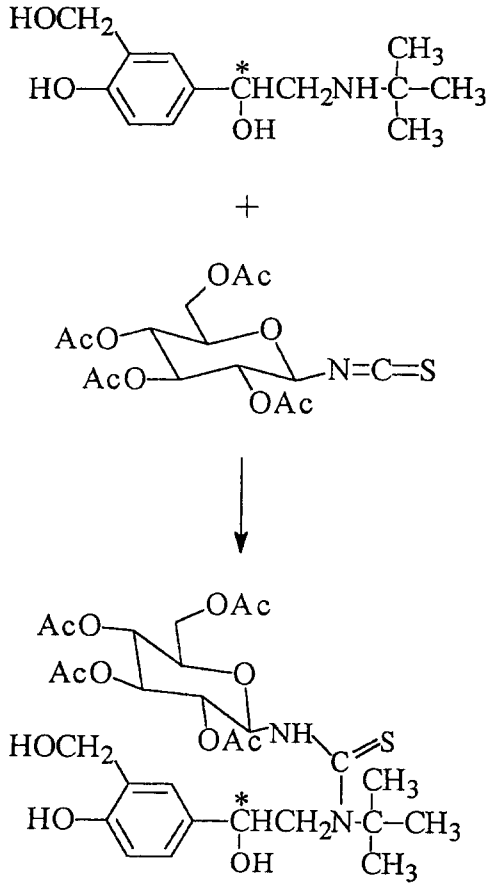

Fig. 1. Proposed reaction of GITC with a secondary amine of a salbutamol to a diastereomeric thiourea.

the preparation of diastereomers of salbutamol (Fig. 1) with the chiral derivatizing reagent 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC), followed by achiral reversed-phase high performance liquid chromatography with electrochemical detection.

MATERIALS AND METHODS

Materials and equipment

Salbutamol sulfate was acquired from Glaxo Korea (Ansan, Korea). Bamethane sulfate, triethylamine (TEA) and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Trifluoroacetic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). HPLC grade methanol, acetonitrile, n-hexane and 1,2-dichloroethane were obtained from J. T. Baker (Phillipsburg, NJ. USA). Water was purified with a Milli-RO 15 Water system (Nihon millipore, Japan) and was filtered through a 0.2 μm membrane filter. All other reagents were of analytical reagent grade. The mobile phase was filtered through a 0.2 μm filter and degassed by sonication under vacuum before using it.

High performance liquid chromatograph was consisted of Shimadzu Model LC-9A pump, SPD-6AV spectrophotometric detector, RF-535 fluorescence detector, SCL-6B system controller, C-R4AD chromatopac (Kyoto, Japan). The ESA Coulochem II Electochemical detector (Bedford, MA, USA) was consisted of model 5020 guard cell operated at a potential of 300 mV and model 5010 dual electrode analytical cells which were operated at potentials of -400 mV and 800 mV, respectively. The separations were carried out on a Inertsil ODS-2 column, 4.6 mm I.D.×150 mm, with 5 μm particle size (GL Science Inc, Japan) at ambient temperature. Semi-preparative chiral chromatography was performed with Sumichiral OA-4700, 8.0 mm I. D.×250 mm, with 5 μm particle size (Osaka, Japan). Bakerbond-spe (silica gel, 1 ml) and vacuum manifold box which holds 12 solid-phase extraction cartridges were obtained from J. T. Baker Inc. (Phillipsburg, NJ, USA).

Determination of the order of elution

Salbutamol racemate 40 mg was dissolved in 10 ml of mobile phase. This solution was injected into the semi-preparative chiral HPLC system and resolved into each enantiomer on the Sumichiral OA-4700 column (5 μm, 8 mm×25 cm) by the n-hexane, 1,2-dichloroethane, methanol and trifluoroacetic acid (240:140:15:1, v/v/v/v) as a mobile phase at room temperature and flow rate of 4 ml/min monitoring at UV 278 nm. Fractions containing single enantiomers were collected and evaporated to dryness under nitrogen stream. The direction of rotation (+/-) was determined using a Jasco DIP-1000 digital polarimeter. R-(-)-salbutamol and S-(+)-salbutamol were derivatized with GITC at room temperature for 30 min, follwed by injected into the achiral HPLC system (column; Inertsil ODS-2, 5 μm, 4.6 mm I.D.×150 mm, mobile phase; 35% acetonitrile in 0.05 M ammonium acetate buffer (pH=6), flow rate; 0.7 ml/min) and the elution order of each isomer was confirmed.

Sample preparation

One ml aliquot of urine sample and 100 μl of internal standard solution of bamethane (1.2 μl/ml) were transferred to a prelabelled culture tube and mixed. The solid-phase (silica) extraction column was preconditioned by washing with 1 ml of acetonitrile followed by 1 ml of deionized water. The washing solvents was allowed to pass through with minimum vacuum (<50 mmHg) which was released immediately after the solvents eluted from all the cartridges. The previously mixed urine sample was transfered into the preconditioned column and minimum vacuum was applied. When the urine sample in the cartridge reservoir had been removed, the vacuum was increased to

500 mmHg for 2 min and then released. Each column was then washed with 1 ml of deionized water followed by 1 ml of acetonitrile under minimum vacuum until all the washing solvents eluted from the cartridges. The column was dried by full vacuum for an additional 5 min. Salbutamol and bamethane were then eluted from the silica adsorbent by rinsing with 3 ml of methanol under minimum vacuum until no effluent was observed. The effluent was evaporated to dryness under the nitrogen gas at room temperature.

To the dried extract, 50 μl of GITC solution (6.5 μm/ml in acetonitrile) and 50 μl of TEA solution (1.53 μl/ml in acetonitrile) were added and mixed well at room temperature for 30 min and then the solvent was removed under a stream of nitrogen at room temperature and residue was reconstituted in 200 μl of mobile phase and 100 μl was injected into the achiral HPLC system.

Standard curve samples were constituted by serial dilution of the working standard solution with the drug-free human urine to give final concentration of 50~1000 ng/ml.

Optimization of the mobile phase

The effects of the changes in the mobile phase pH and ionic strength on the resolution of the diastereomers were investigated. pH of the mobile phase, 35% acetonitrile in 0.05 M ammonium buffer, was changed from 3 to 7.5. And the concentration of the ammonium acetate in the mobile phase, 35% acetonitrile in ammonium acetate buffer (pH=6) was changed from 5 to 150 mM.

Optimization of derivatization

A large volumn of solid-phase extract was prepared for the purpose of optimizing GITC concentration by extracting miltiple drug-free human urines spiked with 1000 ng/ml of salbutamol enantiomers. Aliquots of the extract (3 ml) were evaporated to dryness to which 50 μl aliquots of various concentrations of GITC solution and TEA solution were added and stand for 30 min at room temperature. Following evaporation and dissolution of the residue in mobile phase, the resulting samples were injected into the achiral HPLC system and the peak areas quantitated.

The effects of time and temperature on the reaction were investigated by preparing a 1000 ng/ml extract, as outlined above. Aliquots of the extract (3 ml) were evaporated to dryness and incubated with 50 μl of GITC and TEA solutions at room temperature, 45°C, 65°C for 30 min, 1 hr, 2 hr, 3 hr, or 4 hr. They were evaporated to dryness, the residue dissolved in the mobile phase and the resulting samples quantitated by achiral HPLC.

Assay validation and calculation

The accuracy and precision of the assay were determined by analyzing five replicates from the two samples spiked with 500 and 1000 ng/ml of salbutamol enantiomers to drug-free human urine. Standard curves were constructed by plotting the concentration versus the peak area ratio (peak area of component/peak area of internal standard) and fitting a least squares linear regression analysis line to the data. Concentration of R-(-)-salbutamol and S-(+)-salbutamol in the samples were determined by extrapolating the peak area ratio to the least squares line of the standard curve.

RESULTS AND DISCUSSION

Chiral semi-preparative HPLC of R-(-)- and S-(+)-salbutamol and dermination of elution order of the derivatives

On the chiral semi-preparative HPLC system, S-(+)-salbutamol was eluted first (Fig. 2). After fractionation of the eluent containing each enantiomer and derivatization with GITC, The derivative of R-(-)-salbutamol was found to be eluted faster than that of S-(+)-salbutamol on the achiral reversed-phase HPLC system (Fig. 3). During derivatization racemization was not occured.

Optimization of the mobile phase

Changes in capacity factors followed a simlar pattern for the two derivatives salbutamol enantiomers (Fig. 4). At pH 4 the capacity factors somewhat decreased but over pH 5 the capacity factors were not changed significally. Fig. 5 shows that capacity factors increased as the concentration of ammonium ace-

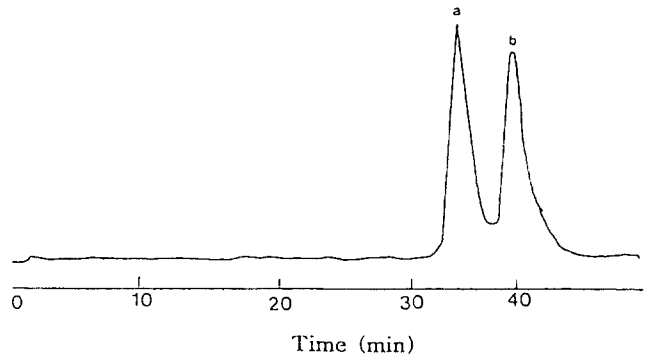

Fig. 2. Chiral semi-preparative HPLC chromatogram of salbutamol racemate. Column; Sumichiral OA-4700 (5 μm, 8× 250 mm), mobile phase; n-hexane: 1,2-dichloethane:methanol: trifluoroacetic acid (240:140:15:1, v/v/v/v), flow rate; 4 ml/min, detection; UV at 278 nm. Peak (a); S-(+)-salbutamol, peak (b); R-(-)-salbutamol.

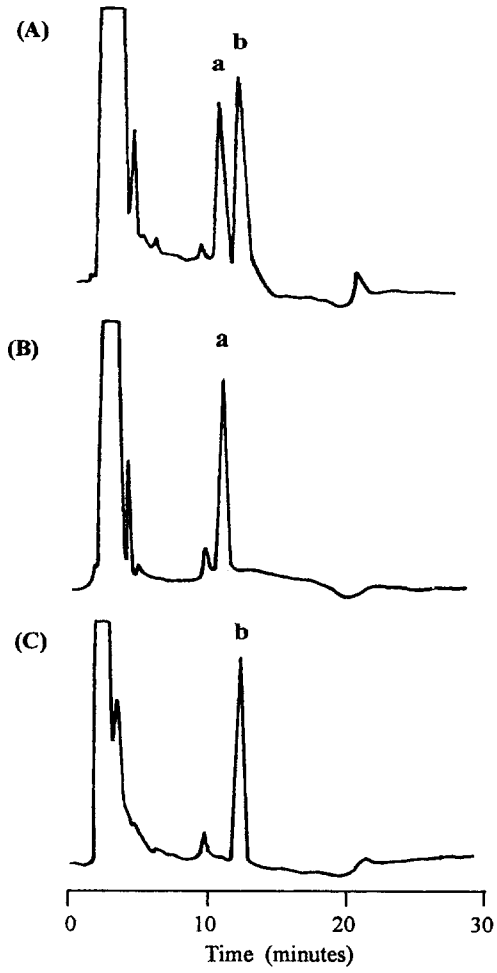

Fig. 3. HPLC chromatograms of (A) salbutamol racemate, (B) R-(-)-salbutamol and (C) S-(+)-salbutamol after fractionation from the chiral semi-preparative HPLC and dervatization with GITC. Column; Inertsil ODS 2 (5 μm, 4.6×250 mm), mobile phase; 37% acetonitrile in ammonium acetate buffer (pH=6.0), flow rate; 0.8 ml/min. Peak (a); derivative of R-(-)-salbutamol, peak (b); derivative of S-(+)-salbutamol.

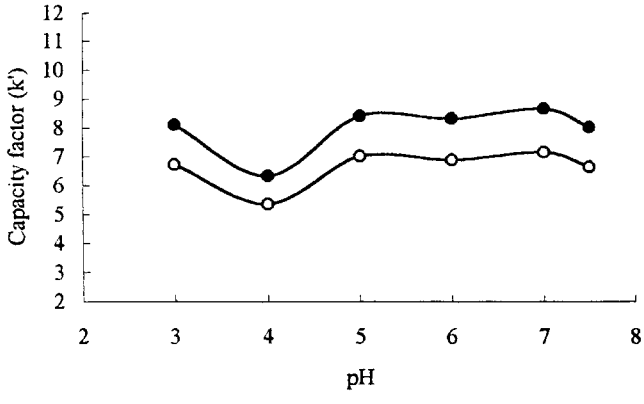

Fig. 4. The effect of pH on the capacity factors of derivatives of salbutamol enantiomers. Mobile phase is 35% acetonitrile in 0.05 M ammonium acetate buffer. ○: Derivative of R-(-)-salbutamol, ●: Derivative of S-(+)-salbutamol.

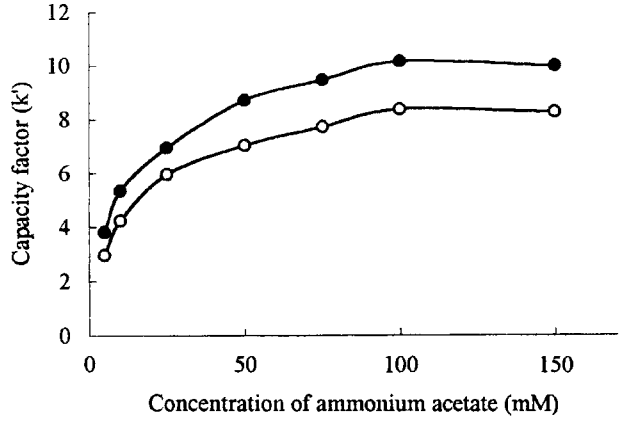

Fig. 5. The effect of ionic strength on the capacity factors of derivatives of salbutamol enantiomers. Mobile phase is 35% acetonitrile in ammonium acetate buffer (pH=6.0). ○: Derivative of R-(-)-salbutamol, ●: Derivative of S-(+)-salbutamol.

tate increased. At 50 mM of ammonium acetate the resoution was better and the capacity factors were less, relatively.

Optimization of derivatization

As shown in Fig. 6, the maximum peak area response for the enantiomers was seen at the 150 times molar excess of GITC. In the final analytical conditions, the GITC concentration was chosen 200 times molar excess in order to provide an adequate excess of reagent.

As the reaction temperature was increased from room temperature to 45°C or 65°C, the peak areas of enantiomers were decreased and other impurity peaks supposed to emerge from side reaction were increased. The formation of the derivatives of enantiomers increased with the reaction time up to 30 min at room temperature and reached a plateau (Fig. 7).

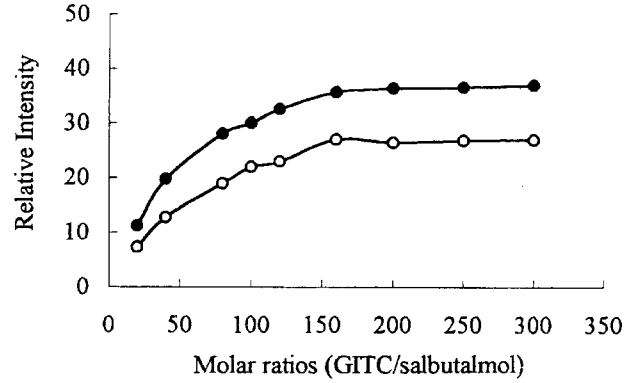

Fig. 6. The effect of GITC concentration on the peak areas of the derivatives of salbutamol enantiomers. ○: R-(-)-salbutamol, ●: S-(+)-salbutamol.

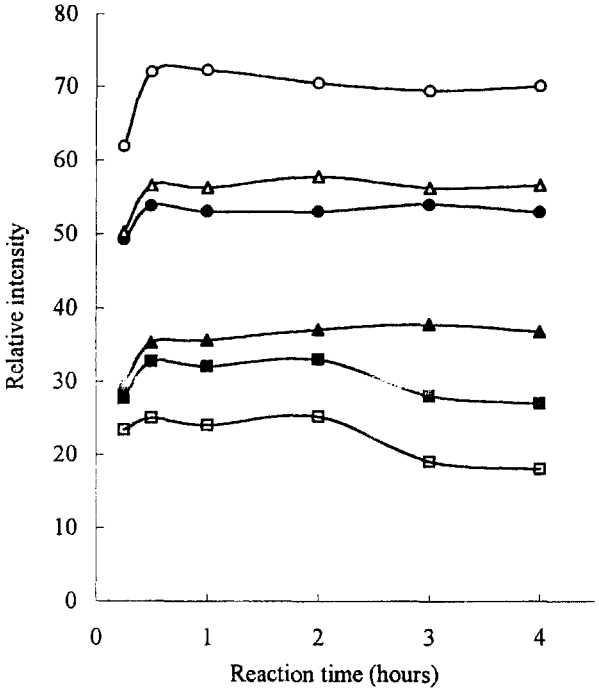

Fig. 7. The effect of reaction temperature and time on the peak areas of the derivatives of salbutamol enantiomers. ○: R-(-)-salbutamol at room temperature, ●: S-(+)-salbutamol at room temperature, ▽: R-(-)-salbutamol at 45°C, ▼: S-(+)-salbutamol at 45°C, □: R-(-)-salbutamol at 65°C, ■: S-(+)-salbutamol at 65°C.

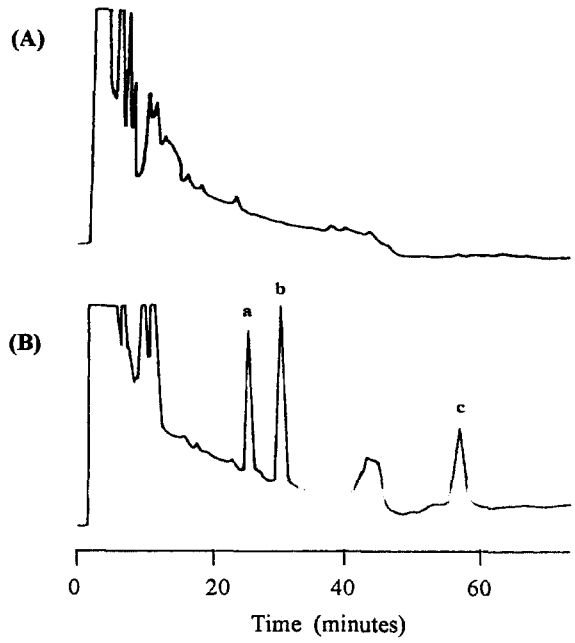

Fig. 8. Typical chromatograms of (A) extract of blank human urine; (B) extract of 500 ng/ml stantard salbutamol enantiomers spiked to human urine. Column; Inertsil ODS-2 (5 μm, 4.6×150 mm), mobile phase; 35% acetonitrile in 0.05 M ammonium acetate buffer (pH=6.0), flow rate; 0.7 ml/min. Peak (a); derivative of R-(-)-salbutamol, peak (b); derivative of S-(+)-salbutamol, peak (c); derivative of bamethane.

Resolution of salbutamol enantiomers in urine

Fig. 8. depicts representative chromatograms of drug-free blank human urine and urine containing 100 ng/ml of R-(-)-salbutamol and S-(+)-salbutamol analyzed by the procedure described above. The peaks corresponding to derivatives of salbutamol enantiomers and derivative of bamethane, internal standard, were easily resolved from coeluting endogenous substances. The retention times of the diastereomeric derivatives of R-(-)-salbutamol and S-(+)-salbutamol were 25.53 and 29.49 min. The retention time of the derivative of bamethane was 54.37.

The values determined for the recovery and precision of the assay in human urine are presented in Table I. The recovery was good and the reproducibility was within 10%. The standard curves for the enantiomers gave a linear response with correlation coefficients greater than 0.998.

In conclusion, it was found that the derivatization of salbutamol with GITC was an useful technique for the separation of the enantiomers by reversed phase HPLC. The procedure has several major advantage over previously described techniques: the derivatization is simple and rapid, the chiral reagent (GITC) is commercially available and inexpensive and commercial inexpensive reversed-phase chromatographic column can be used. And good separation of the diasteromeric derivatives was obtained.

Table I. Recovery test for the R-(-)-salbutamol and S-(+)-salbutamol in human urine (n=5)

Amount added	Component	Amount recoverd	Recovery (%)	Precision (C.V.)
500	R-(-)-salbutamol	487.2	97.4	7.2
	S-(+)-salbutamol	459.4	91.9	6.7
1000	R-(-)-salbutamol	1011.0	101.1	9.8
	S-(+)-salbutamol	952.1	95.2	8.6

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