Selected Ion Monitoring Assay for Bromhexine in Biological Fluids

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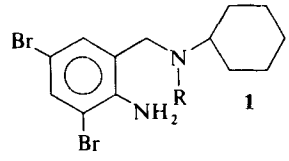
A method has been developed for quantification of bromhexine in plasma using gas chromatography mass spectrometry with selected ion monitoring. A deuterium labelled analogue was synthesized and used as the internal standard. To evaluate the gas chromatographic electron capture detection method described earlier, 23 plasma samples have been analysed by both techniques. Although a good correlation was shown, selected ion monitoring was superior to the electron capture detection method for levels below 3 ng ml^{-1}. The mass spectrometric method has also been used to set up a pharmacokinetic study of bromhexine in horses. Urine extracts were subjected to gas chromatographic mass spectrometric analysis to identify the different metabolites of bromhexine. Three groups of metabolites were detected and identified.

INTRODUCTION

N-Cyclohexyl-*N*-methyl-(2-amino-3,5-dibromobenzyl)-ammoniumchloride (**1**) otherwise known as bromhexine, is used as a mucolytic drug in man.[1,2] For determination of the drug in pharmaceutical preparations, a quantitative gas chromatographic method has been reported.[3] A technique presented earlier, based on



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R = CH_3
R = CD_3

gas chromatography with electron capture detection,[4] proved to be a sensitive and reliable method for the quantitation of bromhexine in plasma. However, the sensitivity for an exact estimate of the concentration was limited to levels above 3 ng ml^{-1}. In view of the requirements of a higher degree of specificity and sensitivity (down to 0.5 ng ml^{-1} plasma) gas chromatography mass spectrometry (GCMS) with isotope dilution appeared to be the technique of choice.

EXPERIMENTAL

Chemicals

All products for the synthesis of the deuterated internal standard were purchased from Aldrich Chemical Co. (Beerse, Belgium). The hydrochloride of bromhexine was provided by Boehringer (Ingelheim, GFR). SE-30 4% on Gas Chrom Q, 100/120 mesh, was obtained from Supelco Inc. (Bellefonte, Pennsylvania, USA). All other chemicals were of analytical grade from Merck (Darmstadt, GFR).

Synthesis of the internal standard

Starting from cyclohexylamine and *o*-nitrobenzyl-bromide, *N*-(2-amino-3,5-dibromobenzyl)cyclohexyl-ammoniumchloride was prepared by methods described in the literature.[5] After alkalinization with 2 N NaOH the base was extracted with $CHCl_3$. The organic phase was removed on a rotary evaporator, and the base (11 g, 0.03 mol) redissolved in sulfolane. One equivalent of CD_3I (4.56 g) was then added and the mixture heated at 70 °C for 2 h. After alkalinization with 2 N NaOH, the solution was extracted twice with *n*-hexane, and the combined organic layers evaporated to dryness, redissolved in absolute ethanol and treated with dry HCl gas to give the hydrochloride. The volume of ethanol was minimized until the solution became turbid; the product crystallized upon refrigeration. The product was identified by mass spectrometry and yielded the following mole percent deuterated species:[6] [$^2H_3$]: 89.81%; [$^2H_2$]: 5.8%; [$^2H_1$]: 3.59%; [$^2H_0$]: 0.79%.

Gas chromatography mass spectrometry

The mass spectra of bromhexine and [$^2H_3$]bromhexine were determined on a Hewlett Packard 5992B system at 70 eV, scan speed 690 u s^{-1}, two samples per 0.1 u.

Prior to the beginning of any series of selected ion monitoring (SIM) measurements, the ion source and quadrupole mass analyser voltage settings were optimized to monitor the centres of each of the selected ions. This was done by injecting 100 ng each of bromhexine and the trideuterated internal standard into the gas chromatograph mass spectrometer and monitoring six channels of 0.1 u difference around the theoretical mass value. The integration of the different peak areas indicated the mass of the most intense signal to the nearest 0.1 u.

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The mass calibration drift within-day was negligible.

The GC separation was made on a 1.8 m × 2 mm silanized glass column packed with 4% SE-30. Gas chromatographic conditions were: injection port 250 °C, oven temperature 230 °C, helium flow rate 25 ml min^{-1}. The transfer line and membrane separator were kept at a constant temperature (270 °C). Under these GC conditions, bromhexine and its trideuterated analog eluted approximately 6 min after injection.

The quantification of bromhexine utilized the integrated peak areas of the fragments m/z 293 for bromhexine and m/z 308 for the trideuterated internal standard.

Peak area ratios were obtained from spiked standard samples and plotted against the concentration of bromhexine, to construct calibration curves.

Spectra of the metabolites were obtained by repetitive scanning (between scan delay of 200 ms and scan speed of 330 u s^{-1}, four samples per 0.1 u) during the GC elution. After data acquisition, the ion chromatograms were reconstructed to identify the metabolites.

Preparation of standard samples

Stock solutions of bromhexine (0.8575 ng μl^{-1}) and [$^2H_3$]bromhexine (1.029 ng μl^{-1}) in methanol were prepared. For quantification purposes plasma samples were spiked with different amounts of bromhexine (1.7–53.6 ng ml^{-1}) and a fixed amount of [$^2H_3$]bromhexine (50 ng ml^{-1}). These samples were extracted according to the method presented earlier[4] developed in this laboratory. The 'extracted' standard curves were compared with standard curves obtained from stock solutions, and prepared freshly for each analysis of the unknown samples.

Preparation of samples

Heparinized samples (1 ml) to which a fixed amount of trideuterated internal standard (50 ng) was added, were extracted as mentioned above. Prior to the injection, the residue was redissolved in 100 μl of n-hexane. Typically 1 μl injections were performed (Fig. 1). Urine samples, collected over 24 h, were obtained from individuals after a single oral dose of 32 mg bromhexine. Urine (60 ml) was hydrolysed at 100 °C with 5 N HCl (1 ml). After alkalinization with 5 N NaOH (1.5 ml), these samples were extracted in the same way as the plasma samples. After evaporation under a gentle nitrogen stream, ethyl acetate (125 μl) and TFAA (25 μl) were added, and held at 40 °C for 20 min. After evaporation to dryness, the samples were redissolved in methanol (200 μl). In addition, urine samples without derivatization were obtained. Then 1 μl of the methanol solution was injected into the gas chromatograph mass spectrometer system.

RESULTS AND DISCUSSION

Synthesis of the internal standard

A deuterium labelled analogue of the drug was chosen as internal standard since the gas chromatographic and mass spectrometric properties were identical to those of bromhexine. The synthesis of the internal standard, according to methods presented in the literature, includes a nucleophilic substitution of the bromine located on the benzyl group, and reduction of the nitro-group followed by bromination of the phenyl nucleus. Finally the trideuterated methyl group was incorporated. In addition, the internal standard which is added to the biological sample prior to the work-up, corrects for losses incurred during the sample manipulation. The incorporation of the trideuterated methyl yields a molecule of acceptable isotopic purity.

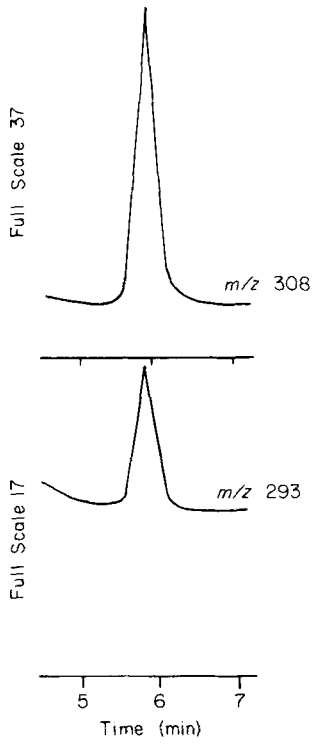


Figure 1. Selected ion current profile from an extract of a horse plasma sample containing bromhexine at a concentration of 0.4 ng ml^{-1}. The upper trace represents the added trideuterated bromhexine (50 ng ml^{-1}) as internal standard.

Mass spectra of bromhexine and [$^2H_3$]bromhexine

The electron impact spectra of the authentic bromhexine and [$^2H_3$]bromhexine have a common base peak at m/z 264 due to the loss of the cyclohexylamine moiety. In addition a weak molecular ion peak is seen at m/z 376 for bromhexine and at m/z 379 for [$^2H_3$]bromhexine. Loss of the cyclohexyl fragment accounts for the ions at m/z 293 and m/z 296, respectively. Opening of the cyclohexyl by α-cleavage with rearrangement of the fragment affords the ions at m/z 305 and 308, respectively (Fig. 2).

Quantification of bromhexine

For quantification by SIM obviously only those ions in which the trideuterated methyl group is still present can be used. Although the ions at m/z 112 and 115 were suitable for SIM analysis, exploratory work indicated that those ions were impractical in the concentration

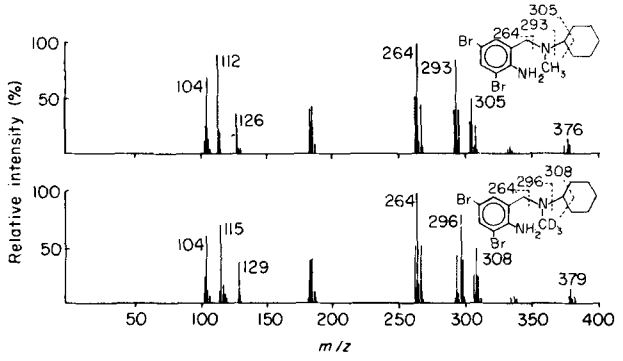


Figure 2. Mass spectra and proposed fragmentation patterns for bromhexine and its trideuterated analogue.

range of interest, due to extensive non-specific interference at low m/z values from co-extracted endogenous material. The ions at m/z 293 and 296 were then chosen for their relative intensity. However, due to bleeding of the SE-30 phase a severe background noise occurred at m/z 296, and therefore the ion at m/z 308 was chosen to monitor the internal standard. Calibration curves were determined for both pure standards and spiked plasma samples. We found no difference between the slope of the two calibration curves, indicating that no isotopic effects were present between bromhexine and its trideuterated analogue. During the analysis of the unknown samples, for each batch spiked plasma samples were analysed to construct a calibration curve. A typical calibration curve is shown in Fig. 3.

Correlation between electron capture GC and GCMS analysis

To compare the GCMS method with the electron capture detection (ECD) method presented earlier, plasma samples covering the concentration range of 3–30 ng ml^{-1} have been analysed by both techniques. A correlation of $r = 0.992$ was found, which seems quite acceptable (Fig. 4). However, for samples below 10 ng ml^{-1} a significant difference exists between both assays. This was shown by calculating the relative sensitivity between both assays. This relative sensitivity is

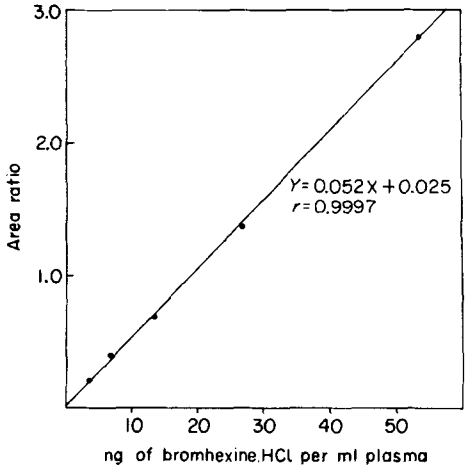


Figure 3. A typical standard curve obtained by spiking plasma samples with varying concentrations of bromhexine and a fixed amount of [$^2H_3$]bromhexine.

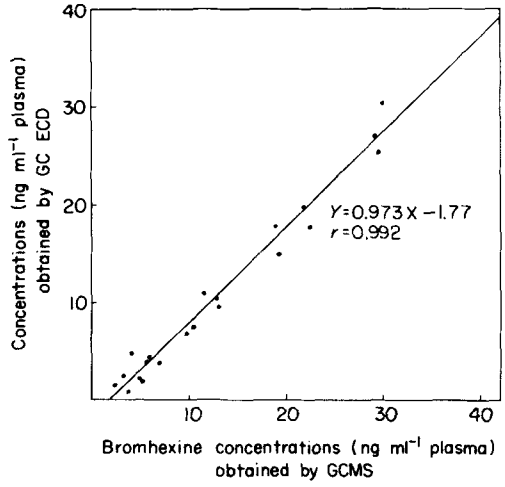


Figure 4. Linear regression of the bromhexine concentrations from drug treated subjects assayed by both GC ECD and GC MS.

defined[7] as the slope of the linear relation between values obtained by two methods on the same sample, divided by the ratio of their respective standard deviations, and is a measure of the relative merit of two procedures. It is expressed as

$$\frac{|dA/dB|}{\sigma A/\sigma B}$$

where A and B are the different methods, dA/dB the slope of the line relating the concentrations obtained by method A to method B, σA and σB, the standard deviation of method A and method B. The ratio $\sigma A/\sigma B$ is determined by taking the ratio of the average variation coefficients (between assay). The relative sensitivity of GCMS to ECD, thus calculated from the values of Table 1, is 4.4916. The square of this value gives then the number of replicates necessary with the ECD technique to obtain the same precision of the GCMS technique. In this case for each single GCMS measurement approximately 20 ECD injections should be made.

Quantification of bromhexine in horses' plasma

In view of the recent discovery of the interesting features[8] of bromhexine for veterinary use, a pharmacokinetic study has been set up for horses. After both oral

Table 1. Between assay precision

GC ECD	Sample concentration ng ml^{-1} ± SD		% CV[a]
	1	4.9 ± 0.71	14.43
	2	1.6 ± 0.35	22.81
	3	7.6 ± 0.64	8.43
	4	2.6 ± 0.57	21.76
	5	9.7 ± 1.20	12.46
GC MS	Sample concentration ng ml^{-1} ± SD		% CV[b]
	1	4.1 ± 0.28	6.90
	2	2.2 ± 0.14	6.40
	3	10.4 ± 0.07	0.68
	4	3.3 ± 0.07	2.18
	5	13.0 ± 0.14	1.09

[a] Average % CV between assay for GC ECD = 15.98.
[b] Average % CV between assay for GC MS = 3.46.

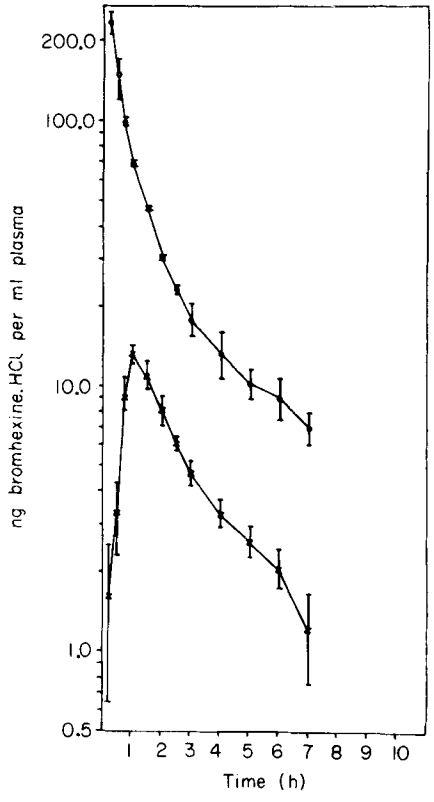


Figure 5. Semilogarithmic plot of the mean bromhexine concentrations (mean ± SEM) vs time after i.v. (○) and oral (×) administration of the drug to three horses.

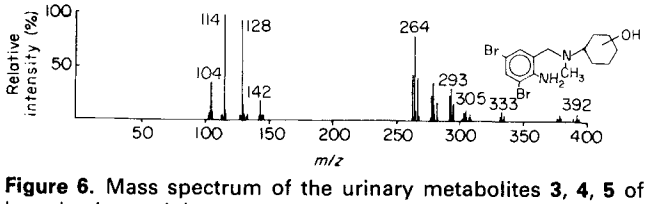


Figure 6. Mass spectrum of the urinary metabolites **3**, **4**, **5** of bromhexine and the proposed structure.

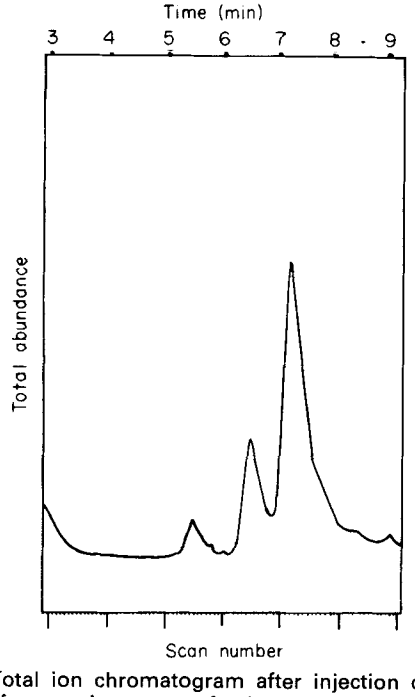


Figure 7. Total ion chromatogram after injection of the TFA derivatives from a urine extract after bromhexine administration.

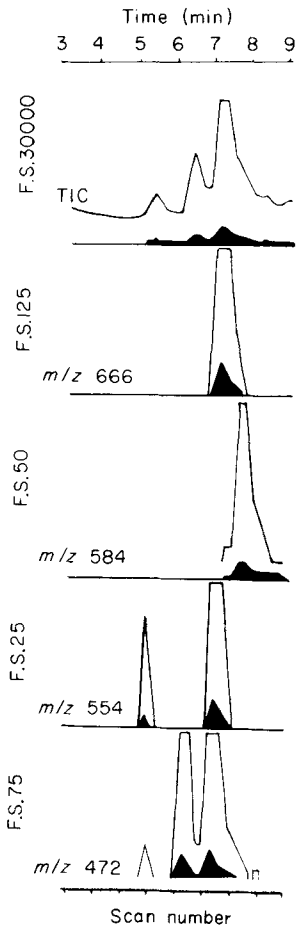


Figure 8. Computer generated ion chromatograms of the molecular ions of the TFA derivatives from urinary metabolites of bromhexine.

and i.v. administration the amount of bromhexine has been monitored. Standard curves from 0.8 ng ml^{-1} to 6.7 ng ml^{-1} were established by spiking plasma samples. The mean curve, related to three horses which received 2 mg kg^{-1} orally or 1 mg kg^{-1} intravenously, is shown in Fig. 5.

It is important to point out that in plasma the half-life of bromhexine is 3.2–4.4 h, and the time of maximum concentration after oral administration is 1–1.5 h. The complete pharmacokinetic study will be published elsewhere.

Metabolites of bromhexine in human urine

It is well known that bromhexine is converted to different metabolites after i.v. or oral administration.[9] So far only thin-layer chromatography and subsequent

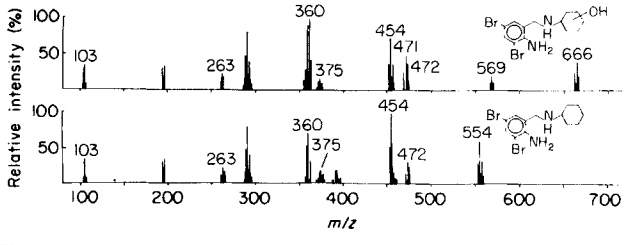
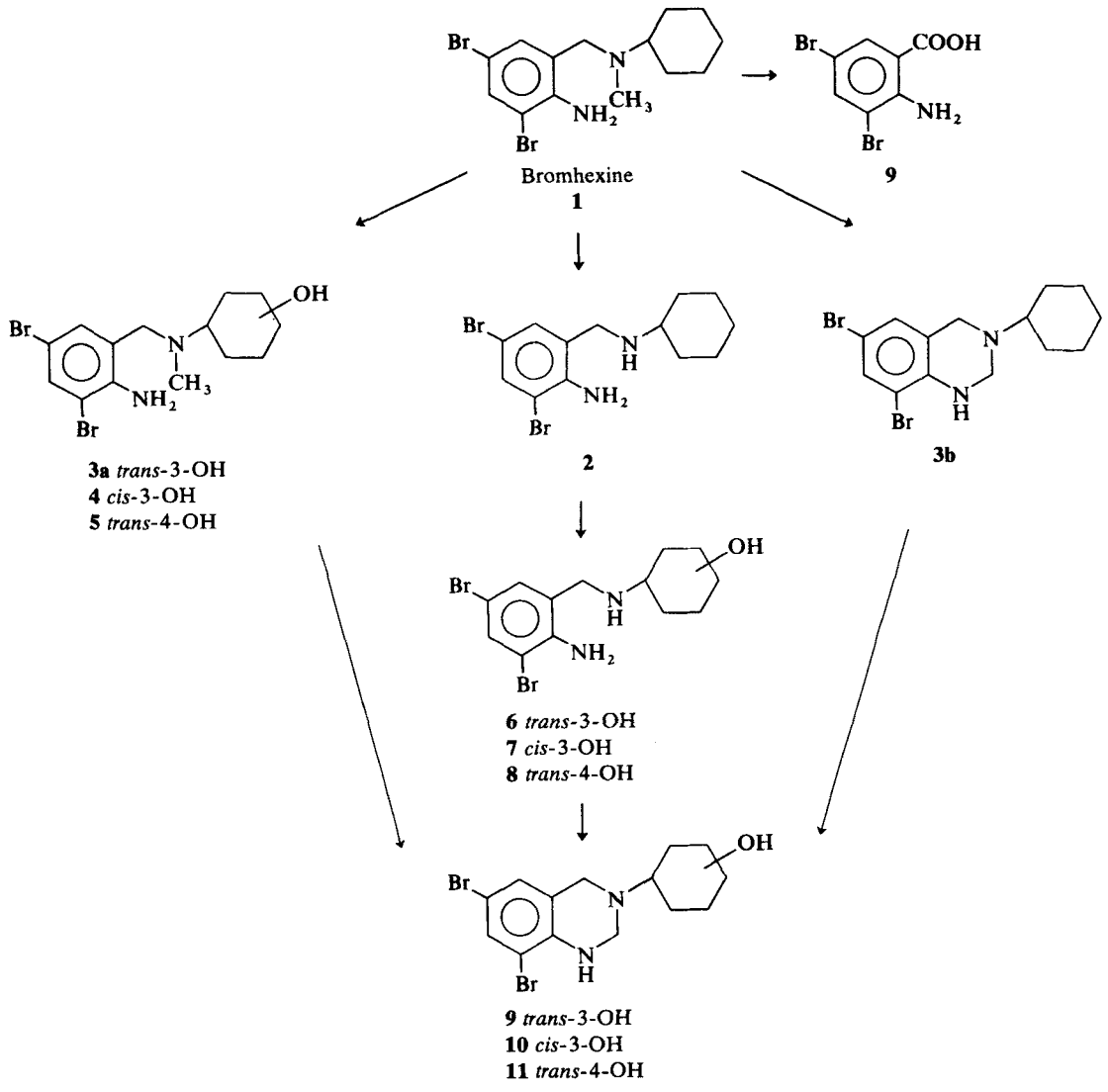


Figure 9. Mass spectra of the urinary metabolites **6**, **7**, **8** and **2** as TFA derivatives and their proposed structures.



Scheme 1. Metabolites of bromhexine as proposed in literature.

analysis by ultraviolet, infrared and nuclear magnetic resonance spectrometry have been used to propose their structural formulae. Four groups of metabolites have been indicated[11] (Scheme 1). Since most of the metabolites are conjugated[10] with glucuronic or sulfuric acid, acid hydrolysis is performed before extraction from urine. After injection into the gas chromatograph mass spectrometer two extra peaks appeared in the chromatogram. By reconstructing the ion chromatograms of the molecular ions of the proposed metabolites, only the major peak contained a metabolite. The spectrum recorded at the top of this peak was highly suggestive of hydroxylated bromhexine, and corresponded to the proposed isomeric metabolites **3a**, **4** and **5** (Fig. 6). After derivatization with TFAA the different metabolites were separated (Fig. 7). The reconstructed mass chromatograms affords the elution patterns of the different metabolites, i.e. at m/z 554 the CF_3CO derivative of metabolite **2** (demethylated bromhexine), at m/z 472 the derivatized bromhexine, at m/z 666 the CF_3CO derivative of metabolites **6**, **7** and **8** (hydroxylated and demethylated bromhexine), and at m/z 584 the derivative of the previously mentioned metabolites **3a**, **4** and **5** (Fig. 8). For each metabolite the complete spectra were run (Fig. 9). They corresponded to the structures of metabolites, proposed earlier in the literature.[9]

Acknowledgments

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