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Characterization of bromhexine and ambroxol in equine urine: effect of furosemide on identification and confirmation*

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Abstract: The purpose of this study was two-fold: (1) to develop a simple and sensitive screening procedure for identifying and confirming bromhexine and ambroxol and, (2) to determine the effect of furosemide on the detection of bromhexine, ambroxol, or their metabolites in urine. Female horses (450–550 kg) treated with bromhexine or ambroxol (1 g, p.o.) were used. Urine samples were collected up to 48 h post-drug administration and analysed. Blind samples were used in evaluating the sensitivity of these methods and reproducibility of the results. Bromhexine and ambroxol were extensively metabolized in the horse. These agents and their respective metabolites were identified and confirmed using thin-layer chromatography (TLC) and gas chromatography–mass spectrometry (GC–MS), respectively. Hydroxy-bromhexine and desmethyl-bromhexine were major metabolites found to be unique to bromhexine-treated horses. These metabolites selectively absent from ambroxol-treated horse urine provide a chemical means to distinguish bromhexine from ambroxol administration in horses. These specific metabolites were similarly identified and confirmed in "blind" horse urine samples. The concomitant presence of furosemide (300 mg, i.v.) with bromhexine or ambroxol did not mask the presence of these agents or alter their metabolite profile. By application of the methods described in this study, bromhexine and ambroxol metabolites in horse urine can be easily identified and confirmed.

Keywords: *Bromhexine; ambroxol; metabolites; identification; confirmation; furosemide.*

Introduction

Bromhexine [*N*-(2-amino-3,5-dibromobenzyl)-*N*-cyclohexyl-methylamine] and its pharmacologically active metabolite, ambroxol [*N*-(*trans*-4-hydroxy-cyclohexyl)-(2-amino-3,5-dibromo-benzyl)-amine] possess both secretagogic and mucolytic actions on the respiratory tract [1–3]. Bromhexine is widely used in Europe for the management of excessive production of mucus as a consequence of respiratory disorders. Investigations of the metabolism of bromhexine in humans [4] and rabbits [5] have shown that bromhexine undergoes rapid and extensive metabolism.

These compounds are not approved for use in the USA and have not been encountered in previous screenings of race horse samples. During a routine screening of urine samples from racing horses, two unknown dibrominated compounds were detected. This study was therefore, undertaken to develop a simple and sensitive screening procedure for identifying and differentiating bromhexine from ambroxol administered to horses, using thin-layer chromatography (TLC) for screening; and gas chromatography–mass spectrometry (GC–MS) for confirmation.

Furosemide is commonly used for the control of exercise-induced pulmonary haemorrhage (EIPH) in race horses [6]. Certified EIPH horses racing in Pennsylvania are treated with furosemide 4 h prior to race time. The general concern over the use of the diuretic, furosemide, is its ability to dilute urinary analytes and therefore, potentially mask the presence of other drugs [7]. For this reason, it was necessary to determine whether the administration of furosemide with bromhexine or ambroxol in horses masks the

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presence of these agents or alters their metabolite profile to such an extent as to render these agents undetectable in the horse urine.

Materials and Methods

Administration of bromhexine or ambroxol

Female horses (450–550 kg) were used in this study. Control urine samples were collected prior to drug administration. A gram of bromhexine or ambroxol was administered orally to horses by adding the drug to oats mixed with molasses. Each horse was observed until all the drug-feed was consumed. Samples were collected hourly for 12 h and at 24, 36 and 48 h via an indwelling urinary catheter (24F). During the study, each horse was allowed free access to food and water.

Administration of furosemide and bromhexine

This portion of the study was designed to determine the effect of furosemide on the detection of bromhexine or ambroxol in urine. For this purpose, three administration schedules were used: (1) 300 mg of furosemide administered intravenously (i.v.) 60 min after oral administration of bromhexine, (2) simultaneous administration of bromhexine and furosemide, and (3) oral administration of bromhexine 180 min after the administration of furosemide.

Preparation of Samples for Screening Using TLC

Urine samples were prepared by an enzyme hydrolysis (EH) procedure. A 5-ml aliquot of urine was combined with 1 ml of *Patella vulgata* (beta-glucuronidase, 5K Fishman Units per ml, Sigma) and 3 ml of saturated phosphate buffer (pH 4.5). The mixture was then incubated in a 65°C water bath for 3 h. Following enzyme hydrolysis, 1 ml of ammonium hydroxide (14%), 200 µl of ascorbic acid (10%), and 5 ml of dichloromethane–isopropanol (10:1) were added. Samples were then mixed for 10 min and centrifuged at 5000*g* for 5 min. The aqueous supernatant fraction from each sample was discarded by aspiration, 3 ml of 1 N sulphuric acid was added and processed as described above. The resultant aqueous supernatant fraction was transferred to a clean test tube. To this aqueous supernatant, 1 ml of ammonium hydroxide (14%), 100 µl of ascorbic acid (10%), and 5 ml of dichloromethane–isopropanol mixture (10:1) were added and re-extracted as described above. The aqueous supernatant fraction was discarded and the organic solvent was transferred to a clean test tube and evaporated at 65°C. The sample residue was then resuspended in 20 µl of dichloromethane and spotted on EM Merck high performance TLC (HPTLC) plates with a baseline to solvent front distance of 5 cm. The sample spotted-HPTLC plate was developed in chloroform–methanol–propionic acid (72:18:10) and visualized with the aid of Modified Ehrlich's (ME; 0.1% *p*-dimethylaminocinnamaldehyde) reagent or Dragendorff/sodium nitrite overspray [D/SN; D = bismuth subnitrate (2 g), glacial acetic acid (25 ml), and distilled water (100 ml) A: potassium iodide (40 g) and distilled water (100 ml) B. Combine 10 ml of A and B with 20 ml of glacial acetic acid and 100 ml of distilled water. SN = 5 g of $NaNO_2$ + 100 ml of distilled water].

Preparation of Sample-HPTLC Plates for Scanning

EH extracts were spotted on HPTLC plates and developed in 72:18:10 with a solvent front distance of 7 cm. Plates were scanned from 1 to 7 cm at 200–360 nm using Camag TLC Scanner II interfaced to a Hewlett–Packard Vectra ES Computer. Scan integration was narrowed to peaks between 1.5–4.5 cm for ambroxol and 1.5–5.0 cm for bromhexine. This narrowing of peaks was necessary to identify, integrate and spectrum scan those spots specific to the drug extracts only.

Preparation of Samples for Confirmation Using GC–MS

A 60-ml aliquot of the urine sample was extracted as described above for GC–MS analyses. The sample residue was resuspended in dichloromethane and streaked on HPTLC plates, developed in 72:18:10 and visualized as described above. The authentic drug standard was prepared in the control urine and similarly extracted. The presence of the primary amino group in these agents allowed the use of specific developing agents as an aid in visualizing the HPTLC "fingerprint" for the respective drug administrations. Fluorescamine was used in developing the HPTLC plate for UV fluor-

escence examination at 366 nm prior to using a non-specific overspray (D/SN) or a specific visualizing agent (ME).

Analyses of Samples on GC–MS

Two types of analyses were conducted on GC–MS (Hewlett-Packard, model 5985). First, unsprayed HPTLC zones corresponding to ME visualized zones were eluted from the silica gel using HPLC grade methanol and then evaporated. Second, crude EH extracts were injected without prior separation of the components on HPTLC. A 4-μl aliquot of the sample in HPLC grade ethyl acetate was introduced into a GC–MS by splitless injection and chromatographed using a 30 m × 0.3 mm i.d. fused-silica DB-1 capillary column (bonded phase equivalent of OV-1). This was interfaced directly to the ion source of the mass spectrometer and programmed from 120°C to 280°C at 5°C min^{-1} with an injection port temperature of 240°C. The carrier gas was helium at a flow rate of 1 ml min^{-1}. EI mass spectra were obtained with a source temperature of 280°C.

Verification of Results Using Blind Samples

In order to evaluate the reproducibility of the results and the sensitivity of these screening and confirmation procedures, "blind" samples were utilized. To obtain "blind" samples, horses were treated with the same dose of the agents and sample collected. The samples were indistinguishably labelled and intermingled with the official race samples for routine drug screening. It should be emphasized that the screening laboratory had no prior knowledge of either the treatment of the horses or the inclusion of a bromhexine or ambroxol blind sample in a set of the official race samples. By totally "blinding" the laboratory out of this phase of the study, we were better able to provide an unbiased evaluation of the sensitivity of the screening procedure employed and the results obtained.

Source of Drugs

Bromhexine and ambroxol, in their hydrochloride salts each with 99.8% purity, were generously supplied by Boehringer Ingelheim (FRG). Additional bromhexine hydrochloride was commercially obtained from Sigma Chemical Company (USA).

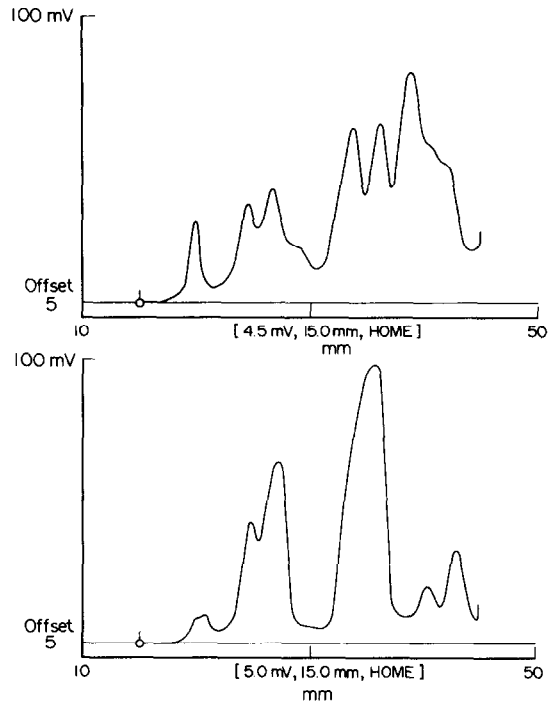

Figure 1
Full spectrum scan of the unsprayed HPTLC plates of bromhexine (top panel) and ambroxol (bottom panel). The plates were prepared using urine sample extracts from bromhexine- and ambroxol-treated horses. Each plate was developed in 72:18:10, dried and scanned without spray reagents on the plate using Camag Scanner (II).

Results

Figure 1 shows the chromatograms for bromhexine (upper panel)- and ambroxol (lower panel)-treated horse urine samples. Samples were extracted and spotted on HPTLC plates, developed in 72:18:10 and scanned without visualizing overspray. However, on sprayed plates, the reaction to Ehrlich's reagent spray on the separated zones was a characteristic rose-pink colour matching that of the standard drug. The samples prepared from ambroxol-treated horse urine (lower panel) showed three major zones, of which two zones were easily resolved by HPTLC. The bromhexine-treated horse samples (upper panel) showed at least four easily resolved major zones. These patterns of separation form the basis for a presumptive qualitative identification, as well as an easily recognizable means of discriminating urinary metabolites of the two compounds when administered to horses. The separation patterns were unique to each drug administration and were consistently observed in all samples post-drug administration, and their

integrity was not compromised by the administration of furosemide.

The respective R_f values and the molecular ions for the principal HPTLC zones obtained from ambroxol- and bromhexine-treated horse urine samples are summarized in Table 1. By GC–MS analysis of crude enzyme hydrolysis extracts, hydroxy-bromhexine (Fig. 2, bottom right panel) and desmethyl-bromhexine (not shown) metabolites, were observed to be unique to bromhexine-treated horse. These urinary metabolites provide a chemical means to distinguish bromhexine- from ambroxol-treated horse urine.

HPTLC plates were also prepared using urine samples collected from horses administered either bromhexine or ambroxol before or after furosemide administration. Compared with the standard, a decrease in the density of the sample spot was observed which was consistent with the expected drug dilution resulting from furosemide-induced diuresis. However, the diuresis did not mask the presence of either bromhexine or ambroxol "fingerprints" to such an extent as to render their presence undetectable or indistinguishable in the horse urine.

The chemical structures and the corresponding mass spectra of bromhexine (upper left panel), ambroxol (upper right panel), and two minor bromhexine metabolites, desbromo-bromhexine and deshexyl-bromhexine, characterized by a molecular weight of 298 *m/z* (lower left panel) and 306 *m/z*, respectively (lower right panel) are shown in Fig. 3. Ambroxol (378 *m/z*) is chemically identical to desmethyl-hydroxy-bromhexine which was detected as one of the major bromhexine metabolites in horses. Bromhexine administration in horses results in at least nine possible bromhexine metabolites in urine as observed from full spectrum scan of the unsprayed HPTLC plates. The results of the GC–MS analyses of the HPTLC spots resulting from extracts of ambroxol-treated horse urine show that when the HPTLC zone 1 with reaction to ME spray was eluted and analysed, a merged GC peak was detected consisting of two distinct compounds with spectra and proposed chemical structures shown in the upper and lower left panels (Fig. 2). The proposed chemical structures and mass spectral characteristics of the two major ambroxol metabolites (methylene-dioxy-ambroxol [dien-one] and hydroxy-ambroxol, upper and lower left panels, respectively) and those of the minor (bromhexadienone, upper right panel) and the major (hydroxy-bromhexine, lower right panel), bromhexine metabolite suspects, are shown in Fig. 2. The silica-gel scrapings from zone 3 on the bromhexine-HPTLC plate when eluted with methanol produced a mass spectrum with an apparent molecular weight of 386 *m/z* (bromhexadienone) as well as those matching *N*-desmethyl-hydroxy-bromhexine (ambroxol).

Selected Ion Monitoring (SIM)

Since the cyclohexyl moiety fragment peak of ambroxol was found to be 114 *m/z* and that of hydroxy-bromhexine to be 128 *m/z*, the relative abundances of these two ions at similar elution time were monitored. The 128/114 ion ratio of 1:1 to 2:1 is indicative of bromhexine administration while 128/114 ion ratio of 1:10 indicates ambroxol administration. These values were based on reconstructed ion chromatograms derived from EI full spectrum scans of enzyme hydrolysis extracts without prior HPTLC separation.

Discussion

Most studies on the assay of bromhexine have been confined to human plasma [4, 8–10]. This study is the first of its kind to describe a simple, sensitive, and inexpensive method for screening and confirmation of bromhexine and ambroxol in the horse urine. The methods described in this study allow the metabolites of bromhexine and ambroxol in the horse to be readily identified and differentiated. The extensive metabolism of bromhexine reported in this study appears to be similar to what has been reported in humans [4] and rabbits [5]. Our observations on the extensive metabolism of bromhexine agree with those of other investigators [4, 5, 8] who have shown that bromhexine is rapidly and extensively metabolized and that the multiple bromhexine metabolites result from hydroxylation of the cyclohexyl ring, *N*-demethylation and cyclization. However, the distinct difference in the possible metabolites we have predicted in horses as opposed to those in other species [4, 5, 8] is the observation of desbromo-bromhexine and bromhexadienone (Figs 2 and 3). The metabolite resulting from cyclization with ambroxol administration in horses is also dif-

Table 1
The R_f* value and molecular ion for ambroxol and bromhexine metabolites in horse urine

Treatment/Metabolite	R_f value	Molecular ion (*m/z*)
Ambroxol (1 g, p.o.)		
Hydroxy-ambroxol	0.30	394
Methylene-dioxy-ambroxol	0.33	402
Ambroxol	0.50	378
Bromhexine (1 g, p.o.)		
Hydroxy-bromhexine	0.40	392
Ambroxol	0.50	378
Bromhexadienone	0.63	386
Desmethyl-bromhexine	0.90	362

*Ratio of the distance from the origin of the samples on the TLC plate to the mid-point of the separated zone divided by the distance from the origin of the sample solvent front line.

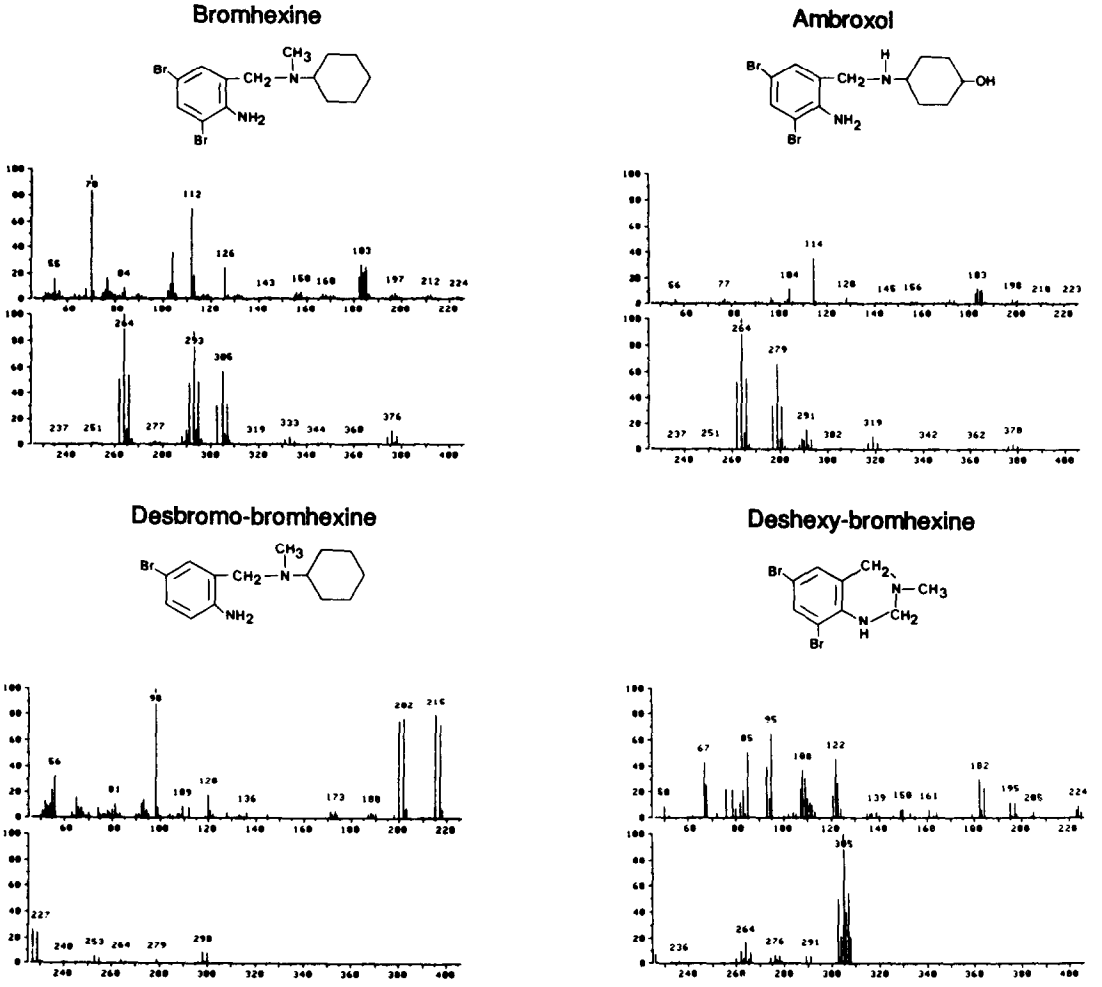

Figure 2
The chemical structures and mass spectral characteristics of bromhexine (top left panel) and ambroxol (top right panel) prepared in control urine samples and extracted as described under Methods and Materials. The bottom (left and right) panels show the chemical structures and spectral characteristics of two minor bromhexine metabolites (298 *m/z* and 306 *m/z*). These minor bromhexine metabolites were obtained from zone 4 on the HPTLC plate for bromhexine-treated samples. No major GC peaks besides the minor components (306 *m/z*, 298 *m/z*) were produced.

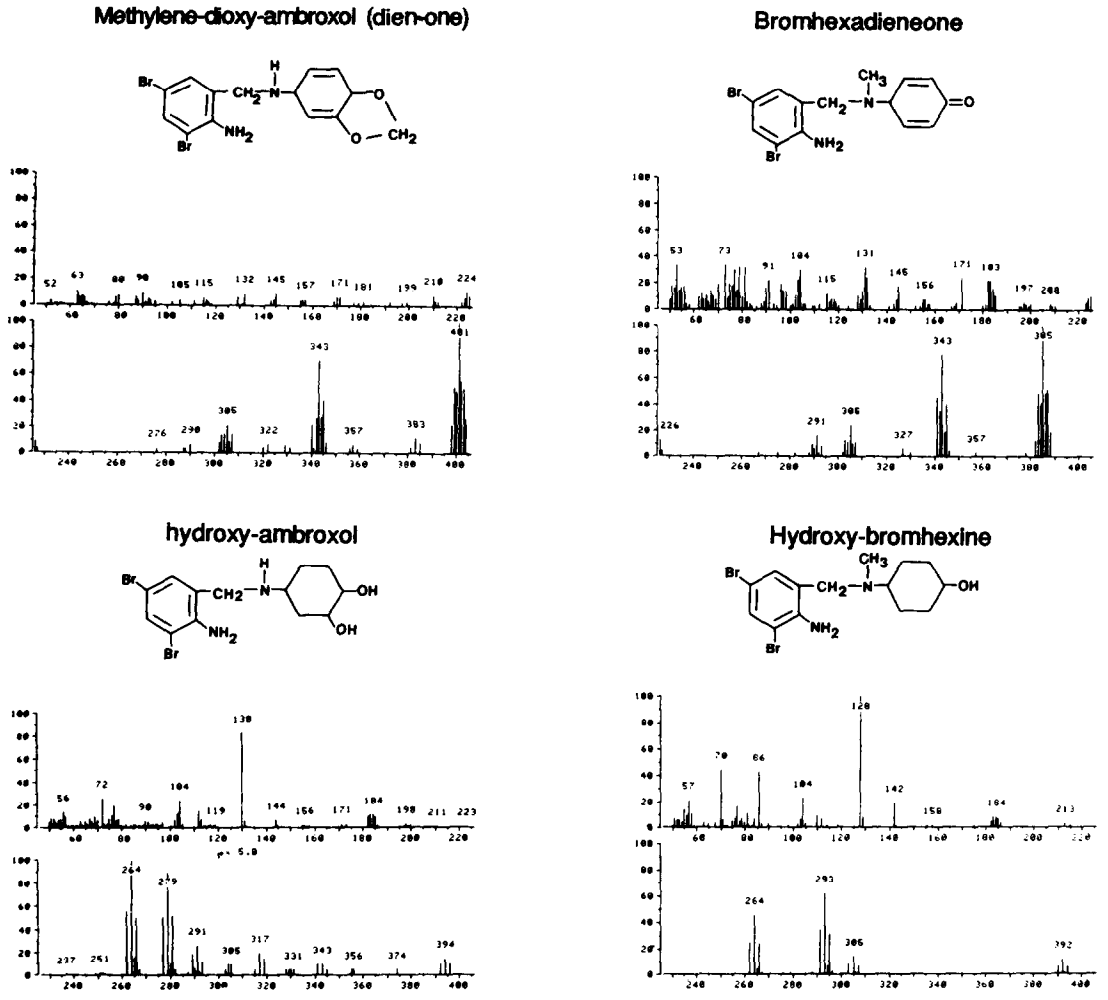

Figure 3
Top left panel is the proposed chemical structure of methylene-dioxy-ambroxol (402 *m/z*) and its characteristic mass spectrum. Top right panel is the proposed chemical structure and spectral characteristics of bromhexadienone (386 *m/z*). Bottom left panel is the chemical structure and mass spectrum of hydroxy-ambroxol (394 *m/z*) observed as ambroxol-specific metabolite. Bottom right panel is the chemical structure and mass spectrum of hydroxy-bromhexine (392 *m/z*) which was observed as a bromhexine-specific metabolite.

ferent from that in other species [4, 5]. The chemical structures of the multiple metabolites of bromhexine and those of ambroxol detected in the horse urine were proposed, based primarily on comparing the mass spectral characteristics of the drug standards with those of the observed products (metabolites). In examining the proposed structures of these metabolites, it is important to note that besides ambroxol, none of the other identified metabolites of bromhexine was available for confirmational or specificity studies. By application of the methods described in this study, bromhexine and ambroxol-specific metabolites in the "blind" horse urine samples were similarly identified and confirmed.

Bromhexine metabolites have been detected in human plasma samples collected post-bromhexine administration and stored frozen at −70°C for 60 days. In this study, bromhexine metabolites were detected in unfrozen urine samples up to 48 h post-administration and as late as 120 days in frozen samples. From the identifiable sample spot on HPTLC, a valid inference as to the detection of bromhexine specific metabolites at times greater than 48 h post-administration of bromhexine (1 g, p.o./horse) can be made. Whether the plasma concentration of bromhexine detected after 48 h following administration of 1 g, p.o., is of any pharmacologic significance or merely a representation of the residual plasma concen-

tration is not known. Based on the lipophilic character of the drug and the large volume of distribution, very low plasma levels of the drug can be expected with increase in time following drug administration. Detection of these agents in plasma can be tedious because the lower the blood levels of the drug, the greater the chance of interference produced by endogenous compounds of various origins [4]. Although the focus of this study was on urine samples, plasma samples at 4-h post-administration were also screened for bromhexine, ambroxol, and/or their metabolites. Identifiable levels of the parent drugs and their metabolites were detected in plasma samples but were not further studied.

Bromhexine is a lipophilic weak base with pK_{a_1} 8.5 (benzylamino group) and pK_{a_2} −0.3 [4]. The partition behaviour of bromhexine is pH dependent and, thus, well-resolved chromatographic patterns are also dependent on the extraction methods. Extraction of bromhexine at a pH of 4.5 as opposed to 5.2 [4, 9] did not significantly affect bromhexine or the characteristics of the metabolites. However, it is not known to what extent, if any, the recovery of bromhexine was affected since quantitation of bromhexine was not required. The chromatographic behaviour of free bromhexine is due to both amino groups which are sterically shielded and because the anilino group has a weak basic character [4]. In this study, bromhexine and its metabolites were detected underivatized. Other investigators [4, 10] have also demonstrated that bromhexine can be separated without derivatization.

Ambroxol is the only pharmacologically active metabolite of bromhexine which is commercially available [1]. Clinically, ambroxol is not as widely used as bromhexine. Data from this study have also shown that ambroxol, in horses, is not metabolized as extensively as bromhexine. However, with the use of the screening procedures presented in this study, ambroxol and its hydroxylated metabolites were also readily identified.

The concomitant presence of furosemide with bromhexine did not interfere with or diminish the sensitivity of the screening procedures described in this study. The diuretic effect of furosemide on the urinary concentration of bromhexine-specific metabolites was evident particularly at the time-of-peak effect of furosemide-induced diuresis. Our data indicate that the presence of furosemide in bromhexine-treated horses should be a matter of concern only when drug quantitation is required. Administration of bromhexine after the time-of-peak effect of diuresis by furosemide is more likely to reflect the true urinary concentration of bromhexine than that which is administered less than 60 min before furosemide.

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