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The isomeric metabolites of doxepin in equine serum and urine

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Abstract

Due to its tranquilizing properties, the tricyclic antidepressant doxepin may be misused as a doping agent in competition horses. Therefore, efficient analytical procedures are required to detect this drug in samples submitted for doping control. To screen for parent doxepin in equine blood and urine, a less specific method has been accepted employing gas chromatography (GC) combined with electron impact (EI) mass spectrometry (MS). The aim of this study was identification of doxepin metabolites providing more specific MS data to verify positives resulting from screening. Thus, after a horse was given doxepin–HCl (1 mg/kg, i.v.), blood and urine were analyzed for free or conjugated metabolites using GC combined with EI- and positive chemical ionization (PCI) MS. In both of the sample materials, *cis*- and *trans*-isomers of desmethyldoxepin were detected for up to 48 h after treatment using trifluoracetylation and GC/EI-MS. Following enzymic hydrolysis of urine and propionylation of extracts, each four isomers of hydroxy desmethyldoxepin and hydroxydoxepin were recovered for up to 24 and 48 h, respectively. These compounds were characterized by their EI- and PCI-mass spectra. Although distinct positions of the hydroxyl groups could not be determined, the presence of each two *cis*/*trans*-isomeric pairs of differently monohydroxylated metabolites may be assumed. Results reported here suggest, that screening horses for parent doxepin should be completed by analysis of its major isomeric metabolites, desmethyldoxepin and hydroxydoxepin, providing MS data specific enough for confirmatory analysis.

Keywords: Doxepin; Antidepressants; Metabolism; Horse; Doping in sports; Mass spectrometric analysis

1. Introduction

Doxepin (Fig. 1; **I**), a tricyclic drug of the dibenzoxepin type, represents a 1:5 mixture of *cis*/*trans*-isomers and has been used in humans as an antidepressant [1]. Like other drugs possessing tranquilizing properties [2,3], this compound may be given to horses even at subtherapeutic doses in order to calm excited or inattentive individuals. However, with respect to competition horses, doxepin has been classified as a banned drug, and hence, is an important target for doping control. Therefore, sensitive analytical techniques as well

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as knowledge on equine doxepin kinetics and metabolism are needed to reliably detect administration of this drug to sport horses.

Recently, a screening procedure was communicated to test equine body fluids for unchanged doxepin isomers [4]. The method made use of GC/MS operating in the selected ion monitoring (SIM) mode at m/z 58. Unfortunately, analytical selectivity of this technique is limited and appears less suitable for verification analysis. This drawback caused us to focus on the biological transformation of doxepin in the horse, as identification of metabolites may provide more specific MS data for confirmation analysis, give additional evidence of illegal drug use, and/or extend detection times.

Although nothing has been reported on doxepin metabolism in the horse, biological fate of this antidepressant should be predictable from theoretical considerations. In particular, these are based on known doxepin metabolites in other species and on equine metabolism of chemically related drugs. For instance, desmethyldoxepin (Fig. 1; **II**) is the main metabolite in blood of rats [5] and humans [6]. Furthermore, in urine of rats and dogs, desmethyldoxepin, hydroxydoxepin, hydroxy desmethyldoxepin (Fig. 1; **II**, **III**, **IV**), and their glucuronides were identified [5]. The chemically related antidepressant amitriptyline is metabolized in the horse to yield desmethylamitriptyline and glucuronides of the hydroxylated drug, all excreted in urine [7].

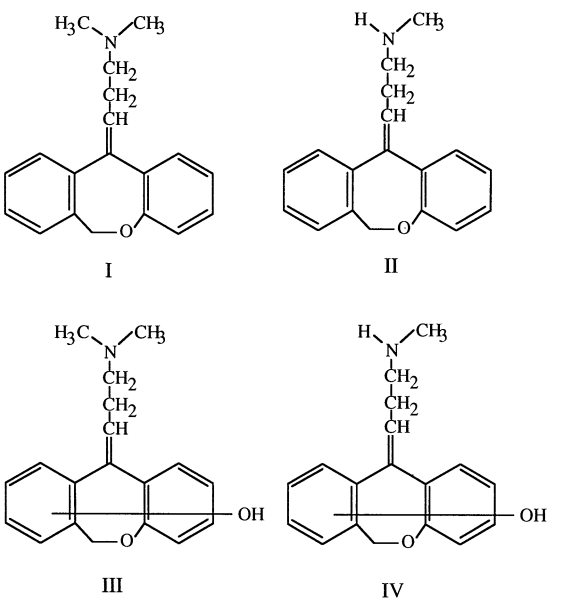

Fig. 1. Principle structures of doxepin (**I**) and its equine metabolites comprising *cis*- and *trans*-configurations. **II**: desmethyldoxepin; **III**: hydroxydoxepin; **IV**: hydroxy desmethyldoxepin. In **III** and **IV**, positions of the hydroxyl groups are uncertain.

In order to verify these considerations with regard to doxepin metabolism in the horse, suitable analytical procedures must be selected for identification of candidate metabolites. Because GC/MS has been shown to be a powerful means to analyse parent doxepin in blood of humans and animals [8,9], this technique was also used to identify metabolites of doxepin in various biological materials such as urine, blood, and hair [5,10,11]. Besides EI-MS, yielding poor mass spectral data of doxepin itself [12], chemical ionization mass spectrometry (CI-MS) has been suggested to determine molecular weight of metabolites like desmethyldoxepin [13].

The present study informs about the detection of doxepin metabolites in equine blood and urine following intravenous drug administration. GC combined with EI- and PCI-MS was used to confirm the presence of particular metabolites which may also be expected on theoretical grounds. Data presented here are suitable to confirm illicit doxepin administration to competition horses by use of sample material which is normally submitted for drug control.

2. Materials and method

2.1. Chemicals

Organic solvents, acetic acid (99%), ammonia (25%), all analytical grade, and solid phase extraction columns LiChrolut TSC (300 mg) were from Merck (Darmstadt, Germany). Trifluoroacetic anhydride, propionic anhydride, and β-glucuronidase from Helix pomatia (10^5 U/ml) were supplied by Sigma (Deisenhofen, Germany). Doxepin–neuraxpharm® (45.22 mg doxepin–HCl/ml) was from neuraxpharm Arzneimittel (Langenfeld, Germany).

2.2. Animal treatment

One female trotting horse (450 kg, 5 years) was given Doxepin–neuraxpharm® (doxepin–HCl, 1 mg/kg, i.v.). Blood was taken from the contralateral V. jugularis and urine was obtained by catheterization. Samples were taken in intervals (1 h) for up to 48 h after treatment. Urine and serum were stored at −30 °C until analysis.

2.3. Instrumentation

Gas chromatography was conducted by means of a Hewlett–Packard GC 6890 (capillary column HP-1, 12 m × 0.2 mm inner diameter, 0.33 μm film thickness, cross-linked methyl siloxane). The injector and transfer line temperatures were set to 280 °C. Automatic pulsed splitless injections (1 μl) were performed by an autosampler Hewlett–Packard 7683 with the oven at 90 °C. The temperature was stepwise increased to 200 °C (30 °C/min), subsequently to 280 °C (10 °C/min). Final time was 6 min. Helium served as carrier gas using constant flow conditions (0.6 ml/min). The GC was interfaced to a mass spectrometer (HP 5973 MSD) equipped with a computerized work station (HP ChemStation, version B.01.00, Windows NT 4.0).

In the EI mode, the instrument was tuned daily with perfluorotributylamine according to the specifications of the manufacturer. Electron energy was 70 eV. Transferline temperature was set to 280 °C. Full scan mass spectra were acquired by scanning the 50–650 amu range. SIM was performed with m/z 361, 234, and 165. Positive chemical ionization was used with methane as the reagent gas. Under these conditions, temperatures of transfer line, MS quadrupole, and ion source were 320, 150 and 250 °C, respectively. Source pressure was 1.3×10^{-2} Pa. Full scan PCI-mass spectra were obtained by scanning the 50–650 amu range.

2.4. Sample preparation

2.4.1. Urine

Urine was centrifuged ($3000 \times g$, 15 min), the pH adjusted to 9.6 ($NaHCO_3/Na_2CO_3$, 2:1, w/w), and 3 ml sample extracted with diethyl ether (6 ml). While the aqueous phase was left for enzymic hydrolysis, the organic solvent was evaporated under reduced pressure (speed Vac concentrator) and the residue dissolved in 300 μl trifluoroacetic anhydride/acetonitrile (5:95, v/v). After 30 min at room temperature, the solution was evaporated, the residue dissolved in 30 μl dichloromethane/toluene (9:1, v/v), and 1 μl submitted to GC/EI-MS. The aqueous phase was adjusted to pH 5.2 (4 M H_3PO_4), mixed with 60 μl β-glucuronidase solution and incubated overnight (37 °C). After adjustment of pH to 2 (4 M H_3PO_4), the solution was extracted with diethyl ether (6 ml), the organic phase discarded, and the aqueous phase readjusted to pH 10 (4 M NaOH). Following extraction with diethyl ether (6 ml), the organic solvent was evaporated, the residue dissolved in 60 μl propionic anhydride/pyridine (1:2, v/v), the solution heated in a sand bath at 60 °C (30 min), and evaporated under reduced pressure. The residue was dissolved in 30 μl dichloromethane/toluene (9:1, v/v), and each 1 μl was submitted to GC/EI-MS and GC/PCI-MS.

2.4.2. Serum

Three milliliters of serum was adjusted to pH 6 (1 M acetic acid) and placed on a TSC extraction column which was conditioned previously with methanol, water and 1 M acetic acid (each 3 ml). After passing the sample, the column was washed with water, 1 M acetic acid, 50% methanol and 100% methanol (each 3 ml). The column was air dried (10 min) and then eluted with 2% ammonia (v/v) in dichloromethane/2-propanol (3:1, v/v; 3 ml). The solvents were evaporated, and the residue was transferred with diethyl ether (250 μl) in an autosampler vial. Following evaporation of the solvent, the residue was trifluoroacetylated as described above and 1 μl analyzed by GC/EI-MS.

3. Results

3.1. Detection of desmethyldoxepin isomers

Full scan EI-mass spectra of the *N*-trifluoroacetylated *cis*/*trans*-isomers of desmethyl-

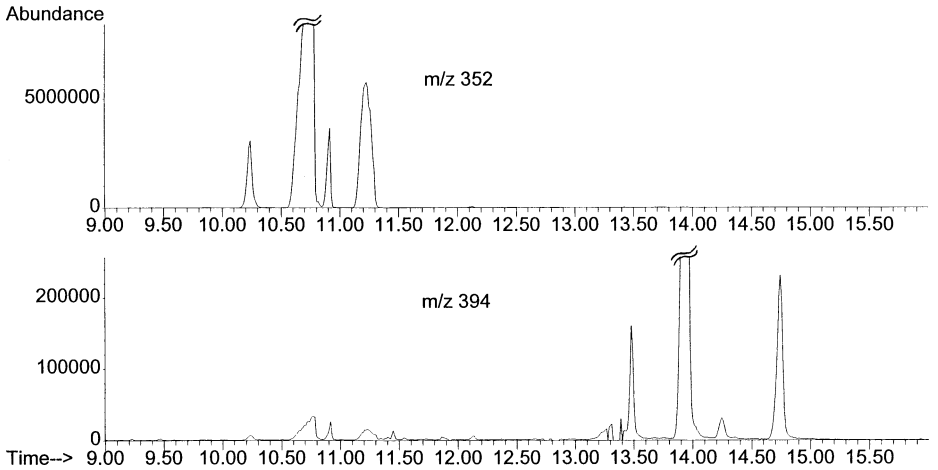

Fig. 2. Selected ion chromatograms (GC/PCI-MS) obtained from hydrolyzed urine 24 h after treatment of a horse with doxepin–HCl (1 mg/kg, i.v.). Four isomers of propionylated hydroxydoxepin (**III**) were detected by monitoring m/z 352 ($M^+ + 1$, top, 10.23, 10.77, 10.92, 11.24 min). Another set of four signals at m/z 394 ($M^+ + 1$, bottom, 13.48, 13.96, 14.24, 14.74 min) refers to isomers of propionylated hydroxy desmethyldoxepin (**IV**). Positions of hydroxylation and identity of cis/trans-isomers could not be determined.

doxepin were virtually identical and corresponded to previously published data [10]. Thus, characteristic mass peaks at m/z 361, 234, and 165 (EI-MS, SIM) were used to monitor *N*-trifluoroacetyl desmethyldoxepin [9] in serum and urine of a horse treated with doxepin. Because authentic desmethyldoxepin was not available in this study, quantification of the isomeric metabolites was omitted.

The *cis*- and *trans*-isomers of desmethyldoxepin were detected in horse serum for up to 48 h after administration of doxepin–HCl (1 mg/kg, i.v.). Chromatographic peaks of the isomers were well resolved, and their retention times were 9.8 (*cis*) and 9.6 min (*trans*). Integration of m/z 234 (mass spectral base peak) revealed maximum levels of the analytes at 3 and 8 h after treatment. Furthermore, an attempt was made to determine the ratio of the *cis*/*trans*-isomers using the peak area ratio of the ion at m/z 234. Interestingly, this ratio appeared to be relatively constant during the observation period (48 h) and amounted to 1: (3.48 ± 0.57) $(n = 10)$.

Following trifluoracetylation, the cis/trans-isomers of desmethyldoxepin were also recovered in unhydrolyzed urine for up to 48 h after doxepin treatment using GC/EI-MS (SIM). Retention times corresponded to those obtained for serum. Integration of m/z 234 indicated maximum analyte concentrations at 3, 8 and 24 h after treatment. During the observation period, ratio of the isomers (*cis*/*trans*) amounted to 1: (24.44 ± 2.41) $(n = 10)$.

3.2. Detection of hydroxylated doxepin metabolites in urine

After enzymic hydrolysis of administration urine and propionylation of extracts, two principal groups of metabolites were detected by GC combined with EI- and PCI-MS. The first group, consisting of four components (10.2, 10.8, 10.9, 11.2 min), was detected 1–48 h following drug administration. Full scan EI-mass spectra of these metabolites were each dominated by base peaks at m/z 58 ($(CH_3)_2NCH_2^+$), suggesting an intact side chain present in the four compounds. Eventually, a very low abundant mass peak was observed at m/z 351 (ca. 1%, relative abundance). PCI-mass spectra of all of the four compounds revealed base peaks at m/z 352 representing the $M^+ + 1$ ions of propionylated hydroxydoxepin. In the extracted ion chromatograms (Fig. 2; m/z 352, top), low- and high-abundant peaks appeared in turn and

had area ratios of 1: (20.90 ± 13.95): (1.80 ± 0.92): (7.90 ± 5.30) in the samples collected between 1 and 48 h after treatment ($n = 10$). PCI-mass spectra of these metabolites (Fig. 3) were completed by typical ions at m/z 380 and 392 as a result of adduct formation ($M^+ + C_2H_5$ and $M^+ + C_3H_5$), thus, confirming the molecular weight of 351. Intense ions at m/z 58 (> 30%, relative abundance) were also present. No information could be obtained on the position of hydroxylation. Furthermore, chromatograms were searched for m/z 424, 496, and 568 representing putative $M^+ + 1$ ions of per-propionylated di-, tri- and tetrahydroxy doxepin. However, there was no evidence for such metabolites.

The second group consisted of another four metabolites (13.5, 14.0, 14.3, 14.7 min) which were detected 3–24 h following application of doxepin. Full scan EI-mass spectra of these compounds were consistent with *N*,*O*-bis-propionyl derivatives of hydroxy desmethyldoxepin and all exhibited prominent ions at m/z 393 (M^+), 306 ($M^+ - CH_3NHCOC_2H_5$), 250 (306-$COCHCH_3$), 237 ($M^+ - COCHCH_3 - CH_2NCH_3COC_2H_5$), 100 ($^+CH_2NCH_3COC_2H_5$), and 57 ($^+COC_2H_5$). No signal was observed at m/z 58. PCI-mass spectra of these four compounds each displayed base peaks at m/z 394, also represented in the extracted ion chromatograms (Fig. 2; m/z 394, bottom). Low- and high-abundant peaks were separated in turn and had area ratios of (3.28 ± 0.50): (39.17 ± 15.50): 1: (8.57 ± 1.67) in the samples collected between 3 and 24 h after treatment ($n = 7$). Additional ions appeared at m/z 422 and 434 (Fig. 4). The PCI-mass peaks in question were referred to the $M^+ + 1$, $M^+ + C_2H_5$ and $M^+ + C_3H_5$ adducts, by this way confirming a molecular weight of 393. No signals of any poly-hydroxylated desmethyldoxepin were detected when searching the chromatograms for corresponding $M^+ + 1$ peaks at m/z 466, 538 and 610. Again, positions of hydroxylation could not be deduced from EI- or PCI-mass spectrometry.

4. Discussion

Due to its tranquilizing properties, the tricyclic antidepressant doxepin is likely to be misused as a doping compound in horses. Consequently, competition horses should be controlled for this drug employing suitable analytical methods. It was shown, that such methods may include selection of appropriate specimen and screening for doxepin isomers using GC/EI-MS [4]. Thus, because of lower concentrations in urine, only serum was recommended to detect the parent isomers of doxepin in the horse [4]. In this study, serum and urine of a treated horse were tested for doxepin metabolites, which can be used to confirm appli-

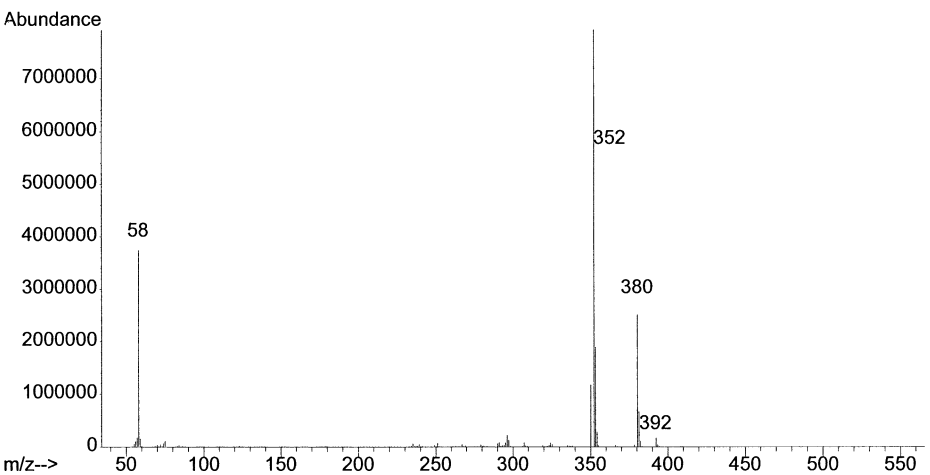

Fig. 3. PCI-mass spectrum of a propionylated isomer of hydroxydoxepin obtained from the peak at 10.77 min (m/z 352, Fig. 2).

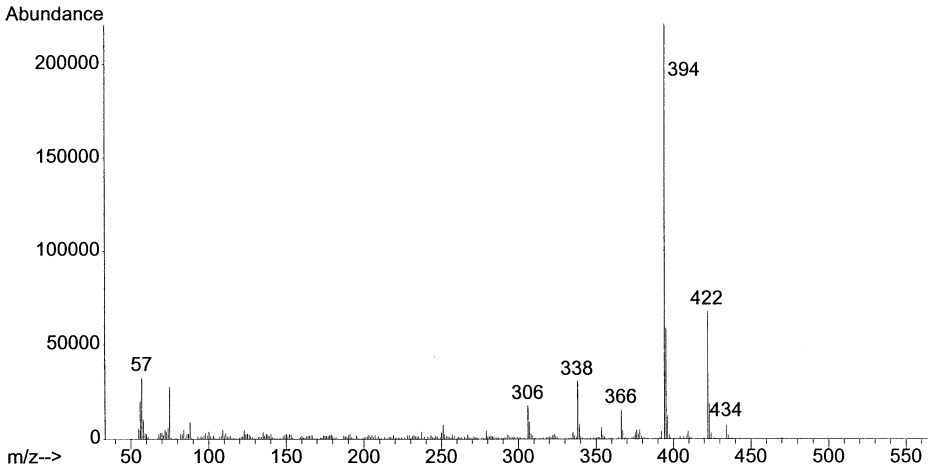

Fig. 4. PCI-mass spectrum of a propionylated isomer of hydroxy desmethyldoxepin obtained from the peak at 13.96 min (m/z 394, Fig. 2).

cation of the antidepressant to sport horses subjected to doping control.

Previously, desmethyldoxepin was reported to be an unconjugated metabolite of doxepin in various species [14]. Its antidepressant activity was found similar to that of doxepin itself [15]. Because parent doxepin constitutes a mixture of *cis*- and *trans*-isomers [5], desmethyldoxepin has actually been found in form of its two isomers [13]. For instance, these compounds were monitored in human blood by means of GC/MS (SIM) after derivatization with trifluoroacetic anhydride [9]. In this study, the same technique was used, and serum and urine of a horse were screened for desmethyldoxepin isomers following drug administration (doxepin–HCl, 1 mg/kg, i.v.). In fact, both isomers were detected for up to 48 h at constant ratios in each of the body fluids. In serum, ratio of desmethyldoxepin isomers was found to be 1:3.5 (*cis*/*trans*), which corresponds roughly to the ratio of parent doxepin isomers (1:4.7) detected for 24 h after drug administration [4]. In contrast to previous results obtained in humans [16], concentrations of the desmethyldoxepin *cis*-form never exceeded those of the *trans*-form. In urine, desmethyldoxepin isomer ratio was 1: 24. Obviously, the *trans*-isomer was preferably eliminated from the horse as compared to the *cis*-form. Again, a relative enrichment of the *cis*-isomer was observed in human urine possibly resulting from interconversation between the isomers [16].

Since aromatic-ring hydroxylation followed by glucuronidation is a known biological transformation of doxepin in other species [5], extracts of hydrolyzed equine administration urine were propionylated and examined for corresponding hydroxydoxepin derivatives. Using GC/EI-MS, four compounds were separated which had base peaks at m/z 58. This prevalent fragment derives from cleavage of the dimethylaminopropyl chain. Due to the lack of other diagnostic ions, EI-MS was unable to better resolve structural details of propionylated hydroxydoxepin isomers. Molecular weights (351) of these four derivatives, however, were confirmed by GC/PCI-MS.

Additionally, another metabolic pathway of doxepin was examined, which relates to combined demethylation and hydroxylation followed by glucuronidation [5]. In propionylated extracts of hydrolyzed administration horse urine, four compounds were detected exhibiting molecular ions at m/z 393 (EI-mass spectrum). No mass peak was found at m/z 58, thus, pointing to metabolic modification of the side chain. Fragmentation pattern deduced from these EI-mass spectra was consistent with *N*,*O*-bis-propionyl derivatives of

hydroxy desmethyldoxepin. When these metabolites were analyzed with PCI-MS, their molecular weight of 393 was actually confirmed by a series of typical ions.

With respect to the isomers of hydroxydoxepin and hydroxy desmethyldoxepin detected in horse urine, neither individual positions of the hydroxyl groups nor identity of *cis*- and *trans*-forms could be determined from the present mass spectrometric data. As a preliminary interpretation, each two pairs of *cis*/*trans*-forms with two different sites of aromatic-ring hydroxylation [5] should be acceptable. On the other hand, only *trans*-2-hydroxydoxepin and *trans*-2-hydroxy desmethyldoxepin were detected in human urine, while the corresponding *cis*-forms were missing [16]. Moreover, in human microsomal incubation mixtures, apparently no hydroxylated metabolites were obtained from the *cis*-forms of doxepin and desmethyldoxepin [17]. If this was true with respect to equine doxepin metabolism, the presence of even four differently hydroxylated isomers of *trans*-hydroxydoxepin and *trans*-hydroxy desmethyldoxepin must be postulated. Additional work is required to elucidate this problem.

In principle, isomeric mixtures of three major doxepin metabolites were detected in equine body fluids: desmethyldoxepin (serum, urine), hydroxydoxepin (urine), and hydroxy desmethyldoxepin (urine). Generally, all of these metabolic species can be applied to confirm administration of doxepin to horses using GC/MS techniques. In case of serum, only one isomeric metabolite, i.e. desmethyldoxepin, was detectable. In EI-MS mode, however, fragmentation of its trifluoroacetyl derivative was superior to that of free doxepin [10], and this may even be utilized for routine drug screening. Isomers of desmethyldoxepin and hydroxydoxepin appeared in urine as most predominant products of equine doxepin metabolism. Because of characteristic four-peak-patterns they were easily identified in the GC/MS chromatograms. Analysis of these compounds was more sensitive as compared with parent doxepin [4], and, moreover, allowed to extend detection times from 12 to 48 h after treatment. Finally, isomers of hydroxy desmethyldoxepin were found to be minor urinary metabolites. Thus, for verification of positive results obtained by screening for parent doxepin [4], samples should be tested for the presence of the isomers of desmethyldoxepin (blood, urine) and hydroxydoxepin (urine).

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