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Application of an enantioselective LC-ESI MS/MS procedure to determine *R*- and *S*-hyoscyamine following intravenous atropine administration in swine

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***S*-hyoscyamine (*S*-hyo) is a natural plant tropane alkaloid acting as a muscarinic receptor (MR) antagonist. Its racemic mixture (atropine) is clinically used in pre-anaesthesia, ophthalmology and for the antidotal treatment of organophosphorus (OP) poisoning with nerve agents or pesticides even though *R*-hyo exhibits no effects on MR. Further investigative research is required to optimize treatment of OP poisoning. Swine are often the animal model utilized due to similarities in physiology and antidote response to humans. However, no studies have been reported that elucidated differences in the kinetics of *R*- and *S*-hyo. Therefore, the concentration-time profiles of total hyo as well as both enantiomers were analyzed in plasma after intravenous administration of atropine sulfate (Atr_2SO_4, 100 μg/kg) to anaesthetized swine. For quantification plasma samples were incubated separately with human serum (procedure A) and rabbit serum (procedure B). The rabbit serum used contained atropinesterase, which is suitable for stereoselective hydrolysis of *S*-hyo, while human serum does not hydrolyze either enantiomer. After incubation samples were precipitated and the supernatant was analyzed by RP-HPLC-ESI MS/MS. Procedure A allowed determination of total hyo and procedure B remaining *R*-hyo concentrations. *S*-hyo was calculated as the difference of the two procedures. Concentration data were regressed by a two-phase decay according to a two-compartment open model revealing similar kinetics for both enantiomers thus indicating distribution, metabolism and elimination without obvious stereoselective preference in tested swine. Copyright © 2011 John Wiley & Sons, Ltd.**

Keywords: atropine; enantiomers; LC-ESI MS/MS; *S*- and *R*- hyoscyamine; swine

Introduction

S-hyoscyamine (*S*-hyo, also known as L-hyoscyamine or (-)-hyoscyamine, Figure 1C) is a toxic tropane alkaloid, (8-methyl-8-azabicyclo[3.2.1]octan-3-yl) (2S)-3-hydroxy-2-phenylpropanoate, that is stereoselectively produced in plants of the solanaceae family, for example, deadly nightshade (*Atropa belladonna*), thornapple (*Datura stramonium*) or henbane (*Hyoscyamus niger*).[1] *S*-hyo acts as an antagonist of muscarinic receptors (MR) thus inhibiting acetylcholine-mediated signal transduction. Rising plasma concentrations cause tachycardia, mydriasis, CNS excitations, hallucinations, coma and ultimately death.[2] Competitive MR antagonists are used clinically for pre-anesthesia medication,[3] ophthalmologic procedures[4] and the therapy of anticholinesterase poisoning.[5,6] Due to the pharmaceutical extraction procedure of natural *S*-hyo from plants, the active ingredient undergoes racemization producing an equimolar mixture of *S*- and *R*-hyo (Figure 1B). This racemic compound is atropine (Figure 1A). In contrast to the eutomer *S*-hyo, the distomer *R*-hyo does not antagonize acetylcholine at MR.[7–9] Despite these differences, racemic atropine is typically administered for pharmacological purposes and therefore pharmacokinetic (PK) data in man were often obtained for total hyoscyamine without any stereoisomer differentiation.[10–17] This simplifying description might be sufficient if both enantiomers exhibited the same PK behaviour with respect to absorption, distribution, metabolism and excretion. However, if these properties should differ significantly, correlation of total hyo concentrations with clinical parameters would be misleading. Interestingly, reported data show that hyo enantiomers have different pharmacodynamic and pharmacokinetic properties in man.[18–22]

These results indicated that enantioselective quantification of both hyoscyamines in body fluids appears to be advisable when analyzing pharmacokinetics for therapy development and optimization. Detailed elaboration of enantioselective PK parameters has been hampered by the lack of appropriate analytical methods that allow unambiguous quantification of both hyo variants.

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Abbreviations: *ACN, acetonitrile; AtrE, atropinesterase; AUC, area under the curve; CNS, central nervous system; C_{max}, maximum plasma concentration; ESI, electrospray ionization; FA, formic acid; HPLC, high-performance liquid chromatography; hyo, hyoscyamine; I.D., inner diameter; i.m., intramuscular; IS, internal standard; i.v., intravenous; MR, muscarinic receptor; MS/MS, tandem mass spectrometry; OP, organophosphorus; PBS, phosphate-buffered saline; PK, pharmacokinetic; RIA, radioimmunoassay; RRA, radio-receptor assay; $t_{1/2\alpha}$, half-life of initial distribution α-phase; $t_{1/2\beta}$, half-life of terminal elimination β-phase.*

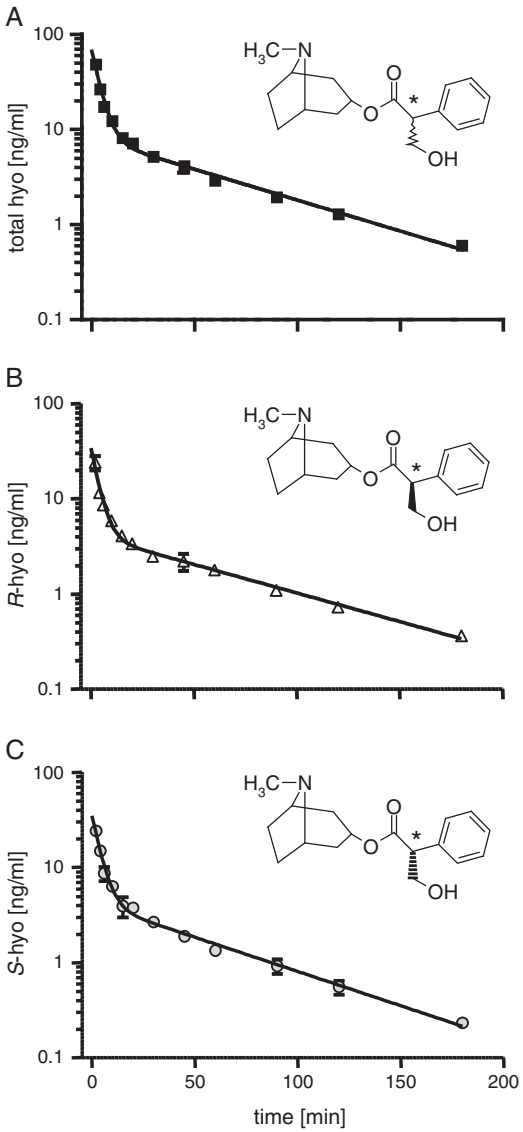

Figure 1. Concentration-time profiles of hyoscyamines in plasma after intravenous administration of atropine sulfate to swine. A: total hyoscyamine. B: *R*-hyoscyamine. C: *S*-hyoscyamine. Atropine sulfate corresponding to a dose of 83.3 µg atropine free base/kg was injected. Data (mean and SD from duplicate measurement of two swine) were regressed according to a two-phase decay ($[hyo] = a\ e^{-\alpha t} + b\ e^{-\beta t}$) following a two-compartment open model ($r^2 > 0.994$ for each curve). No differences of *S*- and *R*-hyo concentrations were obvious.

Aaltonen[18] and Kentala[20] used a combination of both radioimmunoassay (RIA) and radio-receptor assay (RRA) which allowed quantification of total hyo (RIA) and *S*-hyo (RRA), respectively, thus enabling calculation of the *R*-hyo portion by the difference of both results. In addition to these publications only three reports have addressed stereoselectivity in mouse and man in which advanced analytical techniques such as gas chromatography-mass spectrometry (GC-MS)[19] and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) were used.[8,21] Consequently, a detailed description of enantioselective PK parameters has not been reported. Recently, we introduced a novel, precise, and accurate enzymatic enantioselective HPLC-MS/MS procedure for sensitive quantification of both hyo variants in plasma, which allows for the stereoselective analysis of concentration-time profiles in *in vivo* studies.[21]

Since several current concepts on the optimal therapy of organophosphorus (OP) poisoning, in which atropine is administered as the symptomatic antidote, make use of swine, it is important to characterize atropine behaviour in this model.[6,23–25] Therefore this study has applied the recently published enantioselective HPLC-MS/MS procedure to monitor hyoscamine concentrations in swine plasma after i.v. administration of atropine sulfate.

Materials and methods

Chemicals

Atropine (CAS No. 51-55-8, racemic mixture of *R*- and *S*-hyoscyamine, free base) for HPLC-MS/MS analysis, atropine sulfate monohydrate for treatment of animals and triply deuterated cocaine (d3-cocaine as internal standard for LC-MS/MS) were purchased from Sigma (St Louis, MO, USA) in a purity ≥ 98% (TLC). Acetonitrile (ACN, gradient grade), water (LiChrosolv), formic acid (FA, Uvasol), and salts (guaranteed reagent) used for phosphate-buffered saline (PBS) were delivered by Merck (Darmstadt, Germany).

LC-ESI MS/MS system

For chromatographic separation two pumps, an autosampler, column oven, and a controller were used (all PE 200 series, Perkin Elmer, Rodgau-Jügesheim, Germany). A triple quadrupole (linear ion trap) mass spectrometer (API 4000 QTrap, Applied Biosystems, Darmstadt, Germany) with an electrospray ionization interface and nitrogen as collision and spray gas was applied for detection. Both components were controlled by the Analyst 1.4.2 software (Applied Biosystems, Darmstadt, Germany).

Enantioselective enzymatic HPLC-MS/MS assay

Enantioselective analysis of plasma samples was carried out as described recently.[21] Plasma samples from swine (85 µl) were incubated separately (20 min at 37 °C) with PBS-diluted human serum (15 µl, 33% *v/v*, not containing atropinesterase, AtrE, procedure A) and with PBS-diluted rabbit serum (15 µl, 33% v/v, containing AtrE, procedure B). Following plasma sample precipitation with ACN (200 µl), 200 µl of supernatant were diluted with 400 µl solvent A (see below) containing the internal standard (IS) d3-cocaine (0.16 ng/ml).

Hyoscyamines were quantified from this dilution (100 µl) by a validated reversed-phase high-performance liquid chromatography-electrospray ionization-tandem mass spectrometric method (RP-HPLC-ESI-MS/MS) in the positive ion mode.[26]

Separation was carried out on an Atlantis T3 C18 column (150 mm x 4.6 mm I.D., 5 µm) at 30 °C using solvent A (0.1% FA in water) and solvent B (ACN/water 80:20 v/v, 0.1% v/v FA) in gradient mode (1 ml/min): [min]/B [%]: 0/23; 5/38; 6/80; 7/80; 7.5/23; 8.5/23. Detection was performed in multiple reaction monitoring mode (MRM) using the transition from *m/z* 290.3 to *m/z* 124.3 for hyoscyamine and *m/z* 307.2 to *m/z* 185.2 for d3-cocaine as IS with the following settings for collision energy (CE), declustering potential (DP) and collision cell exit potential (CXP): 35 V, 76 V, 6 V and 29 V, 61 V, 12 V, respectively. Parameters for ionization were set to 4800 V (ionization spray voltage), $1.72*10^5$ Pa (25 psi) for curtain gas, $4.83*10^5$ Pa (70 psi) for heater gas (GS1), $4.14*10^5$ Pa

(60 psi) for turbo ion spray gas (GS2), 700 °C gas temperature, 10 V as entrance potential (EP), and a dwell time of 50 ms.

Following procedure A the concentration of total hyo and following procedure B remaining *R*-hyo were determined by an external calibration curve. *S*-hyo was calculated by the difference between these concentrations.

Control experiments (analyzing swine samples after fortification with atropine to test the *R*-/*S*-hyo ratio after incubation with AtrE) were performed as previously recommended to assure the absence of any AtrE-inhibiting compounds.[21]

Atropine study in swine

Studies using anesthetized domestic swine were carried out at Defence Research and Development Canada Suffield and adhered to the guidelines established by the Canadian Council for Animal Care. Male castrated York-Landrace cross pigs weighing 20.0 ± 1.5 kg at the time of treatment were purchased from a local supplier. The animals were housed in a temperature controlled area with a 12 h light/dark cycle with free access to water and were fed twice per day. Swine were allowed to acclimatize for at least one week prior to experimental use.

The animals underwent an inhalation induction with 5% isoflurane in a carrier gas of 100% oxygen at a flow of 8 l/min and received 0.9% normal saline via an intravenous line for fluid replacement. After arterial catheters for blood sampling were securely established, animals were allowed to stabilize at ~2% isoflurane in 30% oxygen supplemented medical air for at least 30 min to establish a level of steady state anaesthesia (SSA).

Two swine received an i.v. injection of 100 μg/kg atropine sulfate monohydrate (corresponding to 83.3 μg/kg atropine free base) without any additional drug treatment. Blood samples were collected from a catheter placed in a branch of the saphenous artery into EDTA tubes (BD Vacutainer K2 EDTA, BD, Franklin Lakes, NJ, USA) at distinct time points during the 180 min experimental time period. Samples were centrifuged at 3000 x g for 10 min at 4 °C. Plasma was aspirated into 2 ml cryovials and snap frozen in liquid nitrogen and stored at −80 °C prior to shipment on dry ice to Munich, Germany.

Data analysis

Biphasic concentration-time profiles of each animal were analyzed by non-linear two-phase exponential decay regression ($[hyo] = a\, e^{-\alpha t} + b\, e^{-\beta t}$) using the appropriate feature of the GraphPad Prism software (vers 5.03). Area under the curve (AUC) was computed using the trapezoid rule. Half-lives of the initial distribution phase ($t_{1/2\alpha}$) and terminal elimination phase ($t_{1/2\beta}$) were calculated from ln2/α and ln2/β, respectively.

Results

Analytical performance

The HPLC-ESI MS/MS procedure was demonstrated to be precise (RSD 2–7%) and accurate (89–109%) for plasma analysis over the entire linear range from 0.05–50 ng/ml.[21,26]

Chromatograms of swine plasma blank samples did not show any interference with hyoscamine (t_R 3.3 min), neither in the presence of human serum (procedure A, Figure 2A) nor in the presence of rabbit serum (procedure B, Figure 2B). Furthermore, hyoscyamine content detected in swine samples 20 min after Atr_2SO_4 injection following procedure A (Figure 2C) was reduced to its half by means of AtrE from rabbit serum (procedure B, Figure 2D).

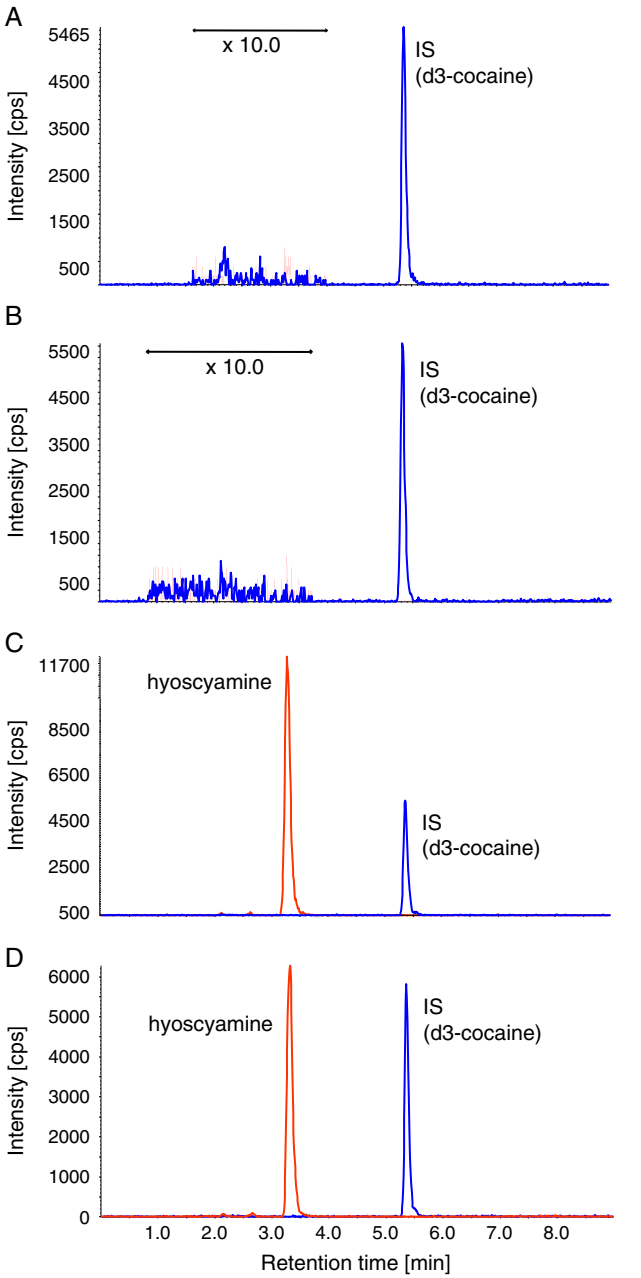

Figure 2. Representative chromatograms of swine plasma samples. A: blank plasma mixed with human serum (procedure A) and internal standard. B: blank plasma mixed with rabbit serum (procedure B) and internal standard. C: plasma sample taken 20 min after i.v. injection of atropine sulfate (procedure A). D: plasma sample taken 20 min after i.v. injection of atropine sulfate (procedure B). Separation was carried out on an Atlantis T3 C18 column (150 mm x 4.6 mm I.D., 5 μm) applying a gradient mode of solvent A (0.1% FA in water) and solvent B (ACN/water 80:20 *v/v*, 0.1% *v/v* FA) at 1 ml/min. Hyo (t_R 3.3 min) and IS (d3-cocaine, t_R 5.4 min) were detected in multiple reaction monitoring mode (MRM), hyo: *m/z* 290.3 to *m/z* 124.3 (red trace) and IS: *m/z* 307.2 to *m/z* 185.2 (blue trace).

Reliability for swine samples from the present *in vivo* study was also proven by the recommended analytical control experiments.[21] Results show the absence of interfering effects of swine plasma ingredients on atropinesterase-mediated hydrolysis of *S*-hyo during the sample preparation procedure (data not shown).

Total hyo and enantiomers after i.v. administration

Concentration-time curves in an lg/linear plot revealed a biphasic profile for total hyo as well as both stereoisomers (Figure 1). Data were regressed by a two-phase exponential decay ($r^2 > 0.994$) to calculate kinetic parameters (Table 1).

Maximum plasma concentrations (C_{max} 48 ± 5 ng/ml for total hyo and half for both *S*- and *R*-hyo) were measured after 2 min (t_{max}, first time point) (Table 1, Figure 1). Within the next 8 min, concentrations of all hyoscyamines decreased rapidly to approximately 20% of C_{max} followed by a much slower decrease over the next 172 min (Figure 1). For total hyo the half-life of the initial distribution α-phase ($t_{1/2\alpha}$) was calculated to be 3.0 ± 1.3 min while 48 ± 7 min was found for the shallower terminal elimination β-phase ($t_{1/2\beta}$) (Table 1, Figure 2).

The equimolar ratio of *R*- and *S*-hyo as present in administered atropine was stable over the entire time of distribution and elimination (Figure 1). Half-life of the initial distribution α-phase ($t_{1/2\alpha}$) was 2.6 ± 0.9 min for *R*-hyo and 2.9 ± 1.2 min for *S*-hyo (Table 1). The shallower terminal elimination β-phase ($t_{1/2\beta}$) also resulted in nearly identical values for total hyo and both enantiomers in the range of 48 ± 7 min (Table 1).

Discussion

Most of the PK studies published report on *S*-hyo or atropine in humans after administration by the ocular,[4] oral,[3,27] sublingual,[28] rectal,[29] inhalational,[14,16] i.m.,[12,17,20,29–34] or i.v.[8,11,12,15,18,21,35] route. The two latter routes are typical for antidotal therapy of anticholinesterase poisoning. According to the cited references atropine or *S*-hyo was found to be rapidly distributed, reaching C_{max} in plasma at a t_{max} of 15–30 min after i.m. and approximately 2–3 min after i.v. injection. However, large individual variations and prolonged times in children and the elderly have been reported.[18,31,36] Biphasic concentration-time profiles after i.v. administration were best described by a two-compartment open model showing a fast distribution phase (distribution half-life, $t_{1/2\alpha} \approx 1$ min) followed by a much slower elimination phase (elimination half life, $t_{1/2\beta} \approx 2$–4 h).

In contrast to the numerous human studies only a limited number of studies using swine have been reported.[6,37,38] None of these reports provides data to demonstrate or compare potential differences in the *in vivo* behaviour of hyo enantiomers.

In general, physiological, anatomical, nutritional, and metabolic similarities between swine and humans make these laboratory animals a good model for biomedical research.[25,39,40] However, when compared to adult humans, swine are characterized by higher plasma volumes, arterial pH, and extracellular space and lower renal blood flow and glomerular filtration rate.[40] Different rates of metabolism in the liver determined by different liver blood flows are probably the most important reason for inter-species variance.[40] To characterize studies using atropine in the treatment of OP poisoning[24,41,42] we determined hyoscyamine enantiomers in the domestic swine model.

Total hyo after i.v. adminsitration

The biphasic concentration-time profile monitored for total hyo in swine plasma is typical for a two-compartment open model (Figure 1). Although similar qualitative profiles were also found in other mammalian species quantitative differences occurred for mice ($t_{1/2\alpha} = 11$ min, $t_{1/2\beta} = 100$ min),[13] dogs ($t_{1/2\alpha} \approx 2$ min, $t_{1/2\beta}$ 120 min),[10] and man ($t_{1/2\alpha} \approx 1$–2 min, $t_{1/2\beta} \approx 120$–240 min).[4,11,15,35] Nevertheless, quite similar values were reported for pigs: $t_{1/2\alpha} = 0.7 \pm 0.1$ min and $t_{1/2\beta} = 36 \pm 0.8$ min.[6,38]

Hyo enantiomers after i.v. administration

Whereas discussions above addressed total hyo behavior the current study examined the novel individual enantiomer data which has not been previously reported.

Identical concentration-time profiles of *R*- and *S*-hyo (Figure 1), which resulted in similar values of half-lives for distribution α-phase and elimination β-phase and AUC (Table 1, Figure 3) indicated identical elimination and distribution properties between the central and peripheral compartment without stereoselective effects.

This fact was quite unexpected as earlier studies conducted in humans revealed striking differences in the PK parameters of *R*- and *S*-hyo after i.v. and i.m. administration.[18,20]

Table 1. Kinetic data after intravenous administration of atropine sulfate to swine

	total hyo	*R*-hyo	*S*-hyo
C_{max} [ng/mL]	48.0 ± 5.4	24.0 ± 5.7	24.1 ± 0.4
a [ng/mL]	60 ± 25	31 ± 13	32 ± 11
α [1/min]	0.25 ± 0.11	0.28 ± 0.09	0.26 ± 0.10
$t_{1/2\alpha}$ [min]	3.0 ± 1.3	2.6 ± 0.9	2.9 ± 1.2
b [ng/mL]	7.7 ± 1.5	4.0 ± 0.8	4.0 ± 0.1
β [1/min]	0.015 ± 0.002	0.014 ± 0.002	0.016 ± 0.001
$t_{1/2\beta}$ [min]	48 ± 7	51 ± 8	43 ± 3
AUC [min*ng/mL]	625 ± 6	325 ± 20	307 ± 26

Concentration-time profiles in swine plasma were regressed by two-phase exponential decay ($[hyo] = a\ e^{-\alpha t} + b\ e^{-\beta t}$) according to a two-compartment open model yielding correlation coefficients of $r^2 \geq 0.994$ for each animal.

α, exponential hybrid constant for α-phase; AUC, area under the curve; β, exponential hybrid constant for β-phase; C_{max}, maximum concentration in plasma; $t_{1/2\alpha}$, half-life of initial distribution phase; $t_{1/2\beta}$, half-life of terminal elimination phase.

Parameters were determined for each animal to calculate listed mean and standard deviation.

Two swine were treated with 100 μg/kg atropine sulfate monohydrate (i.v.) corresponding to 83.3 μg/kg of the pure base and 41.6 μg/kg of each enantiomer. Time to reach maximum concentration in plasma (t_{max}) was found at 2 min for each drug.

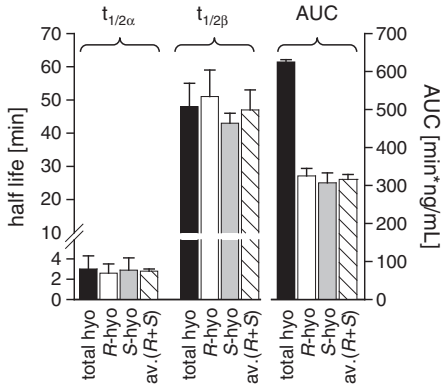

Figure 3. Half-lives and area under the curve of hyoscyamines after i.v. administration in swine. Data were obtained from two-phase exponential decay regression of concentration-time curves of hyoscyamines in plasma of two swine (dose of i.v. administration: 83.3 µg pure atropine base/kg body weight corresponding to 100 µg Atr_2SO_4-H_2O/kg). Bars indicate that within the deviation half-lives and AUC were identical for both enantiomers. AUC, area under the curve; av. (*R* + *S*), averaged data of means from *R*- and *S*-hyo; hyo, hyoscyamine; $t_{1/2\alpha}$, half-life of initial distribution phase; $t_{1/2\beta}$, half-life of terminal elimination phase.

Furthermore, we reported on evidently higher concentrations of *R*-hyo when compared to *S*-hyo (about factor 2) in the plasma of an elderly pesticide-poisoned female patient receiving atropine therapy (single i.v. bolus, 25 mg).[21] The analytical procedure applied for that investigation was the same as used in the present report thus substantiating the assumption that hyoscyamine kinetics in man differ from that in swine.

Conclusions

This study presents the first enantioselective data of atropine in swine following i.v. injection. Results for both animals tested provided no evidence of differences in distribution or elimination properties of distomer and eutomer and therefore stereoselective preferences (Figure 3). This might be an interesting hint that behaviour of hyoscyamines in swine differs from that in man where significant differences between enantiomers have been reported. Nevertheless, a more appropriate PK study using a larger number of animals will be necessary to confirm our assumption with statistical significance in future experiments.

However, the equimolarity of hyo enantiomers clearly demonstrate that the measurement of total hyo by simple non-chiral HPLC-MS/MS, GC-MS or other non-stereoselective procedures will be sufficient to monitor the concentration-time profile of the physiologically active *S*-hyo that represents half of the measured (total hyo) concentration. This is of importance when performing animal studies to clearly define the antidotal effects of atropine in OP poisoning. Furthermore, the applicability of the enantioselective enzymatic HPLC-ESI-MS/MS procedure for plasma analysis was also demonstrated.[21]

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