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Simultaneous determination of the new anticancer agent amidox and its metabolites in rat bile and plasma by high-performance liquid chromatography

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Abstract

A new reversed-phase ion-pair high-performance liquid chromatography method was developed to study the first-pass hepatic metabolism of the anti cancer drug amidox in bile. Separation of the metabolites was achieved on a Spherisorb C_{18} column after liquid–liquid extraction using a linear gradient system of heptanesulfonic acid in potassium phosphate monobasic (pH 4.0) with increasing amounts of methanol (0–40%). The method was further applied to a pharmacokinetic study of amidox in rats after 200 mg kg^{-1} intraperitoneal administration. Using 50 μl of rat bile and 300 μl of rat plasma the limit of detection for amidox was 60 ng and 85 ng, respectively, and the assay was linear from 0.1 to 150 μg ml^{-1}. This method appears to be sensitive enough to be used in further pharmacokinetic studies of amidox in human volunteers.
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1. Introduction

Amidox, 3,4-dihydroxybenzamidoxime (Fig. 1), belongs to a new class of ribonucleotide reductase inhibitors that is currently undergoing clinical trials as a potential drug in cancer therapy [1]. The enzyme ribonucleotide reductase is the rate-limiting step in the de novo synthesis of deoxyribonucleotides and DNA, and because the ribonucleotide reductase activity occurs in increased orders of magnitude in tumor cells compared to the level in non-proliferating cells, the enzyme is a logical target for antineoplastic agents [2–6]. Amidox inhibits the growth of various tumor cell lines more effectively than hydroxyurea, the only clinical approved inhib-

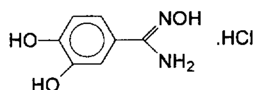

Fig. 1. Structure of amidox.

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itor available at present. Amidox was found to be a good iron chelator as well as a strong radical scavenger [1,3,7]. Co-administration of amidox with anthracyclines like doxorubicin reduces their toxic side effects caused by free radical production and enhances antitumor activity [8]. Didox (3,4-dihydroxybenzohydroxamic acid), like amidox a potent inhibitor of ribonucleotide reductase, was shown to be extensively metabolized in vivo [9,10], so the objective of this study was to develop a reliable and sensitive method of high selectivity for the quantitation of amidox and its metabolites in bile using an in situ perfused rat liver model to study the biotransformation. This assay was further used to determine preliminary disposition of this drug in rats using plasma concentrations.

2. Experimental

2.1. Chemicals and reagents

Amidox was supplied by Dr. B. van't Riet. (Molecules for Health, Richmond, VA, USA). Potassium phosphate, monobasic, 1-heptanesulfonic acid were obtained from Sigma (Munich, Germany). Methanol and water were of HPLC grade (Merck, Darmstadt, Germany). All other chemicals and solvents were of analytical grade and were used without further purification.

2.2. Liver perfusion

Isolated livers of male Wistar rats were perfused in a non-recirculating system using Krebs Hensleit buffer according to literature at an average flow-rate of 37 ± 0.4 ml g^{-1} min^{-1} [11]. After 30 min of perfusion with control medium, amidox in a final concentration of 30 μM was added to the perfusion medium. In order to study time-dependent first-pass metabolism, bile samples were collected every 5 min for a time period of 60 min, immediately frozen on dry ice, and stored at −20°C until analysis.

2.3. Pharmacokinetic study in rats

Male Wistar rats received a bolus dose of 200 mg kg^{-1} via the i.p. route and were killed at 0, 5, 10, 15, 20, 25,30, 45 and 60 min after treatment. Blood (approx. 4–8 ml) was collected from the heart by a heparinized syringe, centrifuged for 5 min at 4000× *g*, plasma collected, immediately frozen on dry ice, and stored at −20°C until analysis.

2.4. Preparation of calibration solutions

A stock solution of 200 μg ml^{-1} amidox in ammonium acetate (10 m*M*, pH 5.0) was used for preparing the calibration solutions. Aliquots of stock solution were diluted further with ammonium acetate to give calibration solutions of 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 100, and 150 μg ml^{-1} for the analysis of whole bile and plasma samples. The calibration solutions were prepared every week. All solutions were stored at −20°C when not in use.

2.5. Extraction procedure

All bile and plasma samples were thawed at room temperature and vortex mixed for 10 s.

After dilution of 10 μl of bile samples with 90 μl ammonium acetate (10 m*M*, pH 6.6), 100 μl of saturated sodium chloride in water was added and the aqueous solution was extracted with 2×1 ml of ethyl acetate. The organic extracts were combined, dried under a stream of nitrogen and the resulting residue was dissolved in 200 μl of methanol–water (1:1; v:v) and transferred to vials for injection onto the HPLC system. The containers for HPLC consisted of conical glass inserts in amberized glass autosampler vials capped with open screw caps with PTFE seals (Chromacol, London, Great Britain). Similarly 100 μl of saturated sodium chloride in water was added to 100 μl of plasma followed by the addition of 2×1 ml of ethyl acetate. The tubes were again vortex mixed and the organic layer dried under a stream of nitrogen as described above.

2.6. Chromatographic conditions

HPLC was performed using a Merck 'La Chrom' System (Merck) equipped with an L-7250 injector, an L-7100 pump, an L7300 column oven (set at 30°C) a D-7000 interface and an L-7400 UV detector, set at a wavelength at 264 nm. The parent drug and metabolites were separated by HPLC on a

LiChrospher RP18 pre-column (10×4.6 mm I.D., 5-μm particle size) and a LiChrospher RP18 column (250×4.6 mm I.D., 5-μm particle size) connected by an Eco-tube cartridge system (all by Bischoff, Leonberg, Germany). The mobile phase A consisted of potassium phosphate (50 m*M*, pH 4.0 with phosphoric acid) and heptanesulfonic acid (5 m*M*) and the mobile phase B consisted of methanol. The mobile phase was filtered through a 0.45 μ*M* filter (HVLP04700, Millipore, Vienna, Austria) and the flow-rate was 1.0 ml min^{-1}. The gradient ranged from 0% (0 min) to 8% B at 15 min, 30% B at 30 min, 40% B at 32 min, kept constant at 40% B till 42 min, and finally decreased linearly to 0% B at 44 min. The columns were allowed to re-equilibrate for 15 min between runs. The sample injection volumes for bile and plasma samples were 100 μl.

2.7. Assay validation

Calibration of the chromatogram was accomplished using the external standard method. Linear calibration curves were performed from the peak areas of amidox and its metabolites to the external standard amidox by spiking drug-free rat bile and plasma with standard solutions of amidox to give a concentration range of 0.1 to 150 μg ml^{-1}. The samples were taken through the extraction procedure described above and the peak areas plotted against the corresponding concentration. As metabolite standards were not available, quantification of metabolite concentrations was based on the assumption that the unknown metabolites have a similar molar extinction coefficient as amidox. The unknown drug concentrations in bile and plasma samples were determined from the equation generated by the least squares regression analysis.

The recovery of amidox from whole bile and plasma was calculated by comparing peak areas for bile and plasma samples spiked with amidox in triplicates with those for aqueous standard solutions over a concentration range of 1–30 μg ml^{-1}. Intraday and inter-day reproducibility of chromatographic data was determined by injecting five replicatives of the standard solutions of 1, 10 and 30 μg ml^{-1} on the same day or for five days, respectively. For freeze–thaw stability, blank bile and plasma samples were spiked with aqueous standard solutions of amidox in triplicates to achieve final concentrations of 1, 10 and 30 μg ml^{-1}, respectively. As freezing time and thawing temperature might influence the stability of amidox, two different protocols were validated. While in one set of experiments samples were frozen at −20°C, stored at the same temperature for one day, and thawed in a water bath (set at 37°C), the other used the following protocol: spiked bile and plasma samples were immediately frozen on dry ice, stored again at −20°C for one day, and thawed more gently at room temperature.

3. Results and discussion

3.1. Sample pre-treatment

Extraction of amidox from rat bile and plasma proved to be very difficult. Precipitation of the proteins using 0.5 *M* perchloric acid or 0.2 *M* TFA with subsequently setting the pH to 4.0 with monobasic potassium phosphate did clear up the chromatogram but also diluted the samples making it difficult to detect minor metabolites. Several attempts failed to extract amidox and its metabolites using endcapped C_2, C_8 or C_{18} solid-phase extraction cartridges possibly due to the persistent strong binding of the basic amino group to free silanol. Addition of triethylamine to methanol for the elution only slightly increased the recovery. Since amidox has pK_a values of 5.2 and 8.2, respectively, liquid–liquid extraction with ethyl acetate at pH 6.8 after addition of saturated sodium chloride resulted in excellent recoveries and almost quantitatively eliminated both polar and non-polar endogenous impurities.

3.2. Chromatograms

The binary HPLC method allowed the separation of amidox and its metabolites with a run time of approximately 30 min. Figs. 2A and 2B show the chromatograms of the extracted blank rat bile and blank rat bile spiked with amidox. By adding amidox at a concentration of 5 μg ml^{-1} to the perfusion medium, additional peaks, mainly unknown metabolites M1–M5 with retention times of 9.0, 10.6, 11.6, 12.7 and 15.1 min, respectively, appeared in the

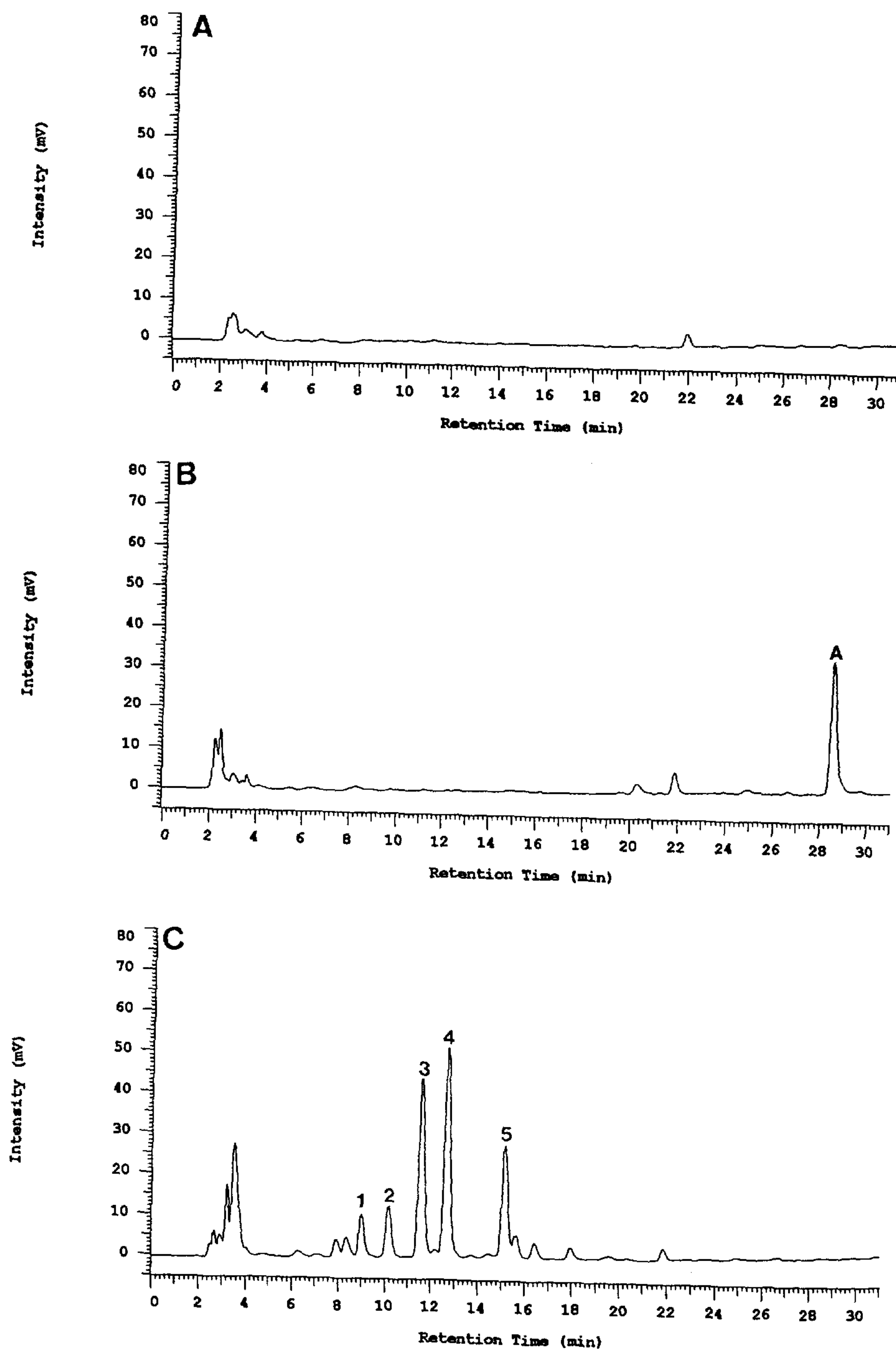

Fig. 2. Chromatograms of amidox in rat bile: (A) extracted blank bile sample; (B) extracted bile sample that has been spiked with 10 $\mu g\ ml^{-1}$ of amidox (A); (C) extracted bile sample taken 45 min after the start of a 5 $\mu g\ ml^{-1}$ perfusion of amidox (1–5, metabolites; concentration: 3.0, 3.8, 12.4, 14.7, 8.1 $\mu g\ ml^{-1}$).

chromatogram, whereas no peak of amidox with an appropriate retention time of 28.6 min could be detected. Fig. 2C represents the chromatogram of a bile sample collected 45 min after adding amidox at a concentration of 5 μM to the perfusion medium. No interferences from the endogenous substances in bile were detected at the retention times of amidox and its metabolites. Figs. 3A and 3B show the chromatograms of an extracted blank rat plasma and drug free plasma spiked with 10 $\mu g\ ml^{-1}$ of amidox. Fig. 3C exhibits the chromatogram obtained from a rat plasma sample after 30 min with a detected amidox concentration of 30.4 $\mu g\ ml^{-1}$. In addition to the drug peak, an endogenous peak appeared with a retention time of approximately 20.4 min. This peak was also seen in blank plasma samples. None of the metabolites found in bile could be detected in any plasma samples. Detection limits, defined as a signal-to-noise ratio of 3, ranged from 60 $ng\ ml^{-1}$ for bile to 85 $ng\ ml^{-1}$ for plasma.

3.3. Standard curves and assay validation

Regression analysis was performed on the calibration curves in bile and plasma. Both calibration curves were directly proportional to drug concentration over the range of 0.1–150 $\mu g\ ml^{-1}$ with linear regression equations of $y=3.17x+0.09$ ($r=0.999$) for bile and $y=2.13x+0.34$ ($r=0.997$) for plasma, respectively, where y=concentration and x= peak area$\cdot 10^{-3}$. In the extraction from bile the recoveries (mean±S.D.) of amidox were found to be 92.7±3.2, 96.3±2.8 and 97.0±2.1% at concentrations of 1, 10, and 30 $\mu g\ ml^{-1}$, respectively, whereas the recoveries (mean±S.D.) for the same concentrations from plasma were slightly reduced (91.8±3.0, 94.5±1.8 and 95.3±2.1%). Intra-day values ranged from 1.3% to 3.0%, inter-day values from 1.5 to 3.2% (see Table 1). As amidox is stable in aqueous solution at 4°C for 1–2 weeks, polymerization might occur in basic solution. Incubating a standard solution of amidox (10 $\mu g\ ml^{-1}$ of phosphate buffer, 50 mM, pH 7.4) for 24 h at 37°C, 30.7% of unpolymerized amidox remained and could be detected (data not shown). Table 2 exhibits the influence of temperature on a freeze/thaw cycle of spiked bile and plasma samples using two different protocols. While the recoveries (mean±S.D.) of the samples which were immediately frozen and gently thawed at room temperature were identical compared to the unfrozen ones, samples which were thawed at 37°C exhibited a loss in recovery of about 5%, indicating the importance of temperature control during the whole analyzing protocol.

3.4. Pharmacokinetics of amidox in rats

The mean plasma concentration versus time curve of amidox in male Wistar rats following a single 200 $mg\ kg^{-1}$ i.p. dose is depicted in Fig. 4. Surprisingly, instead of the expected almost total availability of amidox in the systemic circulation within few min, maximum plasma concentration of 109±11.9 $\mu g\ ml^{-1}$ was reached after approximately 15 min, indicating a slow absorption process through the membranes of the peritoneum possibly due to the low solubility of the uncharged form of the drug. Amidox was cleared very fast from the systemic circulation, as indicated by the short terminal half-life of 16.8±0.9 min and high systemic clearance, 0.86±0.06 $l\ h^{-1}\ kg^{-1}$. Amidox did not distribute extensively in the body as the volume of distribution was low, 0.27±0.05 $l\ kg^{-1}$.

4. Conclusion

The HPLC assay described here for amidox in rat bile and plasma, pretreated with liquid–liquid extraction, is a sensitive, validated and efficient method for the determination of this drug and its metabolites in biological matrices. The gradient–elution method allowed the separation of amidox and its biotransformation products with a total run time per sample of less than 45 min. This assay was used for pharmacokinetic studies of amidox in rats, and is almost certainly applicable to analyze human samples.

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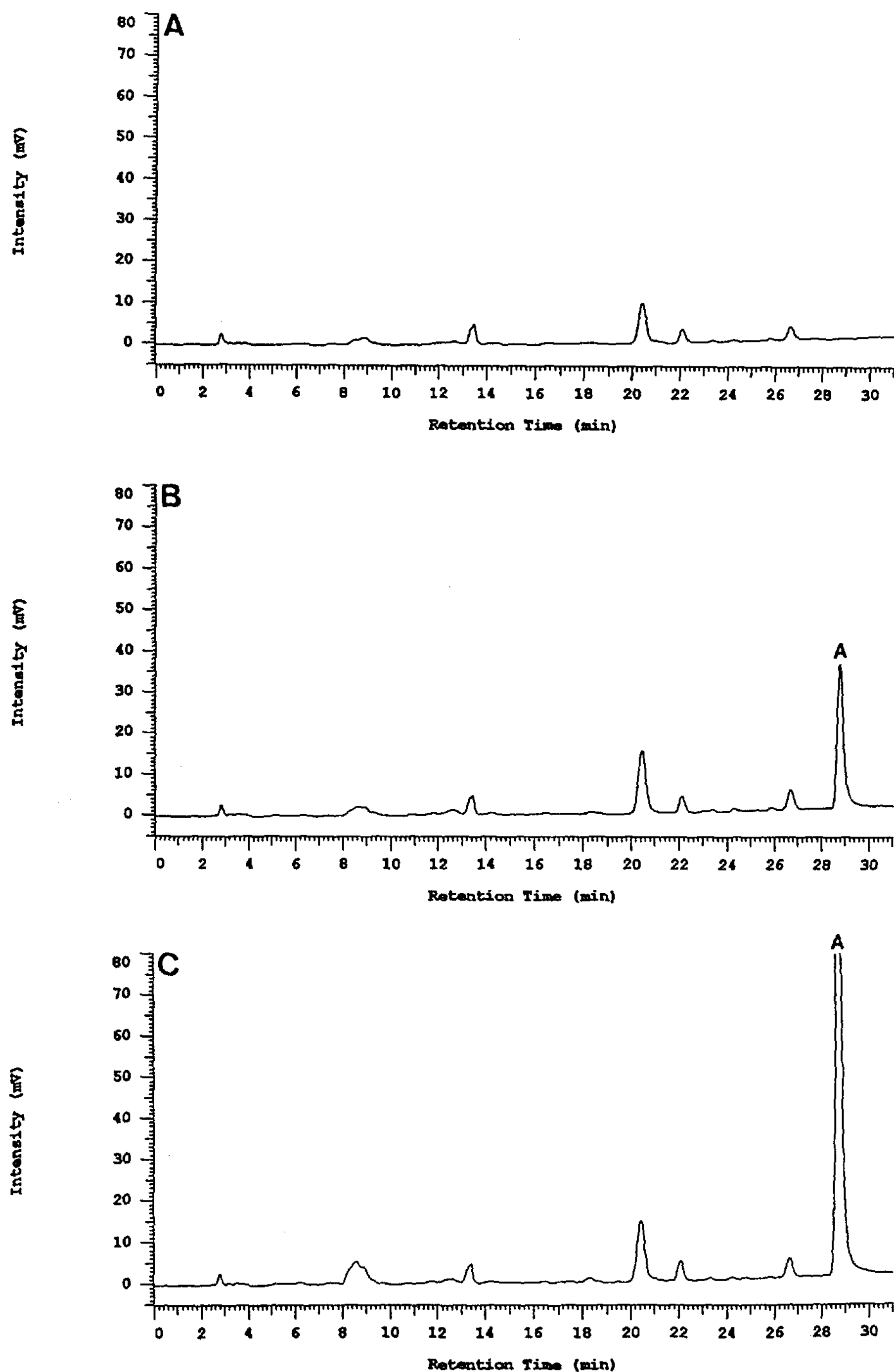

Fig. 3. Chromatograms of amidox in rat plasma: (A) extracted blank plasma sample; (B) extracted plasma sample that has been spiked with 10 $\mu g\ ml^{-1}$ of amidox (A); (C) extracted plasma sample taken 30 min after an i.p. dose of 200 $mg\ kg^{-1}$.

Table 1
Precision of amidox determination by liquid–liquid extraction method

Bile			Plasma		
Spiked concentration (μg ml^{-1})	Mean calculated concentration (μg ml^{-1})	C.V. (%)	Spiked concentration (μg ml^{-1})	Mean calculated concentration (μg ml^{-1})	C.V. (%)
Intra-day (n=5)[a]			*Intra-day* (n=5)[a]		
1	1.190±0.033	2.8	1	0.996±0.030	3.0
10	10.987±0.275	2.5	10	9.825±0.236	2.4
30	28.093±0.478	1.7	30	30.624±0.398	1.3
Inter-day (n=5)[a]			*Inter-day* (n=5)[a]		
1	1.173±0.036	3.1	1	1.047±0.034	3.2
10	10.477±0.283	2.7	10	11.252±0.326	2.9
30	29.217±0.584	2.0	30	28.718±0.431	1.5

[a] Data are expressed as mean±S.D.

Table 2
Validation of freeze–thaw stability of amidox

Bile			Plasma		
Spiked concentration (μg ml^{-1})	Mean calculated concentration (μg ml^{-1})	C.V. (%)	Spiked concentration (μg ml^{-1})	Mean calculated concentration (μg ml^{-1})	C.V. (%)
Protocol 1[a] (n=3)[c]			*Protocol* 1[a] (n=3)[c]		
1	0.961±0.026	2.7	1	0.957±0.036	3.8
10	10.045±0.360	3.5	10	9.573±0.302	3.2
30	29.158±0.557	1.9	30	28.632±0.419	1.5
Protocol 2[b] (n=3)[c]			*Protocol* 2[b] (n=3)[c]		
1	0.927±0.042	3.7	1	0.873±0.039	4.5
10	9.108±0.321	3.5	10	8.852±0.459	5.2
30	28.081±0.487	1.7	30	27.319±0.506	1.9

[a] Sample frozen on dry ice and thawed at room temperature.
[b] Sample frozen at −20°C and thawed at 37°C.
[c] Data are expressed as mean±S.D.

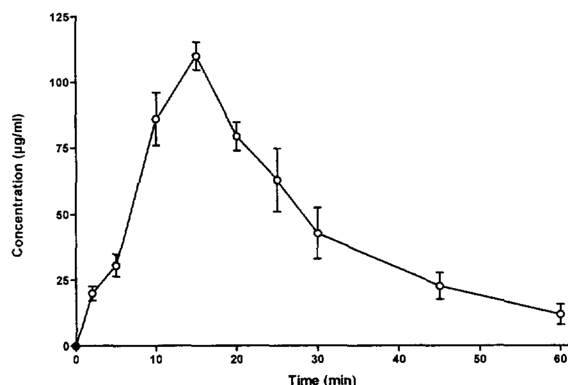

Fig. 4. Mean±S.D. (n=4) plasma concentration versus time curve of amidox in Wistar rats following a single 200 mg kg^{-1} i.p. dose.

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