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Analysis of isobutyl nitrite inhalant in rat and human blood: application for pharmacokinetic investigations

William B. Kielbasa, John Anthony Bauer[1], Ho-Leung Fung*

Department of Pharmaceutics, School of Pharmacy, University at Buffalo, State University of New York, Box 601200, Buffalo, NY 14260-1200, USA

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Abstract

Organic nitrites have been used therapeutically for the treatment of angina pectoris and as diagnostic agents for the evaluation of cardiac heart murmurs. In addition, these highly volatile vasodilators are being used as inhalant drugs of abuse. We developed a gas chromatographic assay using electron capture detection for the analysis of a representative nitrite inhalant, isobutyl nitrite (ISBN), in rat and human whole blood. Unconventional sampling and processing techniques were required because of the high volatility and chemical instability of nitrites in biological fluids. Our method produced a mean recovery of ISBN from rat blood of about 86% over a concentration range of 1.0 to 400 ng/ml. The inter-day coefficient of variation was below 15% at the lowest quantifiable concentration of 1 ng/ml ISBN in rat blood. In this report, we applied the analytical method to obtain new pharmacokinetic information about ISBN. Results show that rats inhaling 900 ppm ISBN for 45 min produced steady-state blood concentrations of about 290 ng/ml, and a rapid elimination half-life of 1.4 min.
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1. Introduction

In 1867, Sir Thomas Lauder Brunton was the first to show that isoamyl nitrite (ISAN) could relieve the chest pain that arose from angina pectoris. Over the last century, inhaled ISAN has been used for the treatment of this disease. In recent years, this organic nitrite has also been used as a smooth muscle relaxant in difficult pre-term cesarean deliveries [1], as a coronary vasodilator prior to thallium imaging [2], as a diagnostic agent for the evaluation of heart murmurs [3], and as an antidote for cyanide poisoning [4]. For pharmaceutical use, organic nitrites are packaged in small, thin glass capsules covered by cloth webbing that are crushed between the fingers to allow breathing of the vapors.

In addition to therapeutic applications, organic nitrites, in particular isobutyl nitrite (ISBN), are used as drugs of abuse in the teenage and homosexual populations. ISBN use was just as common as crack, cocaine, heroin, barbiturates and steroids among the high school graduating class of 1996 in the United

*Corresponding author. Tel: +1-716-645-2842 ext. 222; fax: +1-716-645-3693.
E-mail address: hlfung@acsu.buffalo.edu (H.-L. Fung)
[1]Present address: Division of Pharmacology, College of Pharmacy, Ohio State University, Columbus OH 43210-1291, USA.

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States [5]. Some epidemiological studies have associated nitrite use with Kaposi's Sarcoma, the most common cancer reported in AIDS patients [6,7]. The biological and reported toxicological actions of nitrite inhalants are believed to be due to their ability to produce nitric oxide (NO), although recently the role of NO in nitrite-induced immunotoxicity was disputed [8].

Despite their use as medicinal agents and popularity as ''street'' drugs, the pharmacokinetics of organic nitrites have never been investigated. This lack of information is most likely due to the presence of bioanalytical difficulties associated with these compounds, since nitrites are highly unstable in aqueous solution and biological fluids, due to their hydrolytic lability and volatility. We now have developed special handling procedures to overcome these difficulties, resulting in the successful development of a sensitive and reproducible assay for the determination of ISBN in rat and human whole blood. We further demonstrate that this method can be applied in the examination of ISBN pharmacokinetics in rats.

2. Experimental

2.1. Chemicals and reagents

ISBN and *n*-propyl nitrate (NPN) were purchased from Aldrich (Milwaukee, WI, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO, USA). Pentane was of capillary GC/GC–MS solvent grade (Burdick and Jackson, Muskegon, MI, USA).

2.2. Pentane standard curve

Stock solutions of ISBN (18.7 and 187 μg/ml) and internal standard NPN (235 μg/ml) in pentane were prepared separately in gas-tight glass vials. Calibration standards of ISBN were prepared by adding 0.5–19.3 μl of the stock solution of ISBN to gas-tight vials containing 9 ml of pentane using a gas-tight syringe. NPN was added to the calibration standards by pipetting 3 μl of the stock solution to each calibration standard to achieve a final concentration of 78.3 ng/ml. The final concentration of the ISBN calibration standards ranged from 1.0 to 400 ng/ml. Calibration curves were obtained via the peak area ratio of ISBN vs. NPN using linear least-square regression.

2.3. Aqueous standard curve

Stock solutions of ISBN (1.9 mg/ml) in DMSO were prepared in gas-tight glass vials. Working standards were made by pipetting 4 or 40 μl (depending on the desired concentration of the ISBN standard) of the stock solution into gas-tight glass vials containing 9 ml of DMSO using a gas-tight syringe. Calibration standards of ISBN were prepared by adding 0.5–20.0 μl of the working standard to 0.4 ml of double-distilled water in gas-tight glass vials. The final concentration of ISBN ranged from 1.0 to 400 ng/ml. Samples were immediately extracted with 0.4 ml of pentane (0°C) containing NPN (78.3 ng/ml) under gas-tight conditions. The solutions were vortexed for 5 s and an aliquot of pentane was subsequently removed and placed in glass conical vials for injection. The calibration curves were obtained as described.

2.4. Whole blood standard curve

Stock solutions of ISBN (1.9 mg/ml) in DMSO were prepared in gas-tight glass vials. Working standards were made by pipetting 4 or 40 μl of the stock solution into gas-tight glass vials containing 9 ml of DMSO using a gas-tight syringe. Calibration standards of ISBN were prepared by adding 0.5–20.0 μl of the working standard to 0.4 ml of whole blood. The final concentration of ISBN ranged from 1.0 to 400 ng/ml. The samples were extracted with pentane in the presence of internal standard as described.

2.5. Apparatus and assay conditions

Pentane samples were prepared and injected (3 μl) on the gas chromatograph with a Hewlett-Packard 7673A automatic sampler (San Fernando, CA, USA). The analysis was carried out using a Hewlett-Packard 5890A gas chromatograph equipped with an electron-capture detector. A J&W Scientific (Folsom, CA, USA) DB-1 capillary column was used (30 m×0.32 mm I.D.; 1 μm film thickness). Nitrogen (extra dry grade) was used as the carrier gas (1.0

ml/min) and the detector gas (21.0 ml/min). The samples were chromatographed using gradient temperature elution: 30°C for the initial 9.5 min followed by 45°C for the remaining 8.5 min. The oven temperature was increased at a rate of 60°C/min. The total run time was 18.3 min. The injector and detector temperature were set at 45°C and 195°C, respectively.

The inhalation chamber was constructed with a vaporizer (Cyprane, Keighley, UK) calibrated for ISBN, medical grade air (Strate Welding, Buffalo, NY, USA), and a gas anesthetizing box (Braintree Scientific, Braintree, MA, USA). Volatilized ISBN in the inhalation chamber was determined using gas chromatography–flame ionization detection (GC–FID). Air containing ISBN was analyzed by withdrawing 1.0 ml from the chamber with a gas-tight syringe followed by injection of a 30-μl aliquot on the GC–FID system. Calibration standards ranging from 0 to 1500 ppm were prepared by injecting microliter volumes of ISBN into gas-tight vials that were allowed to vaporize before analysis. Complete vaporization of the standards was verified by heating the vials above its boiling point (67°C). Inter- and intra-day variability was less than 13% (data not shown).

2.6. Recovery of ISBN and NPN

Stock solutions of ISBN (1.9 mg/ml) in DMSO were prepared in gas-tight glass vials. Working standards were made by pipetting 4 or 40 μl (depending on the final concentration of ISBN desired) of the stock solution into gas-tight glass vials containing 9 ml of DMSO using a gas-tight syringe. Standards of ISBN for extraction were prepared by adding 0.5–20.0 μl of the working standard to 0.4 ml of double-distilled water or fresh rat and human whole blood in gas-tight glass vials. The final concentration of ISBN ranged from 1.0 to 400 ng/ml, except for human blood, in which recovery was determined only at 123 ng/ml. Samples were immediately extracted under gas-tight conditions with 0.4 ml of pentane (0°C) by vortexing for 5 s. An aliquot of pentane was subsequently removed and placed in glass conical vials for injection. We also prepared pentane samples (0°C) that were spiked with working standards to achieve the same concentration as the blood and water standards. These samples were also vortexed for 5 s and an aliquot of pentane was removed for injection. To determine the percent recovered, the peak area of the extracted samples were divided by the peak area of the spiked pentane samples and multiplied by 100.

To determine the amount of NPN remaining in pentane after extraction, 0.4 ml of pentane (0°C) containing NPN was extracted with an equal volume of rat whole blood by vortexing for 5 s. An aliquot of pentane was subsequently removed and placed in glass conical vials for injection. Pentane was also spiked with NPN (78.3 ng/ml) and injected. The percent of NPN remaining in the pentane layer was determined by dividing the peak area of NPN following extraction by the peak area of NPN-spiked pentane samples and multiplied by 100. The amount of NPN remaining in pentane after extraction with water was not determined.

2.7. In vitro instability of ISBN

The degradation of ISBN in phosphate buffer (pH 7.4), rat plasma, rat blood and human blood was determined. Fresh samples (0.4 ml) were placed in gas-tight glass vials incubated at 37°C and spiked with ISBN prepared in DMSO to achieve an initial concentration of 94 ng/ml. At different times, samples were extracted with pentane containing NPN and assayed as described.

2.8. Animal studies

Male Sprague–Dawley rats were anesthetized using ketamine/xylazine and the left femoral artery was cannulated for blood withdrawal. The following day, rats were subjected to 900 ppm ISBN for 45 min in the inhalation chamber. Blood samples (0.4 ml) were taken through a heparinized cannula with a 0.5-ml gas-tight glass syringe stored at 0°C just prior to blood sampling. After collection into the ice-cold syringe, the blood was immediately processed by pipetting the sample into a gas-tight vial containing an equal volume of pentane (0°C) with internal standard, and vortexed for 5 s. After vortexing, an aliquot of pentane was removed and analyzed by GC as previously described.

2.9. Statistics

Statistical analysis was performed using analysis of variance (ANOVA). P values <0.05 were considered statistically significant. Data are expressed as mean±standard deviation. The half-life of ISBN was calculated as $0.693/k_{el}$, where k_{el} is the first-order elimination rate constant.

3. Results

Representative assay chromatograms of a pre-dose rat blood sample (A), and rat blood samples taken during the wash-out (B) and apparent steady-state level (C) are shown in Fig. 1. The retention times for ISBN and NPN were 7.6 and 15.9 min, respectively. Extraction of ISBN from rat blood and water was determined at 1.0, 10.4, 41.7, 123 and 400 ng/ml. The recovery appeared to be independent of concentration or the nature of the biological fluids (Table 1; P>0.05, ANOVA). Mean recovery values of 85.9±2.5% and 89.5±2.3% were obtained for rat blood and water, respectively (n=15 each). Extraction of ISBN from human blood was determined at 123 ng/ml and the percent recovery was similar to that observed with rat blood (See Table 1). The amount of NPN remaining in the pentane phase after extraction with rat blood was 95.9±3.8% (n=6).

Table 1
Assay recovery data of ISBN in rat blood, human blood and water (mean±standard deviation; n=3 each concentration)

Concentration (ng/ml)	% Recovery		
	Rat blood	Human blood	Water
1.0	82.4±8.4	–	86.3±7.9
10.4	84.1±8.6	–	89.2±6.6
41.7	87.0±5.7	–	89.2±2.8
123.0	88.3±6.5	85.5±8.4	90.2±6.2
400.0	87.5±4.8	–	92.8±3.5

Calibration curves obtained from blood, water and pentane were linear in the range of 1.0 to 400 ng/ml. The intra- and inter-day coefficient of variations for ISBN in rat blood, water and pentane at concentrations 1.0–400 ng/ml are shown in Table 2. Variability in all sample systems was generally less than 15%. Correlation coefficients were greater than 0.96 in all cases (see footnote in Table 2). The intra- and inter-day coefficient of variation of NPN after extraction with blood was 2.6 and 7.4%, respectively. Stock solutions of ISBN in DMSO and pentane were found to be stable at room temperature for at least 2 h and 24 h, respectively.

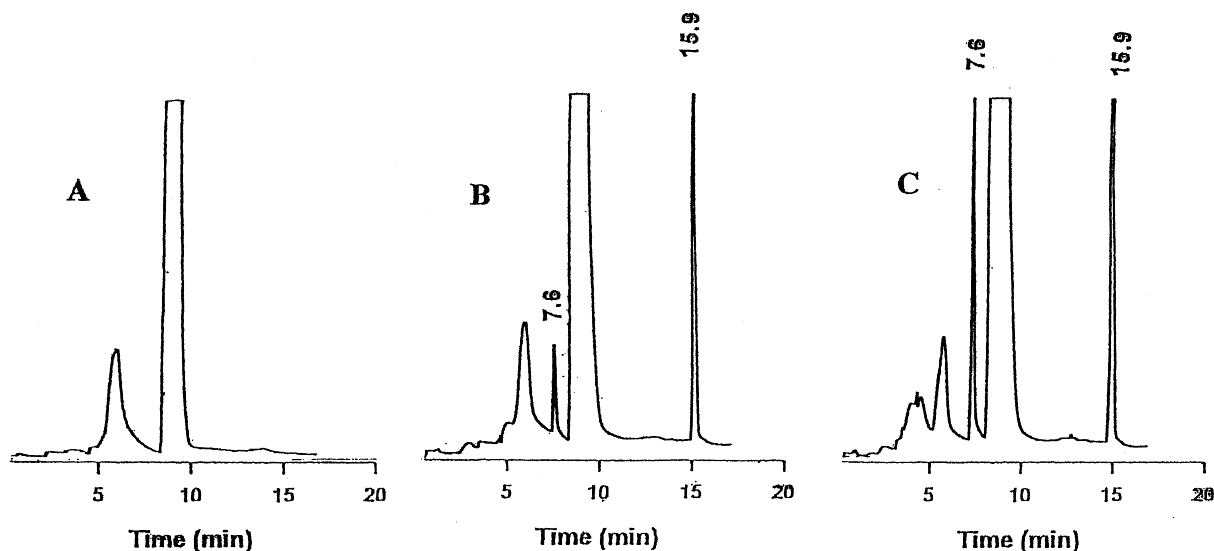

Fig. 1. Representative assay chromatograms of a pre-dose rat blood sample (A), and rat blood samples taken during the wash-out (B; 24.7 ng/ml), and apparent steady-state (C; 259 ng/ml) conditions. The retention times of ISBN and NPN were 7.6 and 15.9 min, respectively.

Table 2
The intra- and inter-day variabilities of ISBN determinations in rat blood, water and pentane (n=6 each)[a]

Concentration (ng/ml)	Coefficient of variation (%)					
	Intra-day			Inter-day		
	Blood	Water	Pentane	Blood	Water	Pentane
1.0	10.6	8.2	6.6	14.4	10.0	9.1
10.4	7.7	4.2	4.8	10.7	8.8	10.2
41.7	4.9	2.1	1.9	9.3	10.1	4.7
77.0	3.4	1.8	2.9	9.4	8.4	6.3
123.0	3.6	1.7	3.3	11.9	4.1	4.9
400.0	3.5	2.2	1.1	3.3	4.6	3.8

[a] Regression equations for ISBN determination in rat blood, water and pentane (y=peak area ratio, x=ISBN concentration); slope and intercept values are expressed as mean±standard deviation: ISBN in rat blood: $y=1.22\ (\pm0.44)\cdot10^{-3}x-1.19\ (\pm3.71)\cdot10^{-3}$; $r^2=0.96\ (\pm0.02)$; ISBN in water: $y=1.39\ (\pm0.30)\cdot10^{-3}x-0.58\ (\pm6.33)\cdot10^{-3}$; $r^2=0.96\ (\pm0.04)$ and ISBN in pentane: $y=1.06\ (\pm0.17)\cdot10^{-3}x-3.09\ (\pm1.88)\cdot10^{-3}$; $r^2=0.98\ (\pm0.01)$.

The degradation of ISBN in phosphate buffer and biological fluids is shown in Fig. 2. The rate of ISBN degradation in all fluids appeared to follow first-order kinetics. The average half-life for ISBN in human blood, rat blood, rat plasma and phosphate buffer were 1.2±0.2, 1.7±0.2, 7.9±1.2 and 20.4±3.4 min, respectively (mean±SD; n=3–4 determinations).

Fig. 3 shows the pharmacokinetic profile of ISBN in rats after a 45-min exposure of 900 ppm ISBN. Steady-state blood concentrations of ISBN were rapidly achieved at approximately 290 ng/ml and remained at this level throughout the entire dosing interval. After ISBN exposure was terminated, nitrite concentrations declined mono-exponentially with a half-life of 1.4±0.2 min, which was unchanged over five half-lives.

4. Discussion

Analytical methods for the determination of nitrites in biological fluids have never been reported, probably because of their unstable chemical and physical properties. We have described a sensitive and reproducible gas chromatographic assay for a volatile nitrite using unconventional sampling and processing techniques to circumvent these problems.

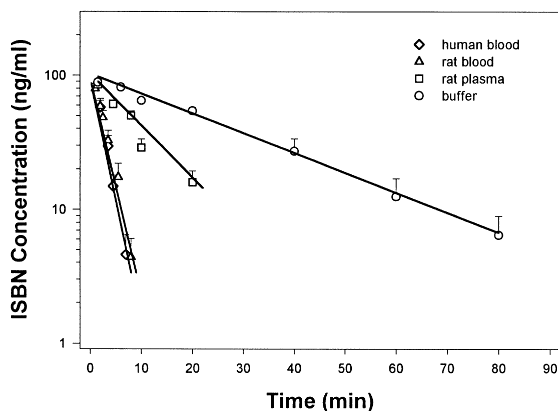

Fig. 2. Degradation of ISBN in several biological media and aqueous buffer. Bars represent standard deviations of mean observations of three to four replicates.

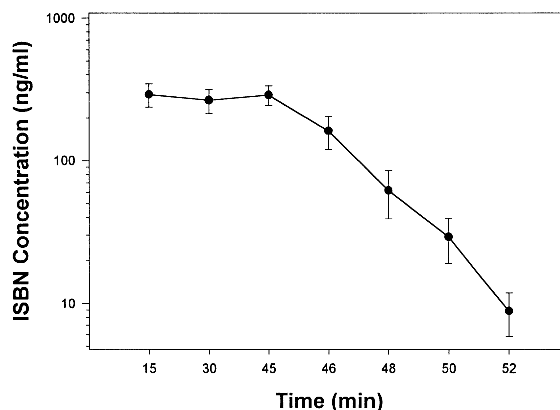

Fig. 3. Whole blood concentration of ISBN in rats during and after inhalation of 900 ppm ISBN for 45 min (n=6).

In preliminary experiments, we determined that enzyme inhibitors such as *N*-ethyl maleimide (a sulfhydryl alkylator), silver nitrate (which denatures protein), and a sodium fluoride–potassium oxalate mixture were not effective in stabilizing inhalant nitrite in blood. Therefore, in our pharmacokinetic study, ISBN blood samples were withdrawn using a gas-tight glass syringe stored at 0°C prior to use. To verify that this process did not lead to substantial loss of ISBN before analysis, either due to blood degradation or volatility, the blood withdrawal procedure was simulated and examined in vitro. Under these conditions, it was determined that ISBN loss during simulated sampling was less than 5% before analysis.

The in vitro instability study indicates that ISBN degradation is rapid in biological fluids at physiological temperature. Because of this property, the sample processing of ISBN needed to be carried out under cold temperatures, rapidly and under gas-tight conditions. Immediate extraction into pentane at 0°C in sealed glass vials efficiently removed ISBN from whole blood and allowed us to quantify it in a solvent in which it did not degrade. Under these conditions, the limits of quantification and detection of ISBN from rat blood were 1 and 0.4 ng/ml, respectively. The latter value was determined at a signal-to-noise ratio of 3:1.

The faster elimination of ISBN in rat plasma and rat blood versus buffer indicates that nitrite degradation is not due solely to chemical hydrolysis, but also to enzymatic degradation. Furthermore, the increased instability in rat blood versus rat plasma suggests that components of red blood cells may also contribute to nitrite degradation. There appears to be no substantial difference in the rates of degradation of ISBN in human blood compared to rat blood (half-life of 1.2 min for human versus 1.7 min for rat).

We illustrated the application of our analytical method in an animal study in which ISBN pharmacokinetics were determined after inhalation. Our results indicate that ISBN is rapidly absorbed through this route, since steady-state blood concentrations were seen at 15 min after dosing. Following termination of inhalation, ISBN levels declined mono-exponentially with an average half-life of 1.4 min. The rapid systemic absorption and elimination of ISBN are consistent with its vasodilative activity observed in rats (unpublished data) and the acute effects of the inhalation of organic nitrites seen in humans [9,10].

In summary, we have developed a specific assay for the determination of ISBN in rat and human blood. Due to the uncommon physicochemical properties of ISBN, our sampling and extraction procedures emphasized low temperature, rapidity and gas-tight processing. Our developed method is capable of efficient and reproducible recovery of ISBN from blood at the 1 ng/ml level. We confirm that this method is suitable for pharmacokinetic studies of ISBN, and may be applied in the examination of the pharmacokinetics and pharmacodynamics of volatile nitrites. Since we show that ISBN undergoes rapid systemic clearance, it is uncertain whether the present method has the requisite sensitivity for quantifying ISBN under human usage conditions. However, deaths due to ingestion of nitrites have been documented [11,12] and it is possible that the present method may also have utility in forensic situations.

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