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Note

Determination of isoxsuprine in equine plasma by high-performance liquid chromatography with electrochemical detection

AYMAN HASHEM*

Department of Pharmacology and Toxicology, School of Veterinary Medicine, Free University of Berlin, Koserstrasse 20, 1000 Berlin 33 (Germany)

and

BENEDIKT LUBCZYK

Department of Equine Diseases and General Surgery, School of Veterinary Medicine, Free University of Berlin, Oertzenweg 19b, 1000 Berlin 37 (Germany)

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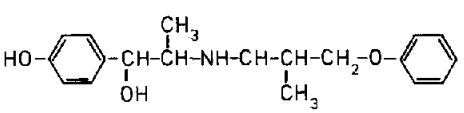
Isoxsuprine (*p*-hydroxy-α-{1-[(1-methyl-2-phenoxyethyl)amino]ethyl}benzyl alcohol) (Fig. 1) is an adrenaline-like agent that acts on the smooth muscles, especially those of peripheral blood vessels, by β-adrenergic receptor stimulation [1]. It is widely used in the pharmacological treatment of premature labour [2] and hypertension [3] and as a peripheral vasodilator [4,5]. Because of its direct action on blood viscosity, isoxsuprine is also used in the treatment of a variety of ischaemic disorders [6,7]. In equine medicine, isoxsuprine is used in the treatment of navicular disease [8]. In equine sport, some trainers have used isoxsuprine prior

ISOXSUPRINE



NYLIDRIN

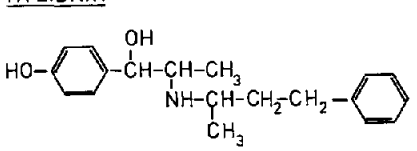


Fig. 1. Structures of isoxsuprine and nylidrin.

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to a race to improve performance [6]. As this doping is prohibited in many countries, a sensitive method to detect isoxsuprine in plasma and urine in racehorses is required. Moreover, a sensitive method is also required to perform kinetic studies, therapeutic drug monitoring and residual analysis of foodstuffs from animals which may have been treated with isoxsuprine.

Among methods used to detect isoxsuprine, only a gas chromatographic method described by Cova *et al.* [2] is sensitive enough for the determination of plasma concentrations of this substance at therapeutic dose levels. This procedure utilizes alkaline extraction from the plasma, derivatization with trichloracetic anhydride and chromatography on a 3% OV-17 column using electron-capture detection. The aim of this study was to develop a simple and sensitive high-performance liquid chromatographic (HPLC) method.

As isoxsuprine is structurally related to catecholamines, it was thought that it would be electrochemically active. Nylidrin (*p*-hydroxy-α-{1-[(1-methyl-3-phenylpropyl)amino]ethyl}benzyl alcohol) (Fig. 1), another adrenaline-like agent with a similar structure, would be useful as internal standard.

EXPERIMENTAL

Chemicals

All chemicals were of analytical-reagent grade and most were purchased from Merck (Darmstadt, Germany). The solvents for HPLC were of LiChrosolv grade (Merck). Octanesulphonic acid was supplied by Fluka (Buchs, Switzerland). Dowex HCR-S resin (20–50 mesh) was supplied by Serva (Heidelberg, Germany). Nylidrin hydrochloride was purchased from Sigma (St. Louis, MO, U.S.A.). Isoxsuprine hydrochloride was supplied by Duphar (Weesp, The Netherlands).

Sample preparation

Equine blood was drawn from the jugular vein into heparinized test-tubes, centrifuged at 2600 *g* for 10 min and the plasma was stored frozen (−30°C) until it was used.

The Dowex resin was washed twice in distilled water and allowed to equilibrate in McIlvaine buffer [0.1 *M* citric acid–0.2 *M* Na_2HPO_4 (29:71, v/v), pH 6.5] prior to use.

In Eppendorf micro-reaction vials, 0.5 ml of plasma supplemented with various amounts of isoxsuprine and nylidrin and 0.5 ml of McIlvaine buffer (pH 6.5) were added and mixed briefly. About 50–75 mg of the equilibrated resin were added to the mixture and the vials were sealed and allowed to equilibrate in an overhead mixer for 30 min (10–15 rpm). After removal of the supernatant with a needle attached to a suction pump, the resin was washed twice by adding 1 ml of buffer and subsequent suction after brief mixing. Finally, the adsorbed isoxsuprine and nylidrin were eluted from the resin by adding 0.5 ml of alkaline methanol and overhead mixing for 30 min. A 20 μl volume of the eluate was

injected into the HPLC system to determine the concentration of isoxsuprine. In the experiments to determine the adsorption and recovery of isoxsuprine and nylidrin, 1 μg of each drug was dissolved in 1.0 ml of McIlvaine buffer and allowed to adsorb on the Dowex resin. A 20-μl volume of the buffer supernatant was injected into the HPLC system to determine the unadsorbed portion of the drugs. Finally, the adsorbed isoxsuprine and nylidrin were eluted from the resin by adding 0.5 ml of each of the eluents described in Table I and overhead mixing for 30 min.

A 20-μl volume of each eluate was injected into the HPLC system to determine the elution capacity of the different eluents. Generally, the adsorption was complete after 30 min but the elution capacity varied widely from one eluent to another (see Results and Discussion).

Chromatographic apparatus and conditions

The chromatographic apparatus consisted of a Type 364.00 HPLC pump (Knauer, Bad Homburg, Germany) equipped with a pulse damper and a Type 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.). A Spherisorb ODS-I (5 μm) reversed-phase (Phase Separations, Queensferry, U.K.) column (250 × 4.6 mm I.D.) was used with a guard column (25 × 4.6 mm I.D.) containing the same material. Both the column and precolumn were supplied by Melz-VDS (Berlin, Germany). The mobile phase was 30 m*M* KH_2PO_4 (adjusted to pH 3.0 with concentrated orthophosphoric acid)–methanol–acetonitrile (520:180:300, v/v). To this mixture octanesulphonic acid was added to a final concentration of 1.8 m*M*. The mobile phase was filtered and degassed in an ultrasonic bath under vacuum and subsequently with helium prior to use. The flow-rate was 1 ml/min.

The HPLC system was connected to an M20 amperometric detector (Gynkotek, Germering, Germany) with a PTFE flow cell and a glassy carbon working electrode. Of various potential settings tested, a potential of 950 mV *versus* Ag/AgCl reference electrode was found to be suitable for the detection of both isoxsuprine and nylidrin. At this potential the offset current was 0.8–1.1 nA. The detector sensitivity was set to a value of 0.08 nA/fs. The detector was linked to a Shimadzu L-R3A integrator. Both peak height and area were used to calculate the results.

RESULTS AND DISCUSSION

Chromatographic procedure

Figure 2 shows representative chromatograms of isoxsuprine and nylidrin after extraction from a buffer solution and from spiked equine plasma.

The combination of a reversed-phase column material with ion-pairing agents in mildly acidic mobile phases provides a good method for separation of basic substances. In the present method, the mobile phase provides sufficient acidity to keep both isoxsuprine and nylidrin ionized. The concentration of octanesulphon-

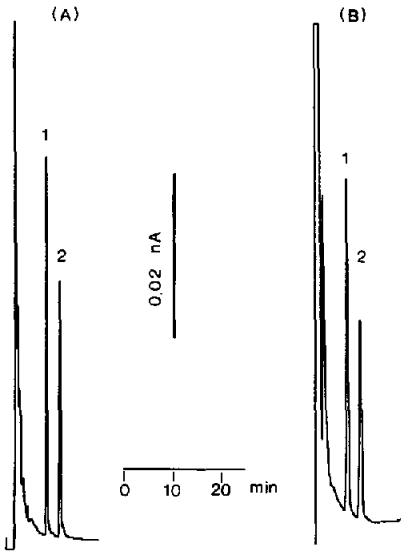

Fig. 2. Representative chromatograms of (1) isoxsuprine and (2) nylidrin (50 ng/ml each) after extraction from (A) buffer and (B) spiked equine plasma.

ic acid correlated well with the retention times and the resolution of both drugs. Concentrations higher than 2 m*M* resulted in longer retention times and broad, tailed peaks. At low concentrations, the peaks were sharper and the retention times shorter, but the resolution was reduced and co-elution of many disturbing peaks between isoxsuprine and nylidrin was observed. The pH of the mobile phase affects the chromatographic procedure markedly, but the ionic strength seems to have no effect over a wide concentration range. We observed no disturbance in the concentration range up to 0.3 *M* KH_2PO_4 in the mobile phase, although the column lifetime was slightly reduced.

Fig. 3 shows current–voltage curves for isoxsuprine and nylidrin. Although at higher voltages the detector response could be increased by almost 100%, the voltage setting of 950 mV *vs.* Ag/AgCl provides sufficient sensitivity without adverse effects due to deterioration of the mobile phase.

Extraction procedure

The selective adsorption of isoxsuprine and nylidrin on a cation-exchange resin represents an effective means for the isolation and purification of these drugs. Compared with alkaline extraction with organic solvents [2,6], the method allows measurement at high sensitivity ranges without interference and disturbance due to co-extractants or an unstable baseline. This is especially important when using an amperometric detector, which is sensitive to interfering factors.

To determine the linearity of the method, calibration graphs were constructed

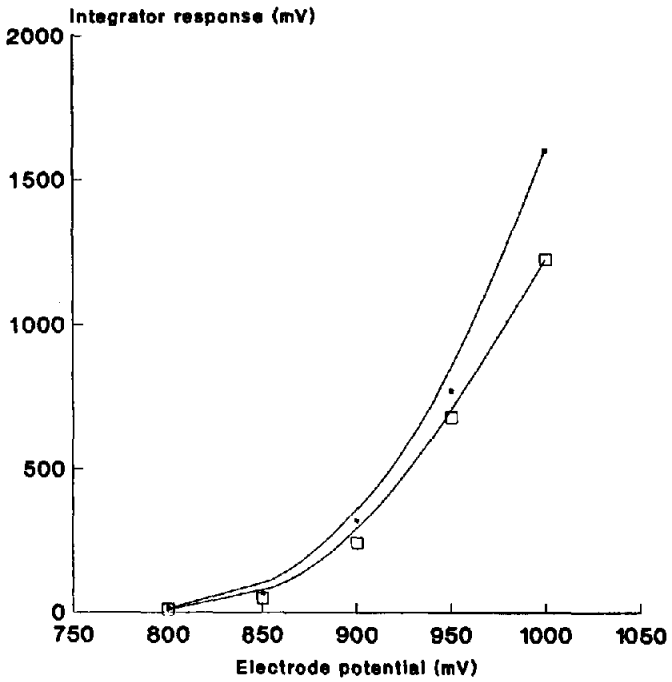

Fig. 3. Current–voltage curves for (■) isoxsuprine and (□) nylidrin. The detector output (current) is expressed as integrator response (peak height).

after extraction of isoxsuprine-spiked plasma in the range 5–200 ng/ml. The graphs showed good linearity in this range. Using least-squares regression analysis, a correlation coefficient of 0.9996 was calculated. The adsorption of isoxsuprine and nylidrin on the Dowex resin was found to be strongly dependent on the ionic strength and the pH of the buffer used. The best adsorption was obtained at pH 6.5. Higher pH values led to markedly less adsorption, whereas at lower pH the adsorption was complete, but the HPLC separation showed disturbances due to a noisy baseline and co-extractants.

Elution from the Dowex resin was best using alkaline methanol (Table I). Other eluents with counter ions having a high affinity to Dowex (Ba^{2+}, Ca^{2+}) led not only to less elution, and hence reduced recoveries, but also to deformed baselines and broad peaks. It is noteworthy that elution from the Dowex resin with alkaline methanol led to sharper and narrower peaks than those obtained by injection of aqueous non-extracted standards. This is not a drawback, however, as sharper and narrower peaks allow a higher sensitivity of the method. This effect on peak shape is probably due to the alkaline nature of the eluate, which cannot be completely suppressed by the mobile phase. The ion pairing with octanesulphonic acid would be then slightly incomplete, leading to this peak sharp-

TABLE I

RECOVERY OF ISOXSUPRINE AND NYLIDRIN FROM DOWEX RESIN AFTER ELUTION WITH DIFFERENT ELUENTS

After adsorption of 1 ml buffer containing 1 μg of both isoxsuprine and nylidrin on the resin and subsequent elution with the same volume of the eluents, 20 μl of both buffer supernatant and eluates were injected. Eluents: (1) octanesulphonic acid in 1 *M* HCl (20 mg/ml)–methanol (1:1, v/v); (2) $CaCl_2$ in water (100 mg/ml)–methanol (1:1, v/v); (3) $Ba(OH)_2$ in water (100 mg/ml)–methanol (1:1, v/v); (4) HPLC mobile phase; (5) KOH in water (100 mg/ml)–methanol (1:1, v/v). Data are expressed as means ± S.D. (n = 6).

Eluent	Recovery (%)	
	Isoxsuprine	Nylidrin
Buffer supernatant	8.9 ± 2.2	10.2 ± 3.5
1	73.9 ± 8.8	67.3 ± 7.9
2	74.4 ± 5.5	68.0 ± 6.9
3	41.5 ± 6.9	42.6 ± 8.3
4	51.4 ± 6.4	70.4 ± 4.1
5	92.6 ± 5.4	90.2 ± 5.2

ness. This alkaline nature is probably also responsible for the instability of the eluates after 6 h (see below).

Inter and intra-assay reproducibility

To determine the inter-assay reproducibility of the method, plasma samples spiked with various amounts of isoxsuprine were divided into 0.5-ml portions and

TABLE II

INTER-ASSAY REPRODUCIBILITY AND RECOVERY OF ISOXSUPRINE FROM PLASMA AFTER EXTRACTION AND DETERMINATION ON FOUR CONSECUTIVE DAYS

The data are expressed as the relative response to the internal standard (50 ng/ml nylidrin). The R.S.D. is expressed as a percentage of the standard deviation relative to the means and the recovery is expressed as a percentage of peak heights relative to those of unextracted standards of the corresponding concentrations. Data are expressed as means ± S.D. (n = 4).

Concentration (ng/ml)	Relative response *vs.* 50 ng/ml nylidrin	R.S.D. (%)	Recovery (%)
0	0	–	–
5	0.19 ± 0.014	7.4	92.1 ± 12.3
10	0.353 ± 0.026	7.4	81.5 ± 9.0
20	0.677 ± 0.039	5.8	82.9 ± 7.7
50	1.558 ± 0.123	7.9	77.1 ± 3.5
100	3.168 ± 0.177	5.6	81.1 ± 9.7
200	6.82 ± 0.355	5.2	83.4 ± 3.8

TABLE III

INTRA-ASSAY REPRODUCIBILITY AND LOSS OF ISOXSUPRINE AND NYLIDRIN DUE TO DECOMPOSITION

Plasma samples were extracted and injected into the HPLC system six times consecutively. One injection was performed 6 h later. The losses are expressed as a percentage of the initial values. The R.S.D. is expressed as a percentage of the standard deviation relative to the means. Data are expressed as means ± S.D. (n = 6).

Concentration (ng/ml)	Relative response *vs.* 50 ng/ml nylidrin	R.S.D. (%)	Loss after 6 h (%)
10	0.327 ± 0.014	4.3	33.1
20	0.657 ± 0.021	3.2	35.9
50	1.652 ± 0.040	2.4	32.7

kept frozen at −30°C. Over the following 2 months, four complete sets of samples in the concentration range 5–200 ng/ml were thawed, supplemented with 50 ng/ml of nylidrin and analysed using the proposed method. Table II gives the results of these experiments. The relative standard deviations (R.S.D.) ranged from 5.2 to 7.9% and the recovery of isoxsuprine and nylidrin from plasma varied from 77.1 to 92.1%.

To determine the intra-assay reproducibility, plasma samples were spiked with 10, 20 and 50 ng/ml of isoxsuprine and 50 ng/ml of nylidrin. After extraction, each sample was injected six times during the first 3 h. After 6 h, the samples were injected again and the peaks compared with those of the initial injections. As shown in Table III, the alkaline eluates were stable for at least 3 h at room temperature (20–22°C). The R.S.D.s ranged between 2.4 and 4.3%. After 6 h, marked losses were observed, which varied between 32.7 and 35.9%. However, these losses can be overcome by freezing the eluates until injection. In the high concentration ranges, this loss should not be dramatic as both isoxsuprine and nylidrin decompose equally. No significant changes in the relative reponse to the internal standard were observed even after 12 h at room temperature.

Applicability and efficiency of the method

The proposed extraction and chromatographic method showed very good precision and resolution. The method has been used routinely in our laboratory to study the pharmacokinetics of orally administered isoxsuprine and its pharmacological effects on blood viscosity. Fig. 4 shows the mean plasma levels of isoxsuprine in each of six horses after intravenous and oral treatment with therapeutic doses. It is sensitive enough to detect plasma levels of isoxsuprine even after long periods after administration. However, if increased sensitivity is desirable, the injection volume can be increased. Injection volumes up to 100 μl have been used without negative effects on the chromatographic process. Another way

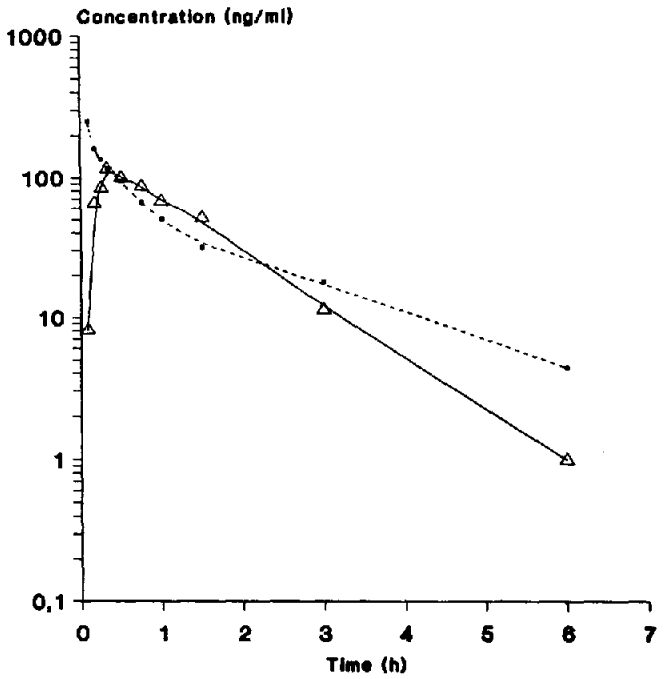

Fig. 4. Mean plasma levels of isoxsuprine after (■) oral (1.2 mg/kg) and (△) intravenous (0.6 mg/kg) administration to each of six horses.

to increase the sensitivity of the method is to increase the volume of plasma to be extracted or to decrease the elution volume from the Dowex resin. Volumes of up to 2 ml of plasma could be extracted using the above method without disturbance. Using a signal-to-noise ratio of 3, as little as 1 ng/ml of plasma could be detected using the above modifications. Hence the sensitivity of the method described by Cova *et al.* [2] can be achieved without using a gas chromatograph, which may not be available in some laboratories.

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