Simultaneous Analysis of Furosemide and Bumetanide in Horse Plasma Using High Performance Liquid Chromatography

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A high performance liquid chromatographic method is described for the simultaneous determination of furosemide and bumetanide in horse plasma. The C_8 (3 μm) reversed phase column (4.8 × 150 mm) provided clear separation of furosemide and bumetanide with other components present in the horse plasma. The detection limit for both the drugs was 10 ng/mL. Both drugs were stable in plasma (at natural or acidic pH) for up to 24 h. The method is sufficiently sensitive to detect furosemide levels in plasma obtained from horses receiving a therapeutic dose of furosemide.

INTRODUCTION

Furosemide (Lasix) is a potent diuretic agent which is used for the treatment of edema and ascites in humans, and epistaxis in race horses. Since the occurrence of epistaxis bleeding during a race can be dangerous (Tobin, 1981), use of furosemide for the purpose of controlling bleeding in race horses is allowed in many racing jurisdictions. However, quantitative analysis of furosemide in plasma is necessary to assure controlled use of this drug in horses. Recent studies have indicated that bumetanide (Bumax), a furosemide analogue, can also be used in horses for diuretic action (Grady *et al.*, 1987). However, the use of bumetanide in race horses is considered illegal. Therefore, it is important to develop assay procedures which will quantitate both the drugs in a single run. The existing assays used for furosemide and bumetanide (fluorometric (Lindstrom and Molander, 1974), gas chromatographic (GC) (Tobin *et al.*, 1978; Lin *et al.*, 1979; Smith *et al.*, 1980), high performance liquid chromatographic (HPLC) (Uchino *et al.*, 1984; Ray *et al.*, 1984; Voller *et al.*, 1976; Smith, 1982; Davies, 1974), and immunological (Dixon, 1976; Rapaka *et al.*, 1982) assays) are specific for furosemide or bumetanide and do not detect both drugs simultaneously. The objective of this investigation was therefore to develop a simple and sensitive HPLC assay for the quantification of furosemide and bumetanide in horse plasma.

EXPERIMENTAL

Materials. Furosemide, bumetanide, sodium phenobarbital, and naproxen were obtained from the Veterinary Pharmacy, University of Minnesota. The HPLC consisted of two Beckman pumps, model 126 UV detector, Spectrophysics autosampler and Spectrovision-300 fluorescence detector. The columns used were (1) reverse phase (RP), 3 μm, C_8 Alltech/Applied Sciences (4.6 × 150 mm), (2) RP, 3 μm, C_{18} (4.6 × 50 mm), and (3) RP, 5 μm, C_{18} (4.6 × 250 mm).

Extraction of plasma. One mL plasma (containing furosemide and bumetanide) was mixed with sodium phenobarbital or naproxen (500 ng) as the internal standard. (500 ng). Plasma was extracted for furosemide and bumetanide by using the following extraction procedures. (1) Plasma samples were deproteinized with acetonitrile and the acetonitrile layer was collected by centrifugation; (2) plasma samples were acidified with 1.0 mL HCl (0.1 N) and extracted with dichloromethane (DCM), ethyl acetate, or petroleum ether (10 mL); or (3) plasma samples were acidified with 1.0 mL HCl (0.1 N), extracted with DCM (10 mL), back extracted with $NaHCO_3$ (1.0 M), the pH of $NaHCO_3$ was adjusted to 3.0 and extracted with DCM. The final extract from each procedure was dried at 50 °C under nitrogen, and the dried residue was redissolved in 1.0 mL of the mobile phase (acetonitrile + phosphoric acid, 0.01 M (35 : 65, v/v)). 20 μL extract was injected into the HPLC.

Chromatographic conditions. In order to establish a suitable chromatographic condition initial experiments were conducted by using the three columns. However, the quantitative experiments were done by using the C_8 column. The mobile phase for the simultaneous analysis of furosemide and bumetanide consisted of acetonitrile + 0.01 M phosphoric acid (35 : 65, v/v). For the C_8 column, the flow rate was 1.0 mL; for the 50 mm C_{18} column, the flow rate was 0.75 mL; and for the 250 mm C_{18} column the flow rate was 2.0 mL per min. The internal standard (phenobarbitol or naproxen) was monitored by the UV detector at 254 nm, and furosemide and bumetanide were monitored by the fluorescence detector at 235 nm (excitation) and 409 nm (emission) wavelengths. Excitation wavelengths 254, 280 and 345 nm were also tested in this study.

Recovery. Known amounts of furosemide and bumetanide were added in 1.0 mL plasma and the plasma was extracted by the extraction procedures described earlier ($n = 5$ for each extraction procedure). Each sample was analyzed by the HPLC equipped with the C_8 column (UV wavelength 254 nm, fluorescence excitation wavelength 235 nm, and emission wavelength 409 nm). The area under the curve for furosemide, bumetanide and internal standard were determined. Standard samples containing known amounts of furosemide and bumetanide (mixed

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with the internal standard) were also injected into the HPLC and the area for each peak was determined. The ratio of (drug standard/internal standard) area as a function of drug concentration was plotted. The recovery was determined by comparing the amount of drug added with that of the amount of drug recovered from plasma.

Precision. Plasma samples were spiked with known amounts of furosemide and bumetanide, and samples were extracted by DCM (extraction 2). Precision of the analysis was determined by using the five samples for each concentration in the presence of sodium phenobarbitol as the internal standard. The coefficient of variation (% CV) and linearity (r) were calculated as described by Lin *et al.* (1979).

Stability of furosemide and bumetanide in plasma. Two 10 mL aliquots of horse plasma were mixed with furosemide and bumetanide (100 ng/mL final concentration). One aliquot was incubated at 25 °C for 24 h. The other aliquot was mixed with 20 mL of HCl (0.1 N) and the mixture was incubated at 25° for 24 h. One mL of plasma was pipetted from each aliquot at 30 min, and 1, 2, 4, 6, 8 and 24 h after the addition of the drugs. Plasma samples were extracted for furosemide and bumetanide as described earlier (extraction 2). The drugs were quantitated by using sodium phenobarbital as the internal standard.

Determination of furosemide levels in horse plasma samples obtained from a lasix exposed horse. A thoroughbred horse (female) was injected with 250 mg furosemide by i.v. injection. Blood samples were collected at various time intervals after the injection. Plasma was separated by centrifugation (10 000 × **g** for 15 min) and stored at −20 °C for further analysis. At the time of analysis, one mL of plasma was mixed with sodium phenobarbitol and extracted as described earlier. For quantitation, a standard curve was prepared by adding 10, 25, 50, 75 and 100 ng of furosemide to plasma and analyzing the plasma samples by the HPLC equipped with a C_8 column. The concentration of furosemide in unknown plasma was determined by using the standard curve.

RESULTS AND DISCUSSION

Chromatographic conditions. Although several HPLC methods are available for the quantification of furosemide and bumetanide, the mobile phase, column, excitation wavelength, and extraction procedure used in different procedures are different (Lin *et al.*, 1979; Smith *et al.*, 1980; Uchino *et al.*, 1984; Rapaka *et al.*, 1982; Swezey *et al.*, 1974). To achieve simultaneous elution of furosemide and bumetanide, and to achieve maximum sensitivity, several columns, extraction procedures, and excitation wavelengths were tested in this study. The mobile phase was acetonitrile + 0.01 M phosphoric acid (35:65, v/v) for the three columns. This study indicated that (1) the retention times of furosemide, bumetanide and sodium phenobarbitol were (i) 10, 40 and 5.8 min respectively for C_{18} (5 μm), 4.6 × 250 mm column (Fig. 1), (ii) 3.8, 3.0 and 28.0 min respectively for C_{18} (3 μm), 4.6 × 50 mm columns (Fig. 2, naproxen was used as the internal standard), and (iii) 5, 3.5 and 12 min respectively for C_8 (3 μm), 4.6 × 125 mm column (Fig. 3); and (2) the excitation wavelength at 235 nm provided maximum sensitivity for both the drugs. The excitation wavelengths 254 nm and 345 nm, which have been used by previous investigators (Lin *et al.*, 1979; Smith *et al.*, 1980), were not sensitive

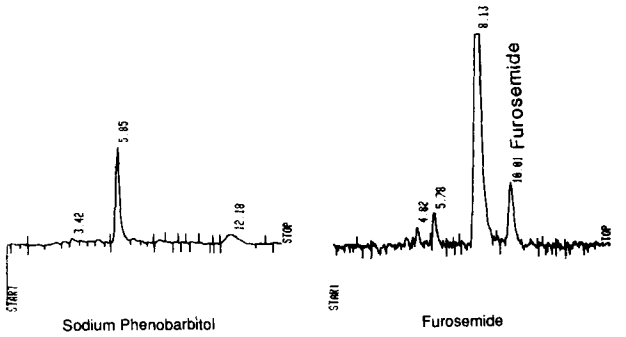

Figure 1. Chromatographic separation of furosemide (100 ng/mL) by using a C_{18}, 4.6 × 250 mm reversed phase column. Sodium phenobarbitol was monitored by a UV detector (254 nm); fluorescence detector, excitation 235 nm, emission 405 nm; mobile phase acetonitrile + phosphoric acid (0.08 M) 35:65, v/v; flow rate 2.0 mL/min.

enough in this study. Based on these observations, it is proposed that, while C_{18} columns were suitable for the individual analysis of furosemide or bumetanide, the C_8 column (3 μm) was suitable for the simultaneous analysis of both the drugs, so it was used for further quantitative experiments.

Chromatograph of blank and drug-containing plasma. The HPLC trace of a blank horse plasma is shown in Fig. 3. Two indigenous peaks (IP) were detected at retention times 4.4 (IP-1) and 13.4 (IP-2) respectively in plasma. IP-1 eluted just before furosemide and IP-2 eluted just after bumetanide (Fig. 3). When the proportion of acetonitrile + phosphoric acid was 30:70 (v/v), the furosemide and IP-1 peaks were well separated, but the bumetanide and IP-2 peaks coeluted. However, when the proportion of the mobile phase was changed to 40:60 (v/v), the furosemide and IP-1 peaks coeluted, but the bumetanide and IP-2 peaks were well separated (data not shown). The acetonitrile + phosphoric acid mixture at 35:65 (v/v) proportions provided clean separation of all peaks (Fig. 3). Because of the unique chromatographic behaviour of IP-1 and IP-2, the choice of different mobile phases was limited for the simultaneous analysis of furosemide and bumetanide.

Internal standards. The use of a proper internal standard is essential for the precise quantitation of drugs. Several

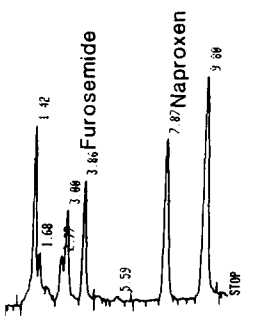

Figure 2. Chromatographic separation of furosemide (100 ng/mL) and naproxen (1.0 μg/mL; internal standard) by using a C_{18} (3 μm), 4.6 mm × 50 mm column. Mobile phase and fluorescence detection as Fig. 1. Flow rate 0.8 mL/min. Extraction: dichloromethane.

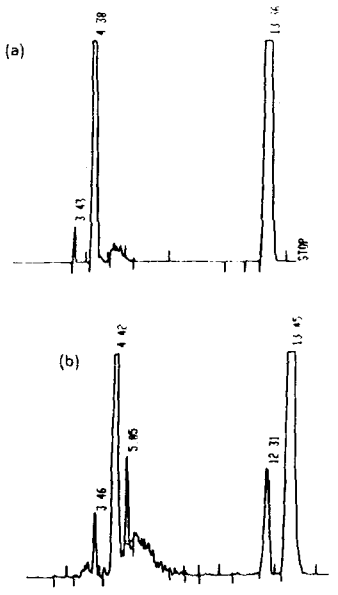

Figure 3. (a) Chromatogram of a blank plasma obtained by using a C_8 (3 μm), 4.6×150 mm column. (b) Chromatographic separation of furosemide (50 ng/mL) and bumetanide by using a C_8 (3 μm), 4.6×150 mm column. Furosemide eluted at 5.05 min; bumetonide eluted at 12.31 min. Mobile phase: acetonitrile+phosphoric acid (0.08 M) 35:65, v/v. Flow rate 1.0 mL/min. Detection as Fig. 1.

studies have used sodium phenobarbitol as an internal standard for furosemide (Lin *et al.*, 1979). However, this compound required both UV and fluorescence detectors, where furosemide or bumetanide was detected by the fluorescence detector, and phenobarbitol was detected by the UV detector. Although certain fluorescent compounds (furosemide analogues) have been used as the internal standards for furosemide (Smith *et al.*, 1980; Uchino *et al.*, 1984), they are available only on experimental basis from drug companies. This study indicated that sodium phenobarbitol or naproxen can be used as the internal standard for both the drugs. Naproxen gives a weak furosemide-like fluorescence and therefore it is also detected by the fluorescence detector (Fig. 2). Since phenobarbital and naproxen are therapeutic drugs, alternate internal standards should be used for quantitation if these drugs are already present in plasma.

Precision and recovery. The assay was evaluated by using phenobarbitol as the internal standard and demonstrated good precision (average coefficient of variation 6.01). The standard curve was linear ($r = 0.99$) over a range of 10 to 500 ng/mL for furosemide (Fig. 4) and bumetanide (Fig. 5). The recovery of furosemide and bumetanide by acetonitrile extraction was approximately 90%, but the chromatogram contained several peaks which interfered with the furosemide peak. The recovery of these drugs by ethyl acetate, DCM and ether extraction procedures were 60, 95 and 80%, respectively. Back extraction of the DCM extract with $NaHCO_3$ reduced the drug recovery to approximately 50%. These observations indicated that DCM extraction was most efficient for the quantitation of these drugs in plasma. As shown in Figs. 6 and 7, the quantitative recoveries of furosemide and bumetanide were linear from 10 ng/mL to 120 ng/mL drug concentration.

Stability of furosemide and bumetanide in normal or acidic plasma. Previous investigators have suggested that low pH causes degradative breakdown of furosemide in plasma (Blair *et al.*, 1975). Contrary to their observation, this study indicated that, under the experimental conditions used in the study, furosemide and bumetanide were stable for up to 24 h in plasma at normal or acidic

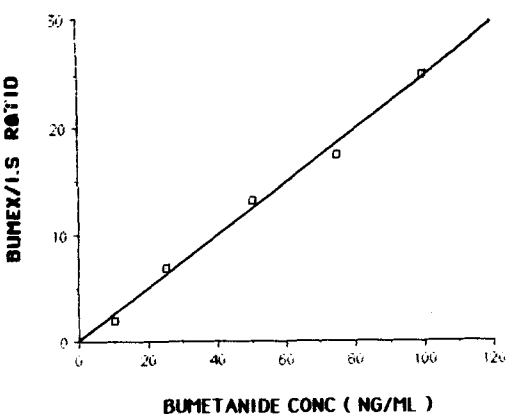

Figure 5. Standard curve for bumetanide by using sodium phenobarbital as internal standard.

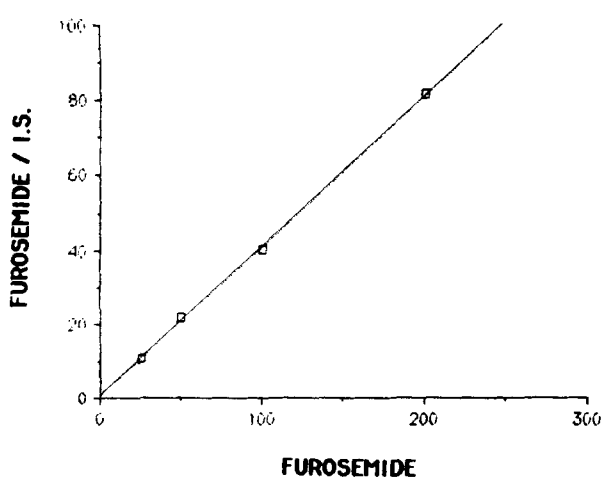

Figure 4. Standard curve for furosemide by using sodium phenobarbital as the internal standard.

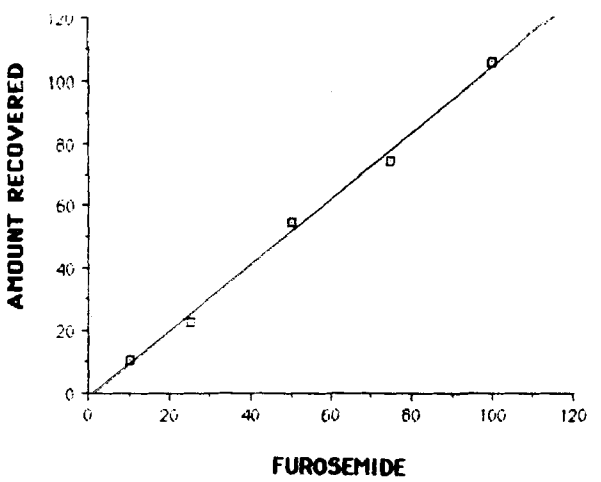

Figure 6. Percent recovery of furosemide from horse plasma by using the dichloromethane extraction procedure.

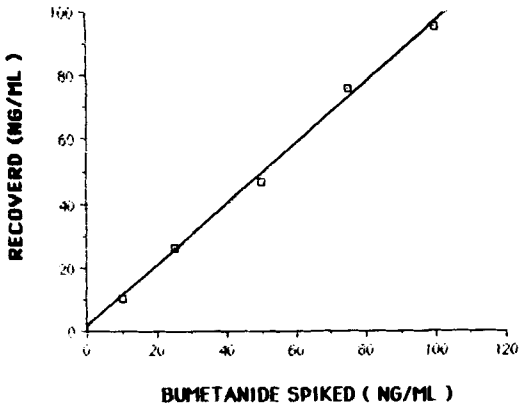

Figure 7. Percent recovery of bumetanide from horse plasma by using the dichloromethane extraction procedure.

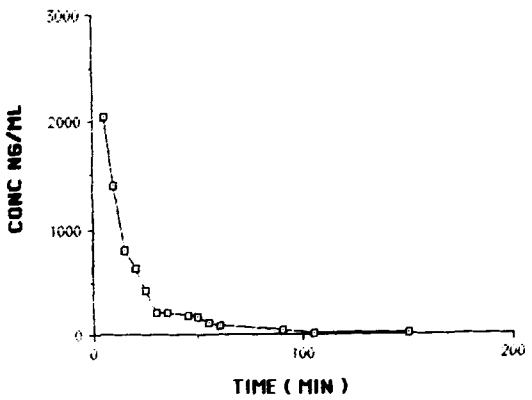

Figure 9. Concentration of furosemide in plasma obtained from a horse injected with furosemide (250 mg/kg i.v.).

pH (Fig. 8). The mean values for each time interval were within the 90% of the overall mean (100.2 ± 17.5 ng/mL). Lin *et al.* (1979) have reported that furosemide was stable for up to 20 days when plasma was stored frozen.

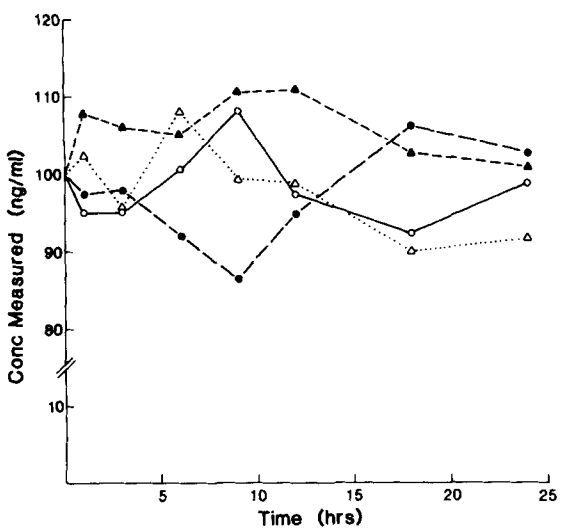

Figure 8. Stability of furosemide and bumetanide in plasma at normal pH and acidic pH. The mean of all values was 100.2± 17.5 ng/mL. △, furosemide at acidic pH; ▲, furosemide at physiological pH; ○, bumetanide at acidic pH; ●, bumetanide at physiological pH.

Analysis of plasma obtained from a furosemide exposed horse. Figure 9 shows plasma level of furosemide versus time curve after intravenous administration of furosemide. The half-life of furosemide was 20 min and the drug was detected in plasma for up to 2 h after the furosemide injection. The analysis time for each sample was 15 min and 20 samples could be analyzed in 5 h excluding the time required for extraction.

Sensitivity of the assay. The conditions of the fluorescence detector were set for moderate sensitivity (high voltage 600, range 5, pulse frequency 50) throughout this study. The detection threshold for both drugs was 10.0 ng/mL. However, the sensitivity of the assay can be substantially increased by increasing the sensitivity of the detector or by derivatizing the drugs with fluorescent derivatives. In a separate study, we compared the sensitivities of HPLC and enzyme-linked immunosorbent assays (ELISA), and observed that the two methods had similar minimum detection threshold values (10 ng/mL), although HPLC was more precise than ELISA (data not shown).

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