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# Short Communication

# Simple microscale high-performance liquid chromatographic method for determination of furosemide in neonatal plasma

Jagdev S. Sidhu and Bruce G. Charles

*Department of Pharmacy, The University of Queensland, Brisbane, Queensland 4072 (Australia)*

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ABSTRACT

A simple microscale high-performance liquid chromatographic method using fluorescence detection for the quantitation of furosemide in neonatal plasma is described. Sample pre-treatment involved protein precipitation of 25 μl of plasma with 100 μl of acetonitrile. The mobile phase consisted of acetonitrile (460 ml) and 0.08 *M* orthophosphoric acid (540 ml) and was delivered at 1.1 ml/min. Calibration curves were linear from 0.1 to 25 μg/ml. Within-day and between-day imprecision (coefficient of variation) was 3.9–6.1, and 6.1–12.2%, respectively. Furosemide was eluted after 6.5 min and naproxen (internal standard) after 11.5 min. The assay was validated for application in neonatal plasma containing a wide range of albumin concentrations.

## INTRODUCTION

Furosemide is an extensively used diuretic in neonates for the management of fluid overload states and is often administered for prolonged periods [1]. However, there is still uncertainty regarding appropriate dosages, adverse effects and plasma drug accumulation with repeated doses. In addition, little is known about its bioavailability from non-parenteral routes in this population.

Previous high-performance liquid chromatographic (HPLC) methods for the analysis of furosemide in biological fluids have involved protein precipitation [2,3], liquid–liquid extraction involving acidification [4,5] or solid-phase extraction [6]. The instability of furosemide in the presence of light, especially under acidic conditions [4], makes deproteinisation procedures the most suitable for rapid analyses. Most of these methods require volumes of plasma that are inappropriate for many critically ill neonates, or for pharmacokinetic evaluations involving multiple furosemide measurements. For instance, recent procedures which have reported limits of quantitation as low as 10 ng/ml require a plasma volume of 1.0 ml [5,7]. In one method which employed 25 μl of plasma the lowest concentration quantified was 1.25 μg/ml [3]. Plasma furosemide concentrations have been reported to range from 1.7 ± 0.3 μg/ml [8] to 4.9 ± 0.5 μg/ml [9] follow-

*Correspondence to:* J. S. Sidhu, Department of Pharmacy, The University of Queensland, Brisbane, Queensland 4072, Australia.

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ing a 1 mg/kg intravenous dose, up to 20.9 ± 9.1 μg/ml following repeated administrations [1].

This paper describes a simple and rapid HPLC assay with fluorescence detection for the analysis of furosemide in neonatal plasma. In addition, the use of naproxen as an internal standard was validated across a wide range of neonatal plasma albumin concentrations.

## EXPERIMENTAL

### *Reagents and materials*

Furosemide was obtained from Sigma (St. Louis, MO, USA) and naproxen from Fisons Pharmaceuticals (Sydney, Australia). All reagents were of analytical grade. Orthophosphoric acid (85%) was purchased from BDH Chemicals Australia (Kilsyth, Australia). Acetonitrile and methanol were HPLC grade obtained from Mallinckrodt Australia (Clayton, Australia).

Master stock (1 mg/ml) solutions of furosemide and naproxen in methanol were prepared freshly each week and kept tightly sealed at 4°C. All solutions and plasma samples containing furosemide were protected from light with aluminium foil at all times.

### *Apparatus*

The chromatographic system comprised of a Model 501 pump, a Model U6K injector and a μBondapak $C_{18}$, 10 μm (30 cm × 3.9 mm I.D.) analytical column, all supplied by Waters Assoc. (Milford, MA, USA). Column effluent was detected by a Model F-1000 spectrofluorometer (Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 270 and 410 nm, respectively. The detector signal was quantitated by a Model C-R6A integrator recorder (Shimadzu, Kyoto, Japan).

### *Sample preparation*

Plasma (25 μl) and 100 μl of acetonitrile (containing 10 μg/ml naproxen) were pipetted into 1.5-ml polypropylene micro test tubes (Bio Rad Labs., Sydney, Australia), vortex-mixed for 30 s and then centrifuged at 11 000 to 12 300 *g* for 7 min. The clear supernatant were decanted into a glass tube and evaporated under air at 55°C. The residue was dissolved in 50 μl of mobile phase of which 20 μl were injected into the HPLC system.

### *Chromatography and quantitation*

The mobile phase consisted of acetonitrile (460 ml) plus 0.08 *M* pH 2.0 orthophosphoric acid (540 ml). Chromatography was performed at room temperature (20–24°C) at a flow-rate of 1.1 ml/min.

Calibrations were established by regressing the peak-height ratio of furosemide to naproxen on plasma furosemide concentration. Furosemide content in plasma was determined by inverse prediction from the calibration equation. Standards, controls and patient specimens were analysed identically. Calibration standards were routinely prepared by supplementing drug-free plasma with furosemide stock solutions over the range 0.1–25 μg/ml. Seeded controls containing target furosemide concentrations of 0.4, 2, 6, 12 and 20 μg/ml were prepared and stored at −75°C.

## RESULTS AND DISCUSSION

Furosemide and naproxen were eluted at 6.5 and 11.5 min, respectively, after injection (Fig. 1B). Peaks from endogenous plasma components did not interfere. There was also no interference from 4-chloro-5-sulphamoylanthranilic acid, a photo-degradation product of furosemide [4], which elutes at 5.2 min, although protection of specimens from light avoided the breakdown of furosemide in the assay procedure. The specificity of the assay was further established by the lack of interference in plasma from neonates who were also being treated with one of more of the following drugs: amikacin, amoxycillin, dexamethasone, gentamicin, indomethacin, morphine, phenobarbitone, theophylline and vitamins.

The detection of furosemide metabolites in neonatal plasma is of little importance since furosemide is predominantly excreted unchanged in urine [10]. The main glucuronide derivative of furosemide has previously been shown to elute in half the time of furosemide under chromato-

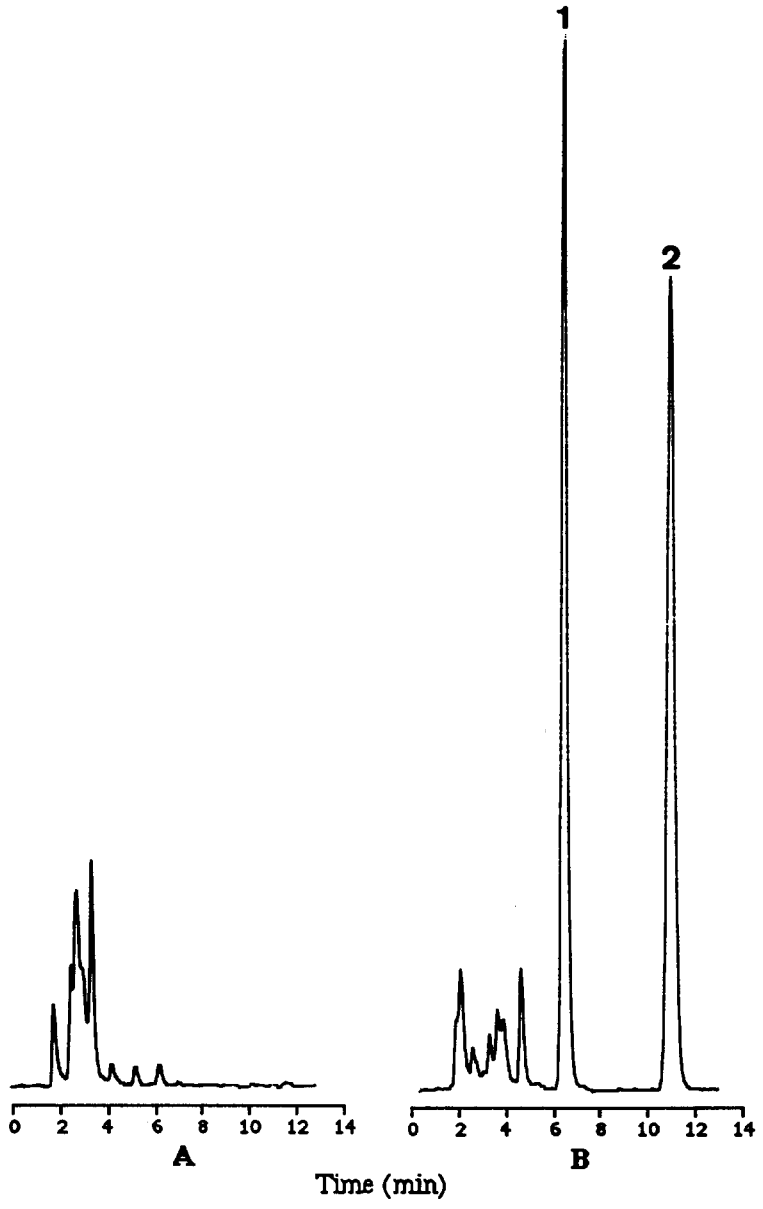


Fig. 1. Chromatograms of (A) drug-free neonatal plasma and (B) a neonate sample (9.8 μg/ml) drawn at 1 h following the sixth intravenous furosemide dose (1 mg/kg every 24 h). Peaks: 1 = furosemide (6.5 min); 2 = naproxen (11.5 min).

graphic conditions similar to those presented here [11].

Calibration plots were linear ($r^2 > 0.999$) and passed close to the origin, as given by the regression equation $y = 0.1272x + 0.0004$. Within-day and between-day coefficients of variation (C.V.) and inaccuracy were satisfactory (Table I). The minimum quantifiable concentration for the assay was 0.1 μg/ml (within-day C.V. = 13.4%).

Recovery from plasma was determined for furosemide and naproxen by comparing peak heights obtained following injection of aqueous standard solutions with those obtained from extracted plasma containing equal amounts of both

TABLE I

IMPRECISION AND INACCURACY OF FUROSEMIDE ASSAY

| Target furosemide concentration (μg/ml) | Imprecision (C.V.) ($n$ = 12) (%) | | Inaccuracy[a] ($n$ = 12) (%) |
|---|---|---|---|
| | Within-day | Between-day | |
| 0.4 | 6.1 | 12.2 | 7.5 |
| 2 | 3.8 | 5.8 | 4.0 |
| 6 | 5.9 | 7.9 | 3.0 |
| 12 | 5.2 | 8.7 | 2.4 |
| 20 | 3.9 | 6.1 | 1.4 |

[a] Defined as (measured mean − target)/target furosemide concentration × 100%.

compounds. The absolute recovery of furosemide was 76.1 ± 5.3%; naproxen recovery was 57.8 ± 6.6%.

The stability of furosemide during sample concentration was assessed by a comparison of furosemide peak heights for samples evaporated under an airstream at 55°C with those injected directly following deproteinisation. Ratios of 1.04 ± 0.05, 1.01 ± 0.07 and 0.97 ± 0.07 for plasma furosemide concentrations of 0.4, 6 and 20 μg/ml, respectively, indicated that furosemide was stable under these conditions.

Naproxen served as a suitable internal standard since it is a stable molecule, it has similar chromatographic properties to furosemide, and is detectable by fluorescence. Further, to our knowledge this drug is not used in neonatal therapeutics. Furosemide and naproxen are carboxylic acids and both are similarly highly bound to plasma albumin. Nonetheless, there is the possibility that structural differences in the molecules may be reflected by differences in their binding to denatured protein formed during sample work-up. Accordingly, drug-free plasma samples from nine newborn babies containing 21–50 g/l albumin (neonatal reference range: 28–48 g/l [12]) were supplemented with furosemide (6 μg/ml) and naproxen (10 μg/ml) and analysed as described. There was very little difference in peak-height ratio between specimens (2.17 ± 0.09, C.V. = 4.0%, range = 2.1–2.3) which establishes the validity of using naproxen as an internal standard in the assay of neonatal plasma samples.

In conclusion, we have developed a rapid and robust method which involves a simple clean-up procedure and short chromatographic run times. The very small volume of sample is well suited for neonatal application, including monitoring for furosemide toxicity in premature infants. If necessary, furosemide can be assayed below the presently reported limit (0.1 μg/ml) by doubling either the injection volume, the plasma sample volume, or both. This approach reduces the minimum quantifiable concentration to 0.02 μg/ml (within-day C.V. = 14.0%), with a limit of detection of approximately 0.01 μg/ml (peak height twice average baseline noise). Unlike several other published methods, the assay is capable of measuring plasma furosemide concentrations usually observed clinically.

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