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Quantification of furosemide from serum and tissues using high-performance liquid chromatography

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Abstract

Since pharmacokinetics may play a significant role in furosemide (FSM) developmental ototoxicity, we developed an assay for the extraction and quantification of FSM in tissue and fluid from neonatal and adult rats. Rats from post-natal day (PND) 10, 30 and 50, were given an intravenous dose of FSM (35 mg/kg). Blood and tissues were analyzed by HPLC. FSM in serum, perilymph and liver was elevated in PND ten rats as was the body burden of FSM. Renal concentrations were higher in older rats. Altered clearance of FSM in developing rats may result in higher concentrations in the cochlea and ototoxicity. © 1997 Elsevier Science B.V.

Keywords: Furosemide

1. Introduction

Furosemide (FSM) is an extensively employed loop diuretic useful in the management of fluid retention (e.g. acute pulmonary edema secondary to congestive heart failure). Loop diuretics inhibit Na^+, K^+ and Cl^- transport in the ascending loop of Henle, resulting in water retention in the tubule. One of the major side effects of FSM administration is ototoxicity. Premature infants and experimental animals appear to be at an increased risk for sensorineural hearing loss when FSM is administered during the period of cochlear development [1–5].

Studies using the rat as an animal model have demonstrated that the ototoxic effect of FSM in newborn animals was greater during the period of post-natal cochlear development [1,5]. A greater reduction of endocochlear potential (EP) and elevation of compound action potential (CAP) thresholds was observed in rats 9–20-days old compared to 30-day-old rats [1]. These functional changes were accompanied by ultrastructural alterations of the stria vascularis. Younger animals demonstrating substantial reductions of the EP exhibited interstitial edema of the stria vascularis. In contrast, older animals having very little or no change in EP had no significant alterations of the stria vascularis ultrastructure. These findings support the concept of a critical period of enhanced susceptibility to ototoxic loop diuretics during development.

Pharmacokinetic factors may contribute to the developmental ototoxic sensitivity [6]. An increased serum elimination half-life could lead to an increase in exposure time of ear tissues to ototoxic levels of FSM, resulting in a more severe or even permanent hearing loss. Another factor which may contribute to

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the sensitivity of younger animals to FSM is an immature blood–labyrinth barrier. This would allow opening of the fluid spaces and thereby granting access of ototoxic agents, including FSM, to the tissues.

The inability of the kidney to metabolize or concentrate FSM is another factor which may contribute to the increased ototoxicity of younger animals. Bräunlich, 1980 has shown that older animals accumulate FSM in renal tissues more efficiently than younger animals [7]. The inability of the kidneys to accumulate FSM in developing rats would increase serum levels. The resulting increased concentration in the central compartment, coupled with the immature blood–labyrinth barrier, would facilitate entry into the ear tissues.

Although high-performance liquid chromatography (HPLC) has been employed to detect FSM in blood, urine and perilymph, detection of FSM from tissues using HPLC has not been previously described. In an effort to examine the influence of changes in the pharmacokinetics and metabolism of FSM in developing verses juvenile rats, we developed an assay (modified from Green et al. [8]) using HPLC for the detection of FSM and FSM-glucuronide in serum, perilymph, renal and hepatic tissues. The assay proved sensitive enough to detect FSM in as little as three microliters of perilymph from 10-day-old rats.

2. Experimental

2.1. Experimental animals

Subjects were Harlan Sprague–Dawley (Indianapolis, IN, USA) albino rats. Experimental procedures followed the NIH Guide for the Care and Use of Laboratory Animals. Subjects were divided into three groups according to age: post-natal day (PND) 10, PND 30 and PND 50.

2.2. Dosing procedure and sample collection

Rat pups were anesthetized with ketamine–xylazine (172.4 and 3.4 mg/kg, respectively). The jugular veins were exposed and a dose of 35 mg/kg of FSM was injected intravenously. Blood samples were collected via cardiac puncture at 30 and 60 min post-FSM. Serum was separated by centrifugation (8874 *g* for 1 min at 4°C) and stored at −80°C. Immediately following blood collection, animals were decapitated, tissue samples collected and their temporal bones removed. Perilymph was collected with a glass micropipet and stored at −80°C.

2.3. Determination of furosemide concentration

Fifty microliters of serum were obtained and 5 μl of *p*-nitrophenol (1100 μg/ml) were added as an internal standard. The cortex and medulla were dissected from kidney samples and the liver was removed. The separate tissues were homogenized in methanol–water (80:20, v/v), centrifuged (500 *g* for 5 min at 4°C), 100 μl of the resulting supernatant were removed and internal standard added. For perilymph, like samples were pooled to yield a 3-μl volume. All samples were then acidified with 5 μl of 2 *M* H_3PO_4 (except perilymph which used 0.5 μl), extracted with 200 μl of ethyl acetate, dried under $N_{2(g)}$ and reconstituted in 30 μl of mobile phase [0.01 *M* KH_2PO_4 (pH 5.5)–MeOH (63:37, v/v)]. Protein concentrations were determined from tissue samples after centrifugation by the Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA).

A 15-μl aliquot of the sample was then injected onto the column. Components of the HPLC system were: mobile phase, 0.01 *M* KH_2PO_4 (pH 5.5) with 37% MeOH; column, C_{18}, reversed-phase, ODS, particle size 5 μm, 250×4.6 mm I.D. (Beckman Instruments, Palo Alto, CA, USA, No. 235329); detector, UV absorption at 235 n*M*; flow-rate, 1.5 ml/min. Retention times for this system are: furosemide–glucuronide conjugate: 4.4 min, furosemide: 5.6 min, *p*-nitrophenol: 8.3 min (Fig. 1). This method produced a linear standard curve from 0 to 500 μg/ml with a detection limit of less than 0.1 μg/ml.

2.4. Concentration calculations

In order to report the amount of FSM in tissue as ng/mg of protein, the amount detected on the column (μg/ml) was converted to absolute ng of the sample. The reported μg/ml from the column multiplied by the injection volume (ml) gave the abso-

lute μg detected which was then converted to ng. After correcting for extraction and sample volumes, the resulting concentration was divided by the protein concentration of the sample to give the reported values as ng/mg of protein.

2.5. Determination of the furosemide–glucuronide conjugate

In order to identify the FSM–GLC conjugate, glucuronidation was induced in the adult chinchilla by anesthetization with 80 mg/kg of pentobarbital preceding a 100 mg/kg intravenous dose of FSM.

2.6. Statistical analysis

A two-factor, repeated measures ANOVA with an alpha level set a priori at 0.05 was used to test for significant differences in furosemide concentrations between rats of different ages. All concentrations are reported as the mean±standard error of the mean.

3. Results

The method described produced a linear curve for the detection of FSM from 0 to 500 μg/ml with a detection limit of less than 0.1 μg/ml. Peak areas were 0.06±0.01. 0.10±0.01., 0.49±0.01, 0.99±0.00 and 1.47±0.01 for 1, 2, 10, 20 and 30 ng, respectively. The equation of the line is: $y=4.888\cdot10^{-2}(x)+3.257\cdot10^{-3}$, with $r^2=1.00$. Data is reported as the mean± s.e.m. ($n=3$ for each). The separation was rapid with all peaks eluting in less than 10 min (Fig. 1). The absorption of the internal standard *p*-nitrophenol (PNP) was linear over the concentrations used (data not shown).

To examine intra-assay precision, the same sample (10 μg/ml) was injected three times yielding a mean area of 8.31±0.07. To evaluate the inter-assay precision, FSM (100 μg/ml) from three individually prepared stocks was injected in three separate trials which yielded a mean area of 74.63±0.60. Recovery of the internal standard was 99.1±0.3%. This meth-

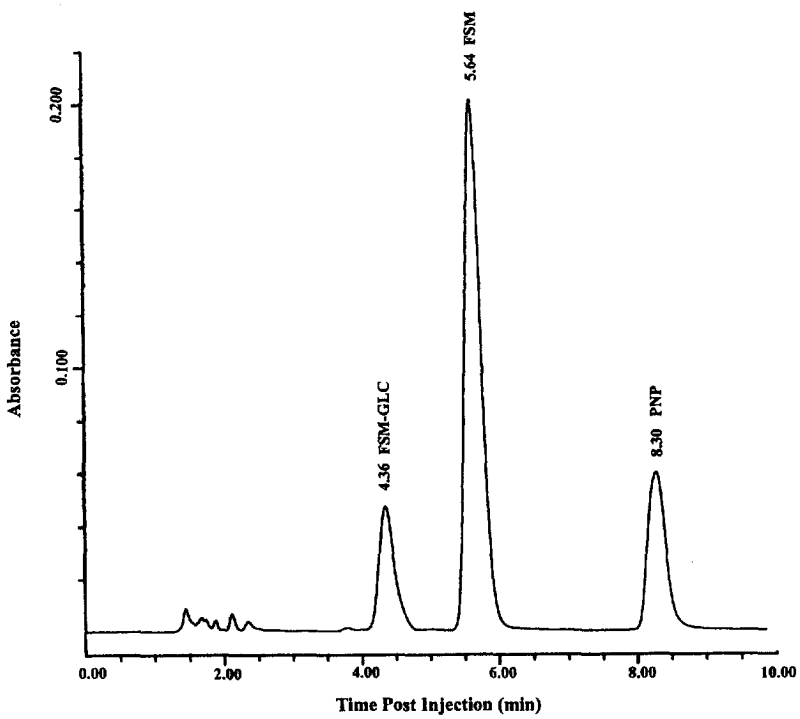

Fig. 1. Typical chromatogram, plotted as absorbance (235 nm) vs. elution time, showing the separation and detection of furosemide (FSM), furosemide–glucuronide conjugate (FSM–GLC) and the internal standard, *p*-nitrophenol (PNP). Retention times (min) are: 4.36 for FSM–GLC, 5.64 for FSM and 8.30 for PNP.

od also produced a recovery of 93.9±0.3% of FSM from spiked kidney tissues. No interfering peaks were detected in any of the samples from untreated control rats.

It has been shown that freeze–thaw has little effect on FSM measurements in serum [9]; however, light has been shown to degrade FSM. Therefore, all extractions were kept in the dark until injection onto the column.

Furosemide was detected in serum from rats of all ages after a single intravenous dose. Serum concentrations of FSM were significantly higher in the younger (PND 10) rats at 30 and 60 min post-injection compared to PND 30 and 50 rats. The body burden of FSM was greatest in 10-day-old rats. One minute after FSM injection, PND 10 rats had serum concentrations of 10.09±1.26 μg/ml/g of body weight (n=10), PND 30 rats had 2.23 μg/ml/g while PND 50 rats had 0.97±0.11 μg/ml/g (n=2). At 30 min post-injection, PND 10 serum FSM concentrations were 6.23±0.47 μg/ml/g (n=4) compared to 0.88±0.04 (n=6) and 0.79±0.03 μg/ml/g (n=4) in PND 30 and PND 50 rats, respectively (Fig. 2). After 60 min post-injection, only PND 10 levels of FSM remained elevated.

Furosemide was detected in perilymph from rats of all ages. Furosemide concentrations in perilymph

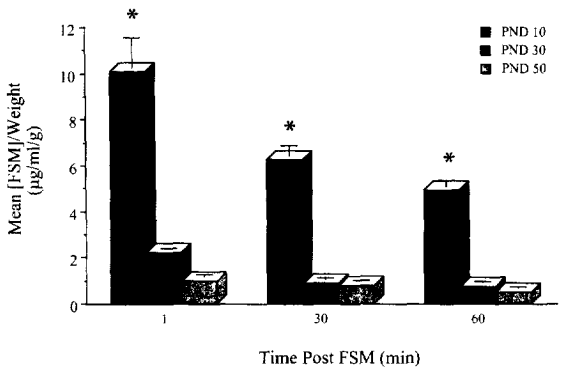

Fig. 2. Furosemide concentrations (μg/ml) in serum normalized to body weight (g) at 1, 30 and 60 min after intravenous administration (35 mg/kg) in 10-, 30- and 50-day-old rats. For PND 30 at 1 min post-FSM, the data represents the mean of 12 FSM values divided by the mean of the 12 body weights. An asterisk indicates statistically significant differences (P<0.05) compared to 30 and 50-day-old rats. Data is expressed as mean±s.e.m.

were higher in 10-day-old rats than 30 or 50-day-old rats. Concentrations for PND 10 at 30 min post-injection were 2.33 ng/μl and 9.75 ng/μl at 60 min post-injection. For PND 30, concentrations were 0.19 ng/μl at 30 min and 0.42 ng/μl at 60 min. PND 50 rats had a perilymph concentration of 0.17 ng/μl at 60 min post-injection.

Furosemide was efficiently extracted from renal cortex and medulla tissues. Renal concentrations of FSM were higher in 30-day-old rats than 10-day-old rats (Fig. 3). For PND 10 rats, renal FSM was 841.27±18.68 ng/mg of protein and 911.73±103.18 ng/mg at 30 min in the cortex and medulla, respectively. At 60 min post-injection, levels were 1060.60±135.18 and 992.47±170.01 ng/mg, in the cortex and medulla, respectively. At 30 min post-FSM injection PND 30 rats had cortex concentrations of 1973.33±45.58 ng/mg and medulla concentrations of 2014.80±150.34 ng/mg. At 60 min post-injection cortex levels were 1756.73±52.96 and medulla levels were 2116.63±111.76 (n=3 for all groups).

Liver concentrations of FSM were highest in PND 10 rats at 30 min post-FSM injection (Fig. 4). For PND 10 rats, liver FSM concentrations were 192.30±18.97 ng/mg of protein and 107.97±27.44 ng/mg at 30 and 60 min post-FSM injection, respectively. For PND 30 rats, liver FSM concentrations were 79.97±9.11 ng/mg and 83.53±17.30 ng/mg at 30 and 60 min post-FSM injection, respectively (n=3 for all groups). No FSM–GLC was detected in any sample.

4. Discussion

We have established a rapid and efficient method for the quantification of furosemide (FSM) and detection of the furosemide–glucuronide conjugate (FSM–GLC) in limited amounts of serum, perilymph and tissue. The benefits of this method are (1) the sensitivity allows for detection of FSM in limited amounts of sample and (2) this is the first method for the extraction of FSM from tissues for HPLC. The method described provides good separation between FSM–GLC, FSM and p-nitrophenol (PNP), the internal standard, without contaminants. Utilizing this method we show that younger rats have

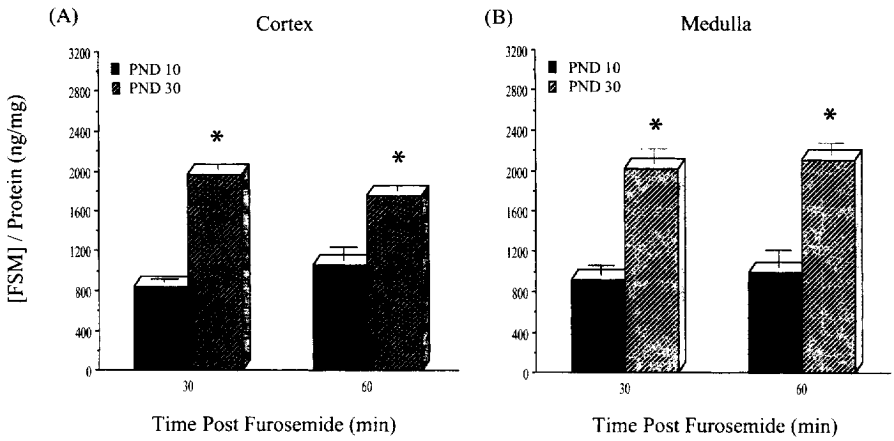

Fig. 3. Renal FSM concentrations (ng/mg protein) in cortex (A) and medulla (B) from 10- and 30-day-old rats, 30 and 60 min after intravenous administration (35 mg/kg). An asterisk indicates statistically significant differences ($P<0.05$) compared to 10-day-old rats. Data is expressed as mean±s.e.m. ($n=3$ for each).

an impaired ability to concentrate furosemide in the kidney. The decreased accumulation in the kidney of younger rats may be attributed to an immature organic acid secretory system [10] and this may lead to the higher serum and perilymph concentrations.

FSM–GLC was not detected in any of the rat tissue or serum samples. The conjugate was detected when adult chinchillas were anesthetized with pentobarbital (80 mg/kg) to induce glucuronidation. The lack of FSM–GLC in PND 10 animals may be explained by the fact that the liver in developing rats has not yet achieved the full capacity to metabolize drugs [11]. FSM in the liver was elevated in PND 10 rats which may reflect impaired metabolism at this stage of development. Also, the liver may not be the primary site of metabolism. For example, patients with severe liver disease (cirrhosis and hepatic ascites) show little change in non-renal clearance. It has been proposed that the kidney is the site of glucuronidation [12]. Perhaps the kidneys in developing rats lack the ability to form the metabolite, which would account for its absence from 10-day-old rats. Absence of FSM–GLC in serum, perilymph and renal tissues of older rats may be due to the fact that pentobarbital was not used as an anesthetic to induce glucuronidation. It has also been suggested that high enough concentrations of FSM may inhibit formation of the FSM–GLC conjugate (substrate inhibition) [11].

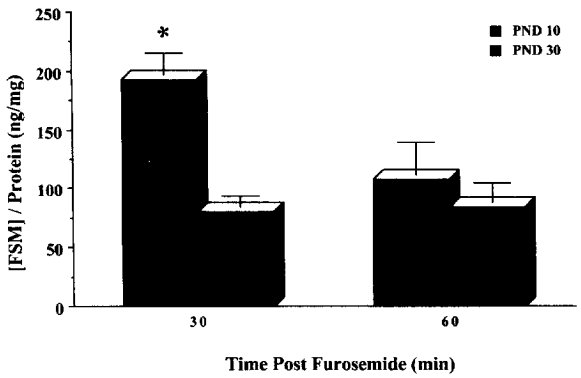

Fig. 4. Liver FSM concentrations (ng/mg protein) from 10- and 30-day-old rats, 30 and 60 min after intravenous administration (35 mg/kg). An asterisk indicates statistically significant differences ($P<0.05$) compared to 30-day-old rats. Data is expressed as mean±s.e.m. ($n=3$ for each).

Our results in the kidney are consistent with those of Bräunlich [7], in that older rats had higher concentrations than younger rats. Also in agreement with this earlier study is the lack of difference between the medulla and cortical concentrations of FSM. However, absolute values differ from Bräunlich for several reasons. First, the dose and route of administration were different. A smaller dose (0.6 mg/100 g of body weight, intraperitoneal) was used in the Bräunlich study, compared to 35 mg/kg body weight, intravenously in the present study. Secondly,

Bräunlich compared tissue levels from 5- and 55-day-old rats whereas we examined 10- and 30-day-old rats. Thirdly, he reports his values as μg FSM/g kidney weight, we report as ng FSM/mg protein.

It is interesting to note that while serum levels are their highest 1 min after injection and appear to level out at 30 and 60 min, perilymph levels are at their highest (of the time points measured) 60 min after injection. The accumulation of furosemide in the perilymph over time in the younger animals may explain the increased ototoxic effects seen with furosemide administration.

There are several factors in the cochlea that could account for the increased FSM concentration and influence the severity of ototoxicity from FSM. Opening of the fluid spaces may allow the access of ototoxic agents to the inner ear tissues. The blood–labyrinth barrier is a concept which has developed by way of analogy to the blood–brain barrier [13,14]. It has been demonstrated that the blood–brain barrier undergoes developmental changes which reduce the penetration of various substances into the brain. The tightening of the blood–brain barrier in mice was found to be mediated by junctional changes between endothelial cells of the brain capillaries [15]. Similar changes are likely to occur within the labyrinthine blood vessels, which are branches of the cerebral circulation. Vessels that may be quite permeable in the immature rat or human may become much less permeable in the adult. Glycerol was found to potentiate the ototoxicity of FSM in the adult, but not in the neonatal guinea pig perhaps by osmotically opening the blood–labyrinth barrier [16].

The inability of the kidney to accumulate and excrete FSM, leading to higher serum concentrations, coupled with the immature blood–labyrinth barrier, may lead to the ear tissues being exposed to ototoxic levels of FSM for an extended period of time (compared to older rats). It is likely that the enhanced ototoxicity of loop diuretics in premature infants and immature experimental animals is caused by a combination of systemic and local factors within the developing cochlea.

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