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Note

Quantitative analysis of furosemide in micro plasma volumes by high-performance liquid column chromatography

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Furosemide is a potent diuretic agent which is widely used for the treatment of various conditions in adult patients. Several reports have appeared in the literature relating to the disposition of furosemide in adults with [1—3] or without [1, 4, 5] renal disease, and in adult patients with heart failure [5]. The analytical method used to measure furosemide in serum or plasma in the majority of those studies [1, 2, 4, 5] was the spectrofluorometric assay of Haussler and Hajdu [6], or one of the modifications of it [7, 8]. Rose et al. [3] used radio-labeled furosemide in their investigation. The spectrofluorometric methods [6—8] use 1 ml of serum or plasma for analysis and suffer from a number of disadvantages, some of which have been discussed elsewhere [5, 8].

In addition to its use in adult patients, furosemide may also be required for administration to infants [9, 10]. One brief report [11] of some pharmacokinetic aspects of the drug in infants has been published. In that study furosemide in plasma was measured by gas chromatography, but the details of the analytical method were not reported. The present authors decided to initiate further pharmacokinetic studies in infants. In reviewing potential methods for the analysis of furosemide in plasma (or serum) it soon became obvious that the spectrofluorometric procedures [6—8] would not be suitable [5, 8]; not the least objection to their use is the relatively large volume of plasma (1 ml) which would need to be collected from small infants. At least one gas chromatographic technique [12] and four methods based on high-performance liquid chromatography (HPLC) [13—16], have been re-

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ported. Four of these five methods [12—14, 16] each require the use of 1 to 2 ml of plasma to achieve quantitation of furosemide in plasma with a lower limit between 0.1 and 1 μg/ml.

Blair et al. [15] have reported an HPLC method for the determination of furosemide in small volumes of serum. In that procedure 5-μl aliquots of serum were injected directly into the high-performance liquid chromatograph. A cation-exchange column maintained at 74° and eluted with buffer was used to achieve chromatographic separation, and furosemide was detected by fluorescence monitoring. The lower limit of quantitation was not stated explicitly, but appeared to be in the range of 0.5 to 1 μg/ml. A precolumn in the chromatographic system was necessary, apparently to avoid plugging the main HPLC column with the proteins contained in the serum. Also, furosemide was not completely resolved from 4-chloro-5-sulfamoylanthranilic acid which is the major breakdown product of furosemide [15] and also is a metabolite of furosemide [6].

The purpose of this paper is to report a more sensitive HPLC assay for furosemide in plasma which utilizes small sample volumes, is specific, and requires only short sample preparation procedures prior to chromatography.

EXPERIMENTAL

Materials

Furosemide was kindly supplied by Hoechst-Roussel (Sommerville, N.J., U.S.A.). 4-Chloro-5-sulfamoylanthranilic acid was purchased from U.S. Pharmacopeia (Rockville, Md., U.S.A.). Glass-distilled acetonitrile was purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.), and phosphoric acid was obtained from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Other drug substances which were tested for potential interference of the assay had, in most cases, been donated by pharmaceutical manufacturing companies.

Apparatus

A Model M-6000A pump was used to deliver the mobile phase to a Model U6K injection loop and a μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm I.D., 10 μm particle size), all of which had been supplied by Waters Assoc. (Milford, Mass., U.S.A.). The chromatography was carried out at ambient temperature (approximately 24°). A Model FS970 fluorescent detector obtained from Schoeffel Instruments (Westwood, N.H., U.S.A.) was used to monitor the column effluent; the excitation wavelength of the detector was set at 225 nm, and an emission filter (KV 389) was used to select the fluorescence emission for detection. The output from the detector was connected to a 10-mV potentiometric 25.4-cm recorder (Houston Instrument, Austin, Texas, U.S.A.). The HPLC mobile phase was acetonitrile—0.05% phosphoric acid (30:70). This was pumped through the HPLC system at a rate of 2 ml/min, and the resulting pressure was approximately 1500 p.s.i.

Procedure

Aliquots of 100 μl of plasma were pipetted into 13 × 100 mm culture tubes followed by the addition of 250 μl of acetonitrile. After sealing with

a PTFE-lined screw cap each tube was vortexed for 10 sec and this was followed by centrifugation at 800 *g* for approximately 1 min. Subsequently, most of the clear supernatant solution was poured into another culture tube, and 10 µl of this solution was then injected into the high-performance liquid chromatograph for analysis. Standard curves were developed by supplementing blank human plasma with known concentrations of furosemide. This was achieved by placing 5- to 10-µl aliquots of methanolic solutions of furosemide in containers and subsequently adding 1-ml aliquots of blank human plasma to each tube. Aliquots of 100 µl of each spiked plasma sample were carried through the procedure described above for the analysis of furosemide in plasma. Peak height measurements were used for quantitation.

RESULTS AND DISCUSSION

The HPLC conditions were selected after appropriate preliminary investigations with a number of different mobile phase compositions and detector conditions (excitation and emission wavelengths).

Chromatograms resulting from the analysis of plasma samples collected from a 2-month old infant who was receiving furosemide therapy are shown in Fig. 1. This infant was receiving theophylline, phenobarbital, and vitamins in addition to the furosemide. Also shown in Fig. 1 is the chromatogram resulting from the analysis of a plasma sample which was collected from a 7-week-old infant who was not receiving furosemide but who was being administered theophylline, phenobarbital, digoxin and vitamins. The small volumes (200—300 µl) of capillary blood samples collected from the infants were obtained by heel-stick. The total analysis time per sample was approx. 10—12 min; furosemide eluted from the HPLC system with a retention time of 8.0 min. No endogenous fluorescent substances with a retention time similar to furosemide were observed during the analysis of plasma collected from infants (Fig. 1) or adults who were not receiving furosemide. The major breakdown product [15] and metabolite [6] of furosemide, namely 4-chloro-5-sulfamoylanthranilic acid, does not interfere with the analysis of furosemide since it has a retention time of approx. 2 min under the conditions of analysis described here for furosemide.

The relationship between the furosemide peak height and furosemide concentration in plasma (Table I) was linear over the concentration range studied as indicated by the high value of the coefficient of determination for the linear regression (r^2 = 0.9999), and the constancy of the response factor (peak height divided by concentration). The maximum sensitivity for furosemide detection was 0.1 µg/ml of plasma since at that concentration the signal-to-noise ratio was ca. 4 or 5 : 1.

The precision of the method was acceptable over the entire concentration range investigated. The coefficient of variation on a given day ranged from a low of 1.03% at 10 µg/ml up to 6.22% at the 0.1 µg/ml concentration. These reproducibility data compare favorably with those of the earlier reported methods of analysis [12—16]. The coefficient of variation for the analysis of the same spiked plasma sample (5 µg/ml) on three days over a period of two weeks was 7.97%. The inclusion of an internal standard in the procedure

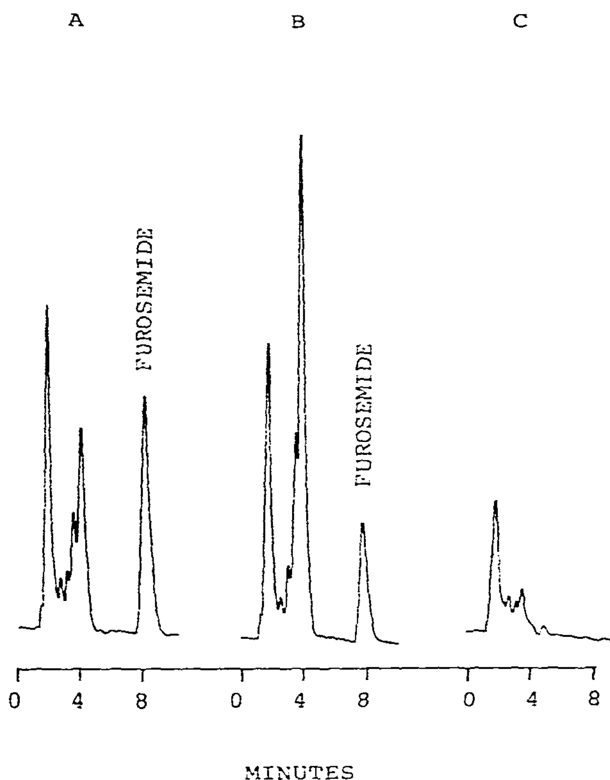

Fig. 1. Chromatograms A and B resulted from the analysis of plasma collected from a 2-month-old female infant (body weight 1.04 kg) who received a 2-mg oral dose of furosemide followed 12 h later by a similar dose. The patient was also being administered theophylline, phenobarbital and vitamins. Sample A, which was collected 2.25 h after the first dose, had a furosemide concentration of 2.51 μg/ml and sample B, which was collected 10.5 h after the second furosemide dose, had a concentration of 1.27 μg/ml. Chromatogram C resulted from the analysis of plasma collected from a 7-week-old male infant (1.36 kg) who was not receiving furosemide but who was being administered theophylline, phenobarbital, digoxin and vitamins. Detector range setting was 0.2 μA for all chromatograms and photomultiplier voltage was 820 V.

may improve the reproducibility on a day-to-day basis. If an internal standard was chosen which had a retention time of approx. 5—7 min, then such an assay based on internal standardization would not be useful for patients receiving salicylates, since salicylic acid elutes in that retention window. On the other hand, an internal standard such as the methyl ester of furosemide would elute after the drug using reversed-phase chromatography [16], hence increasing analysis time. In using the present method based on external standardization it is suggested that one or two standard samples be included on days when patient samples are to be analyzed.

The recovery of furosemide following the deproteinization of plasma with acetonitrile was essentially complete. The mean (± S.D.) peak heights (detector range 0.1 μA), for furosemide obtained by carrying 1 μg/ml aqueous and plasma solutions through the analytical procedure were 3.65 ± 0.097 cm and

TABLE I

STANDARD CURVE DATA FOR FUROSEMIDE IN PLASMA

Linear least squares regression equation for the data is: $y = 4.24\ x - 0.018$ ($r^2 = 0.9999$).

Concentration of furosemide in plasma (μg/ml)	Furosemide peak height ± SD (coefficient of variation, %)*	Mean response factor**
0.1	0.45 ± 0.03 (6.22)	4.50
0.2	0.90 ± 0.06 (6.22)	4.48
0.5	2.14 ± 0.08 (3.93)	4.28
1	4.22 ± 0.07 (1.75)	4.22
2	8.47 ± 0.13 (1.54)	4.23
5	20.91 ± 0.27 (1.29)	4.18
10	42.55 ± 0.44 (1.03)	4.26

*Furosemide peak height in cm when detector range setting was 0.1 μA. Five determinations were performed at each concentration, all on the same day. Photomultiplier voltage was 820 V.

**Peak height divided by furosemide concentration.

3.55 ± 0.069 cm, respectively, when each type of solution was assayed in five determinations. It should be noted that plasma sample volumes as small as 10 or 25 μl may be prepared for analysis by adding 2.5 volumes of acetonitrile for the purpose of deproteinization [17—19].

It is important to establish the specificity of any analytical method which is designed for measurement of drug concentrations in biological fluids, because it is very common for patients to receive other drugs concurrently. The other drugs which are quite commonly administered to infants (particularly in an intensive care setting), include phenobarbital, phenytoin, theophylline, digoxin and ampicillin. None of these drugs interfere with the analysis of furosemide. In addition, ephedrine, caffeine, theobromine, acetominophen, phenacetin, tetracycline and salicylic acid were shown not to interfere.

The method described is rapid and suitable for the quantitative determination of furosemide in small volumes of plasma collected from infants. The limit of detection of furosemide is 0.1 μg/ml of plasma and the reproducibility is satisfactory. The method is specific for furosemide; the main breakdown product and metabolite of furosemide, and a number of other commonly used drugs do not interfere with the analysis.

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