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Note

High-performance liquid chromatographic determination of a new calcium antagonist, fostedil, in plasma and urine using fluorescence detection

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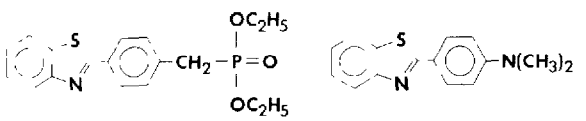
A new calcium entry blocking compound [1—4], fostedil [diethyl-4-(2-benzothiazolyl)-benzyl phosphonate], is currently under investigation at Abbott Labs. as a potential new drug candidate. This compound is being studied under a joint license agreement with Kanebo, Ltd. of Japan and has been reported in the literature as KB-944. We were interested in measuring plasma and urine levels in dogs following administration of the drug. A high-performance liquid chromatographic (HPLC) procedure employing fluorescence detection was developed to measure the concentration of the drug in plasma and urine. The drug was extracted from the biological fluids on Baker 10 Octadecyl (C_{18}) extraction columns (1-ml capacity). This newer extraction system provided a rapid and efficient sample clean-up.

EXPERIMENTAL

HPLC grade acetonitrile and methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) were used. The internal standard (see Fig. 1) was obtained from Abbott Labs. All chemicals and reagents were used as received.

A Waters Assoc. pump and automatic injector were used with a Schoeffel



FOSTEDIL INTERNAL STANDARD

Fig. 1. Structures of fostedil and internal standard.

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FS-970 detector. The column (25 mm × 4.6 mm) was Alltech Octyl (C8), 10-μm particle size. With slight changes in the composition of the mobile phase and/or flow-rate other reversed-phase columns could be used. A Hewlett-Packard recorder was used with peak heights and retention times being determined with a ruler.

The mobile phase was acetonitrile—water (3:2). Minor manipulations of the acetonitrile content may be required to accommodate column efficiency loss or interferences from atypical plasma or urine samples. The mixture was filtered through a 0.4-μm Nucleopore (Pleasanton, CA, U.S.A.) polycarbonate membrane and degassed under house vacuum, while stirring, for about 5 min.

Operating conditions were: flow-rate 1.5 ml/min, chart speed 0.1 in./min. and injection volume was 50–200 μl depending on expected drug concentration. The detector was operated at a range setting of 0.2, a time constant of 6, an excitation wavelength of 290 nm, and a 370-nm emission filter positioned between the sample cell and photomultiplier tube.

The internal standard was 4-(2-benzothiazolyl)-N,N-dimethylbenzenamine. A stock internal standard solution was prepared by dissolving ca. 25 mg in acetonitrile and diluting to 25 ml with acetonitrile. The solution was serially diluted with the mobile phase to a final working concentration of 150 ng/ml.

A methanolic solution of fostedil (1 mg/ml) was diluted (1:10) with methanol and then further diluted with pooled dog plasma or pooled dog urine to concentrations from 10—1000 ng/ml.

Extractions were performed using a Baker 10 Extraction System® with Baker 10 C_{18}, 1-ml columns (Scientific Products, McGraw Park, IL, U.S.A.). This system retains compounds of interest on selected sorbent packings. It has been successfully used by several authors [4, 5]. Solvents and samples are passed through the columns by the application of a vacuum. In this method the disposable extraction columns were first washed with two 1-ml washes of methanol. The vacuum was released and a volume of plasma or urine estimated to contain between 10—1000 ng of fostedil was added to the extraction column. Then 0.1 ml of the working internal standard solution was added to the column. The sample was drawn through the column by vacuum and the drug and internal standard absorbed on the column matrix. The endogenous materials were removed from the matrix by washing three times with a methanol—water mixture. For the plasma samples, the mixture was methanol—water (25:75) and for the urine samples it was methanol—water (55:45). The vacuum was released between each washing.

The drug and internal standard were eluted from the column with 1 ml of methanol. The eluent was collected in a small tube positioned under the column, dried in a water bath (40—45°C) under a gentle stream of air. The residue was reconstituted in 0.3 ml of mobile phase and an aliquot injected into the HPLC system. Peak heights were measured with a ruler and the concentration of fostedil in the unknowns determined by either the internal- or external-standard method.

RESULTS AND DISCUSSION

Originally a solvent extraction procedure was developed to remove the drug

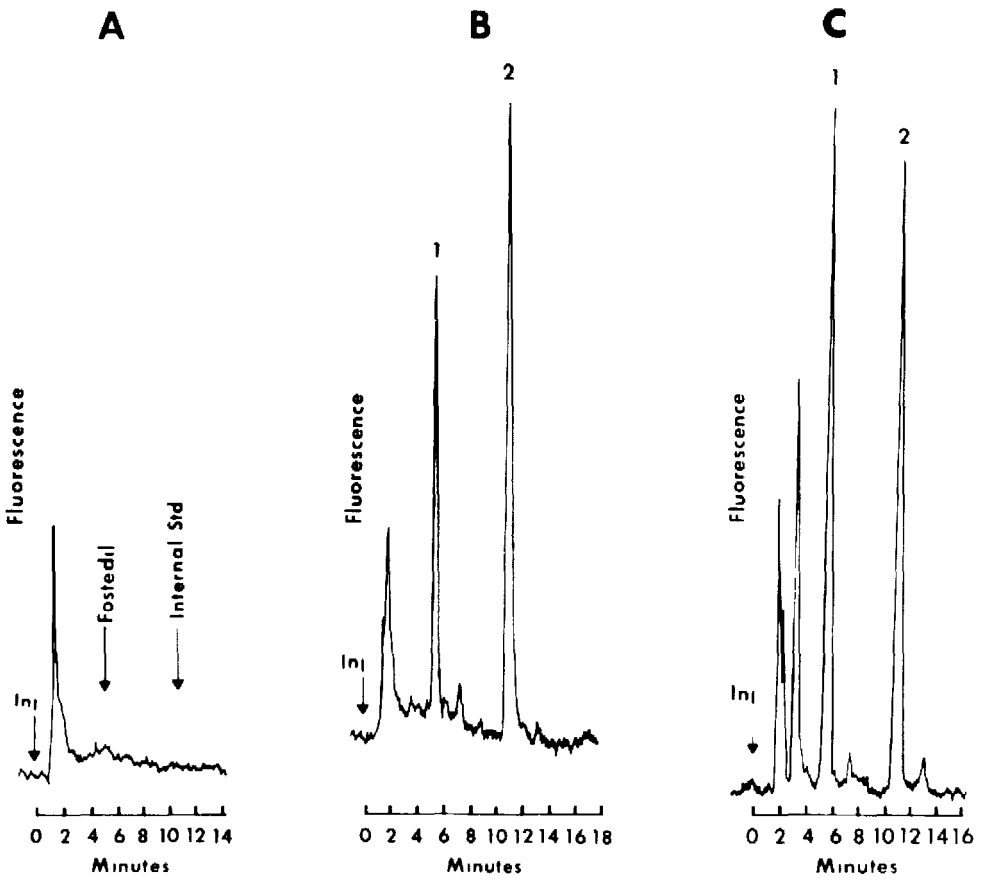

Fig. 2. (A) Chromatogram of extracted blank dog plasma as processed by this method. (B) Chromatogram of extracted dog plasma spiked with fostedil, 50 ng/ml (1) and internal standard (2). (C) Chromatogram of processed dog plasma following p.o. dosing of 12 mg/kg, 6.0 h post dosing.

from the biological fluids. Hexane—isopropyl alcohol (95:5) extracted fostedil quantitatively from the plasma, but resulted in a large background pattern in the chromatogram. Other less polar solvents were tried with hexane and hexane—ethyl acetate (7:3) yielding in the highest recoveries and lowest background pattern in the sample chromatograms.

The Baker 10 Extraction System was also evaluated. This system was found to be simple, fast and reproducible. Cyano, octyl and octyldecyl types of Baker 10 sorbents were screened for extracting efficiency. The following solvents were screened for their eluting properties on these sorbents: methanol, acetonitrile, isopropanol and mobile phase. The best results were obtained with the octyldecyl sorbent with methanol as the eluting solvent.

Typical chromatograms for extracted blank plasma/urine, the 50 ng/ml standard and a dog sample as processed by this method are shown in Figs. 2 and 3 for the plasma and urine assays, respectively. The retention time was 5.5 min for fostedil and 11.0 min for the internal standard. As observed, there may be some variation from sample to sample of the compounds eluting at the void volume.

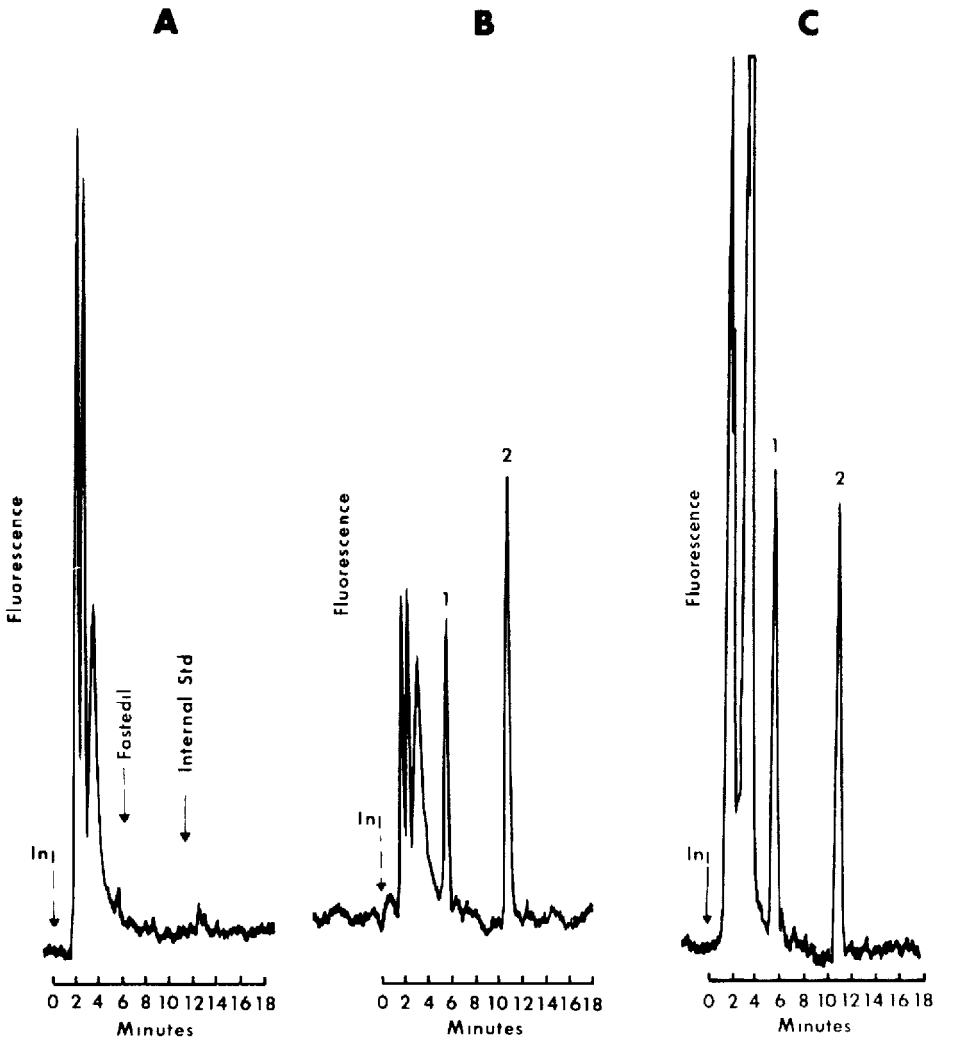

Fig. 3. (A) Chromatogram of extracted blank dog urine as processed by this method. (B) Chromatogram of extracted dog urine spiked with fostedil, 50 ng/ml (1) and internal standard (2). (C) Chromatogram of processed dog urine following p.o. dosing of 12 mg/kg, 0—4 h collection period.

Though an internal standard has been incorporated in this method, we obtained slightly better precision using the external standard technique. Haefelfinger [6] reported that in an HPLC procedure the external calibration is often advantageous. Haefelfinger showed that in HPLC, since the injection volumes are usually large (versus gas chromatography), the precision of the injection is not the main source of variance. Other sources of analytical variance include extraction factors and chromatographic behavior for both the drug and internal standard. If the coefficient of variation (C.V.) of the internal standard is larger than the C.V. of the drug being analyzed then the method will have a higher C.V. value when using internal versus external calibration. In general, samples processed by this procedure have been analyzed by the external

method. However, the use of the internal standard is recommended to guide in peak identification and guard against erroneous results due to sample loss after elution from the column and small within-run changes in HPLC conditions.

The linear response of the fluorescence detector was established by constructing calibration curves from spiked dog plasma and urine. A typical standard curve for the plasma assay yielded a slope of 0.125, a *Y*-intercept of −0.076, and a correlation coefficient of 0.9992. For the urine assay, a typical slope was 0.136, a *Y*-intercept of −0.163, and a correlation coefficient of 0.9990. Analysis of ten standard curves over a six-week period indicated that all correlation coefficients from the linear regression analysis were 0.99 or greater. The detection limit was empirically estimated to be about 5 ng/ml.

The precision of the HPLC assay for fostedil in plasma and urine samples was determined by calculating a mean concentration ± S.D. for each of the six standards from assays of six replicate curves, over a six-week period. The results of this precision study, Table I, show the average C.V. value to be 6.9% for the plasma assay and 7.5% for the urine assay.

TABLE I

PRECISION OF THE ANALYTICAL PROCEDURE FOR DETERMINING FOSTEDIL IN BIOLOGICAL FLUIDS

Theory (ng/ml)	Plasma		Urine	
	Found (ng/ml)	C.V. (%)	Found (ng/ml)	C.V. (%)
10.0	10.6 ± 1.34	12.7	10.1 ± 1.50	14.9
20.0	19.9 ± 1.33	6.7	20.3 ± 0.83	4.1
50.0	46.9 ± 4.27	9.1	47.9 ± 2.71	5.7
200.0	200.1 ± 17.2	8.6	203.6 ± 22.4	11.0
500.0	501.9 ± 12.7	2.5	511.2 ± 22.5	4.4
1000.0	1000.5 ± 14.9	1.5	992.9 ± 47.0	4.7

The recovery of fostedil from the plasma and urine assay procedures was determined by comparing the peak height of the drug from processed samples (plasma/urine) to the peak height of prepared reference samples. The recovery was checked at each standard level (10—1000 ng/ml) for both the plasma and the urine assay. All recoveries were greater than 90%. The recovery of the internal standard was also assessed and found to be greater than 90%.

The stability of room temperature, refrigeration (4°C), and freezer storage (−20°C) of fostedil in dog plasma and urine were assessed. Blank dog plasma and urine were spiked with the drug to 200 ng/ml, and aliquots set at the above stability stations. After 48 h at room temperature and refrigeration storage, no degradation was observed in either the plasma or urine samples.

The frozen samples were tested after one and four months storage. The plasma and urine samples were assayed with freshly prepared reference standards in the corresponding medium. The plasma and urine samples all assayed within the analytical variance of the method. Thus, on storage at −20°C there is no appreciable degradation of fostedil in dog plasma or urine for at least four months.

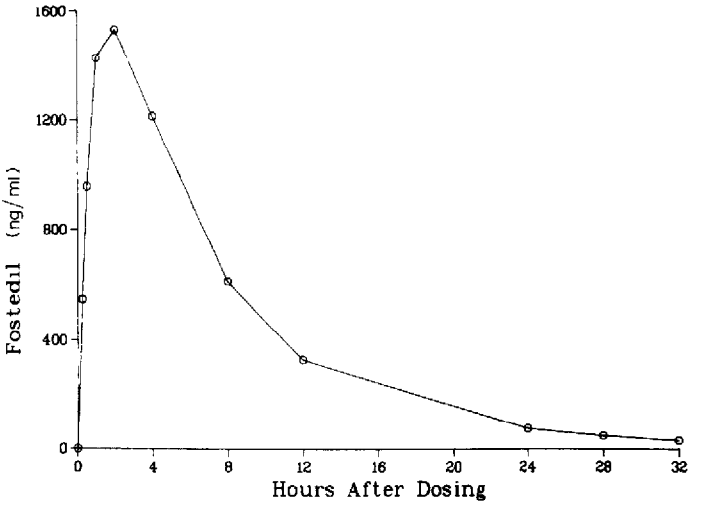

Fig. 4. Average plasma levels of fostedil following oral dose of 12 mg/kg to fifteen dogs.

Several drugs which are often concurrently administered to cardiac patients were also examined for their possible interference with this assay. The following drugs were evaluated on this system: disopyramide, prazosin, methyclothiazide, chlorthiazide, furosemide, hydralazene, procainamide, lidocaine, quinidine, verapamil, flurazepam, prazepam, imiprame and ibuprofen. None were found to interfere with this analysis of fostedil.

This method has been used to analyze over 600 plasma samples and 200 urine samples from dogs. The main metabolites in dog are the 5-, 6- and 7-hydroxy derivatives (i.e. diethyl-4-(5-hydroxybenzothiazol-2-yl)-benzyl phosphonate). Under the chromatographic conditions described here, these metabolites co-elute at a retention of 3.0 min. Following a radiolabeled dose in dogs, over 80% of the radioactivity was recovered in the feces. Thus, renal clearance is not a major route of elimination of the parent compound or the metabolites. A typical plot of plasma concentration versus time post dosing (p.o.) for dogs is shown in Fig. 4. Although all the work for this method was done with dog plasma and urine, generation of acceptable chromatograms and standard curves would serve to validate the procedure for human or other types of samples.

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