*Journal of Chromatography*, 527 (1990) 343–350
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5199

# Determination of belfosdil, a new calcium channel blocker, in human plasma by capillary gas chromatography with nitrogen–phosphorus detection

D.E. MULVANA*, S. KAUL, K.A. DANDEKAR and K.A. PITTMAN

*Department of Metabolism and Pharmacokinetics, Pharmaceutical Research and Development Division, Bristol-Myers Company, Syracuse, NY 13221 (U.S.A.)*

(First received October 10th, 1989; revised manuscript received December 14th, 1989)

## SUMMARY

Belfosdil and the internal standard were extracted from human plasma by a double liquid–liquid extraction. After a concentration step, gas chromatographic analysis of the sample was performed using a capillary fused-silica column and a nitrogen–phosphorus detector. The limit of detection of belfosdil was 0.025 ng/ml and the standard curve was linear over the range 0.05–100 ng/ml. The intra-assay and inter-assay precisions were within 7% (relative standard deviation) and the intra-assay and inter-assay accuracy values deviated by less than 5%. The extractability of belfosdil was 79%. The assay method was successfully used for the analysis of plasma samples from clinical studies with dose ranges of 5–100 mg of belfosdil.

## INTRODUCTION

Belfosdil, tetrabutyl 2-(2-phenoxyethyl)-1,3-propylidenediphosphonate (BMY-21891, Fig. 1) is a structurally novel, highly specific calcium channel blocker that also possesses the ability to inhibit the activity of acyl coenzyme A cholesterol acyltransferase, a key enzyme involved in the formation of cholesterol ester within the arterial wall as well as cholesterol absorption from the gastrointestinal tract [1]. Belfosdil is a heat-stable, odorless and colorless oil that is not sensitive to light. The boiling point is 205–210°C at 0.05 mmHg and 495–500°C at atmospheric pressure. The approximate water solubility of belfosdil is 10 μg/ml and it is freely soluble in ethanol, chloroform and dimethyl

0378-4347/90/$03.50 

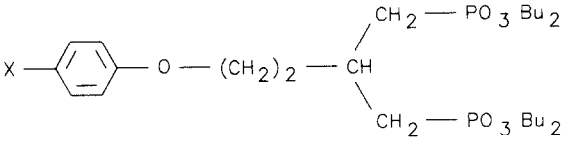

Fig. 1. Structures of belfosdil (X = H) and the internal standard (X = Cl).

sulfoxide (in-house data). Belfosdil is active when administered by oral and parenteral routes. In order to support current clinical studies, it was desirable to have a sensitive and reliable method of quantification so that complete plasma concentration–time profiles could be produced. A method for the quantitative analysis of belfosdil in human plasma has been developed which utilizes capillary gas chromatography (GC) with nitrogen–phosphorus detection and a double liquid–liquid extraction of the compound from the biological matrix.

## EXPERIMENTAL

### *Materials and reagents*

Ethyl acetate (HPLC grade), hexane (Optima), 1-butanol (certified ACS), methanol (Optima) and hydrochloric acid (reagent ACS) were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). The chromatographic gases used were helium (ultra-high purity), hydrogen (ultra-high purity) and air (hydrocarbon-free) and were obtained from Cottets Welding Supply (Syracuse, NY, U.S.A.). EDTA-treated human plasma was obtained from Biological Specialty (Lansdale, PA, U.S.A.). The extraction and evaporation steps were carried out in 125 mm × 16 mm screw-cap glass test tubes and 100 mm × 13 mm borosilicate glass test tubes, respectively, supplied by Kimble (Toledo, OH, U.S.A.). Samples were extracted on a reciprocal shaker purchased from Eberbach (Ann Arbor, MI, U.S.A.). GC autosampler 300-$\mu$l polypropylene microvials were purchased from Sun Brokers (Wilmington, NC, U.S.A.). Samples were centrifuged on a Centra-7 centrifuge from International Equipment (Needham Heights, MA, U.S.A.). Extraction solvent was evaporated using an N-Evap analytical evaporator purchased from Organomation Assoc. (South Berlin, MA, U.S.A.). The drug and internal standard (the *p*-chloryl analogue of belfosdil, BMY-33619, Fig. 1) were obtained from Symphar (Geneva, Switzerland).

### *Instrumentation*

All analyses were performed with a Model 5890A gas chromatograph equipped with a split/splitless capillary inlet and a nitrogen–phosphorus detector purchased from Hewlett-Packard (Avondale, PA, U.S.A.). The analytical column was a 15 m × 0.25 mm I.D. fused-silica capillary column with DB-1 (methylsilicone) bonded phase at 0.25 $\mu$m film thickness from J&W Scientific (Fol-

som, CA, U.S.A.). An HP-7673A autosampler was used to inject sample into the gas chromatograph. A silanized splitless insert was installed in the injector. The control of the GC parameters and injection sequence and all data acquisition, integration and storage were performed by an HP-5895 GC workstation. Integration results were transferred to a Hewlett-Packard 3357 laboratory automation system for data regression and analysis.

*Instrumental conditions*

The injector and detector temperatures were set at 300°C and the oven was operated isothermally at a temperature of 275°C. The split flow through the injector was 67 ml/min and the injector purge was activated 6 s after injection. The helium carrier gas flow-rate was 1 ml/min which gave a column head pressure of 103 kPa. The flow-rates of the detector gases, hydrogen, air and helium make-up, were 3.3, 120 and 24 ml/min, respectively. The detector signal was operated at a baseline of 100 pA.

*Standard and quality control preparation*

Stock solutions of belfosdil (400 μg/ml) and internal standard (40 μg/ml) were prepared in methanol. Working belfosdil and internal standard solutions were prepared at concentrations of 16 and 1.2 μg/ml, respectively. From the working belfosdil solution, plasma spiking solutions at concentrations of 1 and 100 ng/ml were prepared in matrix and used for the preparation of standards. Plasma quality control samples were prepared at concentrations of 1, 15 and 90 ng/ml and stored at −20°C.

*Assay procedure*

Plasma (2.0 ml), working internal standard solution (50 μl), 1 *M* hydrochloric acid (0.5 ml) and 5% (v/v) ethyl acetate in hexane (4 ml) were transferred to a 125 mm×16 mm glass test tube. The tube was capped and shaken for 10 min on a reciprocal shaker at 200 cycles per min and then centrifuged for 10 min at 2000 *g*. The organic layer was transferred by disposable pipet to a 100 mm×13 mm glass test tube. The remaining aqueous layer was then extracted with a second volume of ethyl acetate–hexane and centrifuged and, after freezing the aqueous layer on dry ice, the organic layer was decanted into the same 100 mm×13 mm test tube used for the first extraction. The pooled extract was then evaporated under a nitrogen stream in a 45°C water bath. The residue was reconstituted in 30 μl of 1-butanol and a 3-μl aliquot was injected onto the GC column.

*Validation procedure*

A set of standards consisting of seven plasma concentrations ranging from 0.05 to 100 ng/ml was processed in duplicate for each day of analysis. The standards data, peak-area ratio ($y$) versus drug concentration ($x$), were fit to

a linear regression equation weighting each standard by the reciprocal of its concentration and were tested for outliers by the method of Prescott [2]. The mean ($\pm$S.D.) equation for the resulting line over three days of validation was $y=0.053122\ (\pm 0.000803)x+0.001423\ (\pm 0.000196)$. The correlation coefficients over the three days were $>0.9996$. The area ratios of the unknown or quality control samples were used to calculate drug concentrations from the standard curve.

As a means of determining within-day and between-day assay variability, a set of quality control samples was prepared at concentrations of 1, 15 and 90 ng/ml and assayed in replicates of four on three separate days.

The lower limit of quantitation (LLQ) was established by spiking human plasma from ten individuals at a concentration of 0.05 ng/ml and analyzing the samples versus a standard curve. From these predicted concentrations, the accuracy and precision at the LLQ were established. The limit of detection was established at 0.025 ng/ml as the peak area of belfosdil at this concentration was significantly different from the blanks ($n=6$) as determined by a paired $t$-test. The mean signal-to-noise ratio at this concentration was 3.

Specificity of the assay was examined by comparing the chromatograms of processed blank plasma, plasma containing internal standard and plasma containing belfosdil and internal standard. In addition, a comparison was made between ion scan chromatograms at the retention time of belfosdil obtained from neat drug and an extracted clinical sample which were analyzed on a Hewlett-Packard 5970B mass-selective detector coupled to an HP-5890A gas chromatograph using cool on-column injection.

Drug stability in plasma was evaluated at $-20\,^{\circ}$C, $23\,^{\circ}$C and when submitted to repetitive freeze–thaw cycles. In addition, stability was examined in processed samples at $23\,^{\circ}$C. Extraction recovery was determined from the ratio of the slope of a standard curve constructed from extracted plasma standards to the slope of a standard curve constructed from neat belfosdil in butanol.

## RESULTS AND DISCUSSION

Great structural diversity is found among calcium channel antagonists and, as a result, the bioanalytical methods for these drugs vary greatly [3–7]. The use of splitless injection technique [8], coupled with capillary GC and phosphorus-selective detection, provided a high degree of sensitivity and selectivity for the determination of belfosdil.

The variability of the validation assays, as derived from the quality control sample analysis and the LLQ experiment, is given in Table I. Intra- and inter-assay variabilities were within 7% (Table II). Typical chromatograms obtained from blank plasma, a standard and clinical samples are presented in Fig. 2. No interference from endogenous plasma constituents was observed in the areas of retention of belfosdil or internal standard. The ion scans and chro-

TABLE I

INTRA-ASSAY PRECISION AND ACCURACY FOR PLASMA VALIDATION

| Nominal concentration (ng/ml) | *n* | Mean predicted concentration (ng/ml) | R.S.D. (%) | Deviation from nominal (%) |
|---|---|---|---|---|
| 0.05 | 10 | 0.047 | 21.2 | −6.42 |
| 1.00 | 4 | 0.99 | 6.0 | −0.9 |
| 1.00 | 4 | 1.12 | 6.6 | +12.2 |
| 1.00 | 4 | 1.00 | 3.1 | 0.3 |
| 15.00 | 4 | 14.35 | 1.6 | −4.4 |
| 15.00 | 4 | 14.30 | 2.1 | −4.7 |
| 15.00 | 4 | 14.10 | 4.6 | −6.0 |
| 89.99 | 4 | 86.80 | 2.8 | −3.5 |
| 89.99 | 4 | 84.11 | 1.5 | −6.5 |
| 89.99 | 4 | 85.32 | 1.4 | −5.2 |

TABLE II

OVERALL PRECISION AND ACCURACY FOR PLASMA VALIDATION

| Nominal concentration (ng/ml) | Grand mean observed concentration (ng/ml) | Relative standard deviation[a] (%) | | Inter-assay accuracy[b] (%) |
|---|---|---|---|---|
| | | Inter-assay | Intra-assay | |
| 1.00 | 1.04 | 6.48 | 5.90 | 3.70 |
| 15.00 | 14.25 | 0.78 | 3.02 | −5.02 |
| 89.99 | 85.41 | 1.22 | 2.01 | −5.09 |

[a]Calculated by one-way ANOVA.
[b]Calculated by deviation of grand mean observed concentration from nominal concentration.

matograms obtained from gas chromatographic–mass spectrometric (GC–MS) analysis of a neat drug solution and a 1-h clinical sample are shown in Fig. 3. The abundance of common ions seen indicates method specificity. The molecular ion (548 a.m.u.) is seen in both scans. A slight difference is seen (0.1 min) in the retention time of belfosdil between the two chromatograms. This difference is due to the fact that the injection was done manually.

Belfosdil was found to be stable in plasma when stored for at least 45 weeks at −20 °C and for at least 4 h at 23 °C. Data obtained from repetitively injecting processed samples over time showed the drug to be stable in these samples for at least 69 h. No degradation of drug was observed in plasma samples subjected

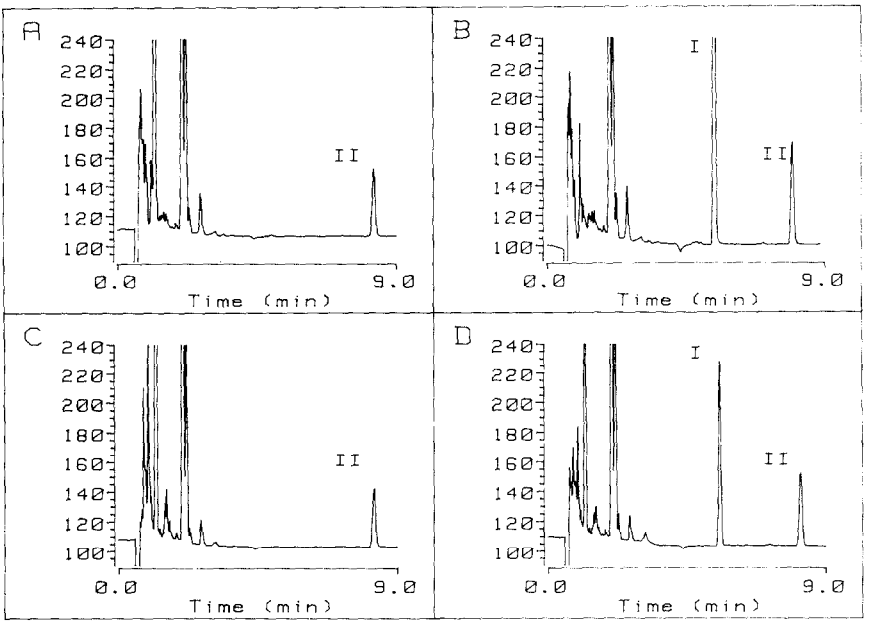


Fig. 2. Representative chromatograms obtained from blank plasma (A), a 100 ng/ml standard (B) and a pre-dose (C) and 1-h (D) clinical sample. Belfosdil and internal standard peaks are labelled as I and II, respectively.

to repetitive freeze–thaw cycles. The extraction recovery of belfosdil from plasma was shown to be 79%.

Several extraction solvents were examined during method development, including methyl *tert.*-butyl ether, ethyl acetate, hexane and diethyl ether. Additionally, various proportions of butanol in diethyl ether and ethyl acetate in hexane were used to modify the polarity of the extraction solvent. It was found that optimum recovery and chromatography were achieved using 5% ethyl acetate in hexane as the extraction solvent. Addition of 1 *M* hydrochloric acid to the matrix further reduced the amount of endogenous plasma constituents in the extract.

Since belfosdil is an extremely hydrophobic compound, preliminary work was performed in order to minimize potential problems in method development. Belfosdil labelled with $^{14}C$ was added to urine, plasma, saline, methanol, diethyl ether and hexane in glass, silanized glass, polypropylene and polyethylene containers. Aliquots were periodically removed and the radioactivity measured by liquid scintillation counting. The experiments showed that solutions of belfosdil in plasma and organic solvents are stable regardless of the container used. However, for urine and saline solutions of belfosdil, only sil-

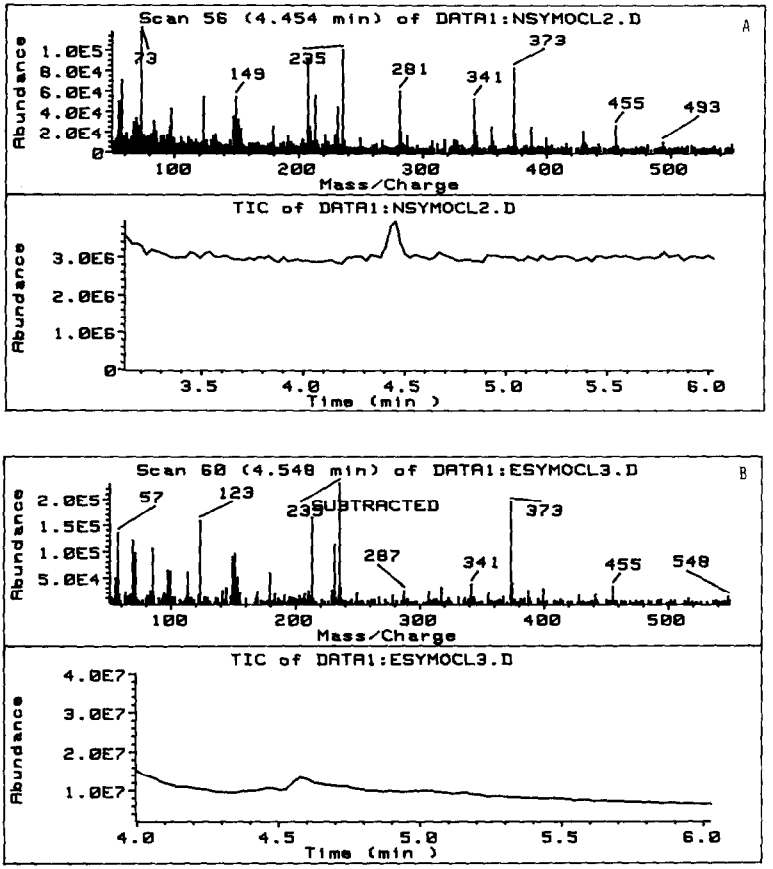


Fig. 3. Mass spectra of belfosdil and ion chromatograms from neat drug solution (A) and a processed clinical sample (B).

anized glass containers should be used. The solution in urine was stabilized in polyethylene and polypropylene containers when Tween 80 (a surfactant) was added to a final concentration of 2%.

## CONCLUSION

The assay method was shown to be sensitive, accurate, precise, reproducible and specific. This method has been successfully used for the assay of over 500 clinical samples. An example of plasma concentration versus time profiles of belfosdil in two subjects is shown in Fig. 4.

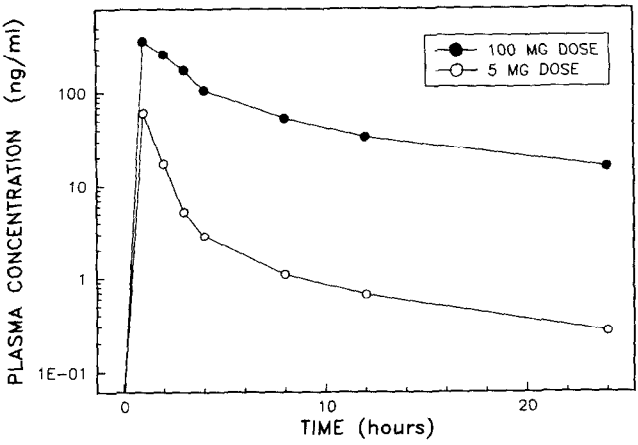


Fig. 4. Plasma concentrations of belfosdil in individual subjects after oral administration of 5- and 100-mg doses.

ACKNOWLEDGEMENTS

The authors thank Mr. M.A. Sciacca for his technical assistance in the GC–MS analysis and Ms. Frances Bertolero for typing the manuscript.

REFERENCES

1 Data on file, Bristol-Myers, Syracuse, NY.
2 P. Prescott, Technometrics, 17 (1975) 129.
3 M.T. Rosseel and F.M. Belpaire, J. High Resolut. Chromatogr. Chromatogr. Commun., 11 (1988) 103.
4 A.T. Kacprowicz, R.O. Fullinfaw and R.W. Bury, J. Chromatogr., 337 (1985) 412.
5 K.-J. Goebel and E.V. Kölle, J. Chromatogr., 345 (1985) 355.
6 B.J. Gurley, R.G. Buice and P. Sidhu, Ther. Drug Monit., 7 (1985) 321.
7 C. Giachetti, P. Poletti and G. Zanolo, J. High Resolut. Chromatogr. Chromatogr. Commun., 10 (1987) 654.
8 K. Grob, Classical Split and Splitless Injection in Capillary GC, Hüthig, Heidelberg, 1986.