Journal of Chromatography, 345 (1985) 402—407
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2793

Note

Sensitive reversed-phase high-performance liquid chromatographic assay for the antitumor agent benzisoquinolinedione (Nafidimide) employing direct on-column injection of plasma and urine, and its use for pharmacokinetic studies in a dog

GARTH POWIS*, DAVID M. KROSCHEL and DAVID J. MOORE

Division of Developmental Oncology Research, Department of Oncology, Mayo Clinic/Foundation, Rochester, MN 55905 (U.S.A.)

(First received March 12th, 1985; revised manuscript received July 26th, 1985)

Benzisoquinolinedione, 5-amino-2-[2-(dimethylamino)ethyl]-1H-benz[*de*]-isoquinoline-1,3(2H)-dione (Nafidimide, Fig. 1) is one of a series of benz[*de*]-isoquinoline-1,3-diones, synthesized by Braña and co-workers [1, 2], that possesses potent anti-viral and cytotoxic activity [2, 3]. When administered by the intraperitoneal, intravenous or oral routes in mice, benzisoquinolinedione has antitumor activity against intraperitoneally implanted L1210 leukemia and activity against two non-leukemic tumors, M5076 sarcoma and the B16 melanoma [4]. Benzisoquinolinedione appears to act as a DNA intercalator [4]. It stabilizes calf thymus DNA to thermal denaturation, produces protein-associated DNA single strand breaks and blocks nucleic acid and protein synthesis. Leukemia P-388 sublines, with acquired resistance to the DNA intercalating agents doxorubicin, mitoxantrone and 4′-(9-acridinylamino)methanesulfono-*m*-anisidine, show cross-resistance to benzisoquinolinedione. Benzisoquinolinedione is to be placed in Phase I clinical trial in the U.S.A. by the National Cancer Institute.

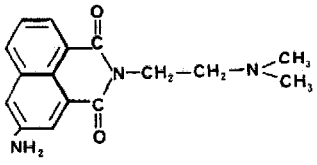

Fig. 1. Structure of benzisoquinolinedione.

0378-4347/85/$03.30

We report the development of a sensitive, reversed-phase high-performance liquid chromatographic (HPLC) assay for benzisoquinolinedione in biological fluids. Plasma and urine is taken without pretreatment and injected directly onto the high-performance liquid chromatograph. Benzisoquinolinedione and its metabolites are concentrated on a precolumn and then automatically displaced onto an analytical column using a switching valve. Because there is no sample preparation samples can be assayed within minutes of their collection. The method has been used to study the disposition of benzisoquinolinedione in a dog.

EXPERIMENTAL

Drug

Benzisoquinolinedione (NSC 308847) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.)

Equipment

The analytical equipment used was a Hewlett-Packard 1090 automated liquid chromatograph with a triple solvent delivery system and an automated column-switching valve. The column effluent was passed first through a Hewlett-Packard diode array variable-wavelength absorbance detector and then through a Schoeffel FS 970 fluorescence detector.

Assay procedure

Plasma, 0.5 ml, was mixed with 50 μl of 1 *M* sodium acetate buffer, pH 5.0. Urine was diluted 1:100 with 0.1 *M* sodium acetate buffer, pH 5.0. In both cases 250 μl of the mixture were injected directly onto the HPLC system using a programmable sample injector at a flow-rate of 0.25 ml/min. The sample passed through a 6-mm disposable RP-8 guard column (Hibar LiChrosorb RP-8; Merck, Darmstadt, F.R.G.) to waste. Alternatively a 50 × 4 mm precolumn packed with RP-8 Perisorb® (Merck) was used. Preliminary studies revealed that pH 5.0 was optimum for adsorption of benzisoquinolinedione onto the precolumn. The precolumn was flushed with water at a flow-rate of 3 ml/min for 3.5 min to remove protein and other material not bound to the column. An automatic switching valve was employed to displace drug and other material adsorbed on the precolumn onto a 25-cm RP-8, 4-μm analytical column (LiChrospher II Super Column, Merck) using a 10-min linear gradient of 0—100% methanol in 10 m*M* sodium phosphate buffer, pH 8.1, at a flow-rate of 1.5 ml/min. The analytical column was protected by a second RP-8 guard column. At the end of the gradient the system was flushed with methanol at a flow-rate of 1.5 ml/min for 5 min, then with 10 m*M* sodium phosphate, pH 8.1, for 1 min, and finally the precolumn was flushed with water for 0.5 min. The flow-rate was reduced to zero when the switching valve was activated and then increased to 1.5 ml/min over 0.5 min to ensure that the analytical column was not subjected to rapid pressure changes. The total time for a chromatographic run was 22 min. Because there was minimal sample preparation and the reproducibility of the Hewlett-Packard automatic sample injector was less than 1%, it was not

necessary to use an internal standard. Eluting compounds were detected by their absorbance at 254 nm or by their fluorescence at an excitation wavelength of 254 nm and with an emission filter of 470 nm. Benzisoquinolinedione has a fluorescence emission maximum of 554 nm. Standard curves were run with benzisoquinolinedione added to frozen, citrated human plasma or to fresh urine.

Animal studies

A female mongrel dog weighing 14.0 kg was injected intravenously with benzisoquinolinedione, 4.6 mg/kg, as a 12.5 mg/ml solution in 0.9% sodium chloride adjusted to pH 5.5 with 0.5 *M* hydrochloric acid, through an infusion set with a 21-gauge needle (Deseret, Sandy, UT, U.S.A.) into a cephalic vein over 1 min. Blood, 5 ml, was collected through a 19-gauge needle into a heparinized tube (Venoject, Terumo, Elkton, MD, U.S.A.) from the other cephalic vein at different times up to 6 h. Tubes were placed in ice and plasma separated as soon as possible and stored frozen at −70°C. The dog was housed in a metabolism cage and urine collected over 24 h. Prior to HPLC analysis and after thawing, plasma and urine samples were centrifuged at 10 000 *g* on a microcentrifuge (Beckman Instruments, Palo Alto, CA, U.S.A.).

Pharmacokinetic analysis

Plasma benzisoquinolinedione concentration data were subjected to non-linear least-squares regression analysis using the NONLIN computer program [5] and pharmacokinetic parameters calculated [6].

RESULTS

Injection of samples of plasma or urine directly onto the HPLC system offers considerable advantages in saving of time and resources over solvent extraction or cartridge extraction for sample preparation. In the method we have developed an RP-8 precolumn is used to adsorb the drug from plasma or urine prior to its displacement onto an analytical column. The precolumn was changed every 300 samples although it would have lasted longer. Because all of the sample is injected onto the HPLC system maximum assay sensitivity for a limited sample volume is obtained.

A typical chromatogram for benzisoquinolinedione added to plasma at 10 ng/ml with fluorescence detection is shown in Fig. 2. The limit of sensitivity of the assay (peak height more than twice background) for benzisoquinolinedione from 0.25 ml plasma employing fluorescence detection was 1 ng/ml. The coefficient of variation of the assay with fluorescence detection at 1 μg/ml was 1.1%, at 100 ng/ml 1.0% and at 10 ng/ml 16.5%. Employing fluorescence detection the assay was linear up to 1 μg/ml in plasma (Fig. 3). Above this level the response of the fluorescence detector was non-linear. Using UV detection the limit of sensitivity of the assay was 50 ng/ml but the response was linear up to 5 μg/ml (Fig. 3). Above 5 μg/ml the response was non-linear apparently due to saturation of binding of benzisoquinolinedione to the precolumn. Similar concentration–response relationships for those seen with plasma were found for benzisoquinolinedione in urine. Benzisoquinolinedione was stable

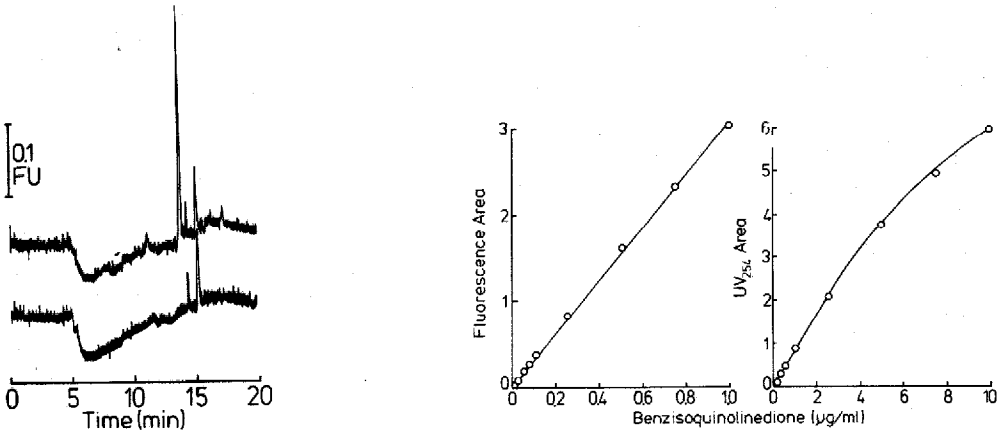

Fig. 2. Chromatogram of benzisoquinolinedione added to human plasma at 10 ng/ml (upper trace) and blank plasma (lower trace) using fluorescence detection. FU is arbitrary fluorescence units.

Fig. 3. Standard curves for benzisoquinolinedione in human plasma. Left panel: fluorescence detection (excitation 254 nm, emission filter 470 nm); right panel: UV detection (254 nm).

in plasma buffered at pH 5.0 with less than 4% loss over 24 h when kept at room temperature in the dark and 10% loss when kept under strong fluorescent room light. There was no loss of benzisoquinolinedione in non-buffered plasma over 24 h at 37°C.

Chromatograms of plasma from a dog administered an intravenous bolus dose of benzisoquinolinedione, 4.6 mg/kg, are shown in Fig. 4. The dose of benzisoquinolinedione is half the highest dose producing no toxic lesions in

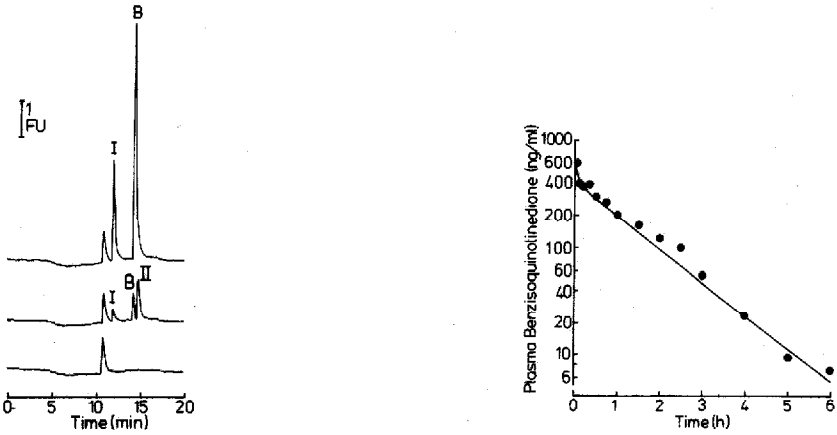

Fig. 4. Chromatograms of plasma from a dog given benzisoquinolinedione, 4.6 mg/kg, by intravenous bolus injection. Upper trace, plasma 1 h after giving drug; middle trace, plasma 4 h after giving drug; lower trace, plasma before giving drug. Peaks: B = benzisoquinolinedione; I and II = metabolites. FU is arbitrary fluorescence units.

Fig. 5. Plasma benzisoquinolinedione in a dog given benzisoquinolinedione, 4.6 mg/kg, by rapid intravenous bolus injection. The continuous line is a computer fit to the data.

dog [4]. Elimination of benzisoquinolinedione from plasma was biphasic (Fig. 5) with an initial half-life of 2.2 min and a terminal half-life of 58.3 min. The steady-state volume of distribution was 10.2 l/kg and the total body plasma clearance of benzisoquinolinedione 128.3 ml/min kg. Two fluorescent metabolites of benzisoquinolinedione were detected in plasma, one a more polar metabolite which reached a maximum after about 30 min and the other a less polar metabolite which was not detected until about 2 h. The two metabolites were also present in urine (results not shown). Urine collected over 24 h contained less than 2% of the dose of benzisoquinolinedione as unchanged drug.

DISCUSSION

A sensitive, reproducible assay for benzisoquinolinedione in plasma and urine has been developed in which the sample is injected directly onto the HPLC system. The essential features of the system are a ternary solvent system, an automatic switching valve and a precolumn for adsorbing the drug from the sample prior to its displacement onto an analytical HPLC column. The RP-8 guard column used as the precolumn is cheap and disposable, but even so it had a life of more than 300 samples as long as the sample was adequately centrifuged to remove particulate matter. A precolumn filled with RP-8 Perisorb functioned equally well to adsorb drug. The method eliminates the need for sample preparation and the total analysis time for each sample is considerably reduced. The method should be applicable to other drugs that can be separated by reversed-phase HPLC and that bind to a precolumn and should be particularly useful for analyses where sample volume is limited or where a short analysis time is desired.

With benzisoquinolinedione the limit of sensitivity of the assay was 1 ng/ml of plasma and the assay was linear up to 1 μg/ml with fluorescence detection and up to 5 μg/ml with UV detection. An advantage of having two detectors in sequence is that non-fluorescent metabolites can be detected, although the metabolites of benzisoquinolinedione that have been detected so far are all fluorescent. The coefficient of variation of the assay with fluorescence detection for benzisoquinolinedione in plasma at 1 μg/ml was 1.1% and at 100 ng/ml 1.0%.

When a dog was administered benzisoquinolinedione intravenously at a dose of 4.6 mg/kg, plasma concentrations of benzisoquinolinedione declined with an initial half-life of 2.2 min and a terminal half-life of 58.3 min. The total body plasma clearance of benzisoquinolinedione was 128.3 ml/min kg. Plasma and urine contained two fluorescent metabolites of benzisoquinolinedione. The metabolites have not been identified. Less than 2% of the dose of benzisoquinolinedione was excreted in the urine in 24 h as unchanged drug.

In summary, a sensitive, reproducible assay for benzisoquinolinedione has been developed where plasma and urine are injected directly onto the HPLC system using a precolumn to adsorb drug and an automatic switching valve to displace the drug onto an analytical column. The assay has been used to study the disposition of benzisoquinolinedione in a dog.

ACKNOWLEDGEMENTS

This work was supported in part by NIH Contract CA 15083. The excellent secretarial assistance of Ms. Wanda Rhodes is gratefully acknowledged.

REFERENCES

1 M.F. Braña, J.M. Castellano, C.M. Roldán, A. Santos, D. Vázquez and A. Jiménez, Cancer Chemother. Pharmacol., 4 (1980) 61.
2 M.F. Braña and A.M. Sanz, Eur. J. Med. Chem., 16 (1981) 207.
3 A.G. Gancedo, C. Gil-Fernandez, P. Vilas, S. Perez, E. Paez, F. Rodriguez, M.F. Braña and C.M. Roldán, Arch. Virol., 74 (1982) 157.
4 Nafidimide (Benzisoquinolinedione) NSC 308847, Clinical Brochure, National Cancer Institute, Division of Cancer Treatment, Bethesda, MD, U.S.A., November, 1984.
5 C.M. Metzler, G. Elfring and A.J. McEwen, Biometrics, 30 (1974) 562.
6 J.G. Wagner, J. Pharmacokin. Biopharm., 4 (1976) 443.