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Two-dimensional high-performance liquid chromatography at low ng/ml levels of the anti-proliferative agent B859-35 in serum with automated sample clean-up, solid-phase trapping and ultraviolet detection

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ABSTRACT

An automated non-chiral high-performance liquid chromatographic method is described for the determination of the new anti-proliferative agent B859-35 in serum. This method employs sample clean-up of 1 ml of biofluid by liquid–solid extraction with the AASP (Advanced Automatic Sample Preparation) system. First separation is achieved on a LiChrospher-60-RP-Select-B column. A fraction of this eluate is then collected by solid-phase trapping. Thereafter, the final chromatogram is developed on a narrow-bore Hypersil-CPS column and quantified with ultraviolet detection at 230 nm. The limit of quantitation of the assay is 250 ng/ml. Linearity was proven in the range 0.25–100 ng/ml. Typical figures for precision at these concentrations are 7.4 and 3.3%, and for accuracy 8.0 and 1.3%, respectively. An application of this method to the study of pharmacokinetics of B859-35 in serum samples of cancer patients is given.

INTRODUCTION

(−)-3-[3-(4,4-Diphenyl-1-piperidinyl)propyl]-5-methyl-1,4-dihydro-2,6-dimethyl-4(*R*)-3-nitrophenyl)pyridine-3,5-dicarboxylate-hydrochloride (B859-35) is the (−)-enantiomer of a dihydropyridine derivative (Fig. 1) with pronounced anti-proliferative activity in *in vitro* as well as in *in vivo* experiments [1,2].

The optical purity of this compound is > 99.5%. There is no evidence so far for a lack of *in vitro* or *in vivo* configurational stability of this enantiomer and, therefore,

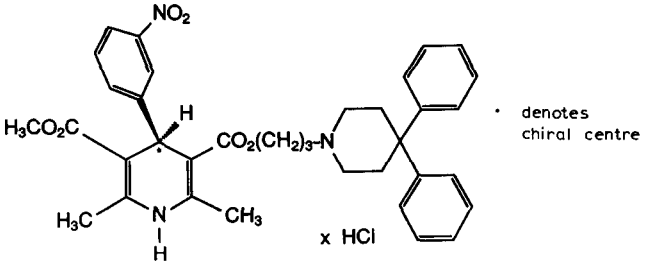

Fig. 1. Structure of B859-35.

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the development of a non-chiral assay seemed to be appropriate. The compound has only negligible cardiovascular effects when compared with the racemate or the (+)-enantiomer [3,4] and its therapeutic usefulness in cancer patients is under clinical evaluation at present.

Due to the very lipophilic nature of B859-35, the volume of distribution (derived from preclinical experiments) is, at 20–40 l/kg in animals, rather high. Consequently most of the administered compound is not in the blood but is distributed into various tissues within the body. Thus for determination of B859-35 concentrations in serum a very sensitive assay has had to be developed.

B859-35 has no functional moieties which can be derivatized to allow for the introduction of a chromophor or fluorophor which will enhance the sensitivity of the assay, nor does electrochemical oxidation contribute to detector response as can be done with other dihydropyridine derivatives [5]. Furthermore it is not sufficiently volatile for gas chromatography.

Direct injection of large volumes (200 μl) of serum or plasma into a fully automated pre-column switching system is used in our laboratory as a bioassay for many compounds [6,7]. To lower the detection limit for B859-35, however, use of 1 ml of serum was necessary. The typical capacity of the pre-column for liquid–solid extraction in the automated pre-column switching device [6] is, however, limited to a minimum of *ca.* 15–20 ml of total injected volume of serum (*e.g.* 75–150 injections of 200 μl each). Therefore, the AASP (Advanced Automatic Sample Preparation) system was introduced for liquid–solid extraction using 1 ml of serum. A new cartridge is used for each serum specimen in this system.

It was the aim of this application to combine the semi on-line technique of liquid–solid extraction provided by the AASP system with a second step of purification of the analyte using two-dimensional chromatography for further removal of matrix components interfering with the peak of interest [8].

EXPERIMENTAL

Chemicals

Standard compound was synthesized by Byk Gulden Lomberg (Konstanz, Germany) and was > 99% chemically pure by high-performance liquid chromatography (HPLC) analysis. Optical purity was > 99.5% as determined by nuclear magnetic resonance (NMR) spectroscopy with shift reagents. All reagents were supplied by E. Merck (Darmstadt, Germany) and were analytical grade for buffer substances and gradient grade for solvents.

Preparation of drug solutions and serum external standards

Stock standard solution of B859-35 at a concentration of 100 μg/ml was prepared by dissolving 10 mg in 100 ml of dimethylsulphoxide (DMSO). DMSO was used to prevent adsorption of B859-35 on glass and synthetic materials. The DMSO stock solution was further diluted to appropriate concentrations with standardized human serum for the external standard calibration and for checking the linearity range.

Apparatus (Fig. 2)

Sample clean-up was performed with the AASP station (Varian, Darmstadt, Germany). This system for liquid–solid extraction of the compound of interest from the serum matrix consisted of an AASP cassette holder for off-line treatment, loading and purging the cartridges, and an AASP sampler to insert the individual cartridges into the mobile phase of the HPLC system. RP-2 extraction cartridges (ICT Handels GmbH, Frankfurt, Germany) were used in this clean-up step.

The first analytical column was a LiChrospher-60-RP-Select-B cartridge (5 μm,

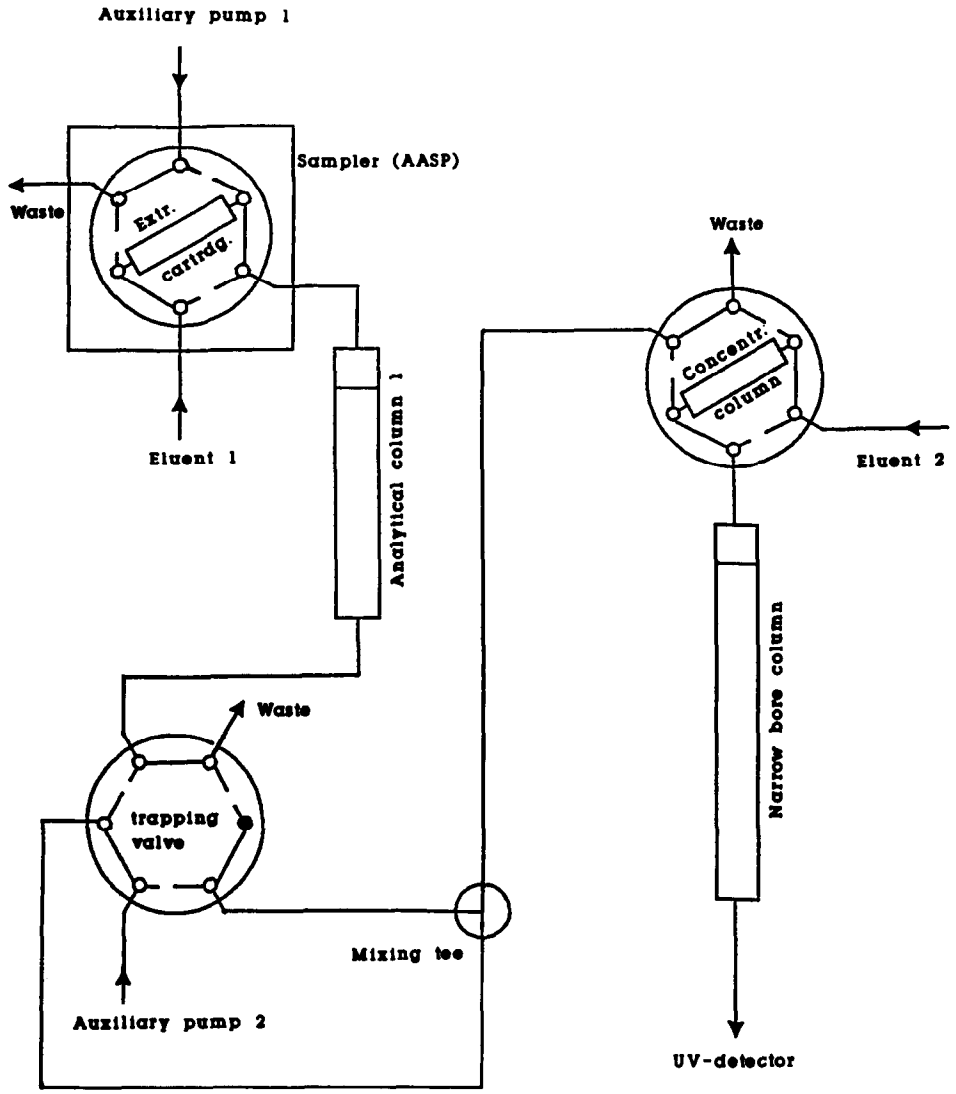

Fig. 2. Column-switching arrangement schematically showing the AASP device, the two analytical columns and the concentration column which precedes the narrow-bore column.

125 × 4 mm I.D.) (E. Merck). A broad fraction of the eluate from the HPLC system was collected by solid-phase trapping using a six-port Rheodyne 7010 pneumatic valve. Further dilution of the trapped fraction to decrease elution strength was necessary using a HKP-11 pump (Labotron Messtechnik, Munich, Germany) to achieve sufficient retention on a RP-8 Nucleosil (5 μm particle size) concentration column (10 × 2 mm I.D.) (Grom, Ammerbuch, Germany). This small column was connected via a Rheodyne 7010 pneumatic valve to the narrow-bore column (200 × 2 mm I.D.) filled with 5 μm Hypersil-CPS (Grom).

A HP 1090 liquid chromatography unit with a built-in filter photometer detector (FPD) and thermostatically controlled column compartment was used (Hewlett Packard, Waldbronn, Germany). Integration was performed by means of the Laboratory Data System HP 3357 (Hewlett Packard).

Sample preparation, column switching, phases

In the first off-line clean-up step liquid–solid extraction of serum samples [6] was carried out by means of AASP cassettes containing ten cartridges filled with alkyl-C_2 bonded phases. The cartridges were placed in a AASP holder which passed liquid through the cassettes by means of pressurized nitrogen and each cartridge was washed with 1 ml of methanol and 1 ml of water for cleaning and wetting. The washed cartridges were conditioned with 2 ml of a 40 m*M* aqueous solution of tetrabutylammonium hydroxide (TBAH) for ionpair formation with the acidic compound B859-35. A 1-ml specimen of serum (centrifuged for 10 min at *ca.* 5000 *g* to remove solid particles) was diluted in a separate step with 800 μl water, then 200 μl of formic acid (100%) were added to this diluted serum to decrease plasma protein binding. This solution was then passed through the conditioned cartridges in 1-ml steps followed by a last wash step with 1 ml of 5 m*M* sodium phosphate buffer pH 7.0. Cassettes with cartridges loaded by this procedure were kept at 4°C until filling of the AASP injection device, thus serving as autosamplers.

Immediately prior to automatic insertion into the analytical mobile phase eluent, each cartridge was purged in the AASP sampler with 500 μl of 5 m*M* sodium phosphate buffer pH 7.0. The connection time of each AASP cartridge to the analytical column was 2 min. Thereafter, the first chromatogram was developed on a LiChrospher-60-RP-Select-B column. The isocratic eluent was premixed and consisted of 5 m*M* sodium phosphate buffer pH 7.0-acetonitrile (35:65, v/v) at a flow-rate of 1.0 ml/min. Collection of a fraction of the eluate by solid-phase trapping started 1 min before the retention time of the compound of interest on the first column. (Retention time is determined before running the first analysis with a standard sample at a concentration of *ca.* 500 ng/ml.) The duration of the trapping window was set to 4 min while the column eluate was transferred to the concentration column for peak compression following on-line dilution with an aqueous 20 m*M* sodium perchlorate buffer pH 2.0, at a flow-rate of 2.2 ml/min to decrease elution strength in order to achieve sufficient retention on the second column [7].

Immediately after the completion of eluate collection, the concentration column with the trapped fraction was connected to the narrow-bore column serving as the second analytical column for 2 min to develop the final chromatogram. A premixed mobile phase of 5 m*M* sodium phosphate buffer pH 7.0 with acetonitrile (32:68, v/v) was used. The flow-rate of this column was adjusted to 0.3 ml/min. The

peak of interest was detected with the FPD at a wavelength of 230 nm. All columns were placed in the HP 1090 column compartment with the temperature adjusted to 40°C.

Before use all eluents were purged with helium to remove dissolved gases. Water for the preparation of solutions and buffers was purified on a Milli-Q-purification unit (Millipore, Eschborn, Germany).

Preparation of standard curves

Standards for the calibration curve were made by spiking control serum at concentrations of 0.25, 0.5, 1.0, 5.0, 25, 50 and 100 ng/ml. Each spiked serum standard was injected ten times. Standard curves were constructed by plotting the chromatographic peak areas against the known concentrations of B859-35 in serum. Individual calibration curves were calculated by ordinary least-squares regression analysis, and the concentration of B859-35 quantified by relating the respective peak area to the appropriate curve.

To calculate loss of compound by solid-phase trapping and DMSO, solutions at concentrations of 0.5 and 25 ng/ml were injected directly on the narrow-bore column and peak areas were compared with those areas at the same concentrations which resulted from chromatography of spiked serum samples through the complete solid-phase system. Each standard was injected five times.

RESULTS AND DISCUSSION

Linearity, precision, accuracy, and recovery

Linearity was examined at concentrations within the range 0.25–100 ng/ml (Table I). Each standard was injected ten times. The calibration curve thus derived was described by the equation: $y = 0.9978\,x + 0.2678$ with a coefficient of correlation $r = 0.9993$. Accuracy was + 8% for the lower limit of quantitation and 1.3% for 100 ng/ml. Values for precision [coefficient of variation (C.V.)] at these concentrations were 7.4 and 3.3%, respectively. Recovery was checked with five subsequent injections each at 0.5 and 25 ng/ml. Values of 47.0 and 53.8%, respectively, showed similar

TABLE I

PRECISION, ACCURACY AND LINEARITY FOR B859-35 (SPIKED SERUM SAMPLES)

Standard regression line: $y = 0.9978x + 0.2678$; $r = 0.9993$.

Set point (ng/ml)	Actual value (Mean ± S.D., $n = 10$) (ng/ml)	Precision (%)	Accuracy (%)
0.25	0.27 ± 0.02	7.4	+ 8.0
0.50	0.58 ± 0.04	6.9	+ 13.8
1.00	1.18 ± 0.05	4.2	+ 18.0
5.00	5.15 ± 0.21	4.1	+ 3.0
25.00	25.10 ± 0.90	3.6	+ 0.2
50.00	46.70 ± 2.90	6.4	− 6.7
100.00	101.30 ± 3.34	3.3	+ 1.3

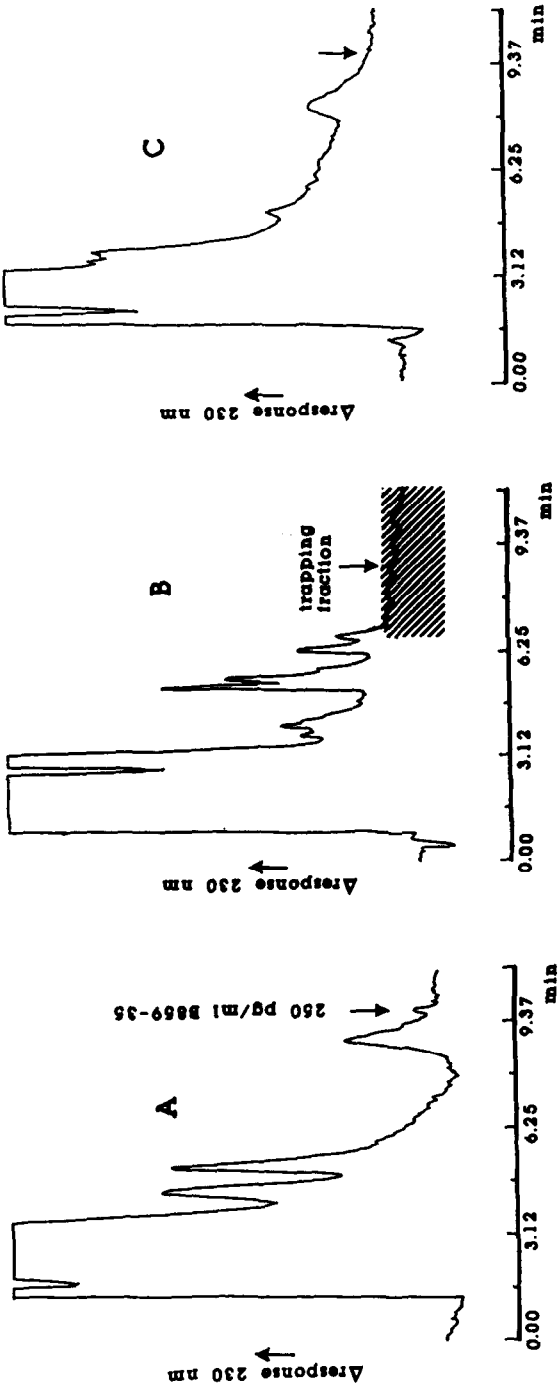

Fig. 3. Final chromatograms of a blank (C) and spiked (250 pg/ml) serum sample (A) eluting from the second (narrow-bore) column. Chromatogram (B) shows elution with the trapping fraction used for concentration and transfer to the second column.

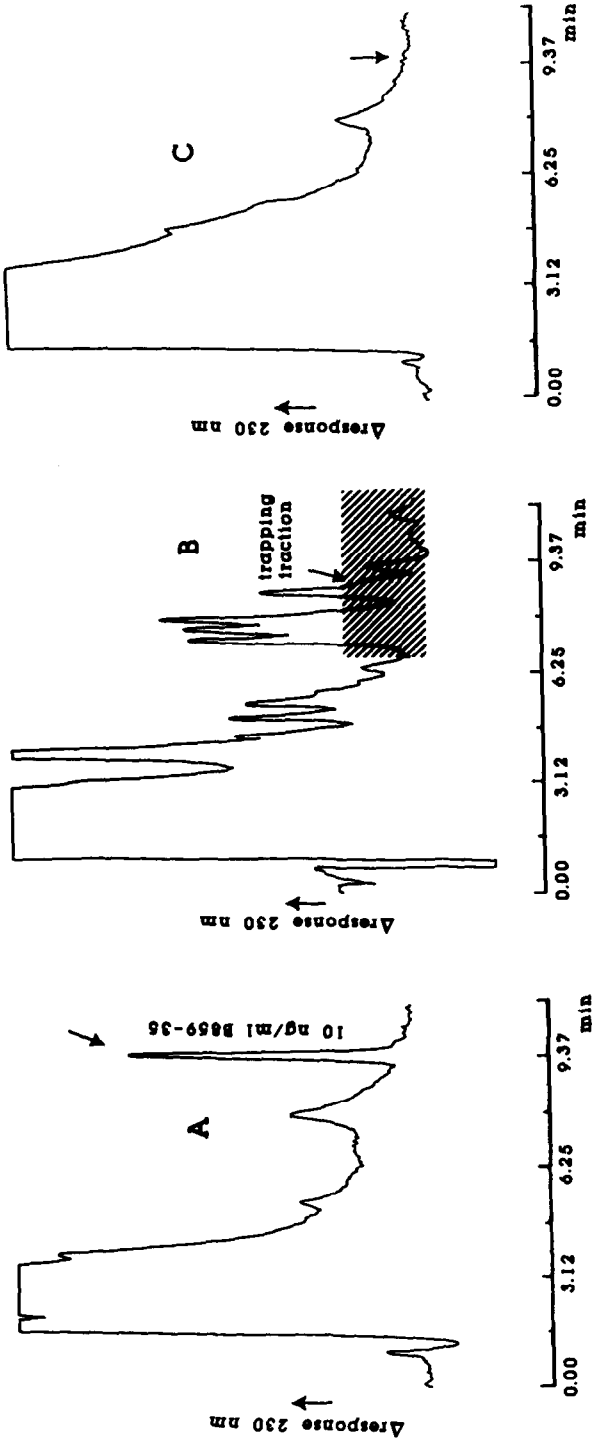

Fig. 4. Chromatograms of a serum sample from a cancer patient 2 h after administration of a 100-mg capsule of B859-35. The serum concentration at this time point is assayed for 10 ng/ml. Chromatograms eluting from the first (B) and second (A) column are given, as well as a pre-dose sample of the same patient (C) eluting from the second column.

recoveries at low and at high concentrations of B859-35 in serum. In these figures a mean recovery of 82% on the AASP cartridge (determined by [^{14}C]B859-35; data not shown) contributes quite considerably to the overall relatively low, but constant, recovery over the entire assay system.

Chromatograms

Fig. 3 shows typical chromatograms of spiked serum standards and Fig. 4 chromatograms of samples obtained from a cancer patient. Comparison of chromatograms from the first and second columns demonstrates clearly the removal of the residual interfering serum matrix achieved by the two-dimensional technique. Therefore, analysis of as much as 1 ml of serum on a narrow-bore column is feasible in order to achieve a high assay sensitivity.

Application

Fig. 5 demonstrates the applicability of the assay. Individual serum B859-35 profiles from four male cancer patients after receiving a 100-mg capsule of B859-35 are shown.

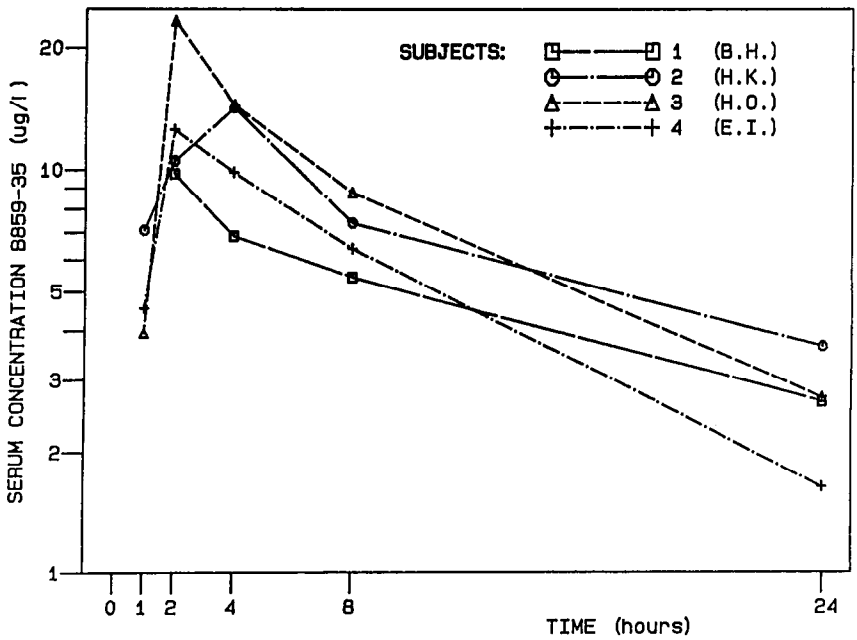

Fig. 5. Individual concentration–time profiles for B859-35 in serum of four cancer patients following single oral administration of a 100-mg capsule of B859-35.

CONCLUSIONS

As the mode of detection for B859-35 is restricted to UV absorption, optimum removal of the interfering plasma matrix was essential to decrease both the detection

limit and the lower limit of quantitation of this bioassay. The automated technique presented enables quantitation of the new anti-proliferative drug B859-35 at very low ng/ml values using 1 ml of serum or plasma. Sample clean-up was obtained in a first off-line step by liquid-solid extraction with the AASP system. Further separation of the interfering matrix was achieved by on-line collection of a fraction with the peak of interest by solid-phase trapping followed by on-line transfer to a second analytical column. For the latter, a narrow-bore column was used to achieve the best sensitivity. Concentration of the collected fraction was therefore a prerequisite for development of the final chromatogram. Up to 40 samples can be handled in one day and for the following series of samples only the concentration column has to be replaced.

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