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Journal of Chromatography B, 658 (1994) 349–353

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Detection and separation of the anthrapyrazole CI-941 and its metabolites in serum and urine by high-performance liquid chromatography

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First received 18 January 1994; revised manuscript received 26 April 1994

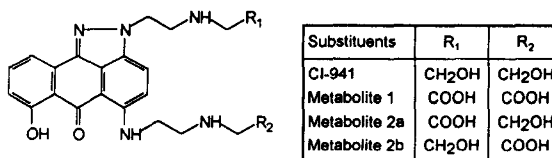
Abstract

A high-performance liquid chromatographic method using ion-pairing chromatography on reversed-phase C_{18} material with a mobile phase of acetonitrile–water (19:81, v/v) containing 5 mM 1-pentanesulfonic acid was developed for the detection and separation of the anthrapyrazole CI-941 (I) and its metabolites. After sample clean-up with solid-phase extraction, I and its metabolites were measurable at a wavelength of 491 nm. A detection limit of 5 ng/ml was achievable for I. The dicarboxylic acid derivative and the isomers of the monocarboxylic acid derivative could be separated. Application of the method to a human pharmacokinetic study showed two and four metabolites of I in serum and urine respectively.

1. Introduction

The anthrapyrazole derivative 10-hydroxy-2-[2-[(2-hydroxyethyl)amino]ethyl]-5-[[2-[(2-hydroxyethyl)amino]ethyl]-amino]anthra[1,9-*cd*]pyrazol-6(2*H*)-one (CI-941, I) is a representative of a new class of anticancer agents which was developed as an alternative to the anthracyclines and anthraquinones to diminish or avoid therapy-related cumulative cardiotoxicity [1–6]. The synthetic DNA-intercalator I has a broad spectrum of antitumour activity, similar to that of doxorubicin, and is currently under clinical phase II evaluation. The structures of the compounds are shown in Fig. 1.

Previous investigations of the pharmacokinetics of I were based on a HPLC method that was not able to separate and determine the metabolites of I [7–11] which were observed by Allan et al. [12] in human urine. However, they did not report the elucidation of the chemical structures or the separation and quantitation of these compounds [12]. Blanz et al. [13] described an other HPLC method for the analysis of urine from patients treated with I using a volatile buffer salt gradient system. This chromatographic system, coupled on-line to an ion-spray mass



Substituents	R_1	R_2
CI-941	CH_2OH	CH_2OH
Metabolite 1	COOH	COOH
Metabolite 2a	COOH	CH_2OH
Metabolite 2b	CH_2OH	COOH

Fig. 1. Chemical structure of I and its metabolites.

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SSDI 0378-4347(94)00235-W

spectrometer, allowed the separation and structural identification of three metabolites of I [13]. Since this HPLC method is limited to urine [13], a rapid, sensitive and specific reversed-phase HPLC assay for the routine analysis of I and its metabolites in serum and urine was developed. The method described here allows the measurement of the parent compound and its metabolites to elucidate the disposition of I.

2. Experimental

2.1. Chemicals and reagents

Compound I (25 mg lyophilized drug/ampoule) was supplied by Du Pont (Bad Homburg, Germany). All solvents were HPLC grade. Water and acetonitrile were purchased from Merck (Darmstadt, Germany). 1-Pentanesulfonic acid (Pic B-5 Reagent) was obtained from Waters Assoc. (Eschborn, Germany). Serum of volunteer blood donors was pooled and used for method development.

2.2. Instrumentation

The chromatographic system consisted of a Waters 501 solvent delivery system, a Model 712 Waters intelligent sample processor (WISP), a Waters Lambda-Max Model 481 LC spectrophotometer and an UV1000 LC spectrophotometer (Spectra-Physics, Darmstadt, Germany). UV-Vis spectra of the peaks were acquired using a fast-scanning spectrophotometer SpectraFocus (Spectra-Physics). Acquired data were processed by the Waters Maxima and the SpectraFocus software. Separation was obtained with a Waters μBondapak C_{18} column (300×3.9 mm I.D., 10 μm particle size).

2.3. Chromatographic conditions

The optimal isocratic system for the resolution of I and its metabolites was found to be acetonitrile–water (19:81, v/v) containing 5 mM 1-pentanesulfonic acid at ambient temperature. The flow-rate was maintained at 1 ml/min. Using the UV1000 spectrometer detection of I and its metabolites at the absorbtion maxima of 491 nm provided a highly selective and sensitive system, showing no interference from endogenous coextracted compounds (see Discussion).

2.4. Standards

Standards of I were prepared with absolute amounts between 1 and 2000 ng by dissolving the drug directly in the HPLC mobile phase. To determine the drug recovery, serum and urine were spiked with I in the concentration range 5–2000 ng/ml.

Since standards of the metabolites were not available, standards of I were used for the measurement of metabolites in serum and urine. The assignment of the metabolites was performed by comparing their UV-Vis spectra with the spectra obtained using the HPLC gradient system coupled to a mass spectrometer [13].

2.5. Sample clean-up procedure

I and its metabolites were isolated from their biological matrix by solid-phase extraction. A Vac-Elut system (ICT, Frankfurt, Germany) equipped with LiChrolut RP-select B (100 mg) disposable extraction columns (Merck, Darmstadt, Germany) was used for sample clean-up. The columns were preconditioned by washing with 1 ml of acetonitrile and 2 ml of water. Serum and urine samples of 1 ml were passed through the cartridge and the columns were washed with 2 ml of 5 mM 1-pentanesulfonic acid. To increase the sensitivity of the assay volumes up to 5 ml of serum and urine could be loaded onto the cartridges. The columns were dried for 2 min by the vacuum stream of the Vac-Elut system. The remaining compounds were extracted with 400 μl of acetonitrile–water (30:70, v/v, containing 5 mM PIC B-5 reagent) directly into micro-glass conical inserts (Ziemer, Mannheim, Germany) by mild centrifugation (2 min at 200 g). The inserts were placed in Waters WISP 4-ml glass vials and volumes of 0.1 ml were injected.

2.6. Quantitative analysis

Quantitation was performed by the external standard method of analysis. The calibration standard curve was obtained by plotting the peak areas of I against the known concentrations of standards prepared as described in section 2.4. The detection limit of the assay was set at a signal-to-noise level of 2 for serum and urine. The amounts of metabolites were expressed as equivalents of I, since no pure standards of the metabolites were available.

2.7. Recovery and precision

Recovery of I was determined by comparing the peak area of the extracted samples to that of the standard injected directly on the column.

Precision and accuracy were determined by assaying duplicate serum samples on ten different days.

3. Results

The calibration curve for I was linear over the range of 5–2000 ng/ml ($r > 0.999$ for serum and urine). The detection limit was 5 ng/ml. The reproducibility (coefficient of variation, C.V.) of the external standard procedure measured on ten different days was 3.4% at 500 ng/ml serum. Volumes up to 5 ml of serum and urine could be extracted with C.V.s less than 7.7%, as shown in Table 1. The precision and recovery data of I are summarized in Table 1. The recovery of metabolites was checked by comparing the peak areas after direct injection of urine containing these

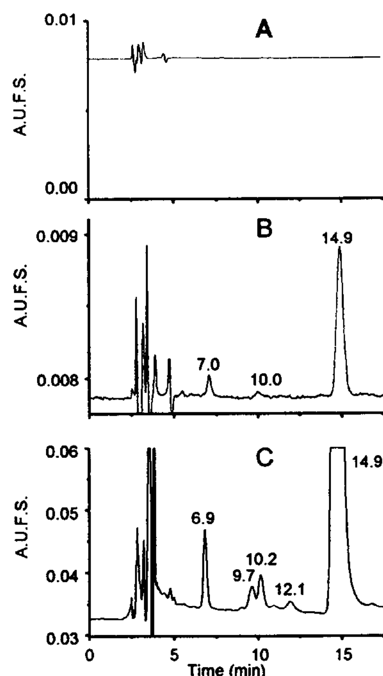

Fig. 2. Chromatographic profiles of extracted serum and urine samples after a 7-min infusion of 64 mg of I: (A) blank, (B) serum after 2 h, and (C) urine collected for 4 h after infusion. Compound I was eluted with a retention time of 14.9 min, the 2-h serum sample shows two more polar metabolites at 7.0 and 10.0 min. In the urine four metabolites were detected at 6.9, 9.7, 10.2 and 12.1 min.

Table 1
Recovery of I from serum and urine

Sample concentration (ng/ml)	Sample volume (ml)	Sample origin	Recovery (mean ± S.D.) (%)	C.V. (%)
1000	1	Serum	103.5 ± 3.0	2.87
100	1	Serum	101.2 ± 4.5	4.47
10	1	Serum	96.7 ± 3.2	3.36
5	4	Serum	95.8 ± 7.3	7.68
2000	1	Urine	98.1 ± 2.5	2.21
1000	1	Urine	97.3 ± 4.1	3.80
100	5	Urine	95.1 ± 4.0	3.88

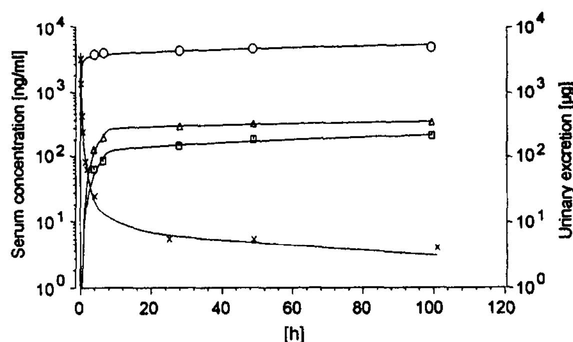

Fig. 3. Computer-fitted curves for I serum concentrations (×), and the cumulative urinary excretion of I (○), and its metabolite 1 (dicarboxylic acid derivative, △) and metabolite 2a/b (isomers of the monocarboxylic acid derivative, □).

metabolites onto the HPLC system with those obtained after the sample clean-up procedure. No loss of metabolites was observed. Fig. 2 shows chromatograms obtained from plasma and urine samples after extraction.

The analytical technique described here permitted the measurement of I with high sensitivity and allows the determination of the terminal half-life. Serum concentration curves and cumulative urinary excretion of I and its metabolites were fitted by use of the Topfit program [14]. The curves are shown in Fig. 3.

4. Discussion

Up to now, pharmacokinetic data of I in human and animal were acquired using a HPLC method developed by Graham et al. [7]. However, their method was not able to separate the metabolites from the parent drug. Since biotransformation is an important factor in the evaluation of pharmacokinetic data of a drug, we developed a HPLC method allowing the separation and detection of I and its metabolites in human urine and in blood serum.

The elucidation of the chemical structures of three urinary metabolites was published recently [13]. However, the HPLC method used in that assay was adapted especially for HPLC–MS coupling and depended on a gradient HPLC system. We now report on a less time consuming isocratic HPLC system applicable for routine analysis of blood samples and urine of patients. Further advantage of the new method is the separation of the two isomers of the monocarboxylic acid derivative of I (chemical structures shown in Fig. 1, retention times 9.7 and 10.2 min) which could not completely be separated using the gradient HPLC method. Furthermore another metabolite ($r_T = 12.1$ min) with unknown chemical structure could be discovered in the patient urine.

The recovery of the drug during solid-phase extraction of serum is excellent as shown in Table 1. It should be noted that the usage of the base-deactivated sorbent LiChrospher RP-select B in the extraction columns greatly improves the recovery compared to conventional sorbent material. The calibration curve obtained is linear over the range 5–2000 ng/ml for plasma and urine with good reproducibility. The search for compounds usable as internal standards failed due to low extraction recovery or unsuited chromatographic behaviour of compounds with appropriate light absorption maxima.

During the development of the HPLC separation, we used a Waters Lambda-Max Model 481 spectrophotometer to detect the drug and the metabolites at their light absorption maximum of 491 nm. However, this widely used spectrophotometer simultaneously detected the light absorption at 245.5 nm (second order diffraction at the grating of the monochromator). Thus, the HPLC chromatograms obtained with this detector consisted of a superposition of the chromatograms detected at two different wavelengths. Without the knowledge of this unexpected behaviour of a HPLC spectrophotometer additional endogenic compounds in serum and urine absorbing in the UV range may be detected and assumed to be drug related. By application of a fast-scanning spectrophotometer, such as the SpectraFocus instrument, the acquisition of the UV-Vis spectra of the chromatographic peaks could be accomplished. This confirmed the presence of only 5 drug-related compounds, exhibiting the typical UV-Vis characteristics of the anthrapyrazole chromophor.

Acknowledgement

The financial support by the Deutsche Forschungsgemeinschaft (SFB 120) is gratefully acknowledged.

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