Journal of Chromatography, 416 (1987) 357–364
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3569

Note

Determination of anagrelide in plasma by capillary gas chromatography–mass spectrometry

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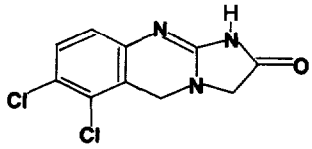
(Received October 24th, 1986)

Anagrelide, 6,7-dichloro-1,5-dihydroimidazo[2,1-*b*]quinazolin-2(3H)-one monohydrochloride, a potent inhibitor of platelet aggregation and intravascular thrombosis in experimental models, is currently undergoing clinical evaluation. The pharmacology of anagrelide has been reviewed [1]. The structure of anagrelide is depicted in Fig. 1.

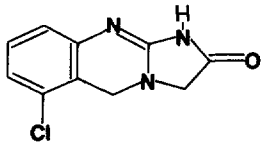
When 1 mg of anagrelide is taken orally by a human, the resulting maximum plasma concentration is less than 10 ng/ml. Therefore, a sensitive method for quantification was required. The lower limit of determination by high-performance liquid chromatography (HPLC) with UV detection was found to be in the 5 ng/ml range. A method based on HPLC separation and radioactive scintillation detection of [^{14}C]anagrelide was used by Gaver et al. [2] to study the pharmacokinetics of anagrelide at plasma concentrations of 0.3 ng/ml and above. A method using on-line chromatography–detection of unlabeled drug is, however, more generally useful.

This paper describes an internal standard method for extraction, clean-up, derivatization, and gas chromatographic–mass spectrometric (GC–MS) determination of anagrelide in plasma. A statistical analysis of the performance of the method is reported. The linear range of quantification is 0.5–100 ng/ml.

0378-4347/87/$03.50



ANAGRELIDE



INTERNAL STANDARD

Fig. 1. Structures of anagrelide and internal standard.

EXPERIMENTAL

Materials

Anagrelide hemihydrate (Fig. 1) and the internal standard, 6-chloro-1,5-dihydroimidazo[2,1-*b*]quinazolin-2(3H)-one monohydrochloride (Fig. 1), were provided by Bristol-Myers (Evansville, IN, U.S.A.). An ethereal solution of diazomethane was prepared with Diazald® and 2-(2-ethoxyethoxy)ethanol from Aldrich (Milwaukee, WI, U.S.A.) [3]. Toluene was HPLC/Spectro grade from Alltech (Deerfield, IL, U.S.A.). Methanol and methyl *tert.*-butyl ether (MTBE) were HPLC grade and diethyl ether was anhydrous grade from Fisher (Fairlawn, NJ, U.S.A.). Prior to use the MTBE was demonstrated to be peroxide-free by an iodine test as follows: 10 ml of MTBE were shaken with 1 ml of freshly prepared 10% (w/v) aqueous potassium iodide and protected from light for 10–20 min. If no color was produced in either phase when observed against a white background, the MTBE was judged to be peroxide-free. Hydrochloric acid was ACS grade and potassium iodide was AR grade from Mallinkrodt (St. Louis, MO, U.S.A.). Sodium bicine (diethylolglycine) was from Calbiochem (LaJolla, CA, U.S.A.) and was prepared as a 2 *M* solution in distilled water which was adjusted to pH 9.3. Human plasma was obtained from Interstate Blood Bank (Memphis, TN, U.S.A.). The plasma was prepared by centrifugation of Na_2-EDTA-treated blood and was stored at −17°C until used.

Primary standard solutions of anagrelide hemihydrate and internal standard were each prepared at 1 mg/ml in methanol which contained about 1% (v/v) of concentrated hydrochloric acid. A secondary solution of internal standard was prepared monthly at 2 ng/μl in methanol and was stored at room temperature. Secondary solutions of anagrelide were prepared monthly at specific concentrations in the range of 0.1–5 ng/μl and were stored at room temperature. All concentrations are in terms of the free base.

Instrumentation

Solvent extractions utilized a Roto-Torque rotator from Cole-Parmer (Chicago, IL, U.S.A.). Solvent removal was accomplished with an N-Evap analytical evaporator from Organomation (Northborough, MA, U.S.A.) with a water-bath temperature of 30°C and a gas stream of extra-dry nitrogen. Reagents were added with repipettes from Oxford Labs. (Foster City, CA, U.S.A.).

A Model 4500 GC–MS system with an INCOS data system from Finnigan MAT (San Jose, CA, U.S.A.) was used. The gas chromatograph was equipped with an on-column injector mounted over the 260°C heated packed column inlet as previously described [4].

The chromatographic column was a 5 m×0.25 mm I.D. DB-1 fused-silica capillary column with a film thickness of 0.25 μm from J & W Scientific. It was routed directly to the ion source. The helium carrier gas velocity was approcimately 200 cm/s. The gas chromatograph to mass spectrometer interface oven was at 260°C. Following injection of 3 μl of sample with a No. 701RNFS syringe from Hamilton, the gas chromatograph oven temperature was held at 100°C for 1 min and then increased at 20°C/min to 300°C.

The analytes were measured by selected-ion monitoring at 268 a.m.u. for anagrelide and 234 a.m.u. for the internal standard. Each mass was monitored at 35 eV for 0.21 s while scanning a 0.25-a.m.u. window about the center of the mass peak.

Procedures

A standard curve, consisting of duplicates at six concentration levels over the range 0.5–100 ng/ml, was prepared daily with each set of samples as follows: into 16×100 mm screw-cap tubes were placed 1 ml each of control plasma; 5–20 μl of anagrelide secondary standards were added to the tubes. The standards were spiked with internal standard and extracted along with the samples. Spiked samples, for characterization of method accuracy and precision, were prepared in multiples in the same manner as the standard curve.

On the day of extraction the plasma samples were removed from the −17°C storage and thawed at room temperature. A 1.0-ml aliquot of each sample was transferred to a 16×100 mm screw-cap culture tube and 10 μl of a 2 ng/μl methanolic solution of internal standard were added. To each tube was added 0.1 ml of bicine buffer, the tube contents were mixed, then 7 ml of MTBE were added. The tubes were capped and extraction proceeded for 10 min by rotation at the maximum speed which allowed complete flow between the top and bottom of the tube. After centrifugation of the tubes at 1000 *g* for 2 min the lower aqueous phase was frozen by placing the lower portion of the tubes in a dry ice–isopropanol bath. The upper phase was decanted into a clean 16×100 mm tube and the solvent was evaporated at ambient temperature under a stream of nitrogen. The aqueous phase was thawed and extracted once more as above and the organic extract was added to the tube containing the residue from the first extraction. Then, 1 ml of 1 *M* hydrochloric acid was added to the combined organic extracts, the tube contents were mixed, centrifuged and frozen as described above. The upper organic phase was decanted and discarded. The aqueous phase was thawed, 0.2 ml of bicine

buffer and 7 ml of MTBE were added. The tube contents were mixed, centrifuged and frozen as above. The organic phase was decanted into a clean 13×100 mm tube, evaporated under nitrogen at ambient temperature, capped and stored below 0°C for up to three days.

After addition of 50 μl of ethereal diazomethane solution to the dried residue at ambient temperature, the tube was immediately capped, the tube walls were rinsed with derivatizing reagent and the tube was kept at ambient temperature for at least 5 min. The derivatizing reagent was evaporated under nitrogen at ambient temperature, 20 μl of toluene were added to each tube and 3 μl were injected into the gas chromatograph–mass spectrometer.

The accuracy and precision of the method were characterized by several tests. Intra-assay variability, or single-day error, was studied by analyzing multiple identically spiked plasma samples at two concentration levels on one day. Inter-assay variability, or long-term error, was studied by analyzing a separate set of identically spiked plasma samples at two concentration levels on three days. Quality control (QC) samples were prepared at the time clinical samples were collected. They were prepared by spiking blank control plasma with a measured amount of anagrelide, stored alongside the clinical samples and analyzed with the clinical samples to provide additional long-term reproducibility data for the method.

A lower limit of determination was determined on plasma from ten individuals. Portions of each sample (1 ml) were transferred to each of two 16×100 mm extraction tubes. To one of each pair were added 0.5 ng of anagrelide. All the samples were extracted and the response from the spiked and blank samples were statistically compared.

RESULTS AND DISCUSSION

The 70-eV electron-impact mass spectrum of anagrelide is shown in Fig. 2. The predominant ions appear in a single cluster in the molecular ion region. The ions at *m/z* 255, 257 and 259 are the chlorine isotope peaks of the molecular ion and the ions at *m/z* 254, 256 and 258 are the chlorine isotope peaks of the $(M-H)^+$ ion. The mass spectrum of the internal standard is also dominated by the same type of cluster in its molecular ion region (*m/z* 220, 221, 222 and 223). Treatment of both compounds with diazomethane shifts each of these clusters 14 a.m.u. higher (Figs. 3 and 4) and confirms that the derivative is a result of single methylation. The 268-a.m.u. ion of the anagrelide derivative and the 234-a.m.u. ion of the internal standard derivative were the most abundant ions and were selected as the ions to be monitored for the quantitative method.

Typical chromatograms of blank, spiked and clinical sample extracts are shown in Fig. 5. No endogenous plasma interferences were observed in the blanks. No interfering metabolites or artifacts from the clinical sample extacts were observed.

Quantification was based on the area under the peak of the selected-ion chromatogram. Unknowns were determined from a linear least-squares fit of the area ratios of the standards versus amount, which typically was described by the equation: $(\text{area}_{\text{anagrelide}}/\text{area}_{\text{I.S.}}) = 0.069 \times (\text{concentration, ng/ml}) + 0.0021$, with a

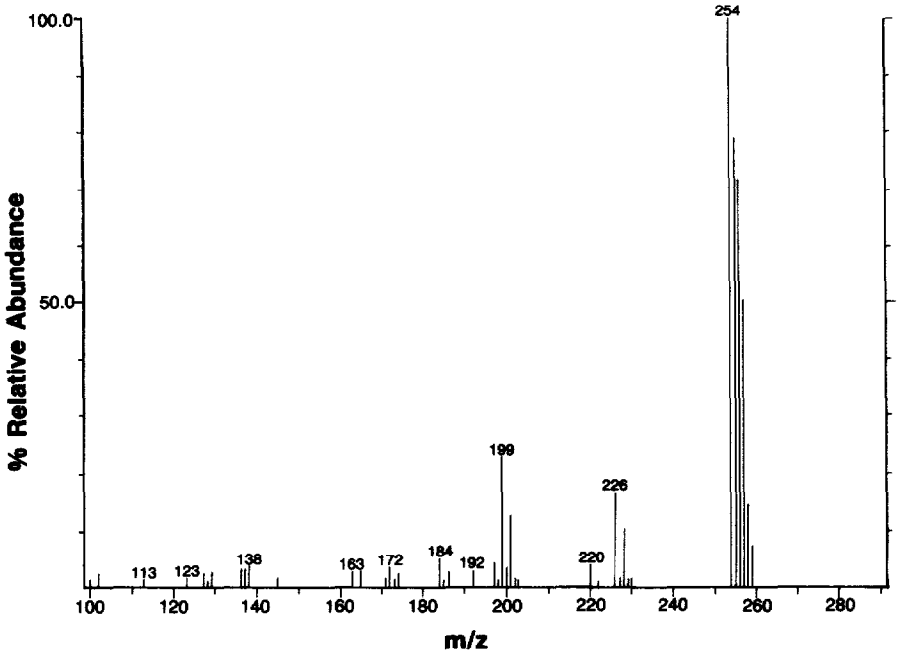


Fig. 2. Electron-impact mass spectrum of anagrelide.

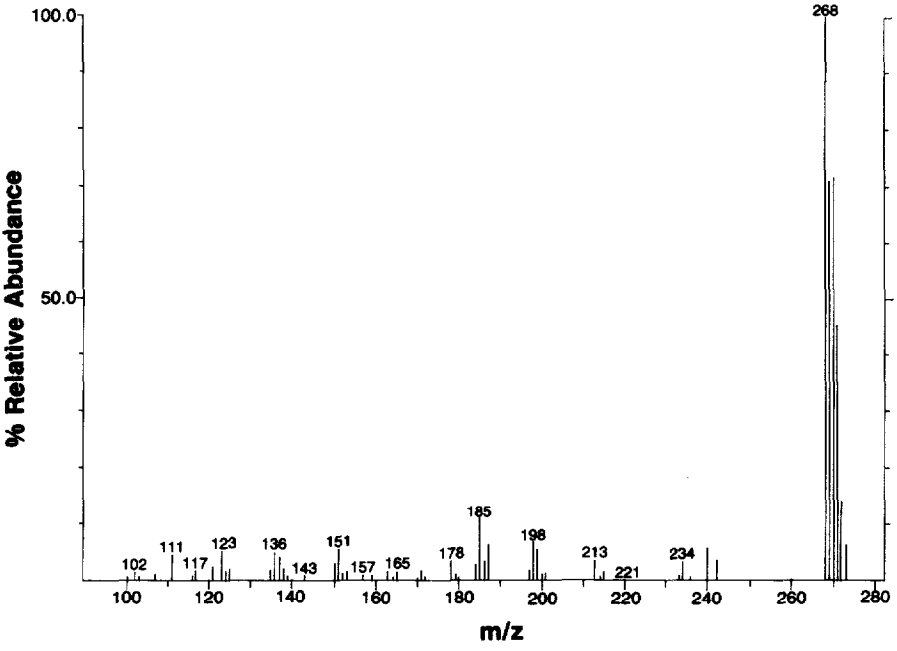


Fig. 3. Electron-impact mass spectrum of derivatized anagrelide.

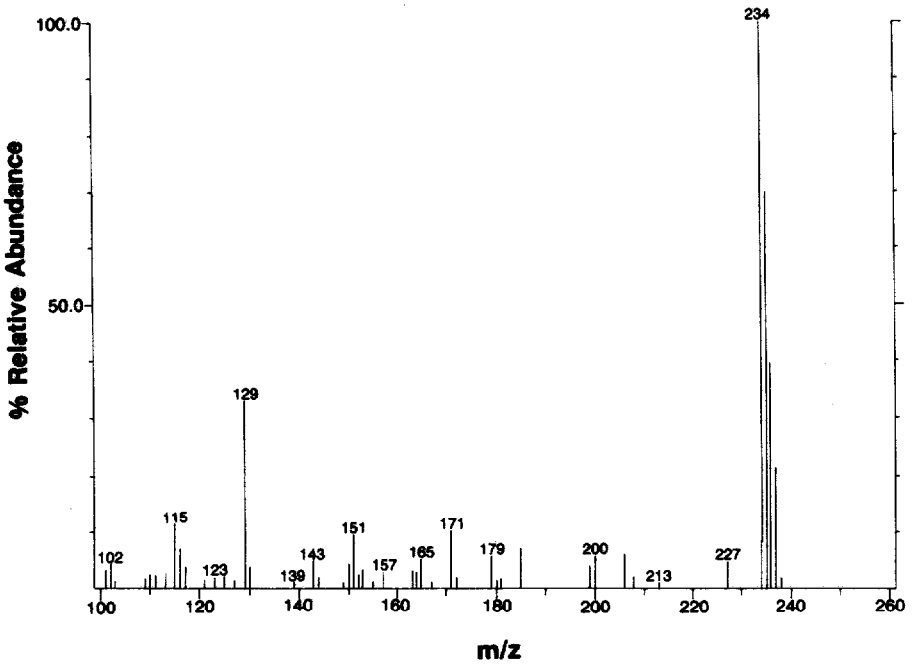


Fig. 4. Electron-impact mass spectrum of derivatized internal standard.

TABLE I

ACCURACY AND PRECISION OF THE METHOD

See text for a description of the tests; n is the number of data points over X days.

Test	n	X (days)	Concentration added (ng/ml)	Concentration found (ng/ml)	Coefficient of variation (%)
Intra-assay	10	1	4.0	3.9	7
	10	1	80	77	3
Inter-assay	5	1	4.0	4.1	6
	5	1	4.0	4.1	7
	5	1	4.0	4.1	4
	15	3*	4.0	4.1	5
	5	1	80	83	5
	5	1	80	78	3
	5	1	80	81	8
	15	3*	80	81	6
QC samples	17	8	2.0	1.9	21
	17	8	20	20	22

*Combination of the three days above.

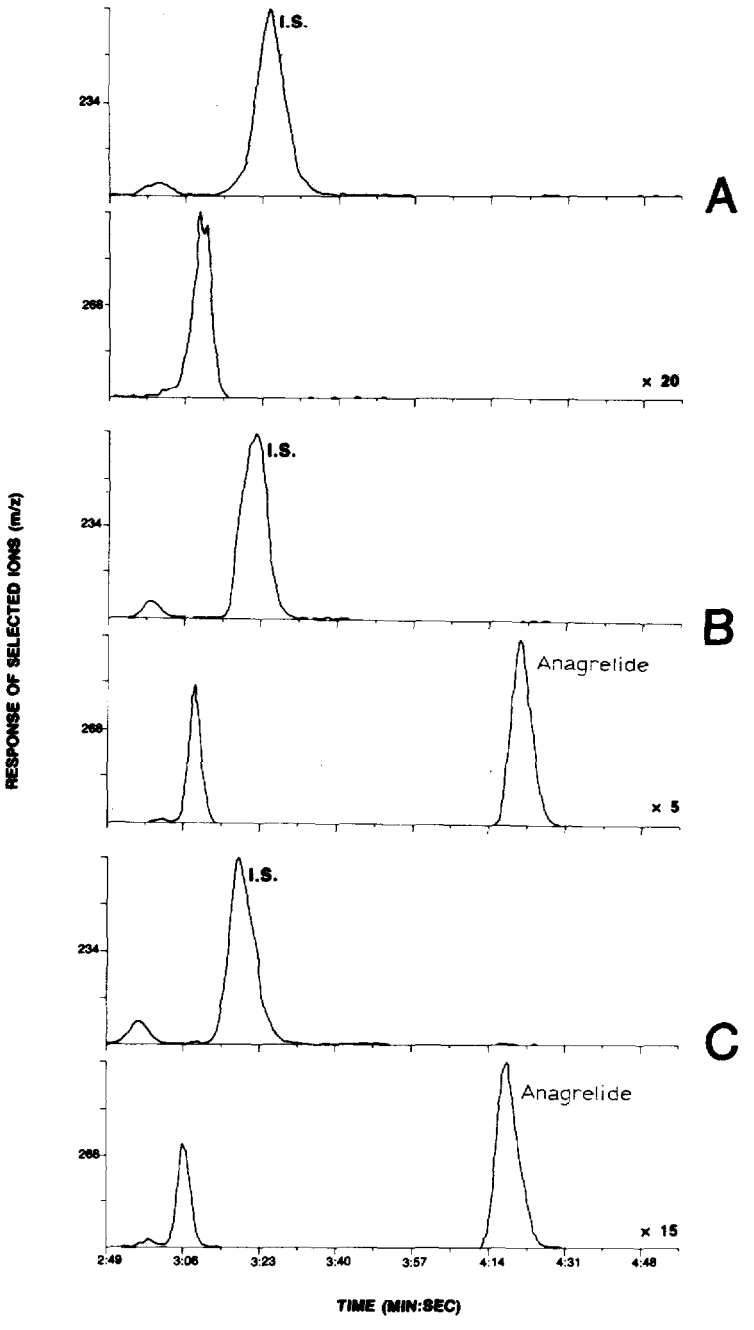


Fig. 5. Selected-ion chromatograms of plasma extracts. (A) Processed blank, spiked with 20 ng/ml internal standard only. (B) Processed standard, spiked with 20 ng/ml internal standard and 5 ng/ml anagrelide. (C) Processed clinical sample, spiked with 20 ng/ml internal standard only.

correlation coefficient of 0.997. Over the range of 0.5–100 ng/ml the data did not deviate significantly from linearity, based on a lack-of-fit statistic [5].

Data from the lower limit of determination study were analyzed by comparing the group of responses of the unspiked samples at the retention time of anagrelide to the group of responses of the same samples spiked at 0.5 ng/ml. A paired t-test indicated that the difference between the means was statistically significant with $t = 6.036$.

Data from the accuracy and precision studies are tabulated in Table I. These data establish the method's performance. The method has been used to quantify anagrelide in samples from clinical studies. QC samples which were stored and analyzed with the samples provide additional data in Table I on reproducibility of the method.

ACKNOWLEDGEMENT

The authors recognize the valuable contribution of William N. Schneider of the Metabolism and Pharmacokinetics Department in developing the extraction and clean-up methodology and in preparing the samples described in this report.

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