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ANALYSIS OF CLOMESONE IN PLASMA BY GAS CHROMATOGRAPHY–ELECTROLYTIC CONDUCTIVITY DETECTION

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SUMMARY

A sensitive and specific gas chromatographic (GC) method with electrolytic conductivity detection (ELD) for the analysis of clomesone (2-chloroethylmethylsulfonylmethane sulfonate), a new experimental antitumor alkylating agent, in plasma has been developed for the first time. Clomesone in plasma containing suitable internal standard was extracted with methylene chloride. After evaporation, the residue was analyzed by GC-ELD. Either a 15-m wide-bore DB-17 or a DB-1 column with the corresponding internal standards of propachlor or butachlor, respectively, was used. For the DB-1 column with butachlor as the internal standard, the routine assay limit was 20 ng/ml with linearity from 10 to 2000 ng/ml monitored. The within-run coefficient of variation of eight replicates at 50 ng/ml was 8.0% and the between-run coefficient of variation was 11% at 120 ng/ml. Using this assay procedure, the stability in several aqueous media and protein binding of clomesone were evaluated. In fresh mouse plasma, the half-life of clomesone was less than 1 h, although in aged pooled human plasma the drug was more stable. The mean protein binding value in mouse and human plasma was about 81–85%.

INTRODUCTION

Analysis of halogenated compounds has frequently been performed by gas chromatography (GC) with electron-capture detection. Although negative-ion mass spectrometry (MS) offers similar sensitivity and better specificity [1], the costs of the instrument for the latter method have hampered its wide application. Relatively recently, GC with electrolytic conductivity detection (ELD) has become a well accepted application, especially in the analysis of pesticides and environmental pollutants [2–5]. This detection system converts halogenated organics into inorganic mineral acids which may be detected by their electrical conductance. Thus, the simplistic nature of this detection system and the relatively low costs of the instrumentation should allow its wide application in many

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laboratories. Despite these potential merits, its application in the analysis of antineoplastic agents has not been reported as yet.

In this communication, we report the use of a GC–ELD method for the analysis of clomesone, a new experimental alkylating agent, for the first time. This agent, synthesized by Fulmer Shealy of Southern Research Institute as an alkylating agent to replace nitrosoureas, has activity against P388 L1210 leukemias and Lewis lung carcinoma systems in experimental animals [6]. The application of this analytical method in the stability studies of clomesone in several solution media and plasma protein binding is also reported.

EXPERIMENTAL

Standards and reagents

Non-formulated clomesone (2-chloroethylmethylsulfonylmethane sulfonate; NSC 338947) was kindly supplied by Drug Synthesis and Chemistry Branch, DCT, National Cancer Institute. Propachlor (Ramrod, N-isopropyl-2-chloroacetanilide) and butachlor [2-chloro-2′,6′-diethyl-N-(butoxymethyl)-acetanilide] were obtained from Chem Service (West Chester, PA, U.S.A.). High-purity hydrogen (99.99%) was obtained from Methason (Cucamonga, CA, U.S.A.). Megabore fused-silica capillary columns were purchased from J & W Scientific (Rancho Cordova, CA, U.S.A.). Centrifree ultrafiltration units were purchased from Amicon (Danvers, MA, U.S.A.). Alzet osmotic pump (Model 2 ML 4) was supplied by Alza (Palo Alto, CA, U.S.A.). CDF_1 mice were supplied by the Division of Cancer Treatment, National Cancer Institute. Pooled human plasma was obtained from the Blood Bank of the Los Angeles County Hospital. Human serum albumin (Cohn fraction V) was obtained from Sigma (St. Louis, MO, U.S.A.). HPLC-grade methylene chloride and *n*-propanol were obtained from CMS (Curtin & Matheson, Brea, CA, U.S.A.). All solvent, chemicals and reagents were used without further purification.

Instruments

A Varian 1400 gas chromatograph (Varian Assoc., Palo Alto, CA, U.S.A.) modified to fit to a Model 4420 electrolytic conductivity detector (O.I., College Station, TX, U.S.A.) was used for the drug analysis. This gas chromatograph was also equipped with a 15-m megabore fused-silica capillary column coated with either 3-μm methylsilicone (DB-1) or 1-μm methyl silicone–phenyl silicone (1:1) (DB-17). High-purity hydrogen gas (99.99%) was used as the reducing gas for the reactor and *n*-propanol was used as the solvent in the detector cell. The operating conditions for the detector were: reaction temperature, 875–950°C; hydrogen gas pressure, 34 bar; solvent pump speed, three to five turns on a ten-turn pot. Helium was used as the carrier gas for the gas chromatograph. The operation conditions for the gas chromatograph were: column temperature, 170°C isothermal; carrier gas flow-rate, 30 ml/min. A Dubnoff incubator (Precision Scientific, Chicago, IL, U.S.A.) was used for all the incubation experiments. A Hewlett-Packard Model 5985A gas chromatograph–mass spectrometer–data system (Hewlett-Packard, Palo Alto, CA, U.S.A.) coupled to an HP 5840 gas chro-

matograph via an all-glass jet separator was used to analyze the structures of the extracted clomesone and internal standards. The GC–MS conditions were: transfer lines, ion source and jet separator temperature, 200 °C; ionization current, 300 μA. Column temperatures were modified slightly from those of the GC–ELD system. Ammonia chemical ionization was used for the analysis.

Extraction procedure

To 1 ml of plasma sample in a 16 mm × 125 mm disposable test tube was added an appropriate amount of internal standard. After brief mixing, 0.3 g sodium chloride was added and the sample was vortexed rigorously for 10 s. Then, 5 ml of ice-cold methylene chloride were added, and the tube was shaken in a horizontal shaker in a cold room for 10 min. Then the tube was centrifuged at 1500 *g* at 0 °C for 5 min. The top aqueous layer was siphoned off and discarded while the bottom organic layer was transferred onto a polypropylene extraction tube (11 ml, Western Analytic, Temecula, CA, U.S.A.) containing 2 g of anhydrous sodium sulfate. The organic layer was allowed to drain in a 12 mm × 75 mm disposable test tube and the organic layer was evaporated to dryness under a stream of nitrogen at room temperature. The residue was reconstituted with 5–50 μl of ethyl acetate, and a 1–2 μl aliquot of the solution was injected into the gas chromatograph.

Stability studies

Clomesone solutions in appropriate media in concentrations of 1–10 μg/ml along with controls were incubated in a Dubnoff incubator at 37 °C for an appropriate length of time. At a pre-determined schedule, typically at 0, 5, 10, 20, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 210, 240, 270, 300, 330, 360, 1350 and 1440 min, appropriate aliquots were removed serially and a known amount of internal standard was added; then the samples were quickly frozen in a dry ice–acetone bath and kept frozen at −20 °C until analysis. For studies in which clomesone degrades more rapidly, the sampling period was carried out at shorter intervals. The analysis was usually performed within two days. Stability of clomesone was evaluated in methanol, water, 0.9% sodium chloride in the presence of Alzet pump material, 5% human serum albumin, pooled human plasma and fresh mouse plasma.

Protein binding

Clomesone at 0.25, 0.50 and 1.0 μg/ml in pooled human and fresh CDF_1 mouse plasma was incubated in duplicate at 37 °C for 0.5 h. At the end of the incubation, the plasma samples were removed and one set of the duplicates was individually loaded onto separate Centrifree tubes. The tubes were centrifuged at 5000 *g* at 0 °C for 1 h, while the other duplicate set was placed in an ice bath and allowed to stand for the same time period. Then an aliquot of each of the ultrafiltrate and the corresponding plasma sample was removed for drug analysis. The drug concentration of the plasma sample served as the total and that of the ultrafiltrate represented the free drug concentration. Each set of the experiment was per-

formed in duplicate, and clomesone concentrations were determined along with appropriate standard curves.

RESULTS AND DISCUSSION

Assay evaluation

A typical gas chromatogram of clomesone and the internal standard butachlor from pooled human plasma eluted on a DB-1 column, along with that of a plasma blank extract, is shown in Fig. 1A and B, respectively. As shown, blank plasma extract exhibited insignificant background interference in the regions of the analytes. The mass spectra of clomesone and butachlor are shown in Fig. 2A and C, respectively. These mass spectra were identical to those of the authentic samples (not shown), thus establishing the identities of the GC peaks being analyzed. The calibration curve was linear from 10 ng/ml to 2 μg/ml for the DB-1 column

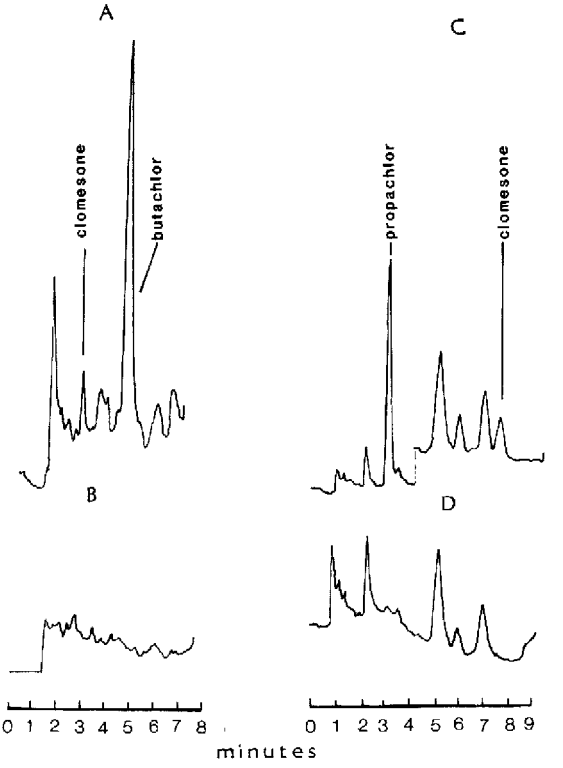

Fig. 1. Gas chromatograms of clomesone and the internal standards in plasma. (A) Clomesone at 30 ng/ml eluted from a 3-μm DB-1 column at 170°C; (B) the corresponding plasma blank for A; (C) clomesone at 20 ng/ml eluted from a DB-17 column at 155°C; (D) the corresponding plasma blank for C.

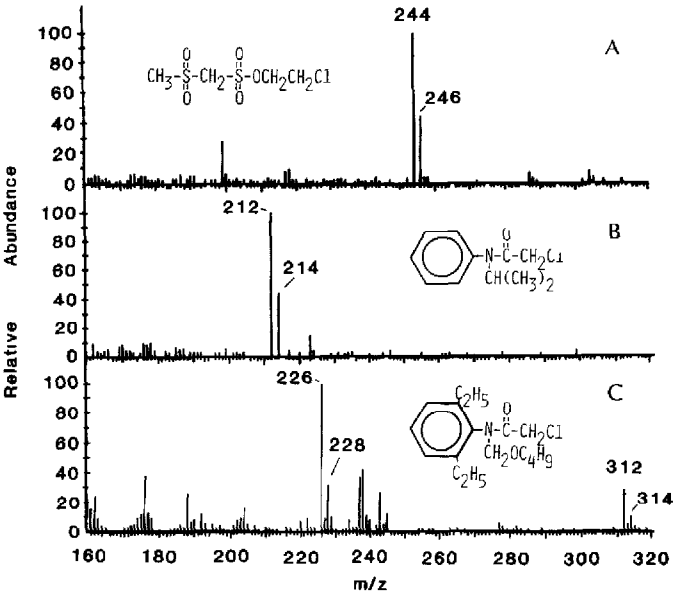

Fig. 2. Ammonia chemical ionization mass spectra of clomesone and the internal standards in plasma. (A) Clomesone; (B) propachlor; (C) butachlor.

with a detection limit in plasma of 10 ng/ml and retention times of 2.2 and 3.5 min for clomesone and butachlor, respectively, at 170 °C. The within-run reproducibility of the assay was found to be 8.0% on eight replicate determinations at 50 ng/ml. Because of the stability problem, the assessment of the between-run coefficient of variation was performed on separate samples freshly prepared each time and was determined to be 11% on eight separate determinations at 120 ng/ml.

The analysis of clomesone was also found to be satisfactorily performed using a 3-μm DB-17 column with propachlor as the internal standard with an assay sensitivity of 30 ng/ml and respective retention times of 3.2 and 7.8 min, at 155 °C. A representative gas chromatogram of a spiked plasma and plasma blank is shown in Fig. 1C and D, respectively. The identities of clomesone and propachlor (Fig. 2B) were also established by GC–MS. Since the conditions using butachlor and DB-1 column gave shorter retention times and better sensitivity, they were selected for most of the determinations of clomesone in biological fluids. The commercial source of the internal standard butachlor contains a small amount of the N-dealkoxylated product which can also be used as the internal standard at low concentration range.

Stability and recovery evaluation

Since clomesone and the internal standards used have structures that are rather reactive toward hydrolysis, their stability in methanol, water and plasma was evaluated to facilitate the development of an assay procedure. Using a DB-17 column and propachlor as the internal standard, clomesone was found to be rather

stable in methanol at room temperature and 37 °C with less than 10% degradation up to 24 h of monitoring, and at 50 °C about 30% degradation was seen after 3 h. The compound was rather unstable in aqueous solution, however. In unbuffered distilled water at 50 °C, clomesone degraded with apparent first-order kinetics and a half-life of 1.6 h (Fig. 3, Table I). Propachlor appeared to be more stable

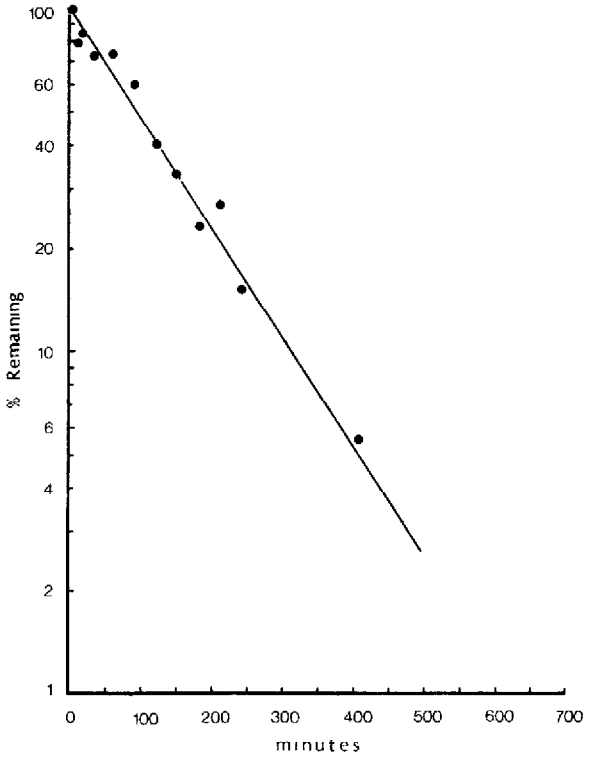

Fig. 3. Stability profile of clomesone in distilled water at 10 μg/ml incubated at 50 °C. Each data point represents the average of duplicate determinations. The line regressed to a monoexponential equation.

TABLE I

STABILITY DATA OF CLOMESONE IN SEVERAL AQUEOUS MEDIA

All stability profiles were of monoexponential decline except for Alzet pump material.

Medium	Temperature (°C)	Half-life (h)
Methanol	0	Stable
Distilled water	50	1.6
Alzet pump material in 0.9% saline	37	α: 1.66 β: 51.3
5% Human serum albumin in 0.9% saline	37	3.15
Pooled human plasma	37	1.5
Fresh CDF_1 mouse plasma	37	0.85

in aqueous solution than clomesone but no quantitative data were obtained. Butachlor was found to degrade in pooled human plasma at 37°C with an apparent half-life of 4.8 h (data not shown). On the other hand, clomesone was found to degrade more rapidly in pooled human plasma with an apparent half-life of 1.5 h at 37°C as measured by GC–MS (Table I) [7]. Thus, the stability of the internal standards did not appear to present a problem under the assay conditions. However, special precaution had to be taken to ensure the integrity of clomesone during the work-up. It was found that processing the sample at low temperature and the use of excess sodium chloride increased the stability of clomesone as was found to be the general case for other chloroethyl alkylating agents [8]. Additionally, the removal of trace water prior to evaporation of the organic extract further increased the stability of the drug. Using the described extraction procedure, the recoveries of clomesone, propachlor and butachlor from water and plasma were similar and approximately 90%.

Instrument evaluation

Two commercial electrolytic conductivity detectors are currently available, the Hall detector (Tracor, Austin, TX, U.S.A.) and the modified Hall detector (O.I.). Although the principles of operation are essentially the same, the former was designed earlier and appears to be more rugged and durable.

Application of the assay procedure

Since the Alzet pump has been frequently used for infusion of drug in animals by implantation, the stability of clomesone in normal saline in the presence of the Alzet pump material was evaluated. As shown in Fig. 4 at 37°C the stability profile of clomesone in 0.9% saline in the presence of two halves of an Alzet pump exhibited biphasic behavior. The data were fitted to a biexponential equation yielding the following parameters: 0.8911 (μg/ml) $\exp[-0.4182\ (h^{-1})\ t]$ + 0.07418 (μg/ml) $\exp[-0.01351\ (h^{-1})\ t]$. This gave apparent half-lives of 1.66 and 51.3 h for the first and second phase, respectively (Table I); the existence of the first phase suggests binding of the drug to the pump material, and the second phase probably represents the degradation of clomesone in normal saline. Thus, the binding of the drug to the pump material would make the use of the Alzet pump for infusion of clomesone in animal experiments unfavorable.

The degradation of clomesone in 5% human serum albumin and in fresh mouse plasma was also investigated. In 5% human serum albumin in saline at 37°C the clomesone concentration was found to exhibit a monoexponential decline. The aparent half-life was 3.15 h (Table I) and this half-life was longer than in pooled human plasma (1.5 h) as was described previously. The degradation of clomesone in fresh CDF_1 mouse plasma at 37°C was also found to exhibit a monoexponential decline with an apparent half-life of 0.85 h (Fig. 5). The increasing value of these half-lives (i.e. fresh mouse plasma, pooled human plasma, 5% human serum albumin) would suggest that human serum albumin might not be the only protein catalyzing the degradation of clomesone. Other material present in whole plasma may also assist degradation of this drug. Additionally, the more rapid degradation of clomesone in fresh mouse plasma may be attributable to an intrinsic difference

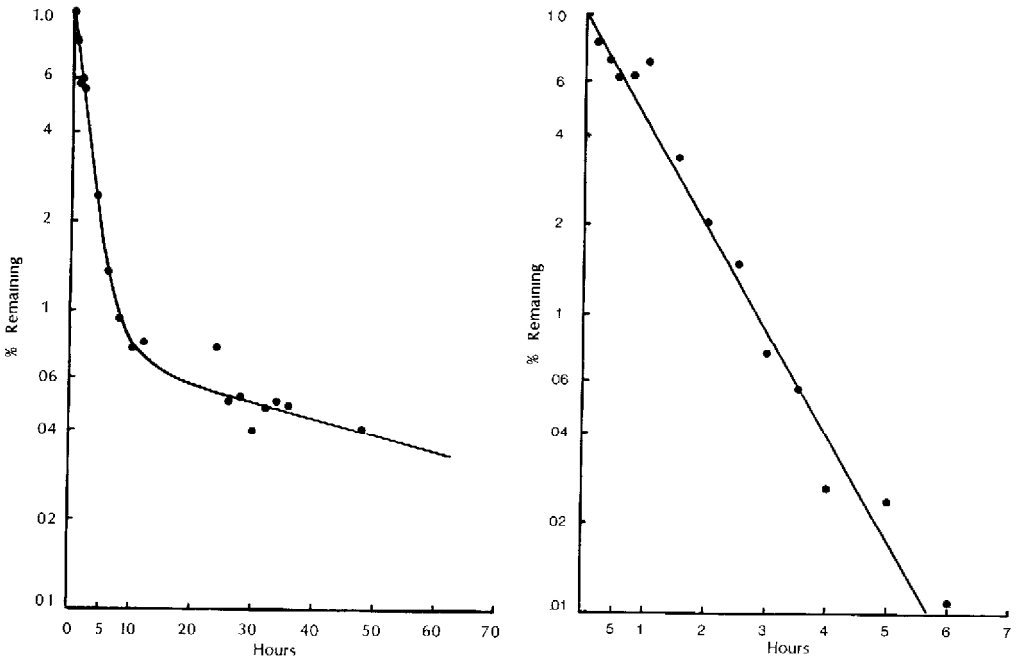

Fig. 4. Stability profile of clomesone in 0.9% saline at 10 μg/ml incubated at 37°C in the presence of two halves of Model 2 ML4 Alzet pump. Each data point represents the average of duplicate determinations. The line regressed to a biexponential equation (see text).

Fig. 5 Stability profile of clomesone in fresh mouse plasma at 10 μg/ml incubated at 37°C. Each data point represents the average of duplicate determinations. The line regressed to a monoexponential equation.

in mouse plasma compared to human plasma or to the presence of a labile substance which lost its activity when the plasma was aged.

Protein binding

Protein binding of clomesone to pooled human plasma as well as to fresh mouse plasma proteins was evaluated using the ultrafiltration technique, since the instability of this drug does not allow the use of equilibrium dialysis. The protein binding values of clomesone in pooled human plasma and in fresh mouse plasma were 85.1 ± 2.5 and $80.9 \pm 1.4\%$, respectively, in the concentration range of 0.25–1.0 μg/ml. However, these values are not statistically different ($P > 0.1$). These concentrations are within the achievable range in rats and mice given an intravenous bolus of 50 mg/kg clomesone (data not shown). Pharmacokinetic studies of clomesone in CDF_1 mice following a single intravenous bolus dose and following intravenous infusion have been accomplished using this assay technique and will be published elsewhere.

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