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Note

High-performance liquid chromatographic determination of spirohydantoin mustard in a clinically acceptable formulation of fat emulsion

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Spirohydantoin mustard (SHM, Fig. 1) is a rationally designed, central nervous system directed drug[1] that is undergoing preclinical evaluation in anticipation of clinical trials against brain tumors. SHM is highly active against murine intracerebral ependymoblastoma[1] and cross-links DNA in rat glioma 9L and bone marrow[2]. In rat 9L cells *in vitro*, SHM both synchronizes cells and blocks DNA synthesis[3].

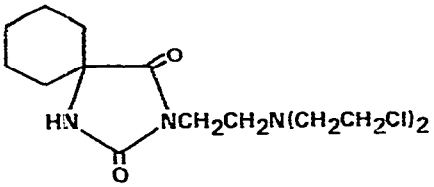

Fig. 1. Chemical structure of spirohydantoin mustard (NSC-172112).

SHM is insoluble in water, and pharmacological studies[4,5] employed dimethyl sulfoxide, a diluent that is not generally acceptable in patients, to solubilize the drug for intravenous (i.v.) administration. Flora *et al.*[6] discovered that SHM is dispensable in 10% fat emulsion, a lipid preparation used clinically as a dispersant for a brain tumor-active nitrosourea[7]. Flora *et al.* devised a gas–liquid chromatographic (GLC) assay to demonstrate uniformity and acceptable stability of the formulation. In preparation for preclinical toxicology studies on SHM, we had occasion to prepare many of these fat emulsion formulations and to perform assays by the reported method[6]. We observed that although the GLC method was satisfactory for a few samples, repeated application of ethyl acetate extracts of the formulations to the column resulted in progressive broadening of the SHM peak as well as an unstable baseline. These results prompted a search for an alternative method, and a satisfactory high-performance liquid chromatographic (HPLC) method was devised. A description of the HPLC method, its application to SHM formulations in fat emulsion, and confirmation of the HPLC method by use of [^{14}C]SHM are included in this report.

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EXPERIMENTAL

Materials

SHM was supplied by the National Cancer Institute. The fat emulsion vehicle (Intralipid, 10%) was purchased from Cutler Medical (Berkeley, CA, U.S.A.). Dimethylacetamide (DMA) and tetraphenylethylene were obtained from Aldrich (Milwaukee, WI, U.S.A.). The column packing used for GLC, 3% OV-17 on 80–100 mesh Gas-Chrom Q, was purchased from Applied Science Labs. (State College, PA, U.S.A.). For HPLC analyses, an ODS-SIL-X-1 (0.26 × 25 cm) column purchased from Perkin-Elmer (Norwalk, CT, U.S.A.) was used. Acetonitrile and ethyl acetate were Burdick and Jackson (Muskegon, MI, U.S.A.) glass distilled, spectroquality. Deionized, distilled, Milli-Q water was used for all analyses.

Apparatus

For GLC analyses, a Hewlett-Packard 5880A microprocessor-controlled gas chromatograph equipped with an automatic sampling system and a flame-ionization detector was used. The glass column (1.83 m × 2 mm I.D.) was packed with 3% OV-17 on 80–100-mesh Gas-Chrom Q.

For HPLC analysis, a Hewlett-Packard 1084B microprocessor-controlled HPLC system equipped with a variable-wavelength detector, an automatic sampling system, and a reversed-phase column was used.

Preparation of extracts

The procedure for preparation of extracts was essentially that described by Flora *et al.*[6], with the exception that SHM in DMA was added to magnetically stirred fat emulsion in a round-bottomed flask while cooling the flask in an ice bath. SHM or [^{14}C]SHM was added below the surface of the fat emulsion by use of a 1-ml pipette at the reported rate[6]. The formulation was stirred for 5 min in the ice bath before the first aliquot was removed for analysis.

GLC and HPLC procedures

For GLC analyses, samples and standards were analyzed isothermally at 240°C with a carrier gas flow-rate of 30 ml/min. The detector and injector zone temperatures were 250 and 230°C, respectively. Sample volume was 3 μl. For sample analyses, authentic samples of SHM were interspersed with extracts of the SHM formulations. The detector output was quantified by the microprocessor, and tetraphenylethylene was used as an internal standard.

HPLC analyses were performed isocratically with a solvent mixture of water–acetonitrile (55:45) at a flow-rate of 1 ml/min. Sample volume was 5 μl. Data for the standard curve were obtained from multiple injections of solutions with concentrations ranging from 0.01 to 2.0 mg/ml.

RESULTS AND DISCUSSION

In preparation for preclinical toxicology studies on SHM, fat emulsion formulations were prepared and analyzed by the reported GLC method[6]. Although this method was satisfactory for a few samples, repeated applications of the ethyl acetate

extracts of the formulations to the column resulted in a loss of column efficiency. The subsequent peak broadening made quantification of the SHM peak unreliable (Fig. 2).

These results prompted a search for an alternative method, and a satisfactory HPLC method was developed. Analysis of ethyl acetate extracts of the SHM formu-

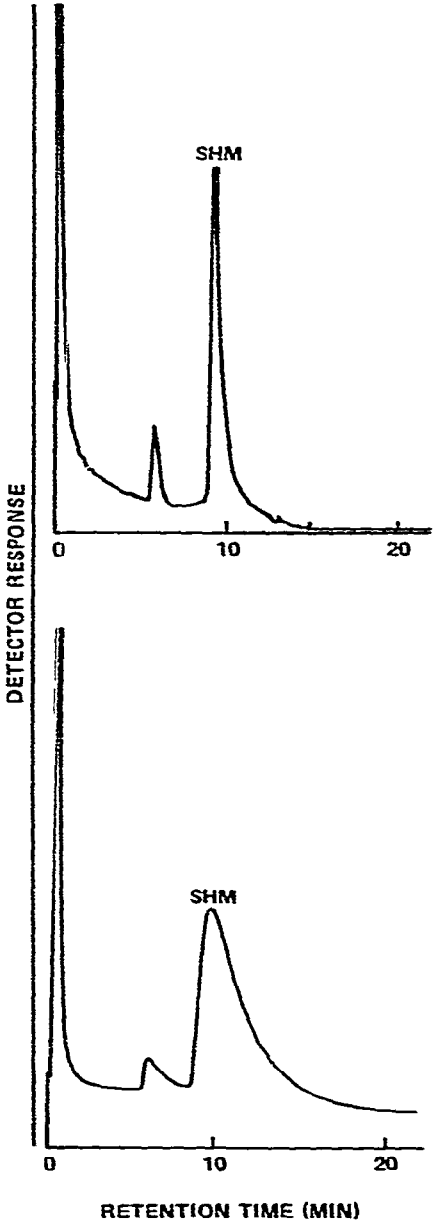
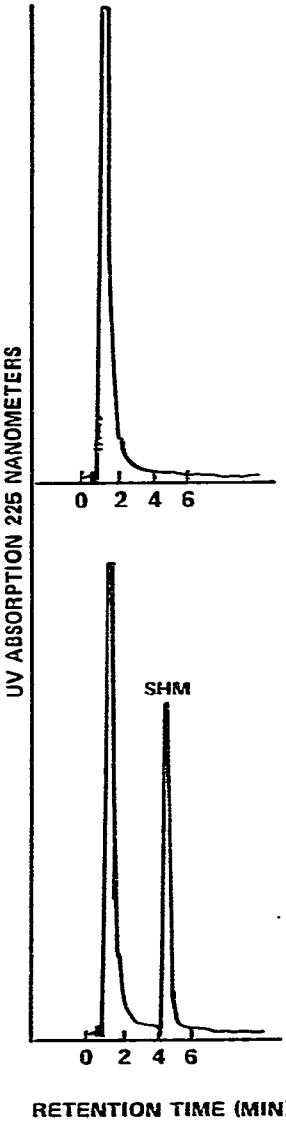


Fig. 2. GLC analysis of ethyl acetate extracts of the SHM formulation with internal standard. The upper panel shows the results obtained on the first injection of this sample. The lower panel shows the results obtained after the sixth injection of this sample.

lations gave satisfactory results with SHM eluting at 4.2 min, well past the large solvent peak at 0.84 min (Fig. 3). Repeated analyses of extracts caused no peak broadening or loss of column efficiency (Fig. 4). Analysis of authentic samples of SHM with concentrations ranging from 0.01 to 2.0 mg/ml gave a straight-line plot with a correlation coefficient (r) of 0.999.



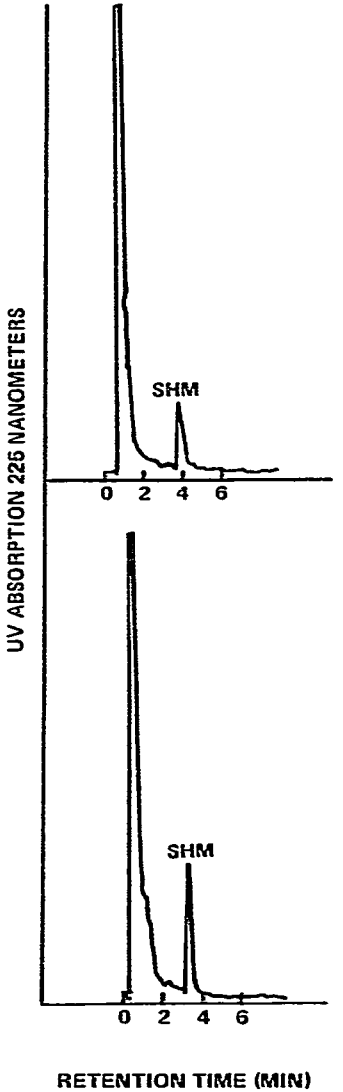


Fig. 3. HPLC analysis of ethyl acetate extracts of the fat emulsion vehicle (upper panel) and the SHM formulation (lower panel).

Fig. 4. HPLC analysis of ethyl acetate extracts of the SHM formulation. The upper panel shows the results obtained on the first analysis of the day on sample 1 (0.38 mg/ml). The lower panel shows the results obtained on the fiftieth analysis of the day on sample 21 (0.72 mg/ml).

TABLE I

EXTRACTION EFFICIENCY OF [^{14}C]SHM in 10% FAT EMULSION

Concentration 1 mg/ml.

Sample	*Radioactivity (cpm)*		*Extraction efficiency (%)*	
	Extract	*Residue*	*Radioassay*	*HPLC method*
1	336	22	94	98
2	320	22	93	98
3	315	40	89	104

To confirm the HPLC method and to determine stability of formulations, [^{14}C]SHM in DMA was added by the usual procedure to 3 ml of 10% fat emulsion to produce a concentration of 1 mg/ml. Three 1-ml aliquots were collected —the first after the 5-min stirring period, the second 15 min later, and the third (total remaining) 15 min after the second. The results (Table I) demonstrate high extraction efficiency and stability of SHM (or other ethyl acetate-extractable degradation products) as well as uniformity. HPLC analysis of the three extracts demonstrated that the extractable component was SHM. These results confirm the HPLC method and demonstrate that time-consuming lyophilization[6] is not necessary to obtain efficient recovery of SHM.

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