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CHROMATOGRAPHIC–FLUOROMETRIC ANALYSIS OF ANTILEPROTIC SULFONES

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SUMMARY

Modifications to the power supply system of a spectrophotofluorometer are described. These modifications stabilize the output of the xenon arc lamp and permit the determination of nanogram quantities of antileprotic sulfones. The sulfones are removed from plasma by a single extraction with ethyl acetate, then separated by high-pressure liquid chromatography on silica, and detected in the effluent by their fluorescence. The method is specific, rapid, and reproducible.

INTRODUCTION

A microbore column chromatographic procedure has been reported[1] that is designed to measure microgram quantities of the antileprotic antimalarial sulfone dapsone (4,4′-diaminodiphenylsulfone, DDS) and its chief plasma metabolite monoacetyldapsone (4-acetamido-4′-aminodiphenylsulfone, MADDS). Several methods that utilize the fluorescent characteristics of these compounds in organic solvents also have been published[2–4]. These fluorescent techniques can be used for measuring as little as 10 ng. The need for greater sensitivity and specificity led us to develop a combined liquid chromatographic–fluorometric procedure that was sensitive to 2 ng of these compounds and, in addition, measured the repository derivative acedapsone (4,4′-diacetamidodiphenylsulfone, DADDS) after diborane reduction[5].

Because clinical trials with DADDS[6], involving hundreds of analyses, necessitated the development of faster, more sensitive procedures, we modified the equipment and techniques used in our original report[5], so that direct chromatographic–fluorometric measurements of DDS, MADDS and DADDS could be performed more rapidly. The procedure described here permits quantitation of as little as 0.1 ng in an injection volume of 0.5 ml —a volume far greater than that used in conventional gas or microbore column chromatography.

EXPERIMENTAL

Reagents and glassware

DDS (Merck, Sharpe and Dohme, West Point, Pa., U.S.A.), MADDS and DADDS (Parke Davis, Detroit, Mich., U.S.A.) were recrystallized, and their purity was assessed as described earlier[5]. Spectrophotometric-grade ethyl acetate (Matheson,

Coleman and Bell, Norwood, Ohio, U.S.A.) was found to be free of extraneous fluorescence and was used without further purification. Silica gel 7 (neutral, irregularly-shaped particles, < 40 μm in diameter) was purchased from J. T. Baker (Phillipsburgh, N.J., U.S.A.). The 1.8-ml vials used for extractions and the disposable pipets were products of Kimple (Toledo, Ohio, U.S.A.). Vials were rinsed with glass-distilled water, dried, used once, and then discarded. The pipets were used as received and discarded. The simple expedient of employing disposable glassware kept fluorescent contamination at a minimum.

Chromatographic apparatus

The chromatographic apparatus consisted of a 60 $\times$ 0.77 cm stainless-steel column with 10-μm frits (Waters Ass., Framingham, Mass., U.S.A.), a six-port injection valve equipped with a 0.5-ml sample loop (Disc, Santa Ana, Calif., U.S.A.) and a 1000-p.s.i. Milton Roy controlled-volume minipump (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) in which Teflon or Rulon O-rings had been inserted. The column (vertically positioned) was packed by adding 5-ml increments of a slurry consisting of 13 g Silica Gel 7 in 55 ml ethyl acetate to the solvent-filled column. Solvent flow was maintained at 150 ml/h for addition of the first two increments but was raised to 180 ml/h for subsequent additions. Each increment was allowed to pack for 5 min. Final additions were made with the aid of a 10 $\times$ 0.77 cm attachment to the main column. When filled, the entire column was compacted at 300 ml/h for 30 min (approx. 950 p.s.i.), after which the 10-cm attachment was removed and replaced by a 10-μm frit. The column was washed with ethyl acetate–water (97.5:2.5) at a flow-rate of 225 ml/h until a stable fluorescent baseline had been obtained (approx. 250 ml). This manner of preparation removed impurities that could cause interference after injections of water-saturated ethyl acetate. Before use, the column was heated for 1 h at 90° (to remove the solvent), followed by 15-h activation at 130°.

Detector

A spectrophotofluorometer (American Instrument, Silver Spring, Md., U.S.A.) equipped with a ratio photometer, a 150-W xenon lamp (Hanovia, Newark, N.J., U.S.A.) and a 0.5-ml quartz flow-cell (Hellma Cells, Jamaica, N.Y., U.S.A.) was used to detect fluorescence in the effluent from the column. The photomultiplier tube (PMT), a selected C7045J (RCA, Palo Alto, Calif., U.S.A.), was attenuated automatically by the ratio photometer to operate between 700 and 850 V when the activation wavelength was 285 nm. In this system, the PMT voltage was determined by the brightness of the lamp. The flow-cell was oriented with the 10-mm window facing the activation grating. Two 5-mm slits were placed on the emission side of the cell compartment, and the PMT slit was opened to the same width. No slits were used on the activation side. The sensitivity was set at 100 while the meter multiplier provided attenuation. Recordings were made on a Dynamaster recorder (Bristol, Waterbury, Conn., U.S.A.) equipped for 50-mV full-scale operation. Because the photometer required 2 h or longer to attain full stability, it was kept on continuously.

Xenon lamp power supply

Several xenon arc power supplies, including units manufactured by Sorensen, Hanovia and Iren (Aminco Nos. 422-818, 422-829 and 422-848, respectively) were tested for long-term stability. Although each unit showed reasonable short-term

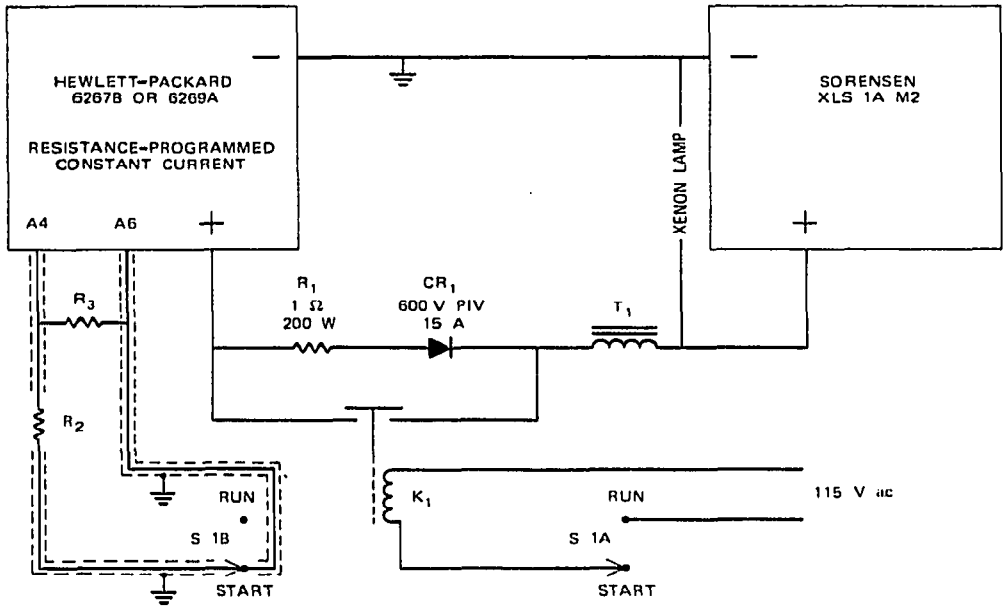

Fig. 1. Scheme of the electronic interface between the constant-current and ignition power supplies. The coil (T_1) consists of twenty turns of mylar-coated No. 10 wire on a TR 73 824336 flyback core. The Potter relay (K_1) is a KA 11 AY rated at 115 V 5 A, and the switch (S1) is a make-before-break type. Diode CR_1 is a HEP R0166. The values of R_2 and R_3, determined by the programming coefficient of the power supply, were 220 and 750 Ω (1 W), respectively, for Type 6267B. Model 6269A requires smaller resistors.

stability, fluorescent baseline traces indicated that unacceptable power changes occurred at 10- to 15-min intervals. Previous investigators had fabricated a lamp starter used in conjunction with a highly regulated, constant-current d.c. source[7]. Using their system as a guide, we designed an electronic unit that allowed interconnection of the standard Sorensen unit (XLS 1A M2) and a stable HP 6267B or 6269A power supply (Hewlett-Packard, Palo Alto, Calif., U.S.A.). Fig. 1 is a scheme of the device. The system uses the Sorensen power supply to ignite the lamp but allows the resistance-programmed HP unit to maintain a stable xenon arc. During ignition, the relay (K_1) is open, and the coil (T_1), diode (CR_1) and resistor (R_1) protect the HP power supply from damage by the 20-kV pulse required to strike the arc. R_2 is a programming resistor that maintains the output of the HP power supply at 2.2 A (start). In the operate position the relay closes, thereby shunting CR_1, R_1 and R_2 and allowing programming resistor R_3 to maintain the output at 7.5 A. Values of R_2 and R_3 are determined by the programming coefficient of each HP power supply. The voltage across the ignited lamp was 18–20 V. When operational conditions had been attained, the Sorensen power supply was turned off. A slightly different device (not shown) also was developed to allow us to use a Hanovia power supply as an igniter.

Extraction and chromatography

Mixtures of DDS, MADDS and DADDS in water or plasma were placed in

small vials, diluted to 0.5 ml with distilled water and made alkaline by addition of 0.5 ml of 1 *N* NaOH. Ethyl acetate (0.70 ml) was added, and the vial was closed with a cap lined with a small piece of aluminum foil (1 × 1 cm, rinsed with ethyl acetate). Extraction was carried out on an Eberbach shaker (Ann Arbor, Mich., U.S.A.) for 10 min at 100 strokes/min. This slow shaking rate retarded formation of an emulsion during extraction of plasma samples. After centrifugation to separate the phases, 0.5 ml of the ethyl acetate phase was injected onto the column, after which elution was accomplished with deaerated ethyl acetate flowing at 225 ml/h. The fluorescence of the column effluent was monitored at 285/375 nm (activation/emission) until DDS and MADDS had been eluted. The emission grating then was changed to 340 nm, and DADDS fluorescence was determined as it was eluted. The baseline was reset after each change of the emission grating.

RESULTS AND DISCUSSION

Fig. 2 presents a typical elution profile obtained when a mixture of DDS, MADDS and DADDS was extracted from water (or plasma) and chromatographed

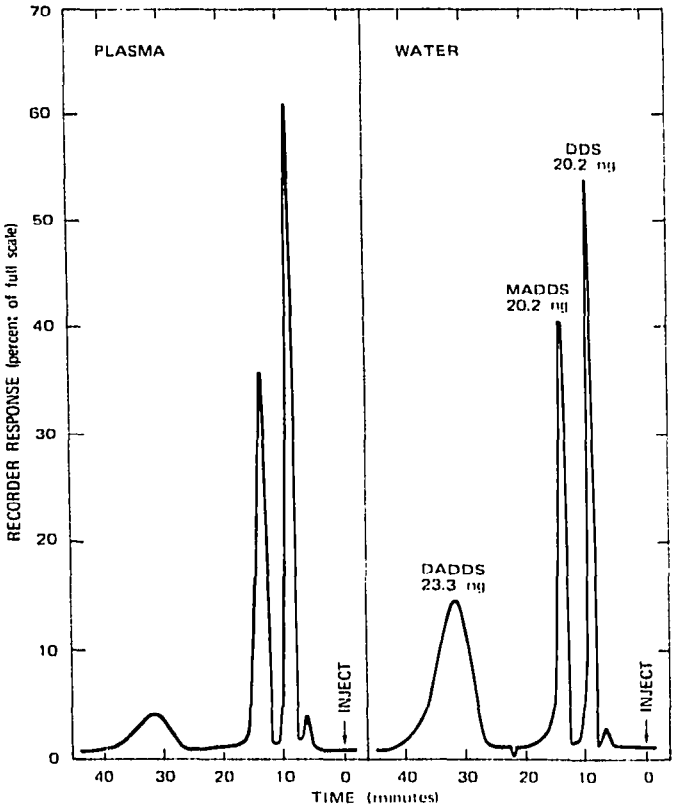

Fig. 2. Chromatographic elution patterns obtained when DDS, MADDS and DADDS were extracted from water (right) and from plasma (left). The plasma was obtained from a patient 61 days after intramuscular DADDS administration. The meter multiplier was set at 1.

on Silica Gel 7. These compounds exhibited retention times of 8.7, 13.4, and 31.5 min, respectively. The peak occurring at 6.3 min was observed in all samples extracted from aqueous media. Although DDS and MADDS exhibited fluorescence maxima at 345 and 415 nm, respectively, we chose to measure them at a common wavelength (375 nm), thereby eliminating an additional parameter change. A two- to three-fold increase in DDS sensitivity can be obtained by monitoring its maximum wavelength. MADDS sensitivity (at 415 nm) also is enhanced, but the increase is smaller. Maximal DADDS sensitivity occurs at 328 nm but, because the signal-to-noise ratio decreases sharply below 340 nm, the latter wavelength was used for measurements. The chromatographic pattern shown in Fig. 2 gave peak height sensitivities of 7.30, 5.50, and 1.65 mm/ng for DDS, MADDS and DADDS, respectively, but computation of peak areas (at one-half peak height) resulted in almost identical sensitivities, *i.e.*, 5.65, 5.55 and 5.70 mm^2/ng. The stability of the fluorometric system allowed ten-fold magnification of these sensitivities while holding the level of short-term noise at 375 nm to <0.1 ng DDS.

Table I presents an evaluation of the daily reproducibility of the chromatographic–fluorometric technique. Aqueous solutions containing a mixture of 20.2 ng DDS, 20.2 ng MADDS and 23.3 ng DADDS were extracted and chromatographed. Replicate assays on six successive days yielded mean peak heights of 7.80, 5.73 and 1.74 mm/ng for the three compounds, respectively. Variability of measurement, expressed as coefficient of variation, was 4.4% for DDS, 3.4% for MADDS and 2.7% for DADDS. Further evaluations, comparing our previous procedure with the current technique, will be presented elsewhere; the results of such a comparison indicate little difference between the two methods.

TABLE I

REPRODUCIBILITY OF PEAK HEIGHT SENSITIVITIES OF DDS, MADDS AND DADDS

The meter multiplier was set at 1.0.

Day	*Peak height sensitivity (mm/ng)*		
	DDS	*MADDS*	*DADDS*
1	8.27	5.99	1.76
2	8.02	5.94	1.78
3	7.92	5.64	1.76
4	7.30	5.50	1.65
5	7.67	5.59	1.74
6	7.62	5.74	1.72
Mean	7.80	5.73	1.74
Standard deviation	0.341	0.196	0.046
Coefficient of variation (%)	4.36	3.42	2.67

Several months of observation reveals that the condition of the xenon lamp is extremely important in maintaining the stability of this fluorometric system. A lamp can be damaged beyond use if ignitions are closely spaced. Thus, if a lamp failed to ignite on the first attempt, it was allowed to cool for at least 15 min before another

attempt. In practice, lamps usually must be replaced after 30 to 40 ignitions. Our observations indicate that xenon lamps, ignited once daily and used for 8–9 h a day, maintained stability on an average of two to three months.

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