Journal of Chromatography, 278 (1983) 193—198
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1840

Note

High-performance liquid chromatographic analysis of 5-chloroaminotoluene in rats

MACK R. HOLDINESS* and LEE ROY MORGAN, Jr.

Department of Pharmacology and Experimental Therapeutics, 1901 Perdido Street, Louisiana State University Medical Center, New Orleans, LA 70112 (U.S.A.)

(First received March 15th, 1983; revised manuscript received June 29th, 1983)

Environmental toxicology, due to uncontrolled use and disposal of chemicals, is becoming a major health management problem. The recent introduction of chlorinated aromatic compounds into open pits and abandoned subterranean oil wells introduces additional health hazards. 5-Chloroaminotoluene (5-CAT) is one of these compounds noted to produce pathological situations in both laboratory animals and man. In 1933 Currie [1] recorded cases of hematuria that occurred from time to time among chemical workers that packaged 5-CAT. Lehmann [2] noted hemorrhagic cystitis and destruction of bladder epithelium in cats exposed to 5-CAT by skin contact. Recently, Folland et al. [3] have reported acute hemorrhagic cystitis in industrial employees who handled the pesticide chlordimeform (CDM); 5-CAT was found in the urine of these workers.

Few analytical procedures have been developed for analysis of 5-CAT in biological media. Kossman et al. [4] reported colorimetric, thin-layer and flame ionization detection gas chromatographic procedures for analysis of 5-CAT content in plant materials. Chemical and enzymatic degradation of CDM yielded the product of 5-CAT which could be analyzed by instrumental methods. The colorimetric and thin-layer chromatographic procedures had detection limits of 1 μg 5-CAT per 50 g of crop material whereas a detection limit of 50 ng 5-CAT per 50 g crop material was obtained by the flame ionization gas chromatographic procedure. Geissbuhler et al. [5] developed an electron capture gas chromatographic procedure for analysis of 5-CAT. This method involved derivatization of 5-CAT via the Sandmeyer iodination reaction giving the derivative electron capturable properties. The extraction procedure was extremely involved and the limit of detection by this method was 50 ng per 50 g of crop material. Folland et al. [3] used a gas chromato-

0378-4347/83/$03.00 © 1983 Elsevier Science Publishers B.V.

graphic method with a Coulson detector specific for halogens for analysis of 5-CAT.

As far as the authors can ascertain this paper represents the first high-performance liquid chromatographic (HPLC) method for analysis of 5-CAT. The compound is detectable in rat urine and feces up to three days following single 100-mg/kg intraperitoneal injections. An internal standard of 2-chloro-4-methylaniline (IS) is used for quantitation and the detection limit is 5 ng per ml of urine or gram of feces. The extraction method is rapid requiring approximately 60 min to process samples for HPLC analysis.

EXPERIMENTAL

Materials

The compounds used in this study, 5-chloroaminotoluene and 2-chloro-4-methylaniline (internal standard), were purchased from Aldrich (Milwaukee, WI, U.S.A.). Solvents of methanol and hexane (HPLC grade) were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Standard solutions of 5-CAT and internal standard were made by adding 20 mg each in 100-ml volumetric flasks and dissolving in methanol—0.05 *M* KH_2PO_4, pH 4.65 (60:40) (Sigma, St. Louis, MO, U.S.A.). The stock solutions were prepared daily.

Instrumentation

HPLC was accomplished using a Beckman 345T ultraviolet—visible system with a Hewlett-Packard 3390A integrator. Separation was performed on a 15 cm × 4.6 mm I.D. 5-μm ODS Altex column. A precolumn of 6 cm × 4.6 mm I.D. contained 5-μm Spherisorb ODS (Supelco, Bellefonte, PA, U.S.A.). The column temperature was maintained at ambient temperature (approx. 22°C). The mobile phase consisted of methanol—0.05 *M* KH_2PO_4, pH 4.65 (60:40) at a flow-rate of 1 ml/min.

Sample collection and extraction

Adult male Sprague Dawley rats (Charles River, MA, U.S.A.) were used in all experiments. Three animals were housed in separate metabolic cages during each experiment for a total of five days and fed food and water ad libitum. Each animal was weighed before experimentation and one was given 100 mg/kg normal saline i.p. (control) and the other two were injected with 100 mg/kg 5-CAT i.p. (test rats). The three animals were given only a single injection at the beginning of each five-day trial period at approximately 9 a.m. Each following morning at 9 a.m. the urine and feces content was collected, recorded and frozen at −70°C and new metabolic collection tubes were replaced for another 24-h collection period.

The extraction scheme is presented in Fig. 1. Analysis was performed after all samples had been collected. To screw-topped PTFE-lined 20-ml glass culture tubes were added 500 μl of internal standard and the solution dried by an air stream. Then either 0.50 ml urine or 1.0 g feces (well homogenized in 5 ml water) was added with 1 ml of 10% sodium bicarbonate and 10 ml hexane. The solutions were shaken for 10 min by hand and centrifuged at 500 *g* for 10 min. The organic layer was transferred to a new tube containing 0.50 ml of

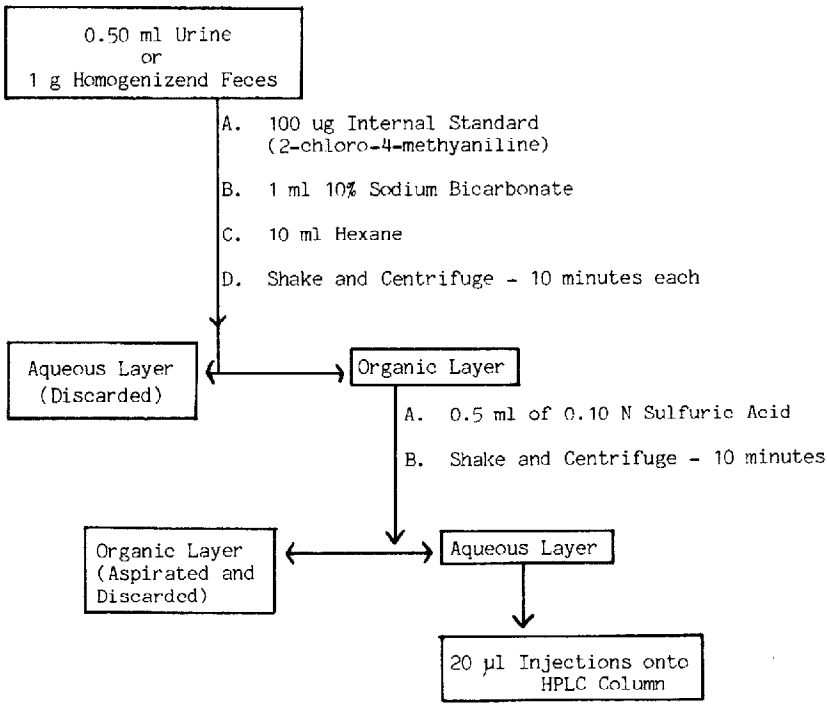

Fig. 1. Extraction scheme for 5-CAT from biological samples.

0.10 *N* sulfuric acid and shaken for 10 min and centrifuged at 500 *g* for 10 min. The organic layer was asperated from the tube and 20-µl injections of the aqueous phase were injected onto the column. The recovery of 5-CAT was found to be 75 ± 4% from spiked biological samples.

Standard curves

Standard curves were constructed by addition of 1, 3, 5, 7, 9 and 11 µg of 5-CAT and 500 µl of internal solution to 20 ml PTFE-lined glass culture tubes. These solutions were then dried by a stream of air. To the tubes was added either 0.5 ml of blank rat urine or 1 g of homogenized feces in 5 ml of water. These solutions were then carried through the above reported extraction procedure and 20 µl of each were injected onto the column. A least-squares fit (r = 0.997) of the data was performed, resulting in the equation: $y = 9.520x - 0.083$. This equation was constructed by plotting µmoles 5-CAT/µmol IS versus the ratio of peak area 5-CAT/area IS. After the concentration (in µmol) of 5-CAT was found the value was multiplied by its molecular weight (142 g/mol) and divided by the ml or g of sample. The final values were reported in µg 5-CAT per ml or g of biological sample.

RESULTS AND DISCUSSION

HPLC analysis

In Fig. 2a and b can be seen the analysis of 5-CAT from urine (a) and a blank sample (b), respectively. Fig. 3a and b represents the separation of

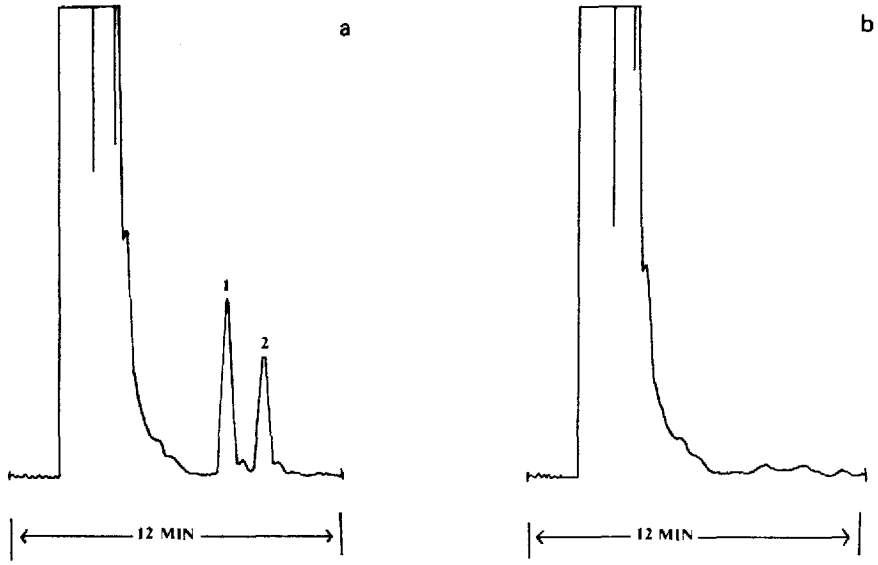

Fig. 2. HPLC analysis of 5-CAT and internal standard from rat urine two days following i.p. injection (a). Analysis of rat urine of a control animal one day following i.p. injection with normal saline (b) without addition of internal standard. Peak 1 is 5-CAT and peak 2 is the internal standard.

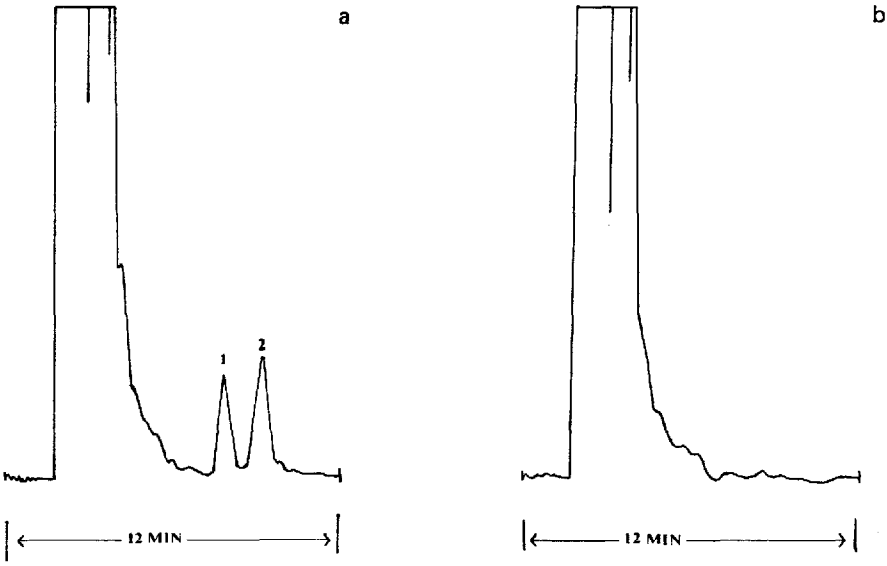

Fig. 3. HPLC analysis of 5-CAT and internal standard from rat feces two days following i.p. injection (a). Analysis of rat feces from a control rat (without addition of internal standard) two days following i.p. injection with normal saline (b). Peak 1 is 5-CAT and peak 2 is the internal standard.

5-CAT and internal standard from feces (sample a) and blank feces (sample b), respectively. The retention times of 5-CAT and IS are 8.20 and 10.53 min, respectively. Positive identification of the samples in question is achieved by peak superimposition (i.e., by injection of 1 to 5 μg of 5-CAT standard with

the previously extracted sample and observing the increase in peak area at its corresponding retention time).

The limit of detection (2:1 signal-to-noise) of this procedure is 5 ng 5-CAT per ml urine or g feces. Repetitive injections of standards gave good reproducibility of retention times (C.V., ± 2.1%) at the 100-ng level. Standard curves were linear in the range of 100 ng to 15 μg 5-CAT and day-to-day reproducibility varied less than 3.0% (C.V.). Standard stock solutions of this compound and its internal standard were stable at least one week when stored at −70°C. Samples extracted from urine or feces gave reproducible results of ±3.5% (C.V.) for two-day duration when stored at −20 or −70°C. However, extracted samples left at room temperature or −4°C had variable losses of up to 25% within 24 h following the extraction and upwards of 50—60% when left at room temperature (approx. 22°C), or −4°C, when analyzed at 48 h. Therefore, it is suggested that all samples should be quantitated immediately within 4—6 h following separation from the biological media unless precautions are taken to store the samples at the lower temperatures as mentioned previously.

Pharmacokinetic study in rats

Three rats were studied during each experiment of five-days duration. One rat was injected i.p. with 100 mg/kg of normal saline and two rats were injected i.p. with 100 mg/kg of 5-CAT as described in the Experimental section. Table I presents the data obtained from this study of the urinary excretion of 5-CAT. This compound was detectable up to three days following injection before its concentration fell below the limits of detection of the analytical methodology. A total of eighteen rats were carried through this experiment and 5-CAT was never detectable on the fourth day of experimentation. Semi-log plots of concentration versus time were constructed and the elimination half-life was determined to be 16.5 h.

TABLE I

AVERAGE URINARY EXCRETION DATA OBTAINED FROM RATS IN THIS STUDY

Day	Urine (μg/ml ± S.D.) (n = 18)
1	161.3 ± 15.9
2	20.8 ± 5.6
3	4.2 ± 1.9

CONCLUSION

A sensitive and selective analytical technique has been developed for the quantitation of 5-CAT in rats. The emphasis of this work was on the rapid extraction and analysis of low-nanogram amounts of this compound in biological tissue. A structurally similar internal standard was used for quantitation and was well resolved from the compound under investigation and any interfering components. A pharmacokinetic investigation carried out demonstrated

the usefulness of this technique and revealed a 16.5-h half-life in rats following a single i.p. injection. Further studies of the pharmacokinetics of 5-CAT in rats and the possible metabolites are currently under investigation in this laboratory.

REFERENCES

1 A.N. Currie, J. Ind. Hyg., 15 (1933) 205.
2 K.B. Lehmann, Arch. Hyg. Bakteriol., 110 (133) 12.
3 D.S. Folland, R.D. Kimbrough, R.E. Cline, R.C. Swiggart and W. Schaffner, J. Amer. Med. Assoc., 13 (1978) 1052.
4 K. Kossmann, H. Geissbuhler and V.F. Boyd, J. Agric. Food Chem., 19 (1971) 360.
5 H. Geissbuhler, K. Kossmann, I. Baunak and V.F. Boyd, J. Agric. Food Chem., 19 (1971) 365.