tyrosol and a corresponding decrease in its oxidation to *p*-hydroxyphenylacetic acid. These results indicate that tyramine metabolism is altered by ethanol in the same way as the metabolism of serotonin and norepinephrine.[1-3] An increase in the NADH/NAD ratio caused by ethanol which increases the reduction of the intermediate aldehyde and decreases its oxidation, and a competitive inhibition of aldehyde dehydrogenase by acetaldehyde have been proposed as possible mechanisms for this effect.[1] The results also suggest a specific effect of ethanol on glycine conjugation, as the decrease in the glycine conjugate of *p*-hydroxyphenylacetic acid is more than twice as great as the decrease in excretion of the free acid. In this connection the observation of Dalgliesh *et al.*,[4] that a large dose of ethanol (6 g/kg B.W.) causes a marked decrease in the excretion of hippuric acid is noteworthy. The increase in tyrosol is represented largely by an increase in the sulfate conjugate there being no increase in free tyrosol and only a 4-fold increase in tyrosol glucuronide. This contrasts with the findings of Davis *et al.*[1] on the effect of ethanol on the metabolism of serotonin-^{14}C in man where a much larger increase in the glucuronide than in the sulfate conjugate of 5-hydroxytryptophol was found.

No change was found in the amount of tyramine-^{14}C excreted as *N*-acetyltyramine. This is in agreement with the observations from this laboratory that ethanol pretreatment does not alter the acetylation of sulphanilamide* in spite of the findings of Ammon *et al.*[5, 6] that acetaldehyde forms a thiohemiacetal with coenzyme A which inhibits its transacetylating ability.

* P. J. Creaven and M. K. Roach, unpublished observations.

Acknowledgement—This work was supported by grant MH 144340-1, U.S. Public Health Service and fellowship No. 5-Fl-MH-29-689 from the U.S. Public Health Service to Martha Tacker.

Division of Biological Research,
Texas Research Institute of
Mental Sciences,
Houston, Tex. 77025, U.S.A.

MARTHA TACKER
P. J. CREAVEN
W. M. MCISAAC

REFERENCES

1. V. E. DAVIS, H. BROWN, J. A. HUFF and J. L. CASHAW, *J. Lab. clin. Med.* **69**, 132 (1967).
2. A. FELDSTEIN, H. HOAGLAND, K. WONG and H. FREEMAN, *Q. Jl Stud. Alcohol* **25**, 218 (1964).
3. V. E. DAVIS, H. BROWN, J. A. HUFF and J. L. CASHAW, *J. Lab. clin. Med.* **69**, 787 (1967).
4. C. E. DALGLIESH, E. C. HORNING, M. G. HORNING, K. L. KNOX and K. YARGER, *Biochem. J.* **101**, 792 (1966).
5. H. P. T. AMMON, C.-J. ESTLER and F. HEIM, *Biochem. Pharmac.* **16**, 789 (1967).
6. H. P. T. AMMON, F. HEIM, C. J. ESTLER, G. FICKEIS and M. WAGNER, *Biochem. Pharmac.* **16**, 1533 (1967).

Biochemical Pharmacology, Vol. 19, pp. 607–612. Pergamon Press. 1970. Printed in Great Britain

Rapid clearance of isosorbide dinitrate from rabbit blood—Determination by gas chromatography*

(*Received* 14 *May* 1969; *accepted* 13 *June* 1969)

IN A RECENT communication Dietz[1] demonstrated, by comparison of R_f values on thin-layer chromatograms, that the metabolites of isosorbide dinitrate (ISDN) in the dog and in man are isosorbide-2-mononitrate and isosorbide-5-mononitrate. DiCarlo *et al.*,[2] studying the biotransformation

* This research was supported by a grant from Ives Laboratories, Inc., New York, N. Y.

of pentaerythritol tetranitrate-1, 2-^{14}C in the rat, showed that during the first hour more than 90 per cent of the absorbed radioactive material was already cleared from the blood and located in the tissues. Repeated attempts in our laboratory, by all available means, to recover ISDN administered intravenously to the rabbit have been unsuccessful.

The purpose of this investigation was to develop a method sensitive enough to detect extremely small amounts of ISDN in rabbit blood, and to determine whether ISDN had already been cleared from the blood when the samples were obtained. Anticipating extremely low levels, a method for the determination of this material by gas chromatography was developed. The results were compared with those obtained by using the thin-layer chromatography method used by Dietz.[1]

Pure isosorbide dinitrate, obtained from the Atlas Chemical Co., was dissolved in absolute ethyl alcohol and diluted with distilled water to a final concentration of 5 mg/ml in 50% ethyl alcohol. A pretreatment blood sample was obtained from the heart of the unanesthetized rabbit and the drug was administered intravenously into the marginal ear vein. The intracardiac needle with syringe attached was held in place until the postadministration sample was obtained. In the first of two experiments, a New Zealand White rabbit weighing 4·4 kg was given a 10-mg dose in the manner described. In a second experiment, a rabbit weighing 4·5 kg received a 20-mg dose. Using this procedure, it was possible to obtain quickly 20 ml blood for both the pre- and post-treatment studies. In both cases, blood was obtained within 90 sec from the time of administration of the drug. Heparinized blood (10 ml) was used for the determination by Dietz's thin-layer chromatographic technique. Stated briefly, the Dietz method calls for extraction of the blood with ethyl acetate, dehydration with sodium sulfate, flash evaporation to a small volume, and application of the concentrated material to silica gel G layers; ISDN is localized by spraying the plates with diphenylamine. Some samples were also sprayed with diphenylbenzidine in sulfuric acid, which is approximately 10 times more sensitive than diphenylamine for nitrate ester determination.[3]

For gas chromatography, 2 μl of the ethyl acetate extract was injected without further purification directly into a 6-ft column packed with SE 30, 3·8% on Gas Chrom P in the F & M Gas Chromatograph model 402. The system was operated isothermally under the following conditions: oven temperature, 110°; flash heater, 130°; detector, 190°; and nitrogen flow rate, 55 ml/min. A flame ionization detector was employed. Identity of the ISDN was supported by injection of the sample into another 6-ft glass column packed with XE-60, 3% on the Gas Chrom Q 100/120 mesh (Applied Science Labs). The conditions were as follows: oven temperature, 150°; flash heater, 160°; detector temperature, 180°; and nitrogen flow rate, 55 ml/min. The recorder speed was $\frac{1}{4}$ in. per min in both systems.

To test the ability of the extraction system to remove ISDN quantitatively from rabbit blood *in vitro*, 10 ml of heparinized blood was incubated in a metabolic shaker for 1 hr at 37·5° with 100 μg ISDN. A control sample without ISDN was run simultaneously. Five ml of each blood sample extracted for 1 min with 50 ml ethyl acetate. The mixture was centrifuged briefly to break the emulsion. The ethyl acetate solution was separated, dried over sodium sulfate, and reduced to dryness at 40° *in vacuo*. The dry residue was redissolved in 0·5 ml ethyl acetate and 4 μl was injected into the gas chromatograph under the conditions described above. For qualitative and quantitative purposes, an internal standard, consisting of 1 mg/ml of *m*-dinitrobenzene, was added to the extracts and to the ISDN standard. The relative elution time was computed by means of the ratio, E_tX/E_tS, where E_tX equals the elution time of the unknown peak and E_tS equals the elution time of the internal standard.

The sample eluting from the column was trapped in a capillary tube and eluted onto a potassium bromide "Wick Stick" (Harshaw Chemical Co.). A KBr pellet was pressed in the micro-die (1·5 mm). The infrared spectrum was run on a Perkin Elmer 421 infrared spectrophotometer using a microbeam condenser with KBr optics.

After intravenous ISDN administration to the rabbit, neither ISDN nor its metabolites were detectable by the thin-layer chromatography system employed. Numerous unidentified fluorescent areas were observed on the chromatograms of this rather crude extract, which made it impossible to distinguish ISDN, if present, in the background. However, when 2 μl of the same extract was injected into the gas chromatograph under the initial conditions (i.e. SE 30, 3·8% column), a small peak with the same relative retention time as pure isosorbide dinitrate was observed. Recovery of intravenous drug after 90 sec averaged 13·7 per cent.

In both column systems employed, a peak was observed that corresponded in retention time and, in the case of XE-60, relative elution time, to authentic ISDN. Figure 1 demonstrates the chromato-

gram resulting from the injection of ISDN standard and *m*-dinitrobenzene on the XE-60 column. The identity of the eluted compound was demonstrated by the nearly identical infrared spectra of the standards and unknowns (Figs. 2 and 3).

To determine whether the low recovery might have been the result of incomplete extraction, the blood sample remaining on filter paper was exhaustively re-extracted by refluxing for 30 min with hot chloroform:methanol (2:1). This extract was flash-evaporated at 45°, re-extracted with ethyl acetate, dried over sodium sulfate, dried again *in vacuo*, redissolved in a minimal quantity of ethyl acetate, and re-injected in the gas chromatograph. No additional ISDN peaks were found on the chromatogram.

The results of the study *in vitro* are shown in Figs. 4 and 5. The relative elution time was 2·59.

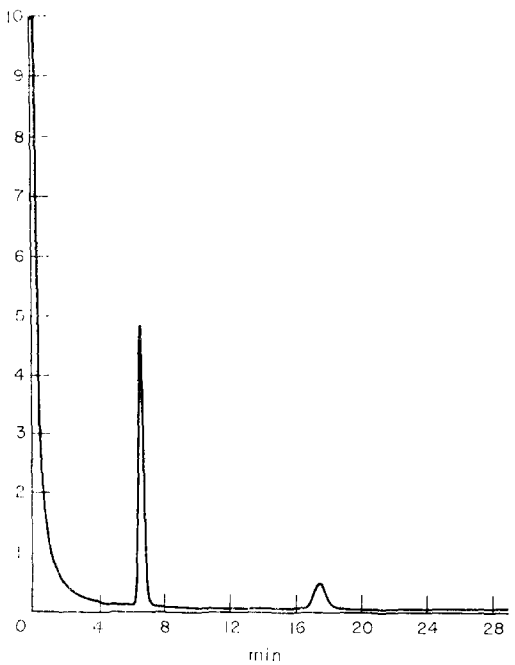

FIG. 1. Retention time of isosorbide dinitrate with *meta*-dinitrobenzene internal standard. Six ft XE-60 column packing. See text for conditions. Internal standard elutes at approximately 7 min.

A study was made of the sensitivity of the diphenylamine reagent by preparing a series of dilutions of ISDN in ethyl acetate which were applied to a thin-layer plate and sprayed with diphenylamine. The limit of detection was found to be 0·5 μg ISDN. However, when 2 μg ISDN was added to a biological extract and subjected to thin-layer chromatography, the sample was not visible against the fluorescent background.

It appears from these results that rabbit blood is cleared of injected ISDN is less than 90 sec. This unusually rapid clearance corresponds qualitatively to DiCarlo's observation in the rat. Goodman and Gilman[4] also state that 60–70 per cent of absorbed nitrite disappears in the body and they suggest that it is converted to ammonia.

The presence of other fluorescing materials on the thin-layer chromatogram probably obscures any ISDN which might be present. This is especially true in view of the extremely low levels present in rabbit blood. Assuming that a 4·4 kg rabbit has approximately 285 ml blood, then the concentration of ISDN in an animal receiving 20 mg would be 70 μg per ml of blood. If 10 ml is extracted and

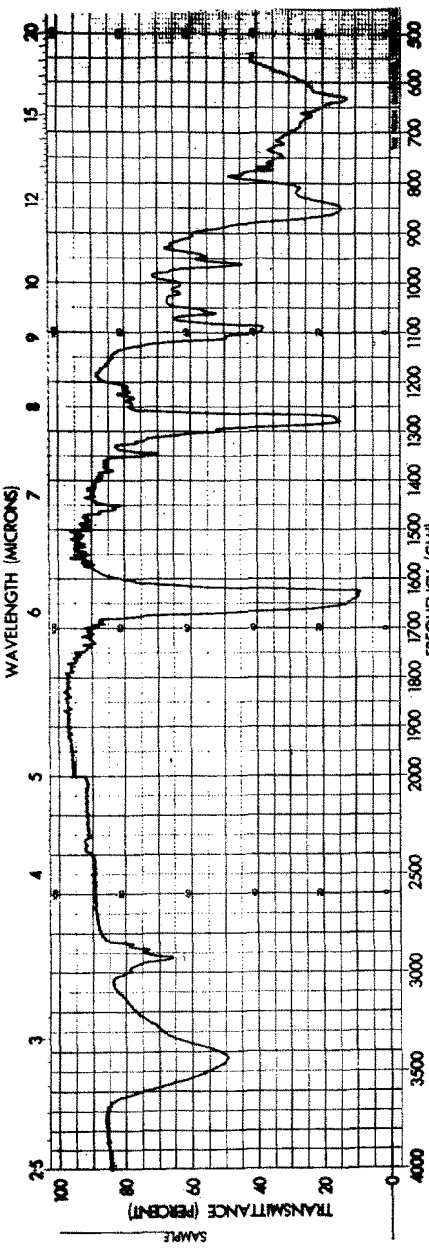

FIG. 2. Infrared spectrogram of authentic isosorbide dinitrate micropellet and micro-beam condenser.

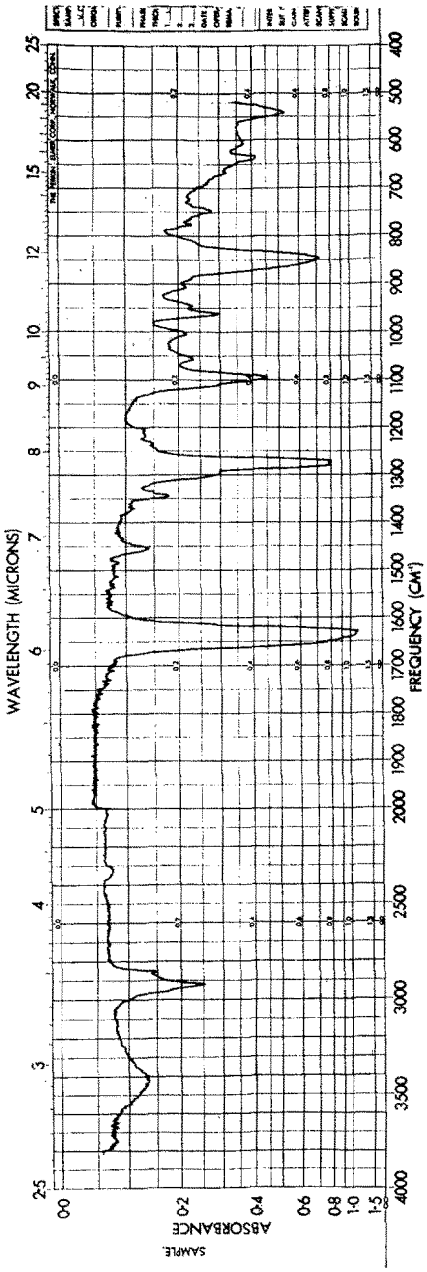

FIG. 3. Infrared spectrogram of sample eluting from gas chromatographic column with same retention time as authentic isosorbide dinitrate. Micropellet and micro-beam condenser.

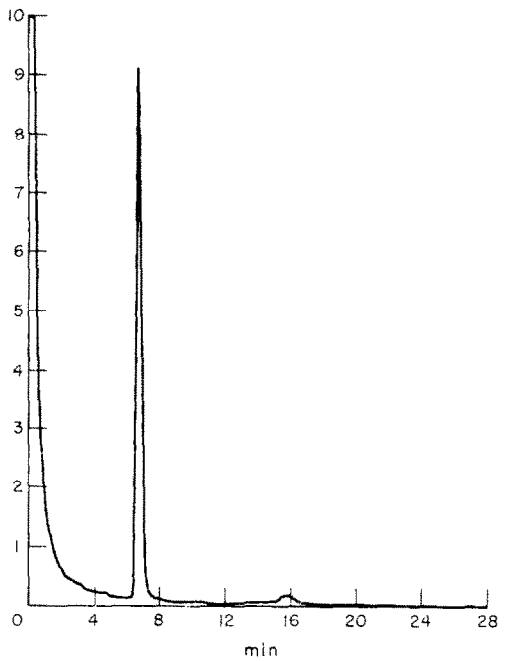

FIG. 4. Study *in vitro*. Ethyl acetate extract of rabbit blood before isosorbide dinitrate incubation.

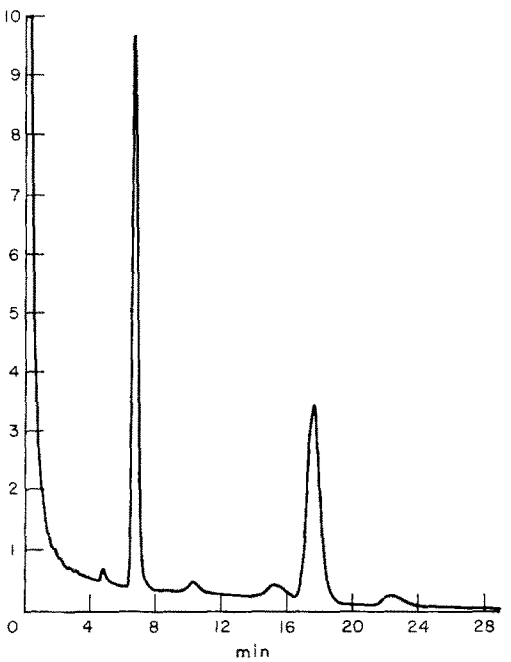

FIG. 5. Study *in vitro*. Ethyl acetate extract of rabbit blood after incubation with isosorbide dinitrate.

recovery is 14 per cent, we would expect to find 98 μg in the extract. When dissolved in 0·5 ml, and 10 μl is applied to the thin-layer plate, as in the Dietz method, then we would expect to find only 1·96 μg ISDN. This is below the amount previously demonstrated to be undetectable in a biological extract containing other fluorescing materials.

On the other hand, the sensitivity of the gas chromatographic method is such that as little as 1×10^{-2} μg may be detected by flame ionization. Some preliminary electron capture experiments have been done and, although these are subject to the problems arising from other contaminating materials, it appears that the sensitivity of the electron capture may be as high as 1×10^{-6}. These values are within the limits of anticipated blood levels and should lend themselves better than previous techniques to the determination of the metabolism of ISDN.

Gas chromatographic separation of ISDN demonstrates that 86 per cent of this drug, intravenously administered, is cleared from rabbit blood within 90 sec.

Thin-layer separation and diphenylamine detection are not satisfactory because of the low levels of ISDN found in rabbit blood.

Metabolic Research Laboratory,
Fordham Hospital,
Bronx, N. Y., U. S. A.

DANIEL A. SHERBER
MARTIN MARCUS
SEYMOUR KLEINBERG

REFERENCES

1. A. J. DIETZ, JR., *Biochem. Pharmac.* **16**, 2247 (1967).
2. F. J. DICARLO, M. C. CREW, C. B. COUTINHO, L. J. HAYNES and N. J. SKLOW, *Biochem. Pharmac.* **16**, 309 (1967).
3. F. FEIGL, *Spot Tests*, 4th edn, vol. II, p. 126. Elsevier, Amsterdam (1954).
4. L. S. GOODMAN and A. GILMAN, *The Pharmacological Basis of Therapeutics*, 2nd edn, p. 736. Macmillan, New York (1958).

Biochemical Pharmacology, Vol. 19, pp. 612–615. Pergamon Press. 1970. Printed in Great Britain

The effect of modifying the structure of Rubratoxin B on the acute toxicity to mice

(*Received* 22 *July* 1969; *accepted* 1 *August* 1969)

PENICILLIUM RUBRUM Stoll was isolated from mouldy foodstuffs which had proved toxic to cattle and poultry.[1, 2] Grown on liquid medium it provided two toxic metabolites, Rubratoxin A (I) and Rubratoxin B (II) the structures of which have recently been described.[3, 4] A remarkable feature of the toxicity of Rubratoxin B, described by Townsend, Moss and Peck[5] was the association of acute liver damage and death as early as 1 hr after injection. As soon as the presence of an unsaturated delta lactone moiety was identified in the structure it seemed important to relate acute toxicity to changes in chemical structure, and these are now described.

The Rubratoxins are closely related to a group of mould metabolites called collectively the nonadrides.[6] For this reason, two members of this group, Glauconic acid (VI) and Byssochlamic acid (VII), were included in these tests. Byssochlamic acid could be examined only as its tetra sodium salt because of its insolubility. With the exception of the zinc/acetic acid reduction product from Rubratoxin B, all have in common a nine membered ring with two anhydride groups, or one anhydride and one gamma lactol, as in Rubratoxin A. Treatment of Rubratoxin B with zinc and