Biochemical Pharmacology, Vol. 35, No. 18, pp. 3003–3006, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

BILIARY EXCRETION OF LINAMARIN IN THE WISTAR RAT AFTER A SINGLE DOSE

EMMANUEL N. MADUAGWU and IMEH B. UMOH*
Department of Biochemistry, College of Medicine, University of Ibadan, Ibadan, Nigeria

(*Received* 13 *September* 1985; *accepted* 7 *February* 1986)

Abstract—The biliary excretion of linamarin (2[β-D-glucopyranosyloxy]isobutyronitrile) was studied in male albino Wistar rats injected i.p. with single doses of 300 mg linamarin/kg following cannulation of the bile duct *in vivo*; 24 hr faeces of uncannulated rats, similarly dosed, was examined for excretory products. Enzymatic and spectrophotometric analyses of the bile exudate showed that glucosidic cyanide (linamarin, and non-glucosidic cyanide were excreted; the elimination of both cyanide forms exhibits biphasic kinetics. Thiocyanate ion was undetectable. T.l.c. of the test bile followed by enzymatic and chemical investigation of the chromatogram confirmed the presence of unchanged linamarin, and four different u.v. fluorescent non-glucosidic cyanide metabolites. Neither linamarin nor cyanide ion was detectable in faeces of the uncannulated rats.

Linamarin (2[β-D-glucopyranosyloxy]isobutyronitrile), a cyanogenic glucoside, occurs widely in plant food substances such as cassava, flax, white clover and lima beans. It produces hydrocyanic acid (HCN), which is toxic [1–5], as a result of enzymatic hydrolysis by an endogenous β-glucosidase enzyme, linamarase, either during plant tissue damage [6] or, by the intestinal microfloral enzyme species, following its ingestion [7].

Cassava roots, which are an important food staple for over 300 million people in the tropics [8], and presumably represent the major dietary source of linamarin, are usually processed by a wide variety of simple traditional methods [9] designed to reduce the cyanide content [10] of the final edible product. Therefore, acute cyanide poisoning, or death, from consumption of cassava seems rare; the hazard being adequately ameliorated by the activity of an efficient thiosulphate-*s*-transferase enzyme (rhodanese) in the liver [11, 12]. This enzyme is known to ensure the detoxication of cyanide by converting it to thiocyanate, a less toxic species and the major excretory product of cyanide metabolism [13].

The toxicity of linamarin, itself, is virtually unknown although a recent experiment [14] indicates that it exhibits toxic properties in chicken embryos not attributable to HCN as a derivative.

Since it is universally accepted that the toxicity of linamarin is due entirely to the release of cyanide, it seems desirable to study the excretion pattern of the cyanogenic glucoside in bile, an elimination route for foreign compounds which has not previously been considered [15] with regard to linamarin.

This paper, therefore, deals essentially with the pharmacokinetics of the biliary excretion of the presumably innocuous linamarin and its traditional noxious metabolic species (HCN), following intraperitoneal administration of a single large dose of the cyanogenic glucoside.

MATERIALS AND METHODS

Chemicals. Pure linamarin (2[β-D-glucopyranosyloxy]isobutyronitrile), mol. wt. 247.2 and m.p. 138.14, was purchased from Calbiochem Ltd (San Diego, CA). Rompum and Ketanest anaesthetics were products of Bayer (Leverkusen, F.R.G.) and Parke-Davies (Munchen, F.R.G.), respectively. All solvents used were of analytical grade and supplied by Merck (Darmstadt, F.R.G.) or BDH Chemicals Ltd (Poole, England).

Pure linamarase [linamarin β-D-glucoside glucohydrolase; EC 3.2.1.21] was obtained from BDH Chemicals Ltd also.

Animals and treatment. Healthy young adult rats (150–200 g) of the albino Wistar strain were kept at room temp (approx 28°) and were fed *ad libitum* on Pfizer pellets for rats (Pfizer Nigeria Ltd, Ikeja, Nigeria). The animals also had free access to drinking water but were starved overnight prior to experimentation.

The test rats were injected i.p. with linamarin (LIN) at the rate of 300 mg/kg following exposure of the bile duct by a mid-line abdominal incision, and collection of bile through a polythene cannula surgically inserted into the bile duct 1 cm from the junction of the duodenum [16, 17]. The linamarin dose was administered to animals weighing approx. 200 g as 0.2 ml aliquots of a solution of 300 mg LIN/ml, in physiological saline (0.9% NaCl), after suturing the abdominal slit.

The control rats were injected with 0.2 ml of the physiological saline while uncannulated test rats were also given i.p. doses similar to the regimen for cannulated rats, and their faeces collected over 24 hr. Five test and five control animals were used and, following dosing with linamarin, bile exudate from the cannulated rats was collected initially at 15 min,

* Present address: Department of Biochemistry, College of Medical Sciences, University of Calabar, Calabar, Nigeria.

30 min and 120 min, respectively and, thereafter, at hourly intervals until the animal was moribund.

All cannulations were done under Rompum/Ketanest anaesthetics, given i.p. also, at the rate of 1 ml of each per animal.

Analysis of bile and faeces. Quantitative determinations of linamarin (glucosidic-bound cyanide) and the non-glucosidic cyanide contents of bile and faeces was by the enzymatic assay of Cooke [18] for total cyanide. An aliquot of 0.1 ml of bile, diluted twice with 0.1 M orthophosphoric acid, or of a filtered slurry of faeces in the acid (1:2 w/v), was mixed with 0.4 ml of 0.2 M phosphate buffer pH 7.0 and incubated with 0.3 ml of 5 units ml^{-1} of the pure linamarase at 30° for 30 min to give the total cyanide content. The enzyme reaction was stopped by adding 0.6 ml of 0.2 M NaOH, and the cyanide present determined spectrophotometrically by making the solution up to 5 ml with 2.6 ml 0.1 M phosphate buffer pH 6.0 followed by the addition of 0.2 ml chloramine T and 0.8 ml of the pyridine/pyrazolone reagent. The blue colour developed was measured at 620 nm after 90 min.

Samples incubated without enzyme addition gave the non-glucosidic cyanide content. The bound cyanide (linamarin) content was then calculated by difference.

The detection limit of this method was <0.1 ppb cyanide and the sensitivity could be extended by using 0.1–0.3 ml of diluted sample, and mixing with 0.4–0.2 ml, respectively, of the 0.2 M phosphate buffer (pH 7.0) to a final volume of 0.5 ml.

Thiocyanate concentration of bile was determined spectrophotometrically [19]. As a precautionary measure ordinary rat bile was spiked with pure linamarin and, together with the test linamarin solution, was examined for CN^- and SCN^- contents, which were undetectable. Recoveries of thiocyanate in bile (0.1 ml) to which 2, 5, 10 and 20 μmol KSCN/L standard aqueous solutions were added were ≈98%, using 1 ml for assay.

Qualitative examination of bile was conducted on t.l.c. plates (0.5 mm silica gel) developed in the solvent of choice, chloroform:methanol (5:1 v/v), following preliminary investigation of the bile by two dimensional t.l.c. in this solvent system and in ethylacetate:acetone:water (4:5:1 by volume), respectively, to ensure that each u.v. fluorescent spot observed migrated as a single band.

The bile was subsequently streaked on t.l.c. plates and processed, and each fluorescent band in the test chromatogram, not also present in the control chromatogram, was scraped and eluted with distilled water, filtered and then assayed for cyanide contents.

Spot tests [20] were carried out on the eluted filtrates of the non-glucosidic cyanide entities in order to determine their general chemical groupings.

RESULTS

The time courses of the appearance in rat bile of glucosidic cyanide (linamarin) and the non-glucosidic moiety, following i.p. administration of pure linamarin (Fig. 1) show that the excretion of both cyanide forms exhibit biphasic kinetics. The differentiation between the two cyanide entities was achieved as a result of the application of the specific hydrolytic activity of pure exogenous linamarase. Linamarase (linamarin β-glucoside hydrolase), in its pure state, is known to be highly substrate specific for linamarin [21]. The only other β-glucoside which is known to be substantially amenable to the hydrolytic activity of the pure enzyme is methyl linamarin [2(β-D-glucopyranoxyloxy) methyl butyronitrile], known as lotaustralin.

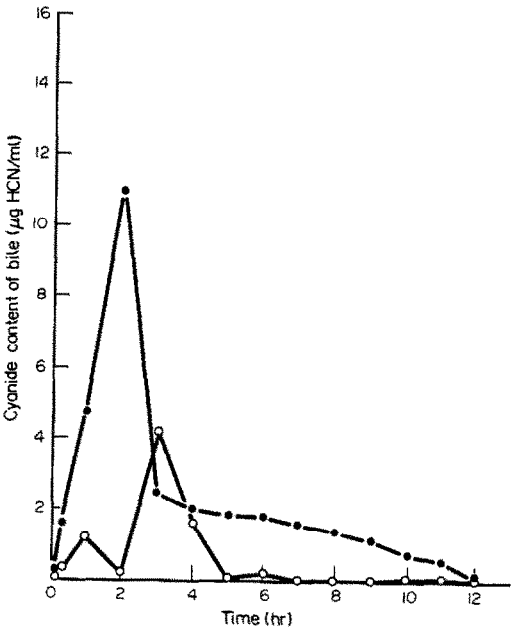

Fig. 1. Mean time courses of the appearance of glucosidic cyanide, linamarin, (○——○) and non-glucosidic cyanide (●——●) in bile following the administration of 300 mg linamarin/kg to cannulated rats.

Our quantitative assessment of the extent of excretion of linamarin, using the estimation [15] which shows that 9.4 mg of linamarin given orally to rats produces 0.25 mg cyanide *in vivo*, indicate that up to 12% of the administered compound will have been excreted unchanged in the bile while 30–32% would appear as non-glucosidic cyanide.

Thiocyanate ion was undetectable either in the test or in control bile samples, and no cyanide entity (glucosidic or non-glucosidic) was found in bile of the control animals. In the test rats, no linamarin or non-glucosidic cyanide was excretable after the 12th hr period of bile flow.

U.v. irradiation of the t.l.c. plates of the test bile revealed a light yellow fluorescent spot of R_f value of 0.063 (identical to the migration of pure linamarin) and four other different light-blue fluorescent areas of R_f values 0.36, 0.47, 0.56 and 0.71, respectively, all of which were absent in the control chromatogram. (A preliminary two-dimensional t.l.c. examination of the test bile had shown that each of the u.v. fluorescent spots migrated as a single band.)

Chemical analysis of pooled aqueous eluates of each fluorescent metabolite showed that it contained a cyanide ion whose concentration did not increase following incubation with pure linamarase. On the

other hand, pooled eluates of the area presumed to contain linamarin responded to the hydrolytic activity of pure linamarase by producing detectable amounts of cyanide ion. No glucosidic cyanide (linamarin) or the non-glucosidic moiety was detected in 24 hr faeces collected from the uncannulated rats.

The spot tests conducted indicated the presence of cyanohydrins in t.l.c. areas of R_f values 0.36, 0.47 and 0.56. (A characteristic violet colour appeared when 0.5 ml extract of each pooled spot was mixed on a white tile with a drop of a 2% alcoholic solution of *p*-phenylenediamine reagent + 2 drops of 2 N acetic acid + 2 drops of 3% hydrogen peroxide. A blank test was done on a drop of double distilled water.) The pooled extract of the area of R_f value 0.71 showed a negative result to the test for cyanohydrins but responded positively to the test for nitriles. (An aliquot of 0.5 ml extract was heated for 5 min with 0.5–1 mg hydrated oxalic acid in a micro test tube immersed in a glycerol bath pre-heated to 200°. Excess oxalic acid was expelled and test tube cooled. An orange colour appeared on further heating the contents of the test tube with 2 mg thiobarbituric acid thereby indicating the presence of a nitrile. The other spots did not respond to this test).

DISCUSSION

The detection of intact linamarin in the bile of rats given i.p. doses of the pure compound, using enzymatic degradation by the substrate—specific exogenous enzyme linamarin β-glucoside glucohydrolase (linamarase) and the identical migration of the bile component, as a single spot, on t.l.c. plates with the pure compound, as the parameters, adequately confirms that the bile is an excretory route for the cyanogenic glucoside.

This finding is in line with the universally recognised prerequisites for the biliary excretion of xenobiotics (foreign chemicals in the system), namely; high polarity and a fairly large molecular weight [22, 23]. Lotaustralin (methyl linamarin), the only other β-glucoside readily hydrolysable by pure linamarin, if present in the test bile as a metabolite, will migrate on t.l.c. plates at a different rate from linamarin.

The chemical forms of the non-glucosidic cyanide entities in bile and their molar ratios, as cyanogens, with excreted linamarin are of toxicological and pharmacological importance. The occurrence of the non-glucosidic cyanide entities, which from preliminary chemical spot tests appear to be cyanohydrins and a nitrile, is consistent with the mode of enzymatic degradation of linamarin [6], and the observation [24] that nitriles are major products in the digestive tract following the ingestion of cassava, or its derivatives, and that release of cyanide ion from them could occur *in vitro* and *in vivo* [25]. The non-glucosidic cyanide moities excreted in bile would, under suitable conditions, dissociate to release cyanide ion which depending on pH would protonate to form hydrocyanic acid. This view is supported by (a) the fact that HCN is a small molecule (mol. wt. ≃ 27) and, therefore, should not be excretable in bile to any appreciable degree, and (b) the observation that thiocyanate ion with an even larger mol. wt. (≃58) was undetectable in bile exudate of the treated animals.

The biphasic elimination kinetics of the excretory products, particularly non-glucosidic cyanide (Fig. 1), shows that in the first phase in which exponential (linear) elimination of cyanide occurred linamarin was still being degraded enzymatically. The subsequent downward trend in the excretion pattern of cyanide, at its origin, is presumed to indicate saturation of the relevant hydrolytic enzyme by linamarin. The hyperbolic curve obtained in this second phase, therefore, would seem to reflect the true elimination kinetics of non-glucosidic cyanide. This rather slow elimination phase suggests that, with repeated administration of linamarin, cumulative toxicity can result.

The biliary excretion of intact linamarin, and its cyanide metabolites, is a detoxication mechanism judging from the well known biochemical effects of cyanide poisoning and the new evidence [14] implicating linamarin *per se* in toxicity in chicken embryos. However, this mechanism would only be valid in the absence of the occurrence of enterohepatic circulation in a given animal species. Otherwise, there is the likelihood that the excreted compounds could be recycled, with the attendant toxic consequences.

It is possible that the excretory products could have appeared in bile as a result of simple diffusion but our estimation of 12% elimination of unchanged linamarin would preclude this possibility.

The excretion of non-glucosidic cyanide metabolites in rat bile following i.p. administration of pure linamarin shows conclusively that the liver contains relevant hydrolytic enzymes for dealing with this compound. The enzymatic method employed in the quantitation of glucosidic and non-glucosidic cyanide is more sensitive, has greater reproducibility, and is much more reliable than other existing methods [18, 26].

REFERENCES

1. M. Dixon and E. C. Webb, in *Enzymes*, 2nd Edn. Longmans, Green, London (1965).
2. R. D. Montgomery, in *Toxic Constituents of Plant Foodstuffs* (Ed. I. E. Liener), pp. 143–157. Academic Press, New York (1969).
3. A. Jakob and S. Diem, *Biochim biophys. Acta* **362**, 469 (1974).
4. G. E. Isom and J. L. Way, *Biochem. Pharmac.* **25**, 605 (1976).
5. G. E. Isom, D. H. W. Liu and J. L. Way, *Biochem. Pharmac.* **24**, 871 (1975).
6. E. E. Conn, *J. Agric. Food Chem.* **17**, 519 (1969).
7. W. O. Winkler, *J. Assoc. Off. Agric. Chem.* **41**, 282 (1958).
8. B. L. Nestel, in *Chronic Cassava Toxicity* (Eds. B. L. Nestel and R. MacIntyre), pp. 11–26, I.D.R.C. Monograph No. 010e, Ottawa (1973).
9. D. G. Coursey, in *Chronic Cassava Toxicity* (Eds. B. L. Nestel and R. MacIntyre), pp. 27–36, I.D.R.C. Monograph No. 010e, Ottawa (1973).
10. R. D. Cooke and E. N. Maduagwu, *J. Food Technol.* **13**, 299 (1978).
11. K. Lang, *Biochem. Z.* **259**, 243 (1933).
12. K. Lang, *Biochem. Z.* **259**, 262 (1933).
13. A. R. Pettigrew and G. S. Fell, *Clin. Chem.* **18**, 996 (1972).

14. E. N. Maduagwu, *J. Plant Foods*, in press.
15. M. D. Barrett, D. C. Hill, J. C. Alexander and A. Zitnak, *Can. J. Physiol. Pharmac.* **55**, 134 (1977).
16. E. Boyland, G. S. Ramsay and P. Sims, *Biochem. J.* **78**, 376 (1961).
17. B. E. Pryor and T. F. Slater, *Biochem. J.* **103**, 49p (1967).
18. R. D. Cooke, *J. Sci. Food Agric.* **29**, 345 (1978).
19. R. G. Bowler, *Biochem. J.* **38**, 385 (1944).
20. F. Feigl, in *Spot Tests in Organic Analysis*, 7th Edn. Elsevier, Amsterdam (1966).
21. G. W. Butler, *Phytochem.* **4**, 127 (1965).
22. R. W. Brauer, *J. Am. Med. Assoc.* **169**, 1462 (1959).
23. R. T. Williams, *Ann. N.Y. Acad. Sci.* **123**, 110 (1965).
24. T. K. Smith and L. D. Campbell, *Poult. Sci.* **55**, 861 (1976).
25. H. Ohkawa, R. Ohkawa, I. Yamamoto and J. E. Casida, *Pest. Biochem. Physiol.* **2**, 95 (1972).
26. R. D. Cooke and E. M. De La Cruz, *J. Sci. Food Agric.* **33**, 1001 (1982).