EVIDENCE FOR THE BIOSYNTHESIS OF A GLUCURONIDE CONJUGATE OF (S)-(-)-NICOTINE, BUT NOT (S)-(-)-COTININE OR (±)-*trans*-3′-HYDROXYCOTININE BY MARMOSET HEPATIC MICROSOMES

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SUMMARY

Recently, the detection of urinary glucuronide conjugates of nicotine and its two major metabolites, *trans*-3′-hydroxycotinine and cotinine, showed that glucuronidation is an important pathway of nicotine metabolism in humans. (S)-(-)-Nicotine-N^+-1-β-glucuronide (quaternary *N*-glucuronide with linkage through the pyridino-nitrogen of nicotine) was shown to be an important nicotine metabolite of humans *in vivo*. The present study was undertaken to develop an animal model for this process, in order to ascertain the factors influencing quaternary *N*-glucuronide formation. (S)-(-)-Nicotine-N^+-1-β-glucuronide was formed *in vitro* when [2′-^{14}C]-nicotine was incubated with Triton X-100 activated marmoset hepatic microsomes in the presence of uridine diphosphoglucuronic acid; it was not formed when activated microsomal preparations of rabbit, guinea-pig, or rat were used as enzyme source. The glucuronide was characterised by comparison with authentic synthetic (S)-(-)-nicotine-N^+-1-β-glucuronide using HPLC. The rate of formation of the glucuronide was almost linear during up to four hours of incubation, but still only accounted for a maximum of 6.0% of the available substrate at the end of five hours incubation. The synthetic and biosynthetic (S)-(-)-nicotine-N^+-1-β-glucuronides were hydrolysed by β-glucuronidase and alkali, but were resistant to acid hydrolysis. The results support the

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concept that the marmoset may be a good animal species to mimic man in studies of nicotine metabolism during exposure to tobacco smoke. *In vitro* studies using (±)-*trans*-3′-hydroxycotinine or (S)-(-)-cotinine (as potential substrate) and [^{14}C]-uridine diphospho-glucuronic acid (as cofactor) failed to produce any new radiolabelled glucuronide when the above microsomal preparations were used.

KEY WORDS

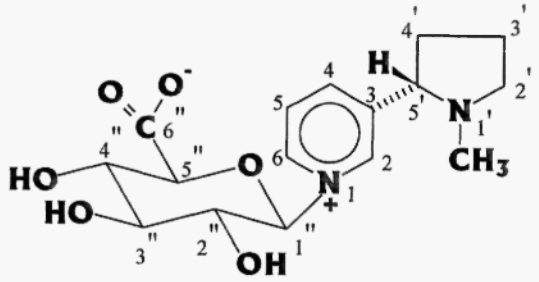
(S)-(-)-nicotine-N^+-1-β-glucuronide, marmoset, hydrolysis

INTRODUCTION

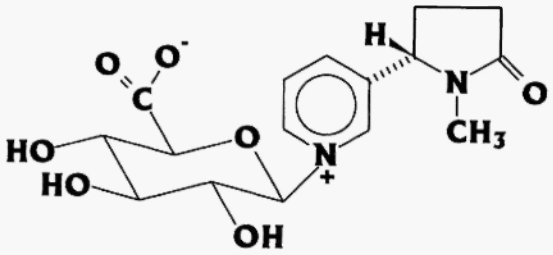
Evidence from studies of nicotine disposition in humans revealed that glucuronides of nicotine, cotinine and 3′-hydroxycotinine were major urinary metabolites /1-3/. The average percentage of glucuronide conjugates of nicotine, cotinine and 3-hydroxycotinine excreted in the urine of 94 tobacco users, determined using a β-glucuronidase hydrolysis method, were approximately 3%, 9%, and 23%, respectively /1/. These results are in agreement with those obtained from another study on smokers' urine /2/. Therefore, an important part of nicotine metabolism in humans is now considered to occur via phase II glucuronidation reactions.

(S)-(-)-Nicotine-N^+-1-β-glucuronide (NNG) /4/, (S)-(-)-cotinine-N^+-1-β-glucuronide (CNG), and (±)-*trans*-3′-hydroxycotinine-N^+-1-β-glucuronides (3-HNG) /5/ have been synthesised and characterised. The chemical structures of NNG, CNG and 3-HNG are shown in Figure 1.

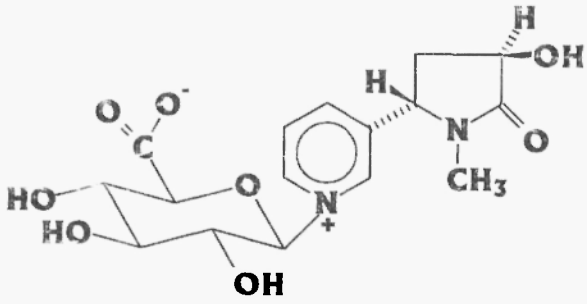
In the present study, the prepared glucuronides were used for the comparative identification of any metabolite formed *in vitro*, using Triton-X-activated hepatic microsomal preparations of marmoset, rabbit, guinea-pig and rat. The substrates used were S-(-)-nicotine, S-(-)-cotinine and (±)-*trans* 3′-hydroxycotinine. The *in vitro* rate of formation of NNG, using Triton X-100-activated marmoset hepatic microsomes ,and its hydrolytic characteristics are reported.



NNG



CNG



3-HNG

Fig. 1: The chemical structures of (S)-(-)-nicotine-N^+-1-β-glucuronide (NNG), (S)-(-)-cotinine-N^+-1-β-glucuronide (CNG), and (±) *trans*-3′-hydroxy-cotinine-N^+-1-β-glucuronide (3-HNG).

MATERIALS AND METHODS

Chemicals

S-(-)-Nicotine-N^+-1-β-glucuronide (NNG), S-(-)-cotinine-N^+-1-β-glucuronide (CNG), and (±)-*trans*-3′-hydroxycotinine-N^+-1-β-glucuronides (3-HNG) were prepared using the method of Crooks *et al.* /5/. (+)-*trans*-3′-Hydroxycotinine was prepared according to the reported method /6/.

(S)-(-)-Nicotine, (S)-(-)-cotinine, uridine diphospho-glucuronic acid (UDPGA, triammonium salt), β-glucuronidase (from *Helix pomatia*, 420,000 units/g), glucosaccharo-1,4-lactone, Dowex-50W, tris-HCl and *p*-nitrophenol were purchased from Sigma Chemical Company, Poole, Dorset, UK. Triton X-100 was purchased from Aldrich Chemical Company Ltd., Gillingham, Dorset, UK. (S)-(-)-[Pyrrolidine-2′-^{14}C]nicotine-di-(+)-tartrate salt, specific activity 58.8 mCi/mmol and radiochemical purity ≥ 98%, was purchased from Chemsyn Science Laboratories, Lenexa, Kansas, USA. [^{14}C]-UDPGA (25μCi/ml in methanolic solution, specific activity 250mCi/mmole) was purchased from ICN Radiomedicals Ltd., UK. Quickszint flow 302 was purchased from Zinsser Analytic, UK.

Instrumentation

The HPLC system consisted of a Beckman pump (model 110A), coupled to a gradient controller (Beckman 420), a Rheodyne injector (7120) fitted with a 20 μl sample loop and a UV detector (Philips Pye Unicam, PU4020). The UV detector, which monitored at 260 nm with a flow rate of 1 ml/min, was further connected to a Flow one Canberra Packard radiochemical detector (model A280, fitted with a 0.5 ml capacity radioactive flow cell) for the radioactivity determination. The HPLC analysis was performed using a strong cation exchange HPLC analytical column (Nucleosil SA 10 μm, 250 x 4.6 mm) purchased from Phenomenex, UK. Two mobile phase systems were employed. Mobile phase A consisted of a mixture of sodium acetate buffer (0.2 M), methanol (70:30 v/v) and triethylamine (0.02%), pH 4.5; mobile phase B consisted of sodium acetate buffer (0.1 M), pH 4.0. The mobile phase was mixed with Quickszint flow 302 cocktail (1:2 v/v), and a flow rate of 3 ml/min was used for the radioactivity detection.

Animals and hepatic microsomal preparations

A male rabbit (New Zealand White, approximately 2.5 kg), two male guinea-pigs (Dunkin-Hartley, 340-400 g) and two male rats (Wistar, 278-300 g) were supplied from King's College Animal Facility, London, UK. Prior to sacrifice, the animals were fasted for five hours and killed by cervical dislocation. The liver was removed and used for microsome preparation. Livers from two male marmosets were donated by King's College Parkinson Research Centre. The ultracentrifugation technique employed to prepare the microsomes was based on the reported method /7/. The concentration of microsomal preparations was adjusted with Tris-buffer (0.25 M, pH 7.4) to approximately 0.5 g original liver/ml. The microsomal preparations were then bubbled with nitrogen gas and stored in a Queue freezer at -80°C until use.

Prior to incubation, the microsomes were thawed and activated with Triton X-100 (0.25% w/v) and sonicated using a Sonicor for 5 min whilst being cooled in ice-water mixture. They were then allowed to stand in ice for another 15 min before being added to the incubation mixture as described previously /8/.

Liver microsomal experiments

Prior to the alkaloid microsomal experiments, the activity of UDP-glucuronosyltransferase towards *p*-nitrophenol in the various hepatic microsomal preparations was assayed using the modified incubation conditions suggested /9/. Incubations (final volume 0.34 ml) contained both the non-labelled substrate, (S)-(-)-nicotine (5 mM, 0.05 ml), radiolabelled nicotine (5 μCi, 0.02 ml), various hepatic microsomal preparations (0.5 g original liver/ml, 0.10 ml, activated [as described above] with Triton X-100, 0.25% w/v, 0.02 ml), Tris-HCl (0.2 M, pH 7.4, 0.05 ml), $MgCl_2$ (5 mM, 0.05 ml). After incubation for 2 min at 37°C in a water bath, UDP-glucuronic acid (cofactor solution, 30 mM, 0.05 ml) was added, and the total mixture incubated for a further 30 min.

Due to lack of radiolabelled cotinine or *trans*-3′-hydroxycotinine, [^{14}C]-UDPGA was used as a radio-marker for the studies. The incubation procedures for the attempted glucuronidation of S-(-)-cotinine and (±)-*trans*-3′-hydroxycotinine (5 mM, 0.05 ml each) were identical to those of S-(-)-nicotine reactions. However, instead of

radiolabelled nicotine, [^{14}C]-UDPGA (0.5 μCi, 0.02 ml) was incorporated into the incubation mixtures of both S-(-)-cotinine and (±)-*trans*-3′-hydroxycotinine.

Control experiments (i.e. either without microsomes or lacking UDPGA cofactor solution) were also carried out. The reaction was terminated by rapidly placing the incubation tubes on ice. The incubates were centrifuged at 4,000 rpm using a MSE bench-top centrifuge for 20 min. Aliquots (20 μl) of the centrifuged nicotine incubates were injected onto the radio-HPLC system for analysis.

In vitro rate of formation of NNG from radiolabelled nicotine by marmoset microsomes

The incubation procedures and materials used were identical to those described above. The glucuronidation reactions of radiolabelled nicotine using Triton X-100-activated marmoset microsomes were terminated after 30, 60, 90, 120, 180, 240 and 300 min of incubation (in triplicate). The incubates were treated as described above for assay.

Hydrolytic studies

In order to determine the susceptibility of the conjugate formed *in vitro* from nicotine, acid, alkali, and β-glucuronidase, hydrolytic studies were carried out. The marmoset nicotine incubation mixtures from the previous experiments (i.e. after 240 and 300 min of incubation) were bulked (1.8 ml), made further alkaline with NaOH (2 M, 50 μl) to pH 10, and extracted with dichloromethane (3 x 5 ml) to remove unreacted nicotine. Aliquots (100 μl) of the dichloromethane-extracted aqueous phase were placed immediately into six separate tubes containing ammonium acetate buffer (0.5 M, 100 μl) at pH 5.0. Two tubes were treated with β-glucuronidase (10,000 units, 150 μl), and the other four tubes served as controls, i.e. with either water (150 μl) replacing the β-glucuronidase or containing glucosaccharo-1,4-lactone (10 mg) together with β-glucuronidase (10,000 units, 150 μl). The total volume of each tube was 350 μl. The three tubes were incubated for 20 h, at 37°C. Aliquots (20 μl) of the incubates were analysed by radio-HPLC as described above.

Aliquots (100 μl) of the remaining dichloromethane-extracted aqueous phase were placed in separate tubes (in duplicate). The samples were then treated as follows: (1) with NaOH (4 M, 250 μl);

(2) with HCl (4 M, 250 µl), and (3) controls (with water only, 250 µl). The pH of the resulting samples (1), (2) and (3) were 14.0, 1.0 and 10.0, respectively. The prepared flasks were sealed with thin plastic film and incubated in a water bath at 70°C for 18 h. Aliquots (20 µl) of the incubates were analysed by radio-HPLC as previously described.

RESULTS

p-Nitrophenol UDPGT assay

The activities for the glucuronidation of *p*-nitrophenol of Triton X-100-activated prepared marmoset, rabbit, rat and guinea-pig microsomes were 5.0, 2.0, 2.0 and 6.0 nmoles/min/mg, respectively. The glucuronidation activity of rat microsomes, when compared to the typical activities reported /9/, was very much lower. However, these results show that all the preparations had viable UDPGT activity.

Microsomal studies

The results of radio-HPLC analysis showed that a new radioactive peak (a) was detected in the marmoset incubates containing nicotine, but this peak was not detected in the other nicotine-containing incubates when rabbit, guinea-pig and rat hepatic microsomes were used (Fig. 2). This radioactive peak (a) had a retention time (Rt; 14.7 min) which corresponded to the Rt of the synthetic NNG standard. The ratio of NNG to unmetabolised nicotine, calculated from their radioactivities, was 1:63, after 30 min incubation. This indicated that the formation of NNG from nicotine was incomplete under these *in vitro* conditions as there was a substantial amount of UDPGA and nicotine left unreacted in the incubates.

Figure 3 shows the chromatograms of Triton X-100 activated marmoset nicotine incubates and the controls (i.e. incubates lacking UDPGA or microsomes), after 30 min of incubation. The peak (a), which corresponded to NNG, was only detected in the incubates that contained both marmoset microsomes and UDPGA (complete system), but not in any of the controls.

Analysis of the incubates of (S)-(-)-cotinine and (±)-*trans*-3′-hydroxycotinine showed no new radiolabelled peak was formed when

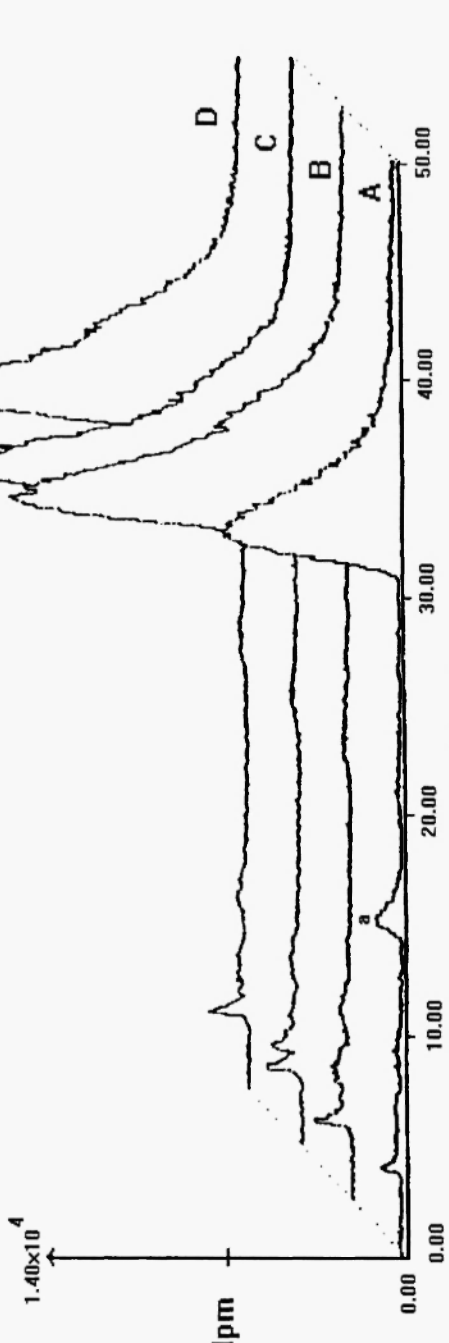


Fig. 2: Radio-HPLC chromatograms of incubates containing radiolabelled nicotine, when Triton X-100-activated microsomes of marmoset (A), rabbit (B), guinea-pig (C) and rat (D) were used as enzyme source. (a) = (S)-(-)-nicotine-N^+-1-β-glucuronide (NNG).

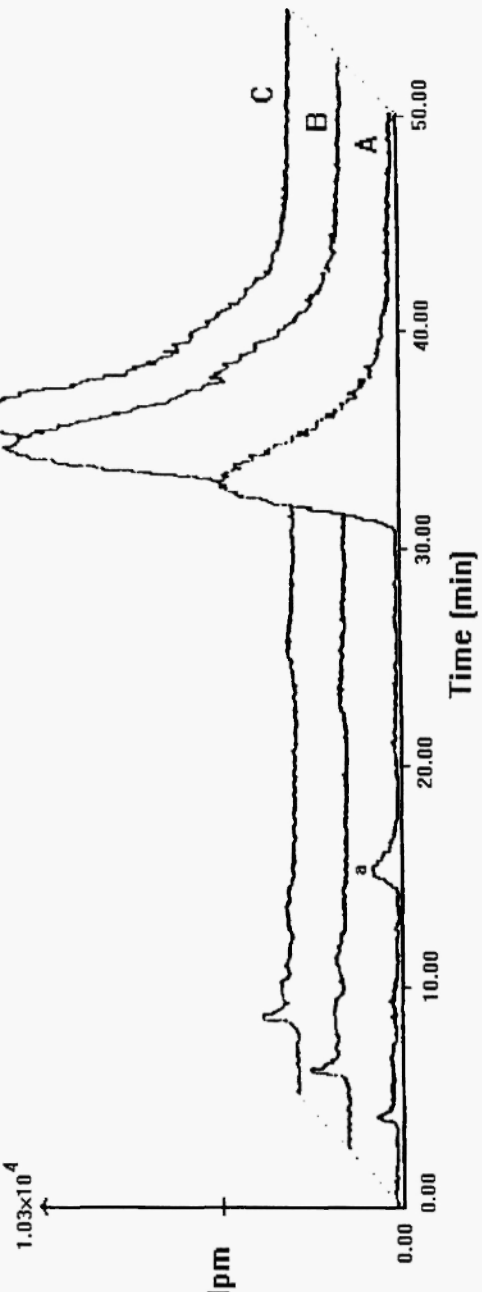


Fig. 3: Radio-HPLC chromatograms of marmoset incubates containing nicotine: complete system (A), and controls [i.e. lacking UDPGA (B) or microsomes (C)]. (a) = (S)-(-)-nicotine-N^+-1-β-glucuronide (NNG).

Triton X-100 activated microsomes of the studied species were used (data not shown).

Rate of formation of NNG

The rate of formation of NNG from nicotine using marmoset hepatic microsomes *in vitro* is shown in Figure 4, and appeared almost linear from 30 min until 4 h, after which it started to decrease slightly. Analysis of the 5 h incubates containing radiolabelled nicotine showed that there was still a large amount of unmetabolised nicotine or UDPGA left in the incubates. At this incubation time, only 6.0% of nicotine was converted to NNG.

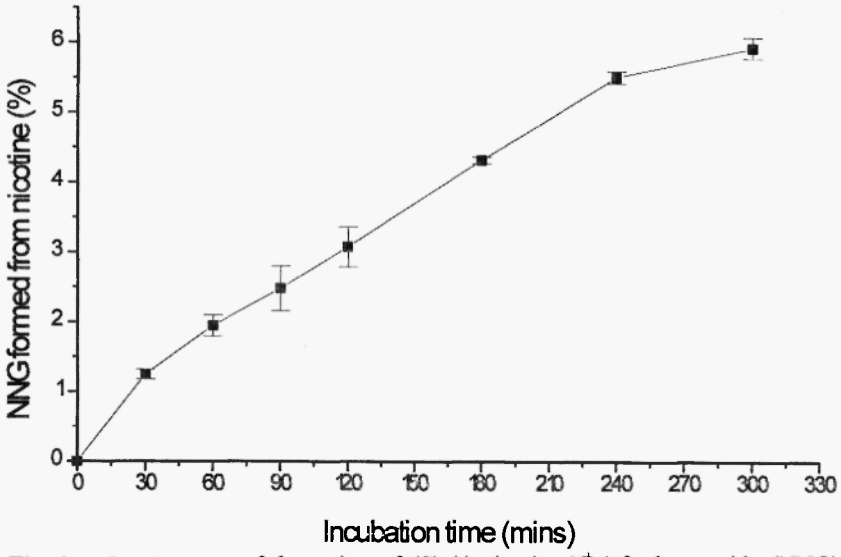


Fig. 4: *In vitro* rate of formation of (S)-(-)-nicotine-N^+-1-β-glucuronide (NNG) from radiolabelled nicotine using marmoset hepatic microsomes. Results are expressed as means ± SEM, $n = 3$.

Hydrolytic studies

Radio-HPLC analysis of "hydrolysis" incubates showed that β-glucuronidase treatment of the 'concentrated' marmoset incubates containing formed NNG, completely hydrolysed the NNG to release free radiolabelled nicotine. The identical amount of radioactivity (dpm) was found in the released nicotine as was originally present in

NNG. However, no radiolabelled nicotine was released and the intact NNG was still detected in both controls, i.e. when glucosaccharo-1,4-lactone was included or water was used instead of the enzyme (Fig. 5).

When a hot, strong alkali treatment (pH 14) was used, neither NNG nor nicotine was detected in the incubates after 18 h. However, when a hot but milder alkali treatment (pH 10) was used, 97% of the NNG was hydrolysed to release nicotine. In the case of hot strong acid treatment (HCl, 0.4 M, pH 1), no hydrolysis of NNG was observed (Fig. 5).

DISCUSSION

In this study, two mobile phase systems, A and B were employed. Mobile phase A was designed specifically for NNG detection, whereas mobile phase B was used for both CNG and 3-HNG. With the former system, UDPGA and 3-HNG were not separable, but using the latter phase, CNG, 3-HNG and UDPGA had respective retention times of 5.7 min, 4.8 min and 2.6 min. The present *in vitro* studies, using TX-100-activated hepatic microsomal preparations from marmoset, rabbit, guinea-pig and rat, showed that a new radioactive compound (**a**) was only observed in the nicotine containing incubates derived from the marmoset, but not in the other three hepatic microsomal preparations. This compound (**a**) had a Rt (14.7 min) which corresponded to that of the NNG standard.

Recently, CNG has been reported to be a major urinary metabolite in smokers /10/. However, when (S)-(-)-cotinine or (±)-*trans*-3′-hydroxycotinine were used as potential substrates, no glucuronide conjugates of (S)-(-)-cotinine or (±)-*trans*-3′-hydroxycotinine were detected in any of the incubates studied. The results also indicated that the biosynthesis (i.e. quaternization) of (S)-(-)-cotinine or (±)-*trans*-3′-hydroxycotinine glucuronides probably utilises UDPGT (UDP-glucuronosyltransferase) isozymes different from that which catalysed the formation of NNG in the marmoset.

Theoretically, the glucuronidation of *trans*-3′-hydroxycotinine can give rise to two types of 3′-hydroxycotinine glucuronide (i.e *O*- or N^+-glucuronides). However, a recent study showed that the *trans*-3′-hydroxycotinine glucuronide present in smokers' urine was the *O*-glucuronide /3/. Although all the studied species have shown *p*-nitrophenol-UPGT activity (i.e. the formation of an *O*-glucuronide),

these activities were not exhibited by the formation of *trans*-3′-hydroxycotinine-*O*-glucuronide *in vitro* as no new conjugate was detected. It is probable that 3′-hydroxycotinine (alcoholic) glucuronide is formed by a UDPGT different from that forming phenolic glucuronides.

The results (Fig. 3) also showed that the *in vitro* glucuronidation of (S)-(-)-nicotine was an enzymatic process and UDPGA was required as a cofactor.

The rate of formation of NNG, using marmoset Triton X-100-activated hepatic microsomal preparations, appeared almost linear up to 4 h under the present incubation conditions. The percentage of NNG formed from nicotine only accounted for approximately 6.0% of the total nicotine substrate used after 5 h. As there was still a substantial amount of UDPGA and nicotine left unmetabolised in the incubates after 5 h of incubation, the failure to form more NNG may be due to denaturation of the enzyme during the incubation.

The result of β-glucuronidase treatment of the 'concentrated' marmoset incubates containing NNG, showed that the quaternary glycosiduronic bond of the NNG conjugate was cleaved to release the nicotine aglycone (100%) after 20 h at 37°C. Furthermore, the inclusion of glucosaccharo-1,4-lactone, a β-glucuronidase inhibitor, prevented the release of nicotine from NNG. These results further confirmed that the compound (**a**) formed *in vitro* in the marmoset incubates was NNG.

These results of chemical hydrolysis indicate that during hot alkali treatment (pH 10) the quaternary glycosiduronic bond of the NNG was cleaved, but complete destruction of NNG and its aglycone occurred when hot strong alkali (pH 14) was used. This is indicated as neither radioactive NNG nor nicotine were detected following the latter treatment. No release of nicotine from NNG was observed when hot strong acid treatment was used.

Generally, there are few reports on the formation of quaternary ammonium glucuronides by laboratory animals *in vitro*. However, aliphatic quaternary N^+-glucuronides were formed from tripelennamine and cyproheptadine using a rabbit hepatic microsomal enzyme system /11/ and from ketotifen in rabbit hepatocytes /12/. The formation of a quaternary glucuronide from lamotrigine, an amino azo heterocyclic compound, has been observed in isolated guinea-pig hepatocytes and activated guinea-pig liver microsomes /13/. The

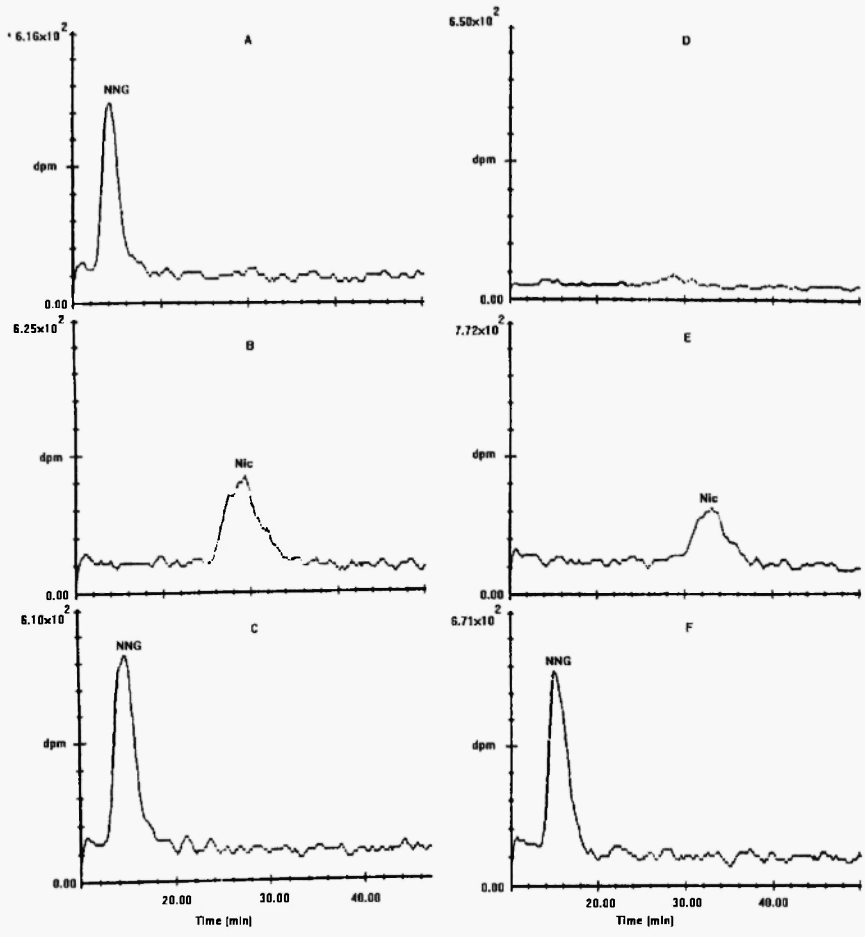


Fig. 5: Radio-HPLC chromatograms of the 'concentrated' incubates containing NNG after various hydrolytic treatments. A = control, i.e. with water, 37° C, 20 h; B = with β-glucuronidase, 37°C, 20 h; C = with β-glucuronidase plus glucosaccharo-1,4-lactone, 37°C, 20 h; D = alkali treatment, pH 14, 70°C, 18 h; E = alkali treatment, pH 10, 70°C, 18 h, and F = acid treatment, pH 1, 70°C, 18 h. NNG = (S)-(-)-nicotine-N^+-1-β-glucuronide and Nic = (S)-(-)-nicotine.

failure to form NNG by rabbit or guinea-pig preparations in our study may indicate that isoforms of UDPGT having different tertiary amine substrate requirements are present in different species /14/. In view of the importance of this metabolic pathway in man, the substrate specificity of various UDPGTs towards tertiary amines of various types needs urgent attention.

Recently, the direct identification of NNG in rat bile, after administration of radiolabelled nicotine, has been reported /4/. However, in our study (unpublished results) we failed to obtain evidence for any nicotine glucuronide conjugates (likely to be NNG) in rat faeces (0-24 h) after enzymic hydrolysis. A possible explanation for this observation might be due to the hydrolysis of any nicotine glucuronides by β-glucuronidase of the gut microflora. At present it is difficult to reconcile our results with these observations and further work is required to determine the source of NNG present in rat bile and establish the enzymology of its formation.

CONCLUSIONS

The results of the present study have shown that the marmoset may be a useful model to represent humans in studies of the glucuronidation of (S)-(-)-nicotine, but not of (+)-*trans*-3′-hydroxycotinine or (S)-(-)-cotinine, *in vitro*.

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