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SHORT COMMUNICATION

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Identification and kinetics of a β-glucuronide metabolite of SN-38 in human plasma after administration of the camptothecin derivative irinotecan

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Abstract Irinotecan (7-ethyl-10-{4-[1-piperidino]-1-piperidino}carbonyloxycamptothecin), also known as CPT-11, is a promising semi-synthetic derivative of camptothecin with significant activity against a range of tumour types. The pharmacokinetic behaviour of its principal and presumedly active metabolite, SN-38 (7-ethyl-10-hydroxycamptothecin), displays wide inter-patient variation. During the high-performance liquid chromatographic (HPLC) analysis of plasma samples collected from a patient given CPT-11, we observed several unidentified peaks that were not present in pre-infusion samples. In this paper we describe the manner in which one of these was determined to be a β-glucuronide of SN-38. The total plasma concentrations of this metabolite were quantified following digestion with β-glucuronidase and were found to be greater than those of SN-38 in the patient studied. The elimination phases of the plasma concentration profile of SN-38 and its glucuronide were parallel, suggesting that the transformation of SN-38 to the glucuronide is the rate-limiting step in the elimination of SN-38 and could play a key role in its pharmacokinetics.

Key words Irinotecan · CPT-11 · SN-38 · Glucuronide

Introduction

Camptothecin (CPT), originally isolated from the Chinese tree *Camptotheca acuminata*, is known to have significant cytotoxic and anti-tumour activity [11]. However, its lack of solubility in water has prompted the development of active hydrophilic derivatives. One of these compounds, CPT-11 (7-ethyl-10-{4-[1-piperidino]-1-piperidino}-carbonyloxy-camptothecin), synthesised at the Yakult Central Institute for Microbiological Research (Tokyo, Japan), appears to have promising activity against a wide range of tumour types [11]. In vivo, CPT-11 is converted to SN-38 (7-ethyl-10-hydroxycamptothecin) by serum and tissue carboxylesterases [5, 12]. SN-38 has 100-fold or greater activity than CPT-11 in vitro and CPT-11 can therefore be considered to be a prodrug of SN-38 [11].

Ideally, administration of a prodrug should result in a reproducible and predictable exposure to the active metabolite. In the case of CPT-11, however, the plasma concentration area under the curve (AUC) of SN-38 is not correlated with the CPT-11 AUC or dose [6, 7, 9], and the pharmacokinetic parameters of both compounds are subject to large inter-patient variation [3]. Camptothecin and its active derivatives possess a lactone ring that is reversibly hydrolysed to an open carboxylate form [1] such that these substances exist as two discrete chemical entities in solution. Because only the lactone forms are active against topoisomerase I, the presumed target of camptothecins [11], efforts have been directed towards a more complete understanding of the pharmacology of camptothecins by quantifying both lactone and carboxylate forms in plasma [8, 9, 11].

During the development of a new technique for the determination of the lactone and carboxylate forms of CPT-11 and SN-38, we became aware of the presence of other metabolites in plasma samples collected from a patient undergoing CPT-11 therapy. In this paper we describe the identification of one of these compounds as a β-glucuronide of SN-38 and present preliminary kinetic data obtained in this patient.

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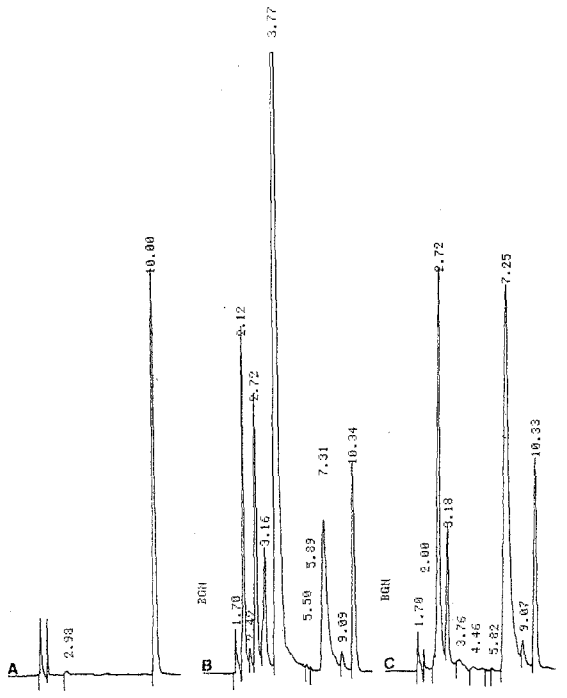

Fig. 1A–C Chromatograms of **A** a blank sample containing only the internal standard, CPT lactone; **B** a plasma extract prepared immediately following the collection of a blood sample at 10 min post-infusion of CPT-11; and **C** the same sample shown in **B** following acidification with 1 *N* HCl. Volumes injected were 10 µl for **A** and 5 µl for **B** and **C**. Peaks identified with pure standards include CPT-11 carboxylate (3.8 min), SN-38 carboxylate (5.5 min), CPT-11 lactone (7.3 min), SN-38 lactone (9.1 min) and CPT lactone (10.3 min). The disappearance of the peaks at 2.1 and 3.8 min indicates a probable open-ring carboxylate structure

Materials and methods

Chemicals

CPT-11, SN-38 and CPT lactone standards were prepared by Yakult Honsha Co. Ltd. (Tokyo, Japan) and were obtained through Bellon (Groupe Rhône-Poulenc Rorer, Neuilly, France). TBAP (tetrabutylammonium phosphate) was obtained from Waters (Millipore, France) as a ready-to-use solution (PIC A). β-Glucuronidase (Type IX-A from *Escherichia coli*) and D-saccharic acid 1,4-lactone were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Sample collection

Blood samples were taken from a 60-year-old woman receiving a third cycle of CPT-11 (420 mg CPT-11 given in a 98-min infusion) for the treatment of a metastatic adenocarcinoma of the colon. These were collected every 30 min during the infusion, at the end of the infusion and then at 5, 10, 20, and 40 min as well as 1, 1.5, 2, 4, 6, 8 and 23 h post-infusion. Each tube was immediately immersed briefly into dry ice/acetone kept at the bedside, with care being taken not to freeze the blood. The samples were centrifuged at 4 °C for 2 min, plasma (200 µl) was transferred to Eppendorf tubes containing 0.5 ml of ice-cold acetonitrile/methanol (50:50, v/v) and 0.2 µg of CPT as the internal standard and the mixture was briefly vortexed and centrifuged (2 min, 4 °C). The supernatant was transferred to fresh Eppendorf tubes and stored immediately at –20 °C and later at –70 °C. The remaining plasma was collected and stored in the same manner. The total time elapsing between collection of blood and cold storage of plasma extract never exceeded 6–7 min.

High-performance liquid chromatography

The high-performance liquid chromatographic (HPLC) assay procedure, developed to separate the carboxylate and lactone forms of CPT-11 and SN-38 simultaneously, will be published separately in full detail [8]. Briefly, the compounds of interest were separated by reverse-phase HPLC with the isocratic delivery of mobile phase containing 5 m*M* TBAP to a NovaPak C-18 radially compressed column (Waters, Millipore, France) and were quantified following fluorimetric detection. Standards for the lactone and carboxylate forms of CPT-11 and of SN-38 were prepared using fresh human plasma, and the standard curves of the area ratio of compound to CPT (internal standard) versus concentration were linear over the ranges investigated.

Enzyme-digestion studies

The frozen plasma aliquots were thawed and 50-µl lots were dispensed into mini-Eppendorf tubes containing 0.05 µg of CPT lactone. The tubes were vortexed briefly and separated into three treatment groups. The first treatment consisted of the rapid addition of 100 µl of cold acetonitrile/methanol (50:50, v/v), vortexing and brief centrifugation (max. 8,000 *g*) at 0 °C. Part of the supernatant (100 µl) was transferred to a new tube and acidified by the addition of 5 µl of 1 *N* HCl prior to HPLC analysis. The second treatment consisted of incubation of the plasma aliquot at 37 °C for 2 h. The third treatment was the addition of 200 units of β-glucuronidase dissolved in 10 µl of water followed by incubation (as described above). Both incubated tubes were then treated as indicated for the freshly thawed plasma. In addition, plasma collected from the patient prior to the CPT-11 infusion and spiked with a 10-µg/ml concentration of CPT-11 was investigated as described above.

Results

Assay of fresh plasma extracts

Chromatograms of the plasma samples treated rapidly with the protein-precipitating mixture of cold acetonitrile/methanol revealed a large number of peaks that were not present in pre-infusion samples (Fig. 1). With the aid of pure standards, we could identify those corresponding to the carboxylate and lactone forms of CPT-11 and SN-38. The remaining four major peaks (2.1, 2.4, 2.7 and 3.1 min, respectively) could be reduced to two (2.7 and 3.1 min, respectively) following the acidification of the sample (Fig. 1C).

Enzymatic treatment and assay of frozen plasma

Treatment of the frozen plasma samples according to the three treatments (cold control, incubated control and β-glucuronidase digestion) revealed that the 3.1-min peak disappeared almost completely in the samples treated with β-glucuronidase, leading to a significant increase in the area under the SN-38 peak (Fig. 2). Only in a few samples did a detectable peak remain. The concentrations of CPT-11 and SN-38 determined for the cold and incubated

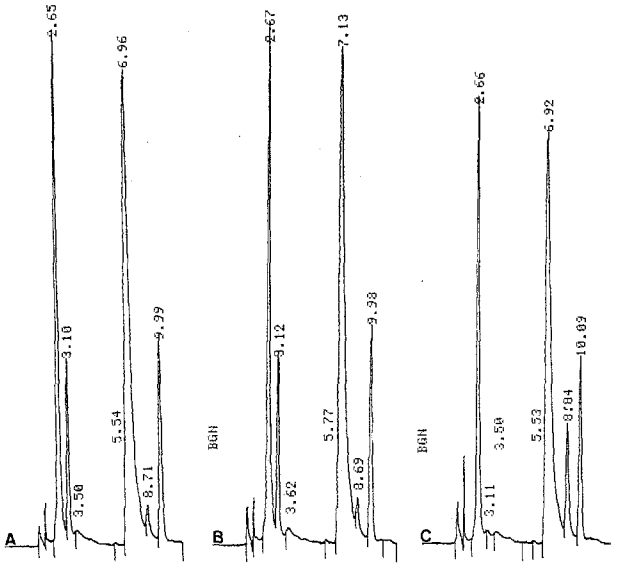

Fig. 2A–C Chromatograms of stored plasma **A** extracted on ice, **B** extracted after incubation for 2 h at 37 °C and **C** extracted after incubation for 2 h at 37 °C in the presence of 200 units of β-glucuronidase. The transformation of SN-38 β-glucuronide lactone (3.1 min) to SN-38 lactone (8.7 min) is almost complete. These plasma extracts were acidified to enable total drug (carboxylate plus lactone) to be quantified

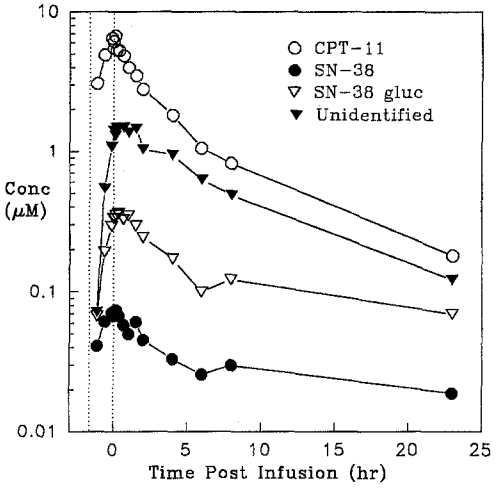

Fig. 3 The kinetics of total CPT-11, SN-38, SN-38 β-glucuronide and a second metabolite in the plasma of a patient. The concentrations of the unidentified metabolite were calculated assuming molar fluorescence equivalent to that of SN-38. The area between the *stippled vertical lines* designates the period of infusion

plasma were in substantial agreement with the post-incubation concentrations, averaging 96.3%±6.5% and 94.6%±7.8% for CPT-11 and SN-38, respectively. There was no measurable production of SN-38 in the blank samples spiked with CPT-11 and incubated either in the presence or the absence of β-glucuronidase. The incubation of the enzyme in 25 m*M* D-saccharic acid 1,4 lactone for 15 min at 37 °C prior to its addition to plasma (final concentration, 7 m*M*) completely blocked the transformation of the metabolite to SN-38 (data not shown).

The quantity of SN-38 produced from the β-glucuronidase incubation was calculated from the difference found in the SN-38 area ratio (SN-38/CPT) between the control and enzyme-digestion incubations. A comparison of the increase in the SN-38 area ratio (enzyme) and the area ratio of the 3.1-min peak (control) yielded a linear regression (r = 0.98) with a slope of 1.59, indicating that the metabolite had only 63% of the molar fluorescence of SN-38 under our assay conditions (assuming equimolar yield; i.e. 1 mol of metabolite produced 1 mol of SN-38). The other principal pair of peaks (reduced to a single 2.8-min peak with acidification) were not affected by the incubation with β-glucuronidase.

Pharmacokinetics

The total plasma concentrations of the β-glucuronide of SN-38 were superior to those of SN-38 itself (Fig. 3) by a factor ranging from 1.8 to 7.0. The AUCs of SN-38 and its glucuronide, calculated to the last plasma sample taken, were 0.70 and 2.87 μ*M* h, respectively, whereas that of CPT-11 was 26.92 μ*M* h. The peak concentration of the glucuronide was seen at 10–20 min after the end of the infusion, somewhat later than was the peak of total CPT-11 and SN-38. The ratio of glucuronide to total SN-38 rose during and after the infusion and peaked at only 1 h after the end of the infusion. This ratio then declined and reached a stable value at some 5–6 h post-infusion. The slopes of the final phase of both SN-38 and its metabolite were estimated graphically to be approximately 0.02 h^{-1}. This corresponds to half-lives on the order of 30–35 h. In contrast, the terminal phase of the concentration-time profile of the as yet unidentified metabolite appeared to be significantly steeper.

Discussion

The pharmacokinetics of CPT-11 and its principal active metabolite SN-38 have not been extensively characterised. In general, the pharmacokinetics of CPT-11 are subject to very large inter-patient variation [3] and the AUC of total plasma SN-38 is not correlated with the CPT-11 dose or AUC [6, 7, 9]. The half-life of the terminal phase of elimination of total SN-38 is long, being on the order of 10–40 h, depending on the schedule of administration, and accounts for much of its AUC [6, 7, 9]. Hence, it is possible that part of the variability of SN-38 kinetics is due to differences in its metabolism and elimination in addition to its variable formation from CPT-11 by carboxylesterases [7, 9].

During the development of an HPLC technique for the simultaneous quantification of the lactone and carboxylate forms of CPT-11 and SN-38 in plasma, we became aware of the presence of potential metabolites in samples collected from a patient treated with CPT-11. We determined that one

of these metabolites is a β-glucuronide of SN-38 that has previously been reported to be present in the urine and bile of both rats and patients receiving CPT-11 [2, 5]. However, this is the first time to our knowledge that a β-glucuronide metabolite of SN-38 has been detected in human plasma, an observation that is likely to be due to the addition of the HPLC ion-pairing reagent TBAP to our mobile phase to increase the retention of the carboxylate forms of CPT-11 and SN-38. Since β-glucuronides possess a carboxyl function, they would be expected to have increased retention and to be less likely to emerge associated with the solvent front. Because the plasma metabolite is present as two separate forms in rapidly frozen extracts and can be transformed into one form immediately following mild acidification, it is unlikely to be an acyl-glucuronide of the carboxylate form of SN-38. Of the two remaining possible sites for β-glucuronidation, the most likely is the hydroxyl function at the C-10 position of the A ring. Further studies will be required to characterise fully the chemical structure of this plasma glucuronide.

The possibility that part of the formation of SN-38 during the incubations was due to the hydrolysis of CPT-11 by plasma esterases can be excluded as a result of (1) the absence of significant SN-38 production in blank samples spiked with CPT-11, (2) the close correspondence between the concentrations of CPT-11 and SN-38 determined in cold and incubated plasma (no β-glucuronidase) samples and (3) the excellent correlation between the loss of the 3.1-min peak area and the accompanying rise in SN-38 area. The plasma time-course of the total concentrations of SN-38 β-glucuronide revealed that this metabolite can be present at concentrations superior to those of SN-38. The terminal elimination phases of these two compounds are very similar and, in fact, the ratio of glucuronide to SN-38 was found to be relatively constant at 5–6 h after the infusion, with the value being approximately 4–5. This ratio corresponds closely to that observed in the bile of both patients [2] and rats [4, 5]. The similarity of the elimination phases of these two compounds suggests that the glucuronidation of SN-38 is slower than the rate of elimination of this glucuronide [10]. That is, the glucuronidation of SN-38 is the rate-limiting step in the elimination of SN-38 in the patient studied.

Whether this observation can be generalised to other patients and schedules of administration remains to be seen. However, the presence of relatively high plasma concentrations of the glucuronide would suggest that its formation and elimination could play an important role in the disposition of SN-38 and may be responsible for part of the large inter-patient variability in SN-38 pharmacokinetics observed in other studies. Further pharmacology studies of CPT-11 and its metabolites are therefore essential. Ideally, this should be combined with tests of hepatic and renal function (e.g. bilirubin levels and creatinine clearance, among others) to identify potential factors influencing SN-38 glucuronide dispostion. The inclusion of patients with biliary stents into such studies could also determine whether appreciable enterohepatic recirculation of SN-38 is likely to occur, although preliminary studies indicate that biliary excretion is of only modest importance in the disposition of CPT-11, SN-38 and SN-38 glucuronide [2]. As our technique of investigation required the use of only 100 µl of frozen plasma, retrospective studies using previously collected samples are possible.

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