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Determination of a new podophyllotoxin derivative, TOP-53, and its metabolite in rat plasma and urine by high-performance liquid chromatography with electrochemical detection

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Abstract

A high-performance liquid chromatographic method was developed for the determination of a new podophyllotoxin derivative, TOP-53 (I), and TOP-53 glucuronide (II) as its major metabolite in rat plasma and urine. For the analysis of I, the sample was chromatographed on a reversed-phase C_{18} column with electrochemical detection after consecutive two-step liquid–liquid extractions. Compound II was determined as I after enzymatic hydrolysis of II. This method was validated sufficiently with respect to specificity, accuracy, and precision. The limits of quantitation for both I and II were 2 ng/ml in plasma and 10 ng/ml in urine. The method is thus useful for the pharmacokinetic study of I.

Keywords: Podophyllotoxin derivative; TOP-53

1. Introduction

TOP-53 (I), a new podophyllotoxin derivative, is a drug that has strong activity against lung cancer and lung metastatic cancer. Etoposide, which is being widely used in cancer chemotherapy at present [1,2], is a glucopyranoside group-coupled podophyllotoxin derivative; whereas I, in which an amino alkyl group is introduced at position 4β of podophyllotoxin, is a non-glycoside, non-ether podophyllotoxin derivative, as shown in Fig. 1. Compound I is a potent topoisomerase II inhibitor and displays significant efficacy equivalent to that of etoposide on solid tumors, e.g., Lewis lung carcinoma [3,4]. In addition, I, a hydrochloride salt form derivative, is more highly water soluble than etoposide, and thus has the advantage of being delivered by the intravenous route. Pharmacokinetic studies in rats with labelled compounds have shown that I is highly distributed in tissues – especially in the lungs – and is mainly metabolized to inactive TOP-53 glucuronide (II, Fig. 1).

A chromatographic method with electrochemical detection was developed previously for the sensitive determination of etoposide in plasma [5,6]. This method could possibly also be used to assay I, as I, like etoposide, contains a phenolic hydroxyl group which is electrochemically active. Furthermore, I can be extracted with an organic solvent in neutral solutions, whereas it cannot be extracted with an organic solvent from an acidic aqueous solution. The extraction behavior of I permits further sample clean-

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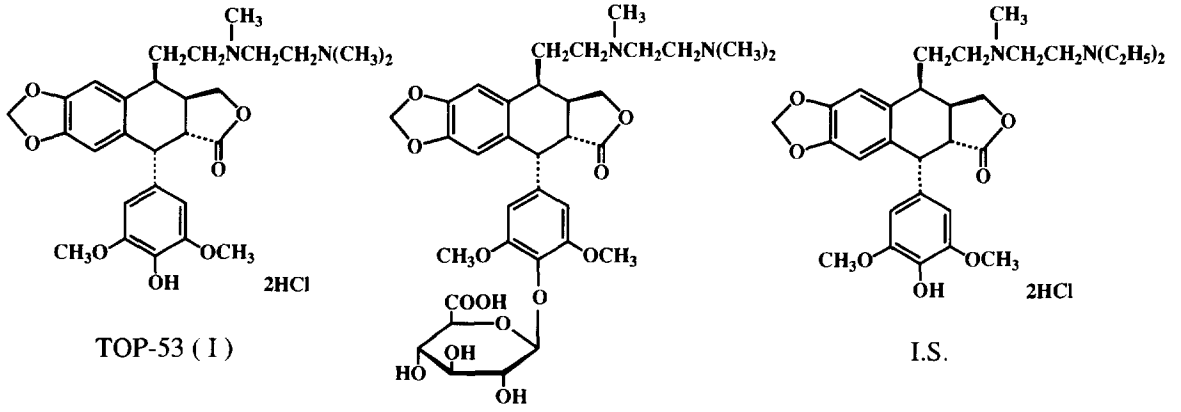

Fig. 1. Structures of TOP-53, TOP-53 glucuronide, and the internal standard (I.S.).

up by back extraction after liquid–liquid extraction as compared with etoposide. On the other hand, II which has no electrochemical activity, needs to be determined as I following enzymatic hydrolysis.

The purpose of our study was to develop a sensitive and selective method for determination of I and II levels for use in pharmacokinetic evaluations. This paper describes a method for the determination of I and II in rat plasma and urine by use of two-step extractions and HPLC with electrochemical detection.

2. Experimental

2.1. Chemicals and reagents

(5*R*, 5a*R*, 8a*R*, 9*S*)-9- [2-[[2-(Dimethylamino) ethyl] methylamino] ethyl]-5, 8, 8a, 9-tetrahydro-5-(4-hydroxy-3,5-dimethoxyphenyl)furo[3′,4′: 6,7]naph-
tho [2,3-*d*]-1, 3-dioxol-6 (5a*H*)-one dihydrochloride (I) and (5*R*, 5a*R*, 8a*R*, 9*S*)-9- [2-[[2-(diethylamino) ethyl]methylamino]ethyl]-5, 8, 8a,9-tetrahydro-5-(4-hydroxy-3, 5-dimethoxyphenyl)furo[3′, 4′: 6, 7] naphtho [2,3-*d*]-1, 3-dioxol-6 (5a*H*)-one] dihydrochloride as the internal standard (I.S., Fig. 1) were synthesized by Taiho Pharmaceuticals (Tokyo, Japan) [3]. β-Glucuronidase (type IX-A from *E. coli*) was purchased from Sigma (St. Louis, MO, USA). Methanol of HPLC-grade and acetic acid of special grade were obtained from Nacalai Tesque (Kyoto, Japan). Potassium dihydrogenphosphate and disodium hydrogenphosphate-12 H_2O of special grade and dichloromethane and ethyl acetate of HPLC-grade were obtained from Wako Pure Chemical Industries (Osaka, Japan). Purified water from a Milli-Q system (Waters, Milford, MA, USA) was used.

2.2. Preparation of standard solution

A stock solution of I (20 μg/ml) was prepared by dissolving a weighed amount of I (2 mg) in 100 ml of 50 m*M* phosphate buffer of pH 3.0. Working solutions of I (20, 50, 200, 500 and 2000 ng/ ml) were prepared by successively diluting the stock solution with 20 m*M* phosphate buffer of pH 3.0. A 20 μg/ml stock solution and a 500 ng/ml working solution of I.S. were prepared in a similar way.

All solutions were stable for at least 3 months at 4°C.

2.3. Instrumentation and chromatographic conditions

The HPLC system consisted of an LC-6A pump, an SIL-6B automatic injector, an SCL-6B system controller, a C-R4AX integrator (Shimadzu, Kyoto, Japan), and an ECD-100 electrochemical detector (EICOM, Kyoto, Japan).

The mobile phase was methanol–0.1 *M* phosphate

buffer of pH 7.0 (43:57, v/v), at a flow-rate of 1.0 ml/min. For the electrochemical detection, a working electrode of glassy carbon and an Ag/AgCl reference electrode were used, with an applied potential of +0.7 V. Either a Develosil ODS-HG-5 (150×4.6 mm I.D.) from Nomura Chemicals (Seto, Japan) or an Inertsil ODS-2 (150×4.6 mm I.D.) from GL Sciences (Tokyo, Japan) were used as analytical column, because they gave nearly the same results for retention time and peak profile of I. The column temperature was kept at 35°C by the water bath of the ECD-100 electrochemical detector.

2.4. Plasma sample preparation

The plasma sample for the analysis of I was prepared as follows: 50 μl of an I.S. solution (500 ng/ml in 20 m*M* phosphate buffer of pH 3.0) and 0.1 ml of 0.5 *M* phosphate buffer of pH 6.8 were added to a volume of 0.5 ml of rat plasma in a glass tube. The solution was extracted with 4 ml of dichloromethane by shaking for l0 min and centrifuged at 1200 *g* for 5 min. The organic layer was transferred into a new tube. The extraction process was performed once more. The organic layer was evaporated under a stream of nitrogen, and the residue was redissolved in 0.2 ml of 0.1 *M* phosphate buffer of pH 3.0. The solution was washed with 0.8 ml of ethyl acetate by vortexing for 1 min and centrifuged at 1200 *g* for 5 min. The organic layer was removed by pipetting and the organic solvent miscible with aqueous layer was removed by evaporation under a stream of nitrogen. A 50-μl sample of the aqueous layer was injected into the HPLC system.

The plasma sample for analysis of II was prepared as follows: the aqueous layer after the extraction process according to the previous procedure of sample preparation for I was concentrated to 1/3 volume by evaporation under a stream of nitrogen. Fifty microliters of the I.S. solution and 0.3 ml of approximately 170 000 units/ml β-glucuronidase in 0.1 *M* phosphate buffer of pH 7.0 were added to the concentrated solution, and the solution was incubated at 37°C for 4 h with gentle shaking. After the incubation, 0.1 ml of 0.5 *M* phosphate buffer of pH 6.8 was added; and the extraction and washing process were performed twice in a similar way to the sample preparation for I. A 50-μl sample of the aqueous layer was injected into the HPLC system.

Plasma from dogs showed the same results as those obtained from rat plasma.

2.5. Urine sample preparation

The procedure for the preparation of I in rat urine was the same as that for I in plasma except that a sample of 0.1 ml of urine and 0.4 ml of water were used instead of 0.5 ml of plasma, and the washing process was performed twice.

The procedure for the preparation of II in urine was the same as that for II in plasma except that a sample of 0.1 ml of urine and 0.4 ml of water were used instead of 0.5 ml of plasma.

2.6. Calibration standard preparation and calibration curve

The calibration standards were prepared by adding 50 μl of each standard spiking solution (20, 50, 200, 500, 2000 ng/ml I) to 0.5 ml of water, and were then processed as described Section 2.4.

The calibration curve was obtained from a weighted ($1/\text{ratio}^2$) least-square linear regression of I/I.S. peak-area ratio versus the I concentration. The curve was used to calculate concentrations of both I and II as I after the enzymatic hydrolysis.

2.7. Pharmacokinetic study in rats

The method was used to measure I and II in plasma after intravenous administration of 3 mg/kg I to male Donryu rats. Blood samples drawn after the administration were heparinized and centrifuged at 1200 *g* for 15 min at 4°C. The resulting plasma samples were stored at −20°C until analyzed.

3. Results and discussion

3.1. Chromatography and specificity

Representative chromatograms for the analysis of I are shown in Fig. 2 and Fig. 3. The retention times for I and I.S. are approximately 17 and 24 min, respectively (Fig. 2C and Fig. 3C). There were

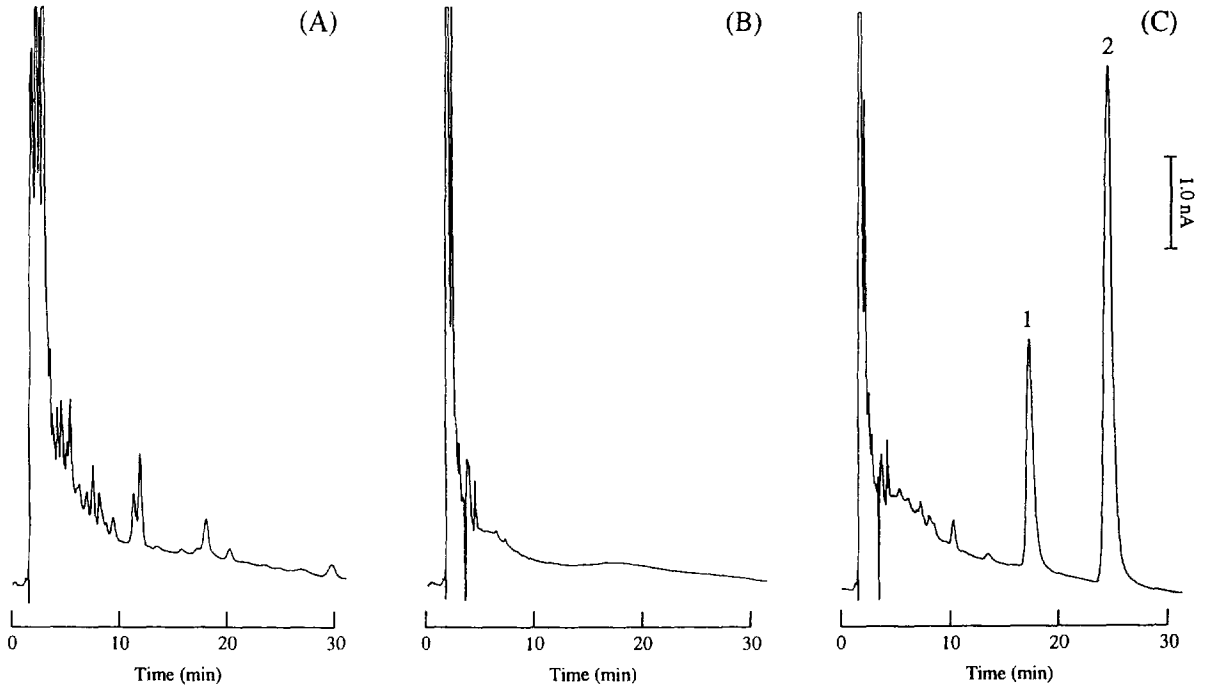

Fig. 2. Chromatograms of (A) a blank plasma sample extracted with dichloromethane, (B) a blank plasma sample prepared by the present method, and (C) a spiked plasma sample prepared by the present method. Peak 1=I (20 ng/ml), 2=I.S.

interfering peaks in the chromatograms of blank plasma and urine that were only extracted with dichloromethane (Fig. 2A and Fig. 3A). To remove the interfering substances, I was back-extracted with 0.1 *M* phosphate buffer of pH 3.0 after the extraction. In the resulting chromatograms, there was no interfering peak, as shown in Fig. 2B,C and Fig. 3B,C. The clean-up by the back-extraction was sufficiently effective for the analysis of II as well.

This method showed no interfering peaks in any of the chromatograms for plasma and urine from six rats.

3.2. Linearity

The calibration curve for both I and II was linear from 2 to 200 ng/ml and from 10 to 1000 ng/ml corresponding to the concentration in plasma and urine, respectively. The weighted least-squares linear regression equation from the curves ($n=5$) for plasma was $y=(0.02004\pm0.00039)x-(0.00230\pm 0.00061)$ with correlation coefficients greater than 0.998.

The calibration standards were not the plasma and urine standards spiked with I; however, the following data on precision and accuracy suggest that the resulting calibration curve was good enough to determine the plasma and urine samples.

3.3. Precision and accuracy

The precision and accuracy in the intra-assay of I were evaluated by the simultaneous determination of spiked plasma and urine samples in replicates of five. The inter-assay precision and accuracy of I were evaluated by determination of spiked plasma and urine samples in five separate runs. Plasma was spiked with concentrations of I of 2, 20 and 200 ng/ml; urine was spiked at concentrations of 10, 100, and 1000 ng/ml in the assay. The intra- and inter-assay precision and accuracy data for of I are shown in Table 1.

In the intra-assay for plasma, the C.V. was ≤5.3% and the relative error (R.E.) ranged from 2.4 to 5.4%. In the inter-assay for plasma, the C.V. was ≤4.3% and the R.E. ranged from −2.7 to 1.4%. In the

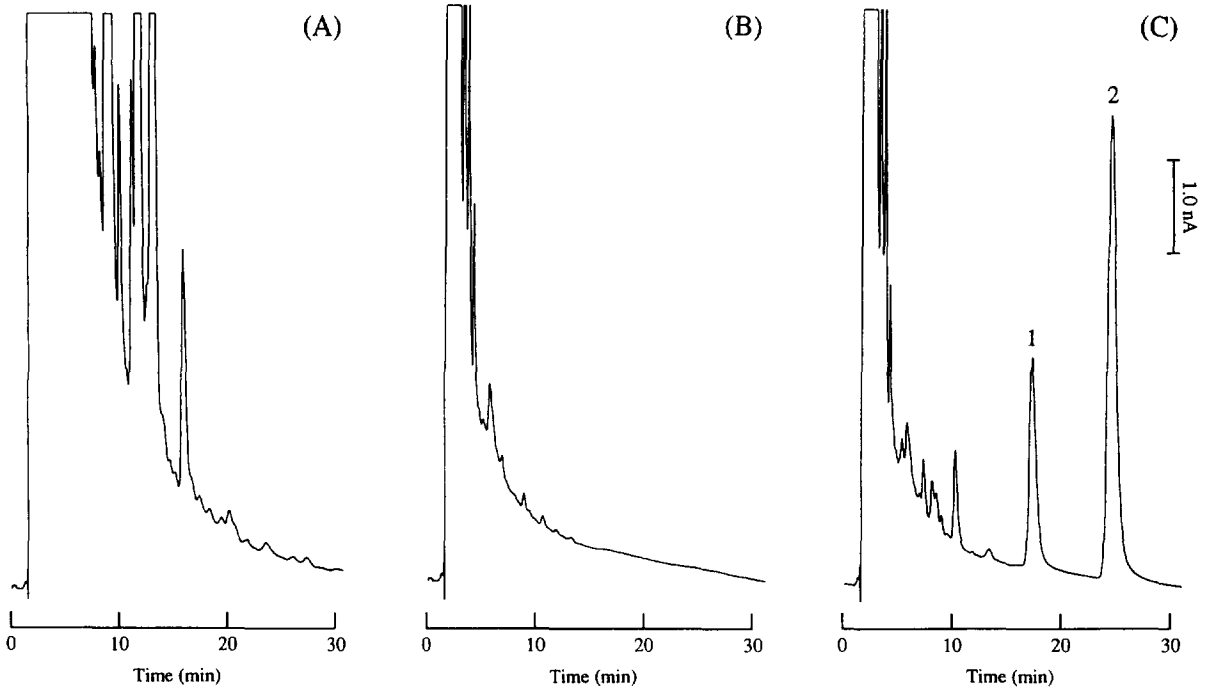

Fig. 3. Chromatograms of (A) a blank urine sample extracted with dichloromethane, (B) a blank urine sample prepared by the present method, and (C) a spiked urine sample prepared by the present method. Peak 1=I (100 ng/ml), 2=I.S.

intra-assay for urine, the C.V. was ≤4.5% and the R.E. ranged from −1.7 to 2.9%. In the inter-assay for urine, the C.V. was ≤5.9% and the R.E. ranged from 1.6 to 4.5%. Based on the precision and accuracy, the limits of quantitation of I in plasma and urine were set at 2 ng/ml and 10 ng/ml, respectively.

As II could not be obtained synthetically, the precision and accuracy in the assay of II were evaluated by both the enzymatic hydrolysis efficiency data on II and the precision and accuracy data on I (Table 1). The efficiency measured preliminarily by use of II in rat bile obtained following the administration of labelled I was 94.2±10.1% for plasma and 90.1±9.9% for urine (n=3).

Table 1
Accuracy and precision of the assay for I in rat plasma and urine

Concentration (ng/ml)	Intra-assay (n=5)		Inter-assay (n=5)	
	C.V. (%)	R.E. (%)	C.V. (%)	R.E. (%)
Plasma				
200	5.3	5.3	2.1	−0.2
20	1.2	2.4	4.3	−2.7
2	4.3	5.4	3.9	1.4
Urine				
1000	2.1	2.9	0.8	1.8
100	1.8	0.8	1.7	1.6
10	4.5	−1.7	5.9	4.5

C.V.=coefficient of variation. R.E.=relative error.

3.4. Stability

The stability of I in rat plasma and urine was investigated. Compound I in plasma and urine was stable at 4°C for at least 12 h; the R.E. from the initial value was −7.7% at 100 ng/ml in plasma and −1.8% at 500 ng/ml in urine. In addition, I in plasma and urine was stable at −20°C for at least 3 months; the R.E. (n=3) from the initial value was −5.9% at 50 ng/ml in plasma and 0.6% at 250 ng/ml in urine. These results suggest that no degradation of I occurred during the handling and storage of plasma and urine samples.

The stability of II in rat plasma and urine was not examined; however, crudely labelled II added to

human plasma or urine was found to be stable at −20°C for at least 3 months (data not shown).

3.5. Analysis of pharmacokinetic study samples

The assay was applied to the determination of I and II in plasma after intravenous administration of 3 mg/kg I to rats. Fig. 4 shows the plasma concentration versus time profiles of I and II. The profiles of I and II on and after 1 h were nearly the same. The method was sensitive enough to measure the concentrations of I and II in plasma up to 24 h after administration.

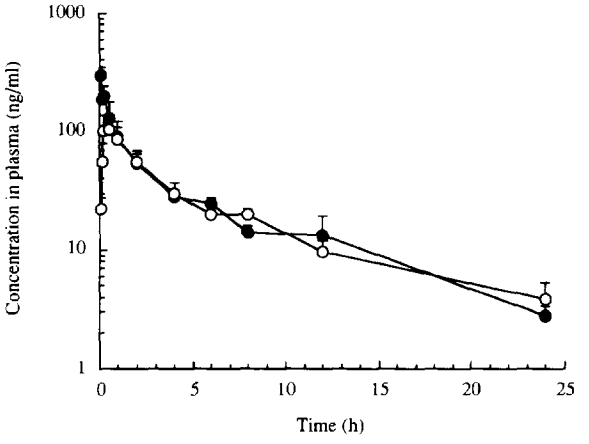

Fig. 4. Plasma levels of I (●) and II (○) after intravenous administration of 3 mg/kg to rats. II concentrations are presented as I concentrations. Each point represents the mean±S.D. ($n=3$).

4. Conclusions

The present report describes an assay to determine I and its metabolite, II, in rat plasma and urine. The assay based on HPLC with electrochemical detection and the sample preparation by two-step liquid–liquid extractions, resulted in high sensitivity and selectivity. The analytical method was sufficiently validated for pharmacokinetic studies in rats.

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