大韓獸醫學會誌(1993) 第33券 第4號
Korean Vet Res (1993) 33(4) : 605～609

Active metabolites in rat bile after intravenous injection of [^{3}H] pteroylglutamic acid

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(Received September 20, 1993)

랫드에 있어서 [^{3}H]pteroylglutamic acid의 靜脈注射後 膽汁中 活性代謝物에 관한 研究

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(1993년 9월 20일 접수)

초록 : 항암치료의 조절인자로서 중요시되는 활성엽산대사물의 腸肝순환동태를 규명하기 위해 랫드에 [^{3}H] pteroylglutamic acid(PteGlu)를 정맥주사한 후 고성능 액체 크로마토그래피법(HPLC)을 이용해 담즙중 활성대사물에 관해 조사하였다. HPLC-liquid scintillation counting법의 분석결과, 현저한 다섯개의 방사활성 피크가 출현, 이들은 H_4PteGlu, 10-HCO-H_4PteGlu, 5-CH_3-H_4PteGlu 와 para-aminobenzoyl glutamate (*p*ABG), PteGlu로 각각 동정되었다. 전자 3물질의 활성엽산대사물의 동정은 표준물질과의 비교분석에 의해 HPLC-전기화학검출기를 이용한 retention time profile 및 hydrodynamic Voltammogram의 일치성 그리고 HPLC-photodiode array 분석에 의한 흡광스펙트럼의 일치성에 근거하였다. 후자 그 물질의(*p*ABG 및 PteGlu)동정은 흡광스펙트럼의 일치성에 근거하였다. 랫드 담즙중 [^{3}H]PteGlu의 활성엽산대사물로서 H_4PteGlu의 존재가 처음으로 밝혀졌다.

Introduction

Folate metabolites have been considerably studied from their pharmacological aspects, for the purpose of reducing the toxicity of antifolates and to enhance the activity of fluorouracil related to one-carbon metabolism in anticancer therapy.[1～7] Whereas kinetics of the metabolites in enterohepatic circulation which takes an important role on folate metabolism has been seldomly investigated. Although a few authors[8～11] demonstrated several forms of folate metabolites in bile after intravenous or extravascular dosing of [^{3}H] pteroylglutamic acid([^{3}H] PteGlu), there have been variations in the forms of metabolites by authors. The variations have probably resulted from the difference in chromatographic techniques used. Recently, we have developed a sensive method to determine active folate metabolites including tetrahydrofolate(H_4PteGlu)using high-performance liquid chromatography with electrochemical detection system(HPLC-ECD).[12～14] In the present study, the qualitative analysis of metabolites in rat bile after an intravenous injection of [^{3}H] PteGlu was examined using HPLC system.

Materials and Methods

[3',5',7,9-^{3}H] PteGlu was obtained from Amersham International Plc.(Buckinghamshire, England)(Fig 1). [6R,S] -H_4PteGlu and disodium salt of [6R,S] -5-methyltetrahydrofolate(5-CH_3-H_4PteGlu)were obtained from Sigma Chemical Co.(St. Louis, USA), *para*-aminobenzoyl glutamate(*p*ABG)from Fluka Chemika(Buchs, Switzerland) and p-

teroylglutamic acid(PteGlu) form Wako Pure Chemical Industries Ltd.(Osaka, Japan). Calcium salt of [6R, S]-5-formyltetrahydrofolate(5-HCO-H_4PteGlu) was a gift from Lederle Japan Co.(Tokyo, Japan). 10-formyltetrahydrofolate(10-HCO-H_4PteGlu)was prepared from [6R,S] -5-HCO-H_4PteGlu using the method described by Scott.[15]

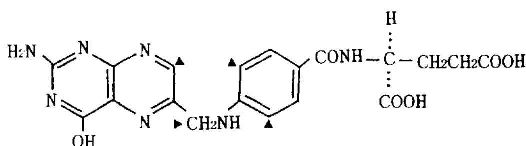

Fig 1. Chemical structure of [^{3}H] PteGlu.

Female Sprague-Dawley rats(about 13 weeks, Clea Japan)were used. The animals were anesthetized with urethan(intraperitoneal, 0,8-1.0g/kg). Abdominal wall was incised and bile duct cannulated with a polyethylene catheter. [^{3}H] PteGlu(specific activity, 20~50 Ci/mmol) was diluted with PteGlu solution and intravenously injected to rat in a dose of 240 × 10^6 dpm/kg of body weight(PteGlu 1.1 mg/kg). Bile samples were collected into ice-cold test tubes containing sodium ascorbate(0.2%) at intervals of 30min. An aliquot of the sample(100 $\mu\ell$)was subjected to the HPLC with a liquid scintillation counting system(HPLC-LSC). The rest of the bile sample was infused into duodenum for enterohepatic recirculation. For the identification of metabolites, bile samples of other rats after intravenous injection of PteGlu were also collected and stored at -80℃ until the HPLC analysis.

Chromatographic analysis was performed using HPLC systems having four detectors including an electrochemical detector(L-ECD-6A, Shimadzu Co., Japan), a photodiode array detector(Multi-340, Jasco, Japan), a liquid scintillation counter(Trace Ⅱ 7150, Hewlett Packard, Penna, USA) and an ultra-violet detector(UV ; SPD-6A, Shimadzu). A pump(LC-9A, Shimadzu) and an analytical column(C_8, RP-8250 X 4mm I.D., E-Merck, Germany) were used. The flow rate was 0.8 mℓ/min.

Retention times and hydrodynamic voltammograms of active metabolites in rat bile and standards(H_4PteGlu,10-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu)were examined using HPLC-ECD method. Retention time profiles were determined for the various pH values(3.5, 4, 4.5 and 5) of acetate buffer, and for the various fractions(3, 4, 6 and 8%) of acetonitrile in mobile phase. The voltammograms were obtained by responses versus applied potentials of the electrochemical detector. The mobile phase was 20 mM acetate buffer containing 0.1 mM EDTA and acetonitrile(94 : 6, v/v).

Spectral curves of UV absorbance of bile metabolites and standards(H_4PteGlu, 10-HCO-H_4PteGlu, 5-CH_3-H_4PteGlu, *p*ABG and PteGlu) were obtained using HPLC with a photodiode array detection system(HPLC-PAD). Mobile phase used in HPLC-PAD analysis was a mixture of 50mM phosphate buffer(pH 5.0) and acetonitrile(95 : 5, v/v).

Results

Radioactive metabolites in rat bile after the intravenous injection of [^{3}H] PteGlu were analyzed using HPLC-LSC system. Fig 2 shows a chromatogram of bile sample collected between 2.5 and 3 hr after dosing. Five predominat radioactive peaks(I-V) were observed on the chromatogram of HPLC-LSC.

For identification of the compounds, HPLC-ECD and HPLC-UV system were employed. Fig 3~5 demonstrates the retention time profiles and the hydrodynamic vol-

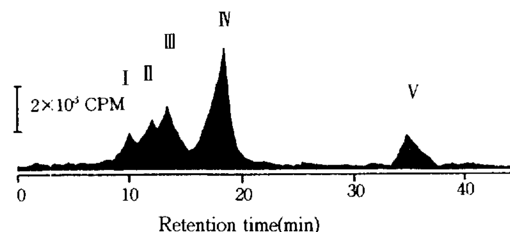

Fig 2. Chromatogram of HPLC-liquid scintillation counting system. The bile sample was collected at 2.5-3 hr after an intravenous injection of [^{3}H] PteGlu. The mobile phase was a mixture of 20 mM acetate buffer(pH 3.5)containing 0.1 mM EDTA and acetonitrile(95 : 5, v/v).

tammograms of active folate metabolites in bile and standards. The consistency of the retention time profiles between the metabolites(Ⅱ , Ⅲ and Ⅳ)and standards(H_4 PteGlu, 10-HCO-H_4 PteGlu and 5-CH_3-H_4PteGlu) was obtained for the various pH values of acetate buffer(Fig 3) and for the various concentrations of acetonitrile(Fig 4) in the mobile phase. The hydrodynamic voltammograms of the bile active metabolites indicating peaks Ⅱ , Ⅲ and Ⅳ were almost identical to those of H_4 PteGlu, 10-

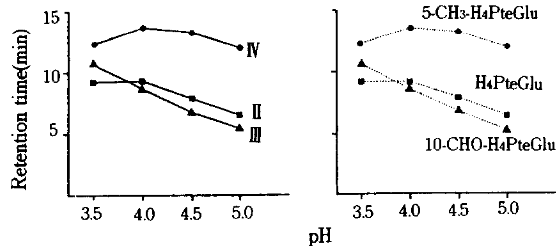

Fig 3. Retention time profiles of bile active metabolites (solid line) and standards(dotted line) under various pH values of acetate buffer in mobile phase. Fraction of acetonitrile in mobile phase was fixed at 6%.

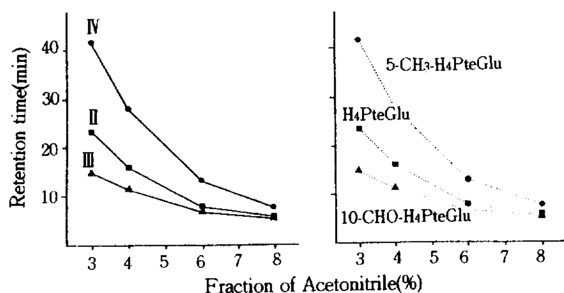

Fig 4. Retention time profiles of bile active metabolites (solid line) and standards(dotted line) under various fractions of acetonitrile in mobile phase.The pH value of acetate buffer in mobile phase was fixed at 4.5.

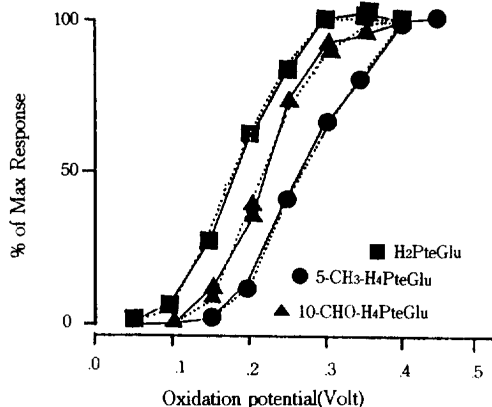

Fig 5. Hydrodynamic voltammograms of bile active metabolites(solid line) and standards(dotted line).

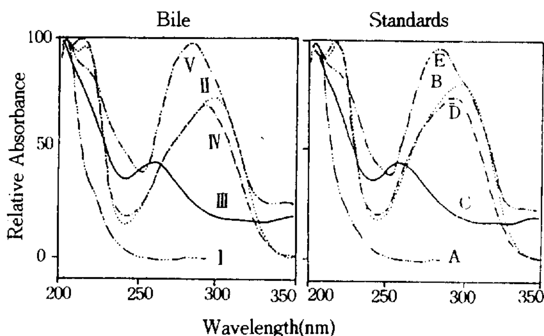

Fig 6. UV absorption spectra of bile metabolites including parent compound and standards(A : *p*ABC, B: H_4PteGlu, C : 10-HCO-H_4PteGlu, D : 5-CH_3-H_4PteGlu and E : PteGlu). These spectra were obtained at pH 5 from HPLC-photodiode array detection system.

HCO-H_4 PteGlu and 5-CH_4-H_4 PteGlu, respectively(Fig 5). Furthermore, the UV absorbance spectral curves of the bile metabolites obtained from peaks Ⅱ, Ⅲ and Ⅳ were very similar to those of H_4PteGlu, 10-HCO-H_4 PteGlu and 5-CH_4PteGlu, respectively(Fig 6). The other peaks I and V in bile also showed almost identical UV absorption characteristics with *p*ABC and PteGlu, respectively(Fig 6).

Discussion

Active folate metabolites of [^{3}H] PteGlu in bile have been investigated by several authors.[8~11] Steinberg et al.[8] and Pheasant et al.[9] demonstrated bile excretion of radioactive 5-CH_3-H_4 PteGlu and 10-HCO-H_4PteGlu after an intravenous injection of [^{3}H] PteGlu in rats. Lavoi et al.[10] and Hillman et al.[11] demonstrated 5-CH_3-H_4PteGlu, 10-HCO-H_4PetGlu and 5-HCO-H_4PteGlu as active metabolites of [^{3}H] PteGlu in bile. Their results therefore revealed some variations in the forms of metabolite of [^{3}H] PteGlu. Most of folate metabolites are susceptible to oxidation, and several kinds of metabolite are easily convery into another metabolite under a given pH or temperature.[16,17] Early studies[8~11], employed mainly the method of gel filtration, DEAE-sephadx chromatography or differential microbiological assay for the identification of the metabolites. These method requires fractionation or incubation of bile sample to separate the metabolites. However, we employed the HPLC-ECD method in which bile sample was not fractionated or incubated.

The present identification for the active metabolite H_4PteGlu, 10-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu in rat bile after intravenous injection of [^{3}H] PteGlu was based on the consistency of retention time profiles(Fig 3, 4), hydrodynamic voltammograms(Fig 5) and UV absorbance spectra(Fig 6) between the bile active metabolites

and standards. These observations indicate that bile metabolites from peaks Ⅱ, Ⅲ and Ⅳ have(1)the same negative logarithm of acid dissociation constant(pKa) and the same lipid solubility, (2) the same electrochemical properties or redox potentials and (3) the same UV absorption characteristics with H_4PteGlu, 10-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu, respectively. The other peaks(Ⅰ, Ⅴ)were also identified as *p*ABG and Parent compound PteGlu, respectively, based on the consistency of UV absorption spectra(Fig 6). Active metabolite 5-HCO-H_4PteGlu was not detected in this study.

In conclusion, the predominant metabolites of [^{3}H] PteGlu in rat bile after intravenous injection were recognized to be H_4PteGlu, 10-HCO-H_4PteGlu, 5-CH_3-H_4PteGlu as active folate metabolites and *p*ABG as degraded metabolite of PteGlu. H_4PteGlu which takes a central role as the acceptor of one-carbon unit in folate metabolism was firstly cleared in rat bile as an active metabolite of [^{3}H] PteGlu.

Summary

Active metabolites in rat bile after an intravenous injection of [^{3}H] pteroylglutamic acid(PteGlu)were studies using high-performance liquid chromatography(HPLC). Predominant four radioactive metabolites and parent compound PteGlu were detected on the chromatogram of HPLC with liquid scintillation counting system. These metabolites were identified as tetrahydrofolate, 10-formyltetrahydrofolate, 5-methyltetrahydrofolate and *para*-aminobenzoyl glutamate. The identification of active folate metabolites was based on the consistency of retention time profiles and hydrodynamic voltammograms which were obtained by HPLC with the electrochemical detection system, and characteristics of UV absorbance spectra obtained by HPLC with photodiode array detection system.

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