Fig. 5 Question 5 : "You think that there is no need to wash hands for examination of the next patient if you pull on disposer gloves."

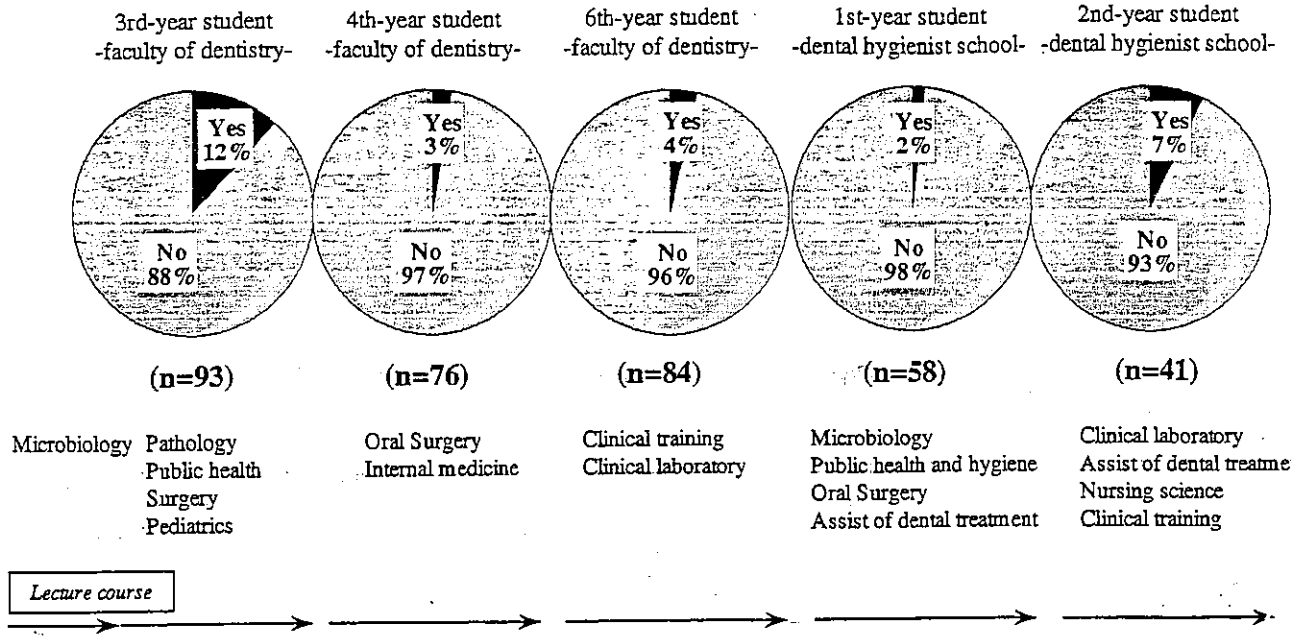
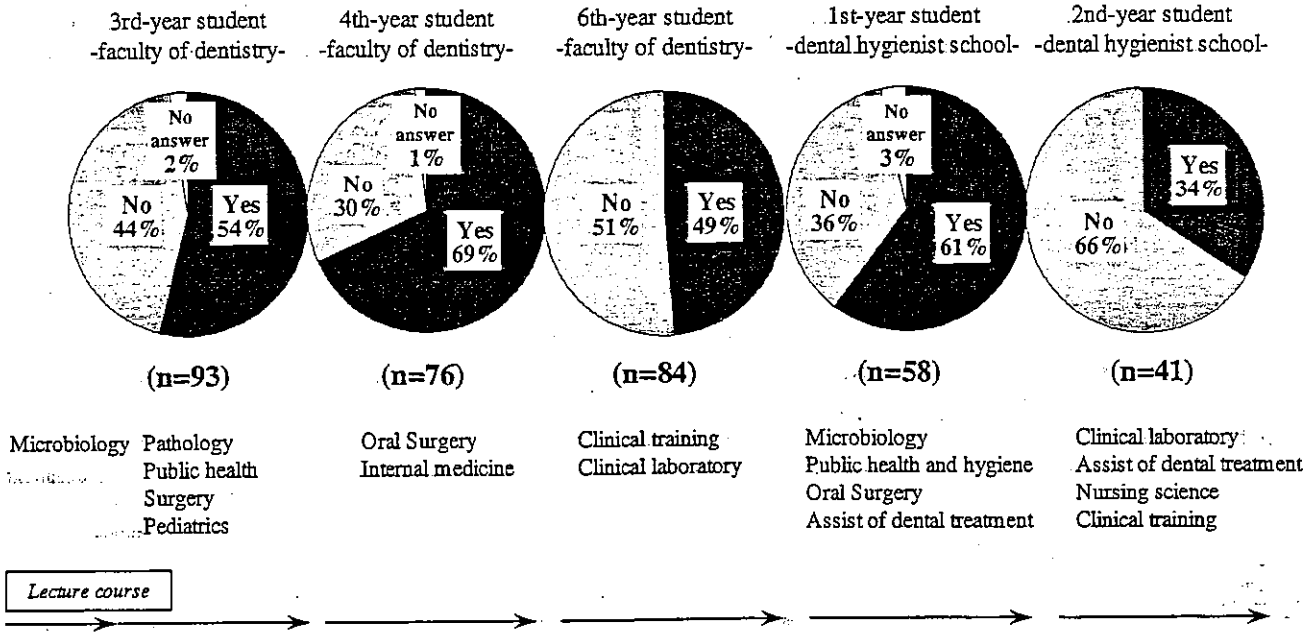



Fig. 6 Question 6 : "You think that you can prevent cross transmission by hand-washing using glutaraldehyde after you examined the patient of hepatitis C."




療用の器具で薬液ビンから薬液を取り出す行為は問題ないと考えていた (Fig. 9). また, Fig. 10 に示すようにHCV抗体を中和抗体として捉える学生が全体の29.5% (104/352名) にも及び, HCV抗体陽性患者を感染症ではないと考えている可能性も明らかになった. インターフェロン療法によってHCVを駆除することのできた所謂ウイルス持続陰性化 (Sustained Viral Response : SVR) 症例でも, 大半の学生が感染症として取り扱うと答えており (82.9%, 299/352名, Fig. 11),

Fig. 7 Question 7 : "You think that chlorhexidine is ineffective in killing the virus."

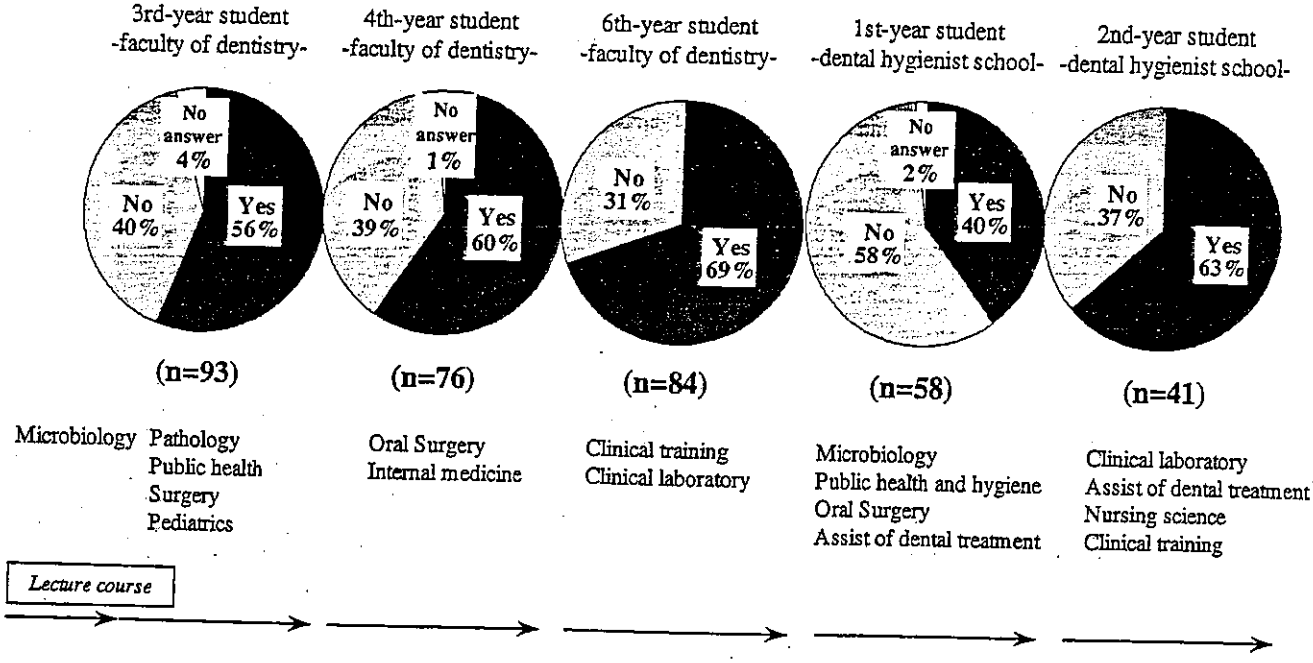
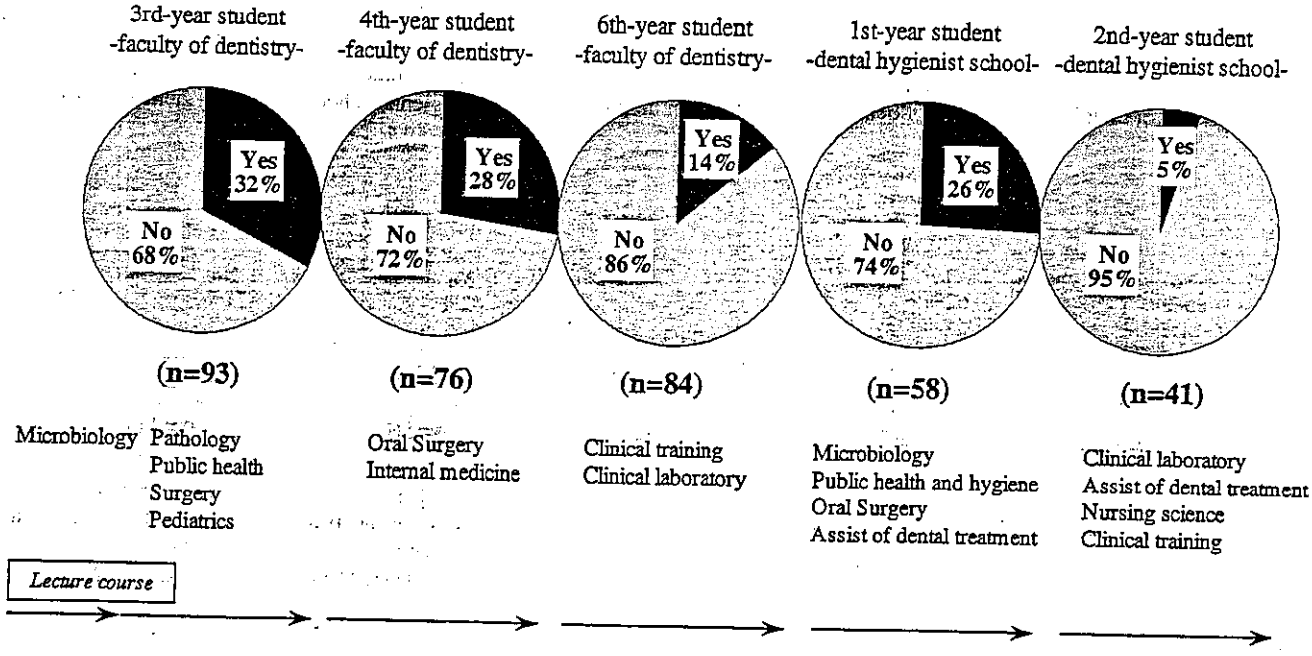



Fig. 8 Question 8 : "You think that you use 100% ethanol as effective sterilization of plastic or rubber instruments which can not sterilize by autoclave among instruments contaminated by HCV or HBV."




HCV RNA が持続陰性化しても歯科治療では感染症として捉えられている可能性が高い．スタンダードプレコーションという言葉を，6年生以外はほとんど聞いたことがないという実態も明らかになった（Fig. 12）．

## 考　察

わが国の肝癌による死亡者数は増加の一途をたどり，この傾向は2015年まで続くと考えられている[5]．肝癌の原因の約80%が，HCV に起因するものであり，HCV による肝癌患者の増加がわが国に

Fig. 9 Question 9 : "You think that there is nothing wrong with catching directly pledget by used pincette, and with getting used pincette into medicine bottle if the patient don't have infectious disease"

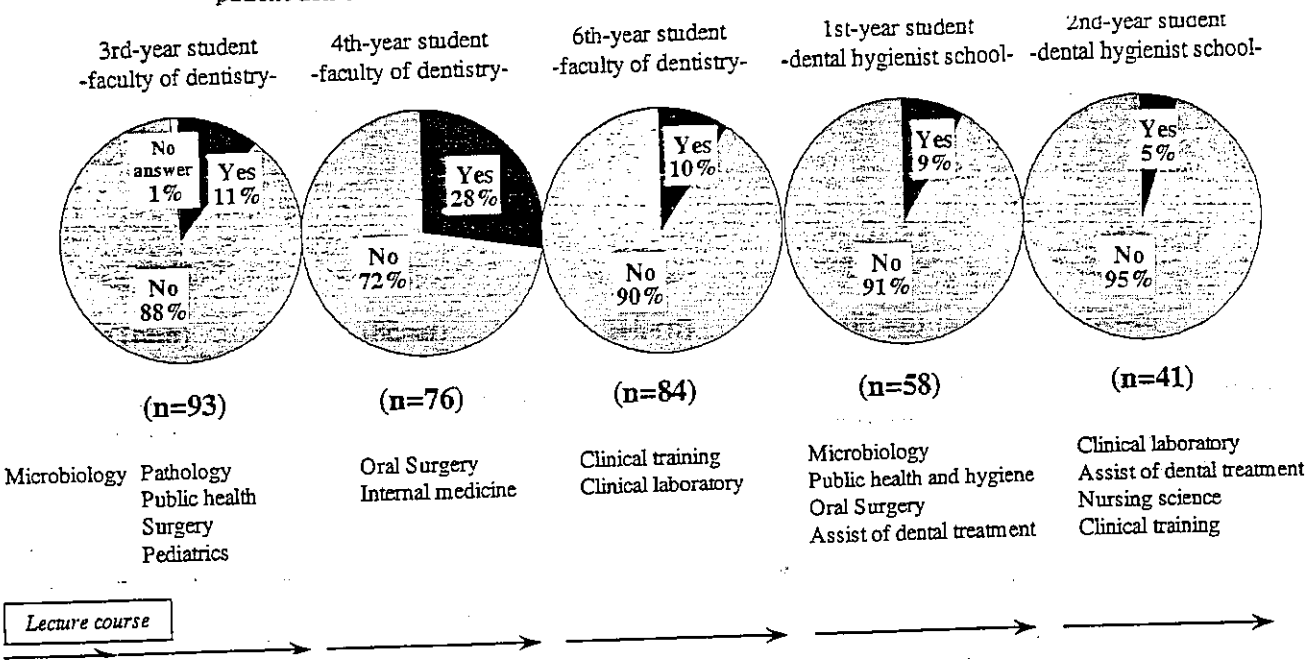
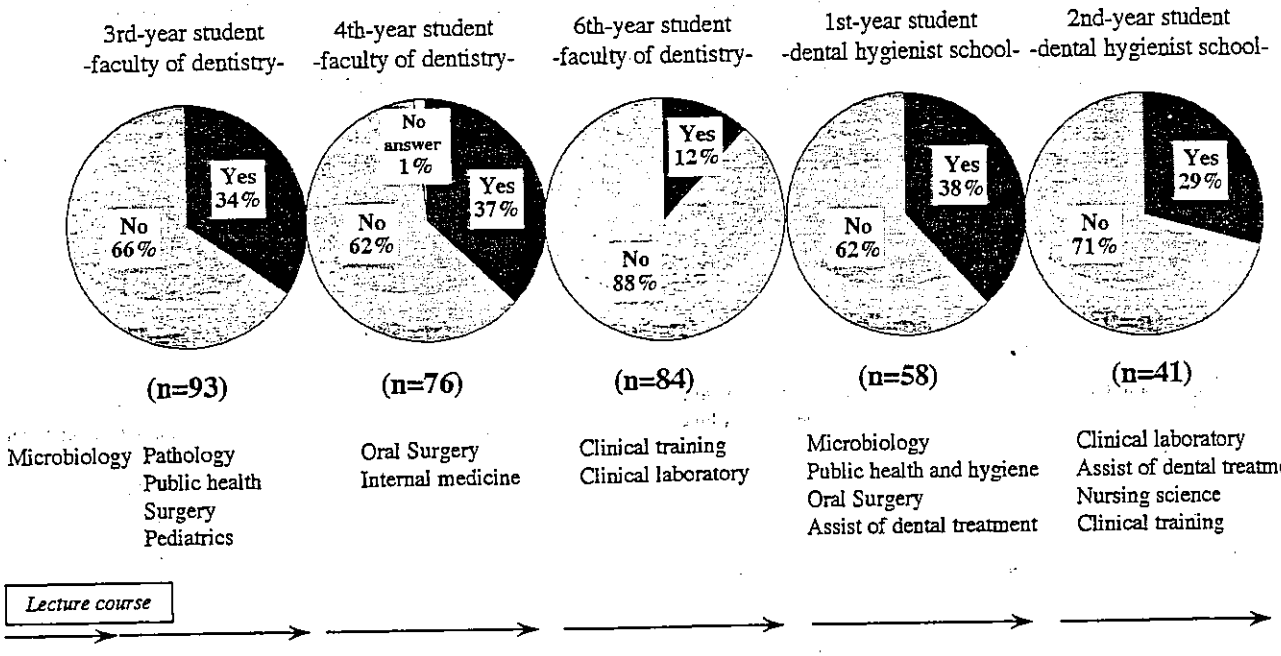



Fig. 10 Question 10 : "You think that you won't need to worry about contraction of hepatitis C if an anti-HCV antibody is detected within blood (in other words anti-HCV positive)."




おける肝癌死亡者数の増加の原因である．現在，わが国では年間 34,000 人が肝癌で死亡しており，日本人の死亡原因の第三位を占めている．平成 14 年度から老人保健法に基づいて 40 歳から 70 歳までの 5 歳刻みの節目の年齢者や過去に肝機能異常を指摘されたことのある者は，節目あるいは節目外検診として HCV 並びに HBV のキャリアの発見と肝発癌抑制を見据えた効率の高い検診を受け

Fig. 11 Question 11 : "You think that you don't need to treat the patient whom exterminated HCV RNA in serum by interferon therapy as high-risk infectious patient in dental treatment"

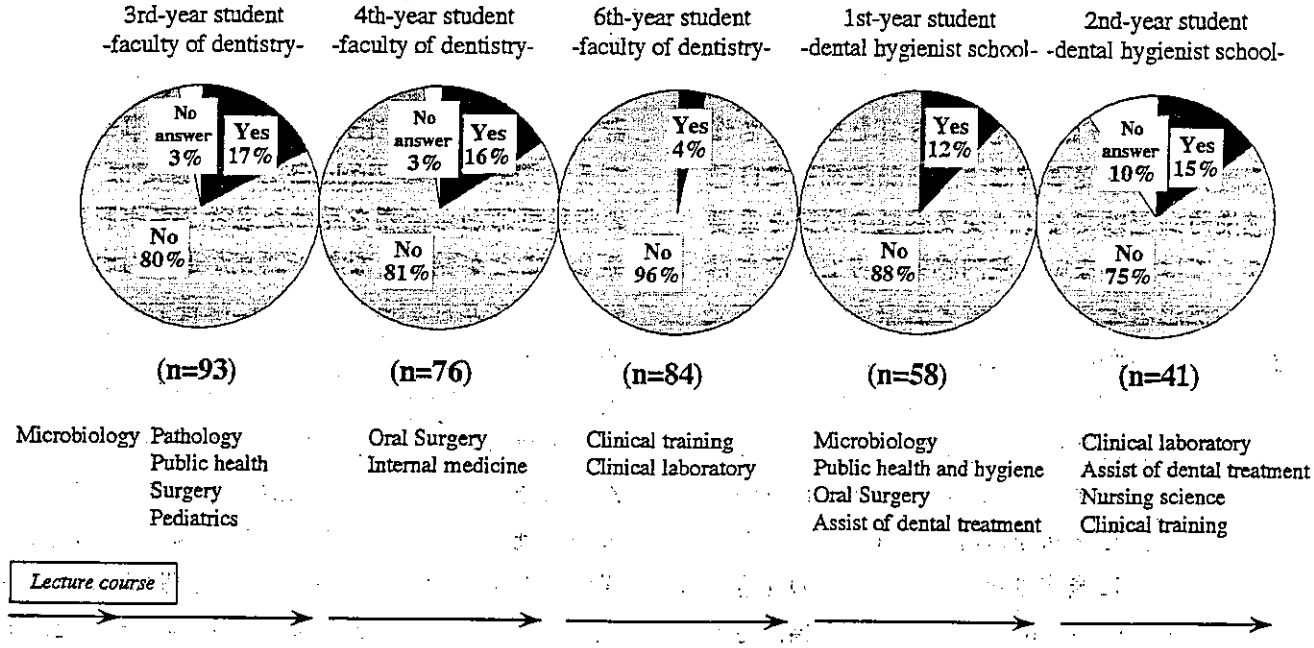
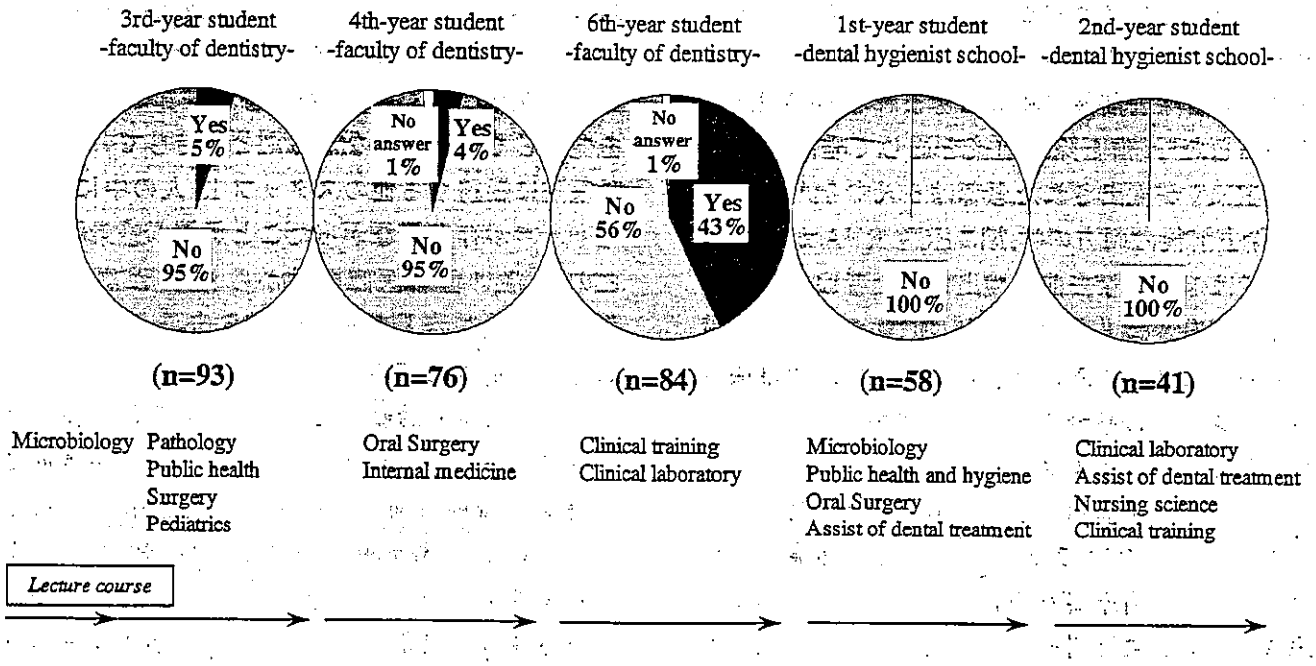



Fig. 12 Question 12 : "Do you know the word of standard precautions?"




られるようになった．現在，肝癌撲滅の事業は国家レベルで推し進められている．日本は，世界的にみてもこのHCV感染が高い国である．そして，その感染率は東日本よりも西日本に高く，したがって西日本は肝癌死亡率も高い．このような社会的背景を，医療に携わる者は周知する必要がある．

平成14年度の厚生労働省の肝炎研究班は，全国調査に基づくC型急性肝炎の感染経路および治療に関する研究報告の中で，その感染ルートとして医原性感染の可能性が30%（33/109名）を占めていることを報告した[3]．この33名の中で感染

ルートを詳細に解明できた症例は存在しないが，感染ルートの内訳として外科手術34%（11/33名），輸血15%（5/33名），静脈注射12%（4/33名），観血手技9%(3/33名)，内視鏡検査9%(3/33名)，歯科治療9%（3/33名），透析3%（1/33名），詳細不明9%(3/33名)であったと報告されている．我々医療従事者は，感染源に対して自己への感染防御だけでなく，交叉感染の防御を重要視しなくてはならない．院内感染の防止策の主目標は，当然患者と患者，患者と医療従事者間の交叉感染防止に力が注がれなければならない．

歯科治療では，鋭利な器具が使用されるだけでなく，歯牙切削時に容易に歯肉から出血するため，血液を扱うことは多い[6]．感染源となる血液が混じった唾液を，高速回転の器具や電気エンジンによって診療室内に飛沫させており，細心の注意が必要である．HBVやHCVが唾液からも検出されるという論文は数多く存在する[7][8]．したがって，歯科医療従事者は，歯科治療を通じて肝炎ウイルスに感染するリスクが高いが，その一方で，交叉感染防止に努める義務がある．我々は，HCVキャリアの歯石除去前後の唾液に含まれるHCV RNAを検出した[9]．歯石除去前後を通じて唾液中からHCV RNAが検出された患者は6人中3人であった．除去前後の両方の唾液にHCV RNAが検出された者は1人，除去前に検出された者は1人，除去後の検出者は1人であった．HCVキャリアの歯科治療では，HCV RNAが歯肉溝滲出液，印象採得時の印象材，診療台の作業台，エアータービンのハンドピース，ホルダー，吸引嘴管，鉗子，デンタルミラー，切削バーからも検出されることが報告されているだけでなく[4][10][11]，HCV感染者に使用された歯科治療器具の表面に付着した器具からHCV RNAが数日間検出され続けたという報告もある[12]．ディスポーザブルの局所麻酔用のカートリッジの残液を再使用することは，逆流させた吸引血液によってHBVやHCVを感染拡大させる極めて危険性の高い行為である．このように卒業を控えた歯学部の6年生や歯科衛生士学校の2年生でさえも，滅菌に対する知識が不足している実態が明らかとなり，早急に汚染した器具の消毒や滅菌の正しい知識を身につけさせる必要がある．肝疾患の病態や感染予防対策に関する講義は，Fig. 1～Fig. 12に示すように講義や実習では行われているが，その理解は完全なものではない．

歯科医師が患者にHCVを感染させた事例は報告されていないが，HBVを感染させた事例は存在する[13]．1977年のRimlandらの報告では[13]，1人の歯科医師から治療を受けた患者2名が肝炎を発症したことが発端となり，調査が進められた．この地方で2カ月から6カ月前に歯科治療を受けた後に肝炎を発症した71名を調査したところ，55名がある特定の口腔外科医から治療を受けていることが判明した．46歳の口腔外科医は，13年にわたり口腔外科を標榜し，治療にあたっていたが，肝炎の既往はなく，後にHBs抗原陽性，HBe抗原陽性であることがわかった．彼は，週250人～300人の患者を診察し，その95%が抜歯であった．彼は指先によく傷をつくることがあったが，手袋をせずに治療にあたっていた．その後，この歯科医師はHBs抗原が陰性になるまで歯科治療を休診し，手袋を着用して治療を再開したところ，1年1カ月の間の約8,000人の患者診察において肝炎を発症した患者はいなかったと報告している．歯科医院に勤務する他のスタッフ13人の中にHBVキャリアが存在しなかったことから，感染経路はこの46歳の歯科医師の手からHBVが患者の抜歯創に侵入したと考えられ，hemo-oral transmissionと推定されている．

わが国の歯科診療では，手袋の着用は義務づけられていないし，たとえ着用していても患者毎に交換する歯科医師は僅か24.6%だと申告されているため[4]，歯科治療を通じてHBVやHCVを交叉感染させる潜在的可能性は高いのではないかと推察される．現在，わが国で手袋を着用する歯科医師の多くは，「破損したら交換する」か「1日2回の交換（午前1回午後1回）」であり（46.2%，167/361名），診察時にマスクさえ着用しない歯科医師は5.8%（21/361名）とされている[4]．1982年チンパンジーの両眼にHBVを滴下させ，9週間後にHBV感染が成立した実験結果が報告された[14]．Bondらは，この論文内で歯科医師のような感染

のハイリスク者は眼をガードする必要があるとよびかけている．したがって，歯科診療時には手袋，マスク，アイガードの使用は必須である．わが国では，篠崎らが主に肝炎やAIDSに関する歯科領域での感染防止とその対策について著書にまとめているが，日本の歯科診療のガイドラインとして普及しているわけではない[15]．米国疾病管理予防センター（CDC，Centers for Disease Control and Prevention）の歯科診療のガイドラインでは，雇用者は従業員にHBVワクチンを打つことを義務づけている．1987年のCDCの医療従事者の感染防止ガイドラインの中では，感染の有無が明らかであるか否かに係わらず，いかなる患者でも血液や体液は感染症の可能性があるということを前提として取り扱いに注意を払うというユニバーサルプレコーションという考えが盛り込まれた[16]．HBVやHCVキャリアの多くは，無症状であり，自分自身がキャリアであることを知らない患者も多い．また感染者かどうかは，患者の自己申告によるため，歯科診療前に全ての患者について感染の有無を把握することは不可能である．したがってユニバーサルプレコーションもしくはスタンダードプレコーションという概念で治療を進めることは大切である[17]．

歯科医療従事者とくに歯科医師の肝炎の罹患率は，どうだろうか？　わが国では，篠崎らが1978年から1982年までに歯科一般開業医998名の採血を行い，HBs抗原陽性率は3.7%（37/998名），HBs抗体陽性率は42.1%（420/998名）であったと述べている[18]．この際，九州よりも北海道の歯科医師の方が，HBs抗体陽性率が有意に高いことも述べている（北海道；47.4%，中国・四国；38.2%，九州36.7%）．その後，篠崎らは1986年～1994年までの間に歯科医師から採取された凍結血清を用いてHCV感染率を分析し，その抗体陽性率が2.6%（10/382名）であったと報告している[19]．しかし当時採取された歯科医師の平均年齢の記載がないため，詳細は不明である．海外での報告では，1991年にKleinらはニューヨークの歯科医師のHCV感染率は（1.75%，8/456名），コントロールよりも（0.14%，1/723名）高く，特に口腔外科医はHCV感染率が高いと報告した（9.3%，4/43名）[20]．このように歯科医師の中でもその専門領域の違いにより，罹患率が異なることを明らかにしている．血液に曝露されやすい職種程肝炎ウイルスの感染を受けやすい．またわが国では東日本よりも西日本にHCV感染率が高いため，この地域で歯科医療に従事する者は自身の健康管理だけでなく交叉感染のリスクに一層の注意を促さなければならない．

2001年8月に日本歯科医師会は，「一般歯科診療　C型肝炎予防対策Q&A」という感染防止マニュアルを作成し，全国の会員約65,000人に配布した．それによると，汚染された器具のうち，オートクレーブにかけることのできない器具に付着したHCVを死滅させる薬液として，次亜塩素酸ナトリウム，グルタールアルデヒド，アルコールの3種を推奨して紹介しているが，消毒用アルコールの有効性についてはその効果が確かめられていないため，HBVと同様に有効でないという前提に立って対処すべきである．したがって，このマニュアルは早急に訂正すべきである．またこの度の歯学部のアンケートや2003年に実施された全国歯科医師のアンケートを通じて，消毒や滅菌に対する知識や感染予防対策が，実際の臨床では実践されていない可能性が非常に高いことも判明した．歯科治療行為がHBVやHCVの感染ルートにならないように歯科医師への教育と啓発普及活動が必要であると共に，学生教育に感染予防についての徹底した教育を導入する必要があると考えられた．さらに，雇用者となる歯科医師は，自己の健康管理だけでなく従業員の健康管理も把握し，適切な注意と指導も必要である．また誤って針刺し事故を起こした場合の事故対応に関してもすぐに行えるだけの知識と能力が必要とされる．現時点の歯科の保険診療内では，厳重な感染対策を盛り込むことは，経済的にも時間的にも困難と思われるが，国内の歯科治療における院内感染防止の標準化を目指したガイドラインを早急に作成すべきである．また，各県市町村の歯科医師会が，院内感染予防対策の充実を図ると同時に，各歯科医療機関が院内感染予防対策に対して認識を深めるこ

平成16年7月20日

とが不可欠である．HCVの高感染地区である佐賀県や福岡県では，県としての歯科診療における院内感染マニュアルは存在しない．

本論文中の対象者には，アンケート実施後にその集計結果だけでなくHCV・HBVと正しい消毒・滅菌法について解説し，各質問に対して各々解答した．歯学部の学生に対しても，感染に対するリスクマネージメントの重要性を教育する統一化されたカリキュラムを作成し，徹底した教育をする必要がある．現行の歯科医師の卒後臨床研修では，「歯科医師は，免許取得後も1年以上，臨床研修を行うよう努めるものとする」という努力義務が定められているが，必修ではない．歯科医師の臨床研修の義務化は2006年（H18）4月からの施行予定である．開業した歯科医師は，感染の知識を得る場が少ない．したがって，学生と歯科医師を対象に生涯教育を目指したinfection controlの教育が最も重要な課題であると思われる．

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## Survey of Hepatitis B and C in Students of Faculty of Dentistry and Dental Hygienist School

Yumiko NAGAO[1], Itsuo CHIBA[2] & Michio SATA[1)3)]
[1)]Research Center for Innovative Cancer Therapy, Kurume University School of Medicine
[2)]Department of Preventive and Community Dentistry,
Health Sciences University of Hokkaido School of Dentistry
[3)]Second Department of Medicine, Kurume University School of Medicine

At present, in Japan, hepatitis B virus (HBV) and hepatitis C virus (HCV) infection by blood transfusion rarely happens. However, according to the national survey, outbreak of sporadic acute hepatitis B and C is reported every year and viral hepatitis induced by iatrogenic infection is also reported. We think that education and enlightenmen for measures of infection control for hepatitis virus in dentisal medical care are important. Therefore, we carried out a questionnaire survey about measures of an infection control including hepatitis B and C for 352 students of a certain faculty of dentistry and a dental hygienist school. 35.5% of the total students thought the defense of oneself against infection was more important than defense of cross infection. Furthermore, the prevalence of the student who thought to permit recycle of a disposer glove and a disposer cartridge of a local anesthesia was 13.1% (46/352), 14.8% (52/352), respectively. The prevalence of students who recognized that HCV and HBV were detected from not only blood but also body fluid such as saliva remained in 65.3%. Consequently, the reality that knowledge of hepatitis virus and understanding about sterilization and disinfection of instruments were low became clear.

In conclusion, immediate making of the guideline that aimed at standardization of prevention of hospital infection in domestic dental treatment and education to introduce the curriculum with a high regard for risk management of infection for students of dentistry will be required. In addition, it is an important problem to spread thoughts of standard precautions for dentistry.

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# Simultaneous determination of dehydroepiandrosterone and its 7-oxygenated metabolites in human serum by high-resolution gas chromatography–mass spectrometry

Yasushi Matsuzaki[a,*], Shigemasa Yoshida[a], Akira Honda[a,b], Teruo Miyazaki[b], Naomi Tanaka[a], Aya Takagiwa[c], Yoshinori Fujimoto[c], Hiroshi Miyazaki[d]

[a] *Department of Gastroenterology and Hepatology, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan*
[b] *Ibaraki Prefectural Institute of Public Health, Mito, Ibaraki 310-0852, Japan*
[c] *Department of Chemistry and Materials Science, Tokyo Institute of Technology, Tokyo 152-8551, Japan*
[d] *Department of Drug Metabolism Disposition, Niigata College of Pharmacy, Niigata 950-2081, Japan*

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**Abstract**

A highly sensitive and specific method has been developed for the simultaneous measurement of free (unconjugated) or sulfate-conjugated forms of dehydroepiandrosterone (DHEA), 7α-hydroxy-DHEA (7α-OH-DHEA), 7β-hydroxy-DHEA (7β-OH-DHEA), and 7-oxo-DHEA (7-oxo-DHEA) in human serum. This method is based upon a stable isotope-dilution technique by gas chromatography–selected-ion monitoring mass spectrometry. Free steroids were extracted from serum with an organic solvent and the sulfate-conjugated steroids remained in aqueous phase. Free steroids were purified by solid-phase extraction, while sulfate-conjugated steroids were hydrolyzed by sulfatase and deconjugated steroids were purified by solid-phase extractions. The extracts were treated with *O*-methylhydroxylamine hydrochloride and were subsequently dimethylisopropylsilylated. The resulting methyloxime-dimethylisopropylsilyl (MO-DMIPS) ether derivatives were quantified by gas chromatography–selected-ion monitoring mass spectrometry in a high-resolution mode. The detection limits of MO-DMIPS ether derivatives of DHEA, 7α-OH-DHEA, 7β-OH-DHEA and 7-oxo-DHEA were 1.0, 0.5, 0.5 and 2.0 pg, respectively. Coefficients of variation between samples ranged from 10.6 to 22.9% for free 7-oxygenated DHEA to less than 10% for DHEA and sulfate-conjugated 7-oxygenated DHEA. The concentrations of these steroids were measured in 18 sera samples from healthy volunteers (9 males and 9 females; aged 23–78 years). Free DHEA, 7α-OH-DHEA, 7β-OH-DHEA and 7-oxo-DHEA levels ranged between 0.21–3.55, 0.001–0.194, 0.003–0.481, and 0.000–0.077 ng/ml, respectively, and the sulfate-conjugated steroid levels of these metabolites ranged between 253–4681, 0.082–3.001, 0.008–0.903, and 0.107–0.803 ng/ml, respectively. The free DHEA-related steroid concentrations were much lower than those previously measured by RIA and low-resolution GC–MS. The present method made it possible to determine simultaneously serum DHEA-related steroid levels with sufficient sensitivity and accuracy.


*Keywords:* Dehydroepiandrosterone; 7α-Hydroxydehydroepiandrosterone; 7β-Hydroxydehydroepiandrosterone; 7-Oxo-dehydroepiandrosterone; Serum; Gas chromatography–mass spectrometry

## 1. Introduction

Dehydroepiandrosterone (DHEA) is an endogenous steroid that is synthesized in the adrenal cortex, gonads, brain [1], and gastrointestinal tract [2]. In addition to its free form, the 3β-sulfate conjugate is produced mainly by both the hepatic and adrenal sulfotransferases [3], with the latter being the predominant form of DHEA in human blood. Serum concentrations of DHEA-sulfate (DHEA-S) increase during adolescence, peak between the ages of 15–25 years, and then decrease continuously after the third decade [4].

* Corresponding author. Tel.: +81 298 53 3138; fax: +81 298 53 3138.
*E-mail address:* ymatsuzaki-gi@umin.ac.jp (Y. Matsuzaki).

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DHEA (in the sulfated form) is probably the second most abundant plasma steroid after cholesterol in humans, especially in young adults (0.1–0.5 mg/dl). Many studies have been published concerning the effects of DHEA administration on the nervous system, immune system, cardiovascular disease, cancers, and metabolic changes [5]. However, its physiological functions have not been clarified.

It has been well established that a significant portion of circulating DHEA is further metabolized to its 7-oxygenated derivatives, i.e., 7α-hydroxy-DHEA (7α-OH-DHEA), 7β-hydroxy-DHEA (7β-OH-DHEA), and 7-oxo-DHEA in several different tissues, including liver and brain [6–11]. These DHEA derivatives seem to be more effective than the parent steroid, in immunomodulatory and antiglucocorticoid actions [12,13], in inducing thermogenic enzymes [14], and in improvement of memory in mice [15]. Despite these interesting findings, the 7-oxygenated DHEA derivatives in body fluids have not been quantified extensively, because the concentrations of the derivatives are much lower than DHEA.

Serum levels of the 7-oxygenated DHEA derivatives have been determined using several methods. The plasma 7α-OH-DHEA concentration was first measured by radioimmunoassay [16]. However, since the antibody used in this assay method recognized all 7α-hydroxylated 3β-hydroxy-5-ene steroids, the levels of 7α-OH-DHEA were overestimated. Recently, Lapcík et al. developed more specific radioimmunoassay methods for determining 7α-OH-DHEA [17] and 7β-OH-DHEA [18]. Measurement of 7-oxygenated DHEA derivatives by high-performance liquid chromatography (HPLC) has also been reported. Marwah et al. [19] have measured plasma 7-oxo-DHEA-S levels after the administration of 7-oxo-DHEA-3β-acetate by HPLC, using 17β-hydroxy-3β-methoxyandrost-5-en-7-one as an internal standard. Identification of blood 7-oxygenated DHEA derivatives by gas chromatography–mass spectrometry (GC–MS) was first performed by Ludwig-Köhn et al. [20]. Recently, Attal-Khémis et al. [21] quantified serum 7α-OH-DHEA levels in mice by GC–MS with selected-ion monitoring (SIM) in the chemical ionization (CI) mode, using [$^3$H]-7α-OH-DHEA as an internal recovery standard. Meanwhile Hampl et al. [22] measured 7α- and 7β-OH-DHEA in human sperm by GC–MS with SIM in the electron ionization (EI) mode calibrated by an external standard, and the method was subsequently applied to human serum and saliva by Hill et al. [23].

This paper describes a more reliable high-resolution GC–MS with SIM method using deuterium labelled internal standards. Free or sulfate-conjugated forms of DHEA, 7α-OH-DHEA, 7β-OH-DHEA, and 7-oxo-DHEA were quantified simultaneously in human serum.

## 2. Experimental

### 2.1. Materials

DHEA, DHEA-S, and sulfatase from *Helix pomatia* (Type H-1: 20,000 units/g solid) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [7-$^3$H(N)]-DHEA-S (592 GBq/mmol) was from New England Nuclear (Boston, MA). *O*-methylhydroxylamine hydrochloride (MOA-HCl), trimethylsilylimidazole (TMSI), dimethylethylsilylimidazole (DMESI), and dimethylisopropylsilylimidazole (DMIPSI) were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Bond Elut $C_{18}$ (octadecyl, 500 mg) and SI (unbonded silica, 100 mg) cartridges were purchased from Varian (Harbor City, CA), and Sephadex LH-20 from Pharmacia (Uppsala, Sweden).

### 2.2. Synthesis of 7-oxygenated DHEA and deuterium labeled internal standards

7α-OH-DHEA (mp 171–173 °C), 7β-OH-DHEA (mp 204–206 °C), and 7-oxo-DHEA (mp 247–248 °C) were synthesized from 3β-(*t*-butyldimethylsilyloxy)-17,17-ethylenedioxyandrost-5-en-7-one, as described previously [24].

[2,2,3,4,4-$^2H_5$]-7-Oxo-DHEA was synthesized from 3β-(*t*-butyldimethylsilyloxy)-17,17-ethylenedioxyandrost-5-en-7-one in 10 steps. Hydroboration-oxidation of the silyl ether with $BH_3$/THF followed by 3N-NaOH/$H_2O_2$ gave the 6β-alcohol, which was protected as the benzyl ether with NaH/$tBu_4N^+I^-$, BnBr. The benzyl ether was desilylated with TBAF and Swern oxidation [25] of the resulting 3-alcohol gave the 3-ketone. Treatment of the ketone with NaOD/$D_2O$ afforded the corresponding [2,2,4,4-$^2H_4$]-compound. This was reduced with $LiAlD_4$ and then protected as the TBS ether. Removal of the benzyl group under hydrogenation conditions ($H_2$–Pd/C) gave the 6-alcohol, which was dehydrated with $POCl_3$ to give the 5-ene compound. This was oxidized with $RuCl_3$ in the same manner, as described above, to give [2,2,3,4,4-$^2H_5$]-7-oxo-DHEA 3-TBS ether. Deprotection of the silyl group under acidic conditions (dil. HCl in methanol/tetrahydrofuran) furnished [2,2,3,4,4-$^2H_5$]-DHEA, mp 248–250 °C.

[2,2,3,4,4-$^2H_5$]-7α-OH-DHEA (mp 173–175 °C) and [2,2,3,4,4-$^2H_5$]-7β-OH-DHEA (mp 203–204 °C) were obtained from the [2,2,3,4,4-$^2H_5$]-7-oxo-DHEA 3-TBS ether in two steps. The TBS-ether was reduced with $NaBH_4$/$CeCl_3$ to give a mixture of the 7α- and 7β-alcohols. The mixture was desilylated under acidic conditions (dilute HCl in methanol/tetrahydrofuran), and the resulting 7α- and 7β-alcohols were separated by silica gel chromatography into the target labeled compounds. All deuterium labeled internal standards were finally characterized by GC–MS.

### 2.3. Subjects and sample preparation

Eighteen healthy volunteers (9 males and 9 females), aged 23–78 years (51 ± 19 years, mean ± S.D.), were used for determination of serum DHEA-related steroids. Blood samples were taken in the morning (between 6:00 and 12:00 a.m.), centrifuged at 1000 × *g* for 15 min to separate serum, and stored at −20 °C until analysis. Informed consent was

obtained from all subjects, and the experimental procedures were carried out in accordance with the ethical standards of the Helsinki Declaration.

### 2.4. Purification of DHEA-related steroids

A flow chart for the purification of DHEA and the 7-oxygenated DHEA derivatives is shown in Fig. 1. We added internal standards for free DHEA-related steroids as a mixture of [$^2H_5$] DHEA (20 ng), [$^2H_5$] 7α-OH-DHEA (2 ng), [$^2H_5$] 7β-OH-DHEA (2 ng), and [$^2H_5$] 7-oxo-DHEA (4 ng) to 1 ml of serum. Free and sulfate-conjugated steroid fractions were separated, according to the method of Attal-Khémis et al. [21]. Free steroids were extracted twice with 3 ml of isooctane/ethyl acetate (1:1, v/v), and the extracts were evaporated to dryness under nitrogen. Sulfated steroids were recovered in the resulting aqueous phase of isooctane/ethyl acetate extraction, and were subjected to enzymatic hydrolysis. After addition of 1 ml of acetonitrile to the aqueous phase and centrifugation at 1000 × *g* for 5 min, the supernatant fraction was collected. The precipitated protein fraction was washed twice with 2 ml of acetonitrile/distilled water (1:1, v/v) and the acetonitrile/distilled water layer was collected and combined with the first supernatant fraction. After evaporating the solvent by heating at 80 °C under nitrogen stream, 3 ml of 0.5 M acetate buffer (pH 5.1), 1000 units of sulfatase, and a mixture of [$^2H_5$] DHEA (1000 ng), [$^2H_5$] 7α-OH-DHEA (10 ng), [$^2H_5$] 7β-OH-DHEA (10 ng), and [$^2H_5$] 7-oxo-DHEA (20 ng) were added as internal recovery standards, and incubated for 3 h at 55 °C [26]. The incubation was stopped by adding 3 ml of 0.5 M sodium phosphate buffer (pH 7.0) and the mixture was applied to a Bond Elut $C_{18}$ cartridge prewashed with 5 ml of chloroform, 5 ml of ethanol and 10 ml of distilled water. After washing with 4 ml of distilled water, deconjugated steroids were eluted with 4 ml of ethanol and the eluate was evaporated to dryness.

Both free and hydrolysed sulfated steroid fractions were further purified by solid-phase extraction according to the method described by Dzeletovic et al. [27], with minor modifications. The dried residue containing unconjugated steroids was dissolved in 1 ml of toluene, and the sample was applied to Bond Elut SI cartridge prewashed with 1 ml of *n*-hexane. After washing with 1 ml of n-hexane followed by 8 ml of *n*-hexane/isopropanol (99.5:0.5, v/v), DHEA-related steroids were eluted with 4 ml of *n*-hexane/isopropanol (1:1, v/v). The resulting eluate was evaporated to dryness.

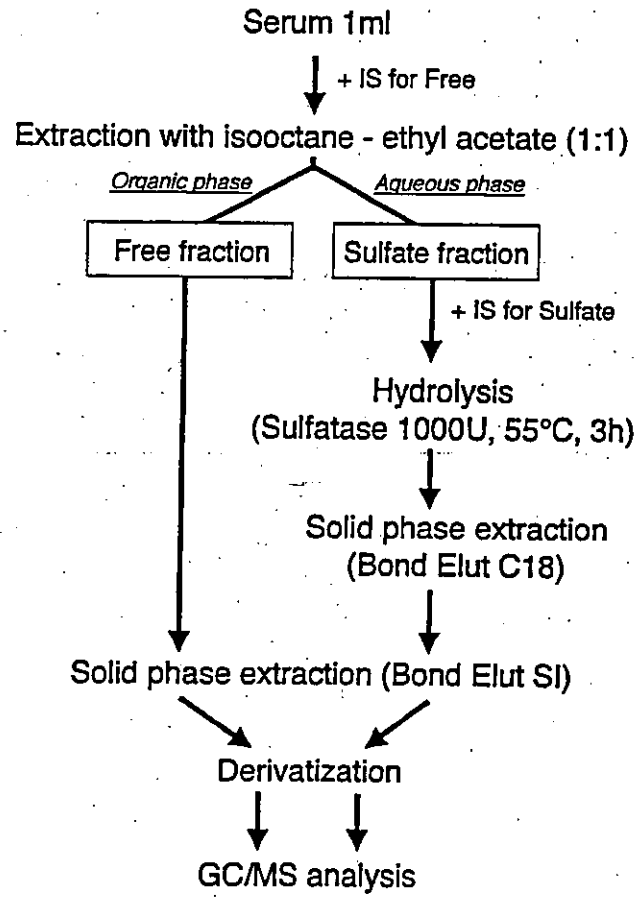


Fig. 1. A flow chart for the quantification of DHEA and 7-oxygenated DHEA derivatives in serum.

### 2.5. Sample derivatization for GC–MS analysis

A 100 μl aliquot of 10% MOA-HCl in pyridine (w/v) was added to the residue and the mixture was heated at 60 °C for 1 h [28]. The sample was then treated with 40 μl of TMSI, DMESI [29], or DMIPSI [30] and heated at 60 °C for 30 min. Excess silylating reagent was removed on a Sephadex LH-20 column (60 mm × 6 mm i.d.) equilibrated with *n*-hexane/chloroform (1:1, v/v). Derivatized DHEA-related steroids were recovered in the first 3 ml of effluent. After evaporation, the residue was redissolved in 50 μl of n-hexane and used for GC–MS analysis.

### 2.6. GC–MS analysis

GC–MS analysis in the EI mode was performed using a JMS-SX102 instrument equipped with a data processing XMS-system (JEOL, Tokyo, Japan). An ultra performance capillary column (25 m × 0.32 mm i.d.) coated with methylsilicone (Agilent Technologies, Palo Alto, CA) was used at a helium carrier gas flow-rate of 1.0 ml/min. The oven was programmed to change from 100 to 240 °C at 30 °C/min, after a 1 min delay from the start time. The accelerating voltage was 10 kV, the separator and ion source temperature 280 °C, the ionization energy 70 eV, and the ionizing current 300 μA. The reconstructed ion profile was obtained by repetitive scanning of the mass range *m/z* 50–600. SIM in the high-resolution mode was performed by focusing multiple ion detector on *m/z* 374.2515 and 402.2828 for MO-DMIPS ether derivative of DHEA, *m/z* 379.2829 and 407.3142 for that of [$^2H_5$] DHEA, *m/z* 415.2906 for MO-DMIPS ether derivatives of 7α- and 7β-OH-DHEA, *m/z* 420.3220 for those of [$^2H_5$] 7α- and [$^2H_5$] 7β-OH-DHEA, *m/z* 311.2123 for MO-DMIPS

ether derivative of 7-oxo-DHEA, and *m/z* 316.2437 for that of [$^2H_5$] 7-oxo-DHEA. The mass spectral resolution was approximately 10,000.

### 2.7. Statistics

Data are reported here as mean ± S.D. Linearity of the calibration curves was determined by simple linear regression. Correlation was tested by calculating Pearson's correlation coefficient, $r$, or Spearman's correlation coefficient, $r_s$. In all statistical tests, significance was accepted at the level of $P < 0.05$.

## 3. Results

### 3.1. Absolute recovery of extraction and purification procedure

The absolute recovery of DHEA-S in the aqueous phase by extraction with isooctane/ethyl acetate (1:1, v/v) was determined by adding $^3$H-labeled DHEA-S (55,000 dpm) to 1 ml of serum, and was 97.8 ± 0.4% ($n = 4$). The recovery of DHEA-S in the subsequent step of the elimination of serum proteins from the aqueous phase was also examined using $^3$H-labeled DHEA-S, and was 98.6% ($n = 2$). The absolute recoveries of DHEA, 7α-OH-DHEA, 7β-OH-DHEA and 7-oxo-DHEA from the Bond Elut SI cartridge were determined by the addition of unlabeled DHEA (288 ng), 7α-OH-DHEA (304 ng), 7β-OH-DHEA (304 ng), and 7-oxo-DHEA (302 ng) to the cartridge. After the purification steps, deuterium-labeled DHEA, 7α-OH-DHEA, 7β-OH-DHEA and 7-oxo-DHEA were added to the eluate as internal standards, and both unlabeled and deuterium-labeled steroids were quantified by GC–SIM. The recoveries of DHEA, 7α-OH-DHEA, 7β-OH-DHEA and 7-oxo-DHEA were found to be 85.0 ± 8.5% ($n = 4$), 86.8 ± 12.1% ($n = 4$), 88.8 ± 12.3% ($n = 4$), and 85.3 ± 8.8% ($n = 4$), respectively.

### 3.2. Hydrolysis of conjugated steroids by use of sulfatase

The completeness of the hydrolysis of sulfate conjugation was examined by thin-layer chromatography (TLC) using $^3$H-labeled DHEA-S. Incubation at 55 °C for 3 h with 1000 units of sulfatase in 0.5 M acetate buffer (pH 5.1) achieved complete cleavage of the conjugated sulfate. The absolute recovery of hydrolyzed DHEA by Bond Elut $C_{18}$ cartridge was 99.7% ($n = 2$).

### 3.3. Comparison of TMS, DMES and DMIPS ether derivatives

The gas chromatographic and mass spectrometric data for the methyloxime (MO) -TMS, MO-DMES and MO-DMIPS ether derivatives of DHEA, 7α-OH-DHEA, 7β-OH-DHEA and 7-oxo-DHEA are listed in Table 1. The mass spectral

Table 1
GC/MS characteristics for MO-TMS, MO-DMES and MO-DMIPS ether derivatives of DHEA-related steroids

| Relative retention time[a] | | Characteristic ions *m/z* (% relative intensities) | | | | |
|---|---|---|---|---|---|---|
| MO-TMS derivatives | | $[M]^+$ | $[M-31]^{+b}$ | $[M-46]^{+c}$ | $[M-90]^{+d}$ | A-ring fragment[e] |
| DHEA | 1.00 | 389 (9) | 358 (67) | 343 (3) | 299 (16) | 129 (100) |
| 7α-OH-DHEA | 1.04 | 477 (1) | 446 (1) | 431 (0) | 387 (100) | 129 (7) |
| 7β-OH-DHEA | 1.16 | 477 (1) | 446 (1) | 431 (0) | 387 (100) | 129 (9) |
| 7-oxo-DHEA | 1.30 | 432 (24) | 401 (70) | 386 (74) | 342 (20) | 129 (100) |
| MO-DMES derivatives | | $[M]^+$ | $[M-29]^{+f}$ | $[M-46]^{+c}$ | $[M-104]^{+g}$ | A-ring fragment[e] |
| DHEA | 1.14 | 403 (17) | 374 (100) | 357 (3) | 299 (11) | 143 (21) |
| 7α-OH-DHEA | 1.34 | 505 (5) | 476 (2) | 459 (1) | 401 (100) | 143 (2) |
| 7β-OH-DHEA | 1.55 | 505 (4) | 476 (1) | 459(1) | 401 (100) | 143 (3) |
| 7-oxo-DHEA | 1.54 | 446 (59) | 417 (10) | 400 (78) | 342 (22) | 143 (100) |
| MO-DMIPS derivatives | | $[M]^+$ | $[M-43]^{+h}$ | $[M-118]^{+i}$ | $[M-149]^{+j}$ | A-ring fragment[e] |
| DHEA | 1.26 | 417 (1) | 374 (100) | 299 (0) | 268 (8) | 157 (12) |
| 7α-OH-DHEA | 1.65 | 533 (5) | 490 (28) | 415 (100) | 384 (18) | 157 (8) |
| 7β-OH-DHEA | 1.95 | 533 (6) | 490 (5) | 415 (100) | 384 (6) | 157 (4) |
| 7-oxo-DHEA | 1.76 | 460 (18) | 417 (47) | 342 (11) | 311 (100) | 157 (79) |

[a] Retention times are expressed relative to that of MO-TMS ether derivative of DHEA. The oven was programmed to change from 100 to 240 °C at 30 °C/min after a 1 min delay from the start time. The retention time of MO-TMS derivative of DHEA was 7.94 min.
[b] $[M-CH_3O]^+$.
[c] $[M-CH_3O-CH_3]^+$.
[d] $[M-(CH_3)_3SiOH]^+$.
[e] $(CH_3)_3SiO^+=CH-CH=CH_2$ (*m/z* 129), $(CH_3)_2C_2H_5SiO^+=CH-CH=CH_2$ (*m/z* 143) and $(CH_3)_2C_3H_7SiO^+=CH-CH=CH_2$ (*m/z* 157) for MO-TMS, MO-DMES and MO-DMIPS ether derivatives, respectively.
[f] $[M-C_2H_5]^+$.
[g] $[M-(CH_3)_2C_2H_5SiOH]^+$.
[h] $[M-C_3H_7]^+$.
[i] $[M-(CH_3)_2C_3H_7SiOH]^+$.
[j] $[M-(CH_3)_2C_3H_7SiOH-CH_3O]^+$.

fragmentation patterns of the MO-TMS, MO-DMES and MO-DMIPS ether derivatives were closely related to each other. However, the MO-TMS ether derivatives were unstable and decomposed within several days. In contrast, the MO-DMES and MO-DMIPS ether derivatives were stable for at least a month. As for gas chromatographic separation, the four DHEA-related steroids were completely resolved when they were derivatized to the MO-TMS or MO-DMIPS ether. However, the separation between the MO-DMES ether derivatives of 7β-OH-DHEA and 7-oxo-DHEA was not satisfactory for simultaneous determination. Therefore, MO-DMIPS ether was chosen as the best derivative for the present analysis.

### 3.4. Calibration curves and detection limits

Calibration curves were established for DHEA, 7α-OH-DHEA, 7β-OH-DHEA and 7-oxo-DHEA (Fig. 2). The weight ratio of a component, relative to the corresponding deuterated internal standard, was plotted on the abscissa, and the peak-area ratio of the component to the internal standard was plotted on the ordinate. The linearity of the standard curves, as determined by simple linear regression, was excellent for weight ratios between 0.01 and 5.0 ($n=6$; $r>0.995$; $P<0.0001$). The minimum amounts of MO-DMIPS ether derivatives detected by the present high-resolution GC–SIM method were 1.0 pg for DHEA, 0.5 pg for 7α- and 7β-OH-DHEA, and 2.0 pg for 7-oxo-DHEA.

### 3.5. Representative SIM

Fig. 3 shows typical SIM chromatograms obtained by analysis of the free and sulfate-conjugated fractions of serum from a 30-year-old healthy man. The peaks of DHEA, 7α-OH-DHEA, 7β-OH-DHEA and 7-oxo-DHEA in the free fraction (Fig. 3A) corresponded to about 66, 2.9, 8.3, and 3.2 pg, respectively, while those in the sulfate-conjugated fraction (Fig. 3B) corresponded to about 182 ng, 12, 14, and 5.0 pg, respectively. When the peaks of 7-oxygenated DHEA were too small to calculate, concentrating the injected sample increased the sensitivity. In this case, *m/z* 402.2828 and 407.3142 were used to monitor DHEA and [$^2H_5$] DHEA, respectively, as the DHEA and [$^2H_5$] DHEA peaks monitored by *m/z* 374.2515 and 379.2829 sometimes became oversaturated. Despite these efforts, 7-oxo-DHEA was undetectable in some cases.

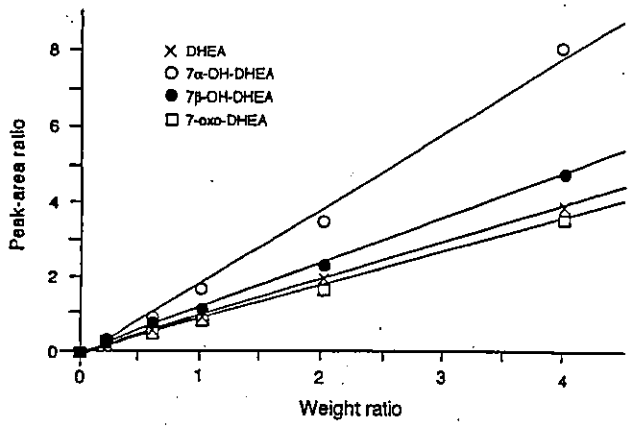


Fig. 2. Calibration curves for DHEA and 7-oxygenated DHEA derivatives. The weight ratio of a component relative to the corresponding deuterated internal standard is plotted on the abscissa and the peak-area ratio of the component to the internal standard is plotted on the ordinate. Linearity of the standard curves was determined by simple linear regression, and the equations for the lines of best fit were as follows: $y=0.963x-0.032$ ($n=6$; $r=0.997$; $P<0.0001$) for DHEA, $y=2.025x-0.151$ ($n=6$; $r=0.996$; $P<0.0001$) for 7α-OH-DHEA, $y=1.189x-0.012$ ($n=6$; $r=0.998$; $P<0.0001$) for 7β-OH-DHEA, and $y=0.935x-0.011$ ($n=6$; $r=0.998$; $P<0.0001$) for 7-oxo-DHEA.

### 3.6. Precision and accuracy of the method

We studied the precision of the method by analyzing spiked and unspiked samples. The DHEA-related steroids in 1 ml aliquots of pooled serum were purified by the present method in quadruplicate, before and after the addition of 10 ng DHEA, 1 ng each of 7α-OH- and 7β-OH-DHEA, 2 ng 7-oxo-DHEA and 3,000 ng DHEA-S; the results are summa-

Table 2
Inter-sample precision (CV) and accuracy (recovery) in the quantification of DHEA-related steroids in human serum

| Compound | Amount added (ng/ml) | Amount measured (ng/ml) | | | | | CV (%) | Recovery (%)[a] |
|---|---|---|---|---|---|---|---|---|
| | | Individual values | | | | Mean ± S.D. | | |
| DHEA | 0 | 4.96 | 4.66 | 4.30 | 4.28 | 4.55 ± 0.32 | 7.0 | |
| | 10 | 14.92 | 14.73 | 14.50 | 14.59 | 14.69 ± 0.18 | 1.2 | 101 ± 2 |
| 7α-OH-DHEA | 0 | 0.047 | 0.050 | 0.064 | 0.057 | 0.054 ± 0.008 | 13.9 | |
| | 1 | 1.179 | 1.208 | 1.117 | 1.089 | 1.148 ± 0.055 | 4.8 | 109 ± 5 |
| 7β-OH-DHEA | 0 | 0.016 | 0.014 | 0.014 | 0.022 | 0.017 ± 0.004 | 22.9 | |
| | 1 | 0.914 | 1.084 | 0.999 | 0.916 | 0.978 ± 0.081 | 8.3 | 96 ± 8 |
| 7-oxo-DHEA | 0 | 0.087 | 0.076 | 0.071 | 0.069 | 0.076 ± 0.008 | 10.6 | |
| | 2 | 2.324 | 1.969 | 2.245 | 2.232 | 2.192 ± 0.154 | 7.0 | 106 ± 8 |
| DHEA-S | 0 | 1693 | 1733 | 1624 | 1684 | 1684 ± 45 | 2.7 | |
| | 3000 | 4470 | 4288 | 4298 | 4394 | 4363 ± 86 | 2.0 | 89 ± 3 |
| 7α-OH-DHEA-S | 0 | 0.820 | 0.841 | 0.725 | 0.705 | 0.773 ± 0.068 | 8.8 | |
| 7β-OH-DHEA-S | 0 | 0.729 | 0.793 | 0.663 | 0.652 | 0.709 ± 0.066 | 9.2 | |
| 7-oxo-DHEA-S | 0 | 1.496 | 1.689 | 1.739 | 1.492 | 1.604 ± 0.129 | 8.0 | |

[a] Calculated as follows: (amount measured in spiked samples − mean amount measured in unspiked samples) divided by the amount added × 100%.

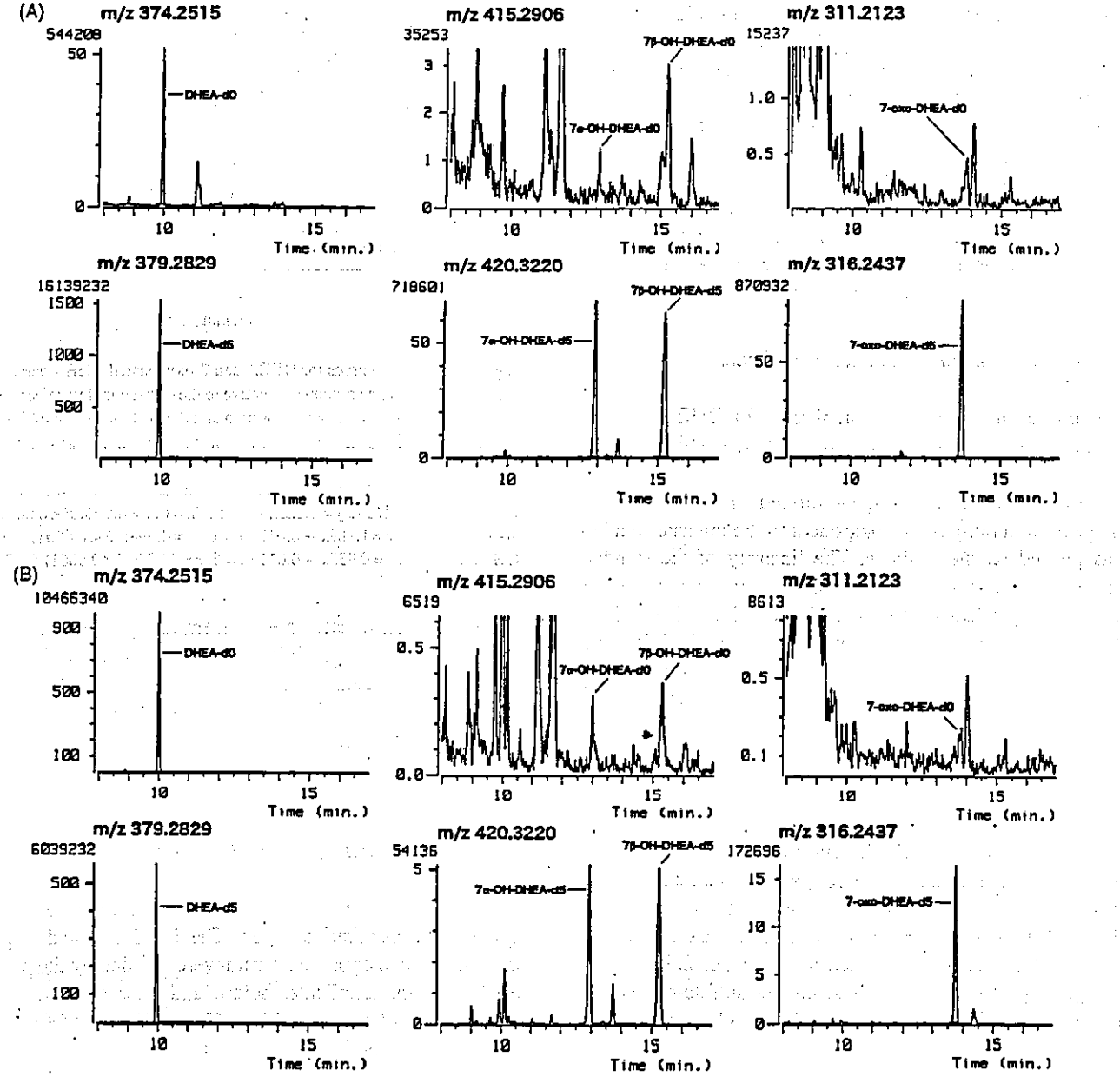


Fig. 3. Representative high-resolution GC–SIM chromatograms of DHEA, 7α-OH-DHEA, 7β-OH-DHEA and 7-oxo-DHEA in the free fraction (A) and sulfate-conjugated fraction (B) obtained from 1 ml of serum.

rized in Table 2. Coefficients of variation between the samples were 10.6–22.9% for unspiked free 7-oxygenated DHEA, and less than 10% for all other unspiked and spiked samples. The recoveries of the spiked known amounts of DHEA-related steroids ranged from 89 to 109%.

### *3.7. DHEA-related steroid concentrations in normal human serum*

Free and sulfate-conjugated DHEA, 7α-OH-DHEA, 7β-OH-DHEA, and 7-oxo-DHEA concentrations were determined in serum from 18 subjects; the results are summarized in Table 3. The concentrations of free 7α-OH-DHEA, 7β-OH-DHEA, and 7-oxo-DHEA were 6.2 ± 8.8%, 3.3 ± 3.9%, and 2.1 ± 1.6% of the free DHEA levels, respectively. In contrast, sulfate-conjugated 7α-OH-DHEA, 7β-OH-DHEA, and 7-oxo-DHEA were only 0.1 ± 0.2%, 0.02 ± 0.02%, and 0.05 ± 0.04% of the sulfate-conjugated DHEA levels, respectively. The concentration ratios of the free to sulfate-conjugated forms were calculated as 0.2 ± 0.2% for DHEA, 8.1 ± 13.7% for 7α-OH-DHEA, 13.5 ± 16.8% for 7β-OH-DHEA, and 8.6 ± 14.8% for 7-oxo-DHEA. Free ($r = -0.61$, $r_s = -0.66$, $P < 0.01$) and sulfate-conjugated DHEA levels ($r = -0.61$, $r_s = -0.61$, $P < 0.01$) correlated negatively with age. However, significant correlations were not observed between age and free or sulfate-conjugated 7-oxygenated

Table 3
Concentrations of DHEA-related steroids in human serum ($n = 18$)

| Compound | Concentrations (ng/ml) | |
|---|---|---|
| | Free | Sulfate |
| DHEA | 1.42 ± 1.02 (0.21 – 3.55) | 1077 ± 1065 (253 – 4681) |
| 7α-OH-DHEA | 0.058 ± 0.066 (0.001 – 0.194) | 0.756 ± 0.861 (0.082 – 3.011) |
| 7β-OH-DHEA | 0.039 ± 0.043 (0.003 – 0.481) | 0.250 ± 0.247 (0.008 – 0.903) |
| 7-oxo-DHEA | 0.019 ± 0.022 (0.000 – 0.077) | 0.324 ± 0.228 (0.107 – 0.803) |

Mean ± S.D. and (range of results) are given.

DHEA levels. The concentrations of free 7-oxygenated DHEA tended to correlate with each other ($r = 0.40$–$0.46$, $0.1 > P > 0.05$) and sulfated 7-oxygenated DHEA levels correlated significantly with each other ($r = 0.49$–$0.67$, $P < 0.05$).

## 4. Discussion

For determination of serum DHEA-related steroids, GC–MS with SIM is considered to be the best method, owing to its superior sensitivity and specificity. In this study, the previously reported GC–SIM methods [21-23] were developed using deuterium-labeled internal standards and measuring in a high-resolution mode. Deuterium-labeled DHEA-related compounds are ideal internal standards for quantification by GC–SIM. Since we were not able to prepare deuterium-labeled DHEA-S, the absolute recovery of DHEA-S from serum and the efficiency of enzymatic hydrolysis of sulfate conjugation were tested using [$^3$H]DHEA-S. The results showed that more than 96% of the serum DHEA-S was recovered in the mixture for enzymatic hydrolysis, and complete cleavage of the conjugated sulfate was achieved under the current hydrolysis conditions. High-resolution GC–SIM was quite effective at eliminating the interfering peaks from the chromatograms. We decreased the resolution to 5000 and attempted to measure the DHEA-related steroids, but 7-oxygenated DHEA derivative peaks were occasionally markedly influenced by impurities that exhibited the same retention times as the authentic compounds. Thus, it was necessary to adjust the resolution to more than 5000 for our purposes.

Analysis by EI–GC–MS after conversion to the MO-DMIPS ether derivatives was another advantage of the present method. GC–MS analysis in the EI mode does not require the tedious maintenance procedure necessary for the use of the CI mode [21]. MO derivatization of each DHEA-related steroid produces both the *syn* and *anti* isomers. However, the retention times of these isomers were completely identical and each steroid always produced a single peak. After MO derivatization, the steroids were further converted to their DMIPS ether derivatives. In comparison with previous TMS ether derivatives [22,23], the DMIPS ether derivatives were markedly stable. The derivatized samples could be stored in n-hexane for at least a month without decomposition.

The serum concentrations of DHEA-S were 1077 ± 1065 ng/ml (2.93 ± 2.90 μmol/l), which were significantly lower ($P < 0.001$) than those previously reported (7.69 ± 4.62 μmol/l, $n = 29$; age, 19–60 years) [17]. However, since the range of serum DHEA-S concentrations, 253–4681 ng/ml (0.69–12.7 μmol/l), was similar to that previously reported (1.2–13.1 μmol/l) [17], the differences between the mean values might have been due to the various backgrounds, particularly ages, of the subjects. In contrast, not only the mean value, 1.42 ± 1.02 ng/ml (4.92 ± 3.54 nmol/l), but also the range, 0.21–3.55 ng/ml (0.73–12.31 nmol/l), of serum free DHEA concentrations were lower ($P < 0.001$) than previously reported values (26.5 ± 25.5; range, 3.2–70 nmol/l) [17]. These results suggest that measurement of serum free DHEA concentrations by conventional radioimmunoassay (RIA) kits may overestimate the levels due to cross-reactivity with other steroids.

Serum free 7α-OH-DHEA and 7β-OH-DHEA concentrations were much lower than those measured previously by RIA and low-resolution GC–MS [23,31]. The mean value, 0.058 ± 0.066 ng/ml (0.191 ± 0.217 nmol/l), and range, 0.001–0.194 ng/ml (0.003–0.637 nmol/l), of serum free 7α-OH-DHEA concentrations were significantly lower ($P < 0.001$), compared with the values reported previously (1.242 ± 1.043; range, 0.070–4.36 nmol/l by RIA and 0.913 ± 0.646; range, 0.016–2.38 nmol/l by GC–MS) [23]. Similarly, serum free 7β-OH-DHEA levels were significantly lower ($P < 0.001$) than the previously reported values measured by RIA and GC–MS [23]. Serum concentrations of sulfate-conjugated 7β-OH-DHEA were 0.250 ± 0.247 ng/ml (0.821 ± 0.811; range, 0.026–2.966 nmol/l) that were also significantly lower ($P < 0.001$) than previously reported values (16.63 ± 9.70 nmol/l, $n = 10$) [18]. The concentrations of serum sulfate-conjugated 7α-OH-DHEA were 0.756 ± 0.861 ng/ml (2.484 ± 2.829; range, 0.269–9.892 nmol/l) that tended to be low, but were not significantly different from previously reported values (5.25 ± 4.14, $n = 9$; range, 0.33–10.8 nmol/l) [17]. Although we do not exclude the possibility that the backgrounds of our subjects were very different from those of the previous investigations, it may also be possible that previous RIA and low-resolution GC–MS methods measured cross-reactive steroids and interfering peaks, respectively.

In contrast to 7α-OH-DHEA and 7β-OH-DHEA, previous data about serum 7-oxo-DHEA concentrations in normal subjects were not available. Using a recent HPLC method, plasma 7-oxo-DHEA–sulfate was successfully quantified in

subjects administered 100 mg of 7-oxo-DHEA as its acetyl derivative [19]. However, since the detection limits of free and sulfate-conjugated 7-oxo-DHEA were 5 and 3 ng/ml, respectively, the method was not applicable to measure background concentrations of 7-oxo-DHEA and 7-oxo-DHEA–sulfate.

In summary, we have developed an improved high-resolution GC–MS method for simultaneous determination of the free and sulfate-conjugated forms of DHEA, 7α-OH-DHEA, 7β-OH-DHEA, and 7-oxo-DHEA in human serum. The concentrations of these steroids were generally lower than those measured previously by RIA and low-resolution GC–MS.

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# Liquid Chromatography Assay for Routine Monitoring of Cellular Ribavirin Levels in Blood

Yoichi Inoue,[1] Masato Homma,[1]* Yasushi Matsuzaki,[2] Minoru Shibata,[3] Takuya Matsumura,[3] Takayoshi Ito,[3] Keiji Mitamura,[3] Naomi Tanaka,[2] and Yukinao Kohda[1]

*Department of Pharmaceutical Sciences[1] and Department of Gastroenterology,[2] Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki, and Second Department of Internal Medicine, Showa University, Shinagawa, Tokyo,[3] Japan*

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**Ribavirin-induced hemolytic anemia is one cause for cessation of combination therapy with alpha interferon 2b and ribavirin for hepatitis C infection. Determining cellular ribavirin levels in blood, including the levels of its phosphorylated metabolites, might be useful for predicting ribavirin-induced anemia, because the metabolites accumulate in erythrocytes. We simplified an assay method developed previously to make it suitable for routine monitoring of cellular ribavirin. Whole blood diluted with a sixfold volume of ice-cold distilled water was subjected to acid phosphatase digestion to convert phosphorylated ribavirin metabolites to free ribavirin. The resulting mixture, spiked with an internal standard, was treated by phenyl boronic acid column extraction, followed by reverse-phase high-performance liquid chromatography analysis. The calibration curve for ribavirin levels in whole blood was linear at concentrations of 5.3 to 1,024 μM ($r^2$ = 0.9999). Validation coefficients of variation for intra- and interday assays were 2.9 to 5.8% and 4.3 to 8.3%, respectively. We tested this method by monitoring blood ribavirin concentrations in two hepatitis C patients receiving alpha interferon 2b-plus-ribavirin combination therapy.**

Ribavirin, a guanosine analog broad-spectrum antiviral agent, is used for hepatitis C virus (HCV) elimination in combination with alpha interferon 2b (2, 3, 13). Polyethyleneglycol-alpha interferon 2a combined with oral ribavirin also brings substantial benefit to HCV patients (12, 14). Despite the strong beneficial effects of these combination therapies in severe HCV infection, loss of hemoglobin occurs in a substantial population of HCV patients. Progressive loss of hemoglobin leads to anemia, which is counteracted by reducing the ribavirin dose or prematurely discontinuing the combination therapy (1, 5, 12). Current studies suggest that the excessive accumulation of ribavirin in erythrocytes is responsible for the anemia (8, 11). Once incorporated into erythrocytes, ribavirin is converted into phosphorylated metabolites by intracellular phosphorylation (9). The phosphorylated metabolites decrease intracellular ATP levels, resulting in the reduction of erythrocyte integrity, which is followed by extravascular hemolysis via the reticuloendothelial system (4). It should therefore be possible to predict the occurrence of ribavirin-induced anemia by determining the ribavirin concentrations in blood cells and plasma.

A high-performance liquid chromatography (HPLC) method was previously developed to determine ribavirin levels in order to assess the disposition of ribavirin in erythrocytes (7). Since phosphorylated metabolites are the main form of intracellular ribavirin (7, 8), whole-blood samples were treated with acid phosphatase prior to column extraction and analysis. However, the dephosphorylation procedure used in a previous study (7) was too tedious to use for routine monitoring of cellular ribavirin. In the present study, we simplified the dephosphorylation procedure. The modified method was tested for therapeutic drug monitoring of cellular ribavirin levels in two patients during the first 8 weeks of HCV treatment by combination therapy with ribavirin and alpha interferon 2b.

* Corresponding author. Mailing address: Department of Pharmaceutical Sciences, Institute of Clinical Medicine, University of Tsukuba, Ten-nodai 1-1-1, Tsukuba, Ibaraki 305-8575, Japan. Phone: 81-298-53-3859. Fax: 81-298-53-7025. E-mail: masatoh@md.tsukuba.ac.jp.

## MATERIALS AND METHODS

**Chemicals and instruments.** All chemicals were of HPLC or reagent grade and were obtained from Wako Pure Chemicals Industries (Osaka, Japan) or Sigma-Aldrich Corp. (St. Louis, Mo.). Ribavirin (1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide), 3-methylcytidine methosulfate (internal standard), and acid phosphatase type 4 prepared from sweet potato were purchased from Sigma. Phenyl boronic acid (PBA) cartridges with a high specificity for *cis*-diol-containing compounds (100 mg; Bond Elute PBA), which were used for solid-phase extraction, were obtained from Varian (Palo Alto, Calif.).

The HPLC system used in this study was the Tosoh (Tokyo, Japan) model 8020 system equipped with a UV detector, an autosampler, and a pump. A $C_{18}$ reversed-phase column (4.6 [inner diameter] by 150 mm; TSK-Gel ODS-80Ts, Tosoh) was used, and UV absorbance was monitored at 225 nm. The mobile-phase solvent, 10 mM ammonium phosphate buffer (pH 2.5), was pumped through at a flow rate of 1.0 ml $min^{-1}$.

**Reference sample preparation.** A stock ribavirin solution (4.1 mM) was prepared by dissolving ribavirin in distilled water. Reference samples were prepared by diluting the stock solution with a human plasma alternative (Twin-Consera H; Nissui, Tokyo, Japan) or with drug-free whole blood collected in heparinized tubes from a healthy volunteer. The concentrations of reference samples were 0.25, 0.5, 1, 5, 10, and 20 μM in the plasma alternative and 5.3, 21, 85, 341, 683, and 1,024 μM in whole blood. The concentration ranges of the reference samples were established in a previous study (8). Reference samples were stored at −30°C until use. An internal standard was prepared as a 250 μM solution of 3-methylcytidine methosulfate in distilled water, and the solution was stored at 4°C.

**Enzyme digestion and column extraction.** A 20-μl sample of whole blood was supplemented with 120 μl of ice-cold distilled water and vortexed intensely for 20 s to accomplish complete hemolysis. The hemolysate was treated with acid phosphatase to hydrolyze the phosphorylated metabolites, according to the method of Homma et al. (7). The reaction mixture, consisting of the hemolysate (140 μl), 30 μM Tris-HCl (200 μl), 1 M sodium acetate (20 μl), and 2 U of acid

phosphatase, was incubated for 1 h at 37°C. The reaction was terminated by neutralizing the reaction mixture with 10 M KOH. That dephosphorylation of the phosphorylated metabolites of ribavirin went to completion was confirmed by monitoring the peak height ratio after various incubation times (30, 60, 90, and 120 min); a plateau was reached at 30 min. Furthermore, addition of another 2 U of enzyme preparation to the reaction mixture after 120 min of incubation did not lead to any change in peak height.

The dephosphorylated samples were supplemented with 20 μl of the internal standard and 500 μl of 250 mM ammonium phosphate buffer (pH 8.5). After being centrifuged at 1,500 × *g* for 5 min, the supernatants were loaded onto PBA cartridges that had been pretreated with 1 ml of 100 mM formic acid, followed by 5 ml of 250 mM ammonium phosphate buffer (pH 8.5). The cartridges were positioned in a 12-port manifold (Alltech, Lexington, Ky.) and washed with 5 ml of 250 mM ammonium phosphate buffer (pH 8.5). Ribavirin and the internal standard were subsequently eluted with 1 ml of 100 mM formic acid into glass tubes. The recovery rate was as almost the same as that described in a previous study (7). The effluents were evaporated to dryness at 40°C under a nitrogen gas flow. The samples were reconstituted with 200 μl of mobile-phase solution, and a 20-μl portion was injected into the HPLC system.

For quantification of plasma ribavirin, a plasma sample (200 μl) was supplemented with 20 μl of the internal standard and 500 μl of 250 mM ammonium phosphate buffer (pH 8.5) and subjected to PBA column extraction. Plasma samples were not treated with acid phosphatase, because phosphorylated ribavirin was not found in plasma in our preliminary study.

**Assessment of cellular ribavirin disposition in blood.** Our modified assay method was tested by assessing cellular ribavirin concentrations in two HCV patients treated with alpha interferon 2b plus ribavirin. The daily dose of ribavirin was 800 mg throughout the study, in accordance with the standard dosing instructions for HCV patients treated in Japan. The daily dose of alpha interferon 2b started at 10 million units intramuscularly for 14 days, followed by 6 million units three times weekly. Kidney function in both patients was normal throughout the treatment. A 10-ml sample of blood was collected in heparinized tubes at 0, 1, 2, 7, 14, 21, 28, 56, and 84 days after the start of the combination therapy. Blood samples (5 ml each) drawn from patients were immediately divided into two portions: one whole-blood portion to be treated by enzyme digestion, and the other portion to be used to obtain plasma by centrifugation.

The concentration of cellular ribavirin was calculated with the formula $C_c = [C_w - C_p(1 - Ht)]/Ht$, where $C_c$ is the ribavirin concentration in blood cells, $C_w$ is the concentration in whole blood, $C_p$ is the concentration in plasma, and Ht is the hematocrit. Hematocrit includes many blood cell types such as erythrocytes, lymphocytes, and neutrophils. Most ribavirin in blood cells is located in erythrocytes, because ribavirin and its phosphorylated metabolites could not be detected in the fraction of white blood cells in our preliminary experiment (data not shown). In this study, therefore, $C_c$ is essentially identical to ribavirin concentration in erythrocytes. The hematological parameters (hemoglobin and hematocrit values) were also measured on each sampling day. Informed consent was obtained from all patients, and the study was approved by the ethics committee of our university.

## RESULTS

A typical HPLC chromatogram for determination of ribavirin levels in phosphatase-digested whole blood samples is shown in Fig. 1B. Peaks representing ribavirin and the internal standard were observed at retention times of 4.7 and 6.1 min, respectively. No interfering peaks generated from endogenous substances were observed on the chromatogram for blank (i.e., untreated) whole blood (Fig. 1A). Similar chromatograms were obtained from plasma samples (data not shown).

Calibration curves for ribavirin levels determined at concentrations of 0.25 to 20 μM for plasma and 5.3 to 1,024 μM for whole blood were linear. The equations for the lines calculated by regression analysis were $y = 0.0387x + 0.0037$ ($r^2 = 1.0000$) for plasma and $y = 0.0034x + 0.0026$ ($r^2 = 0.9999$) for whole blood, where $y$ is the peak height ratio of ribavirin/internal standard and $x$ the concentration of ribavirin.

The analytical precision of determination of ribavirin whole-blood levels was evaluated with whole-blood reference samples

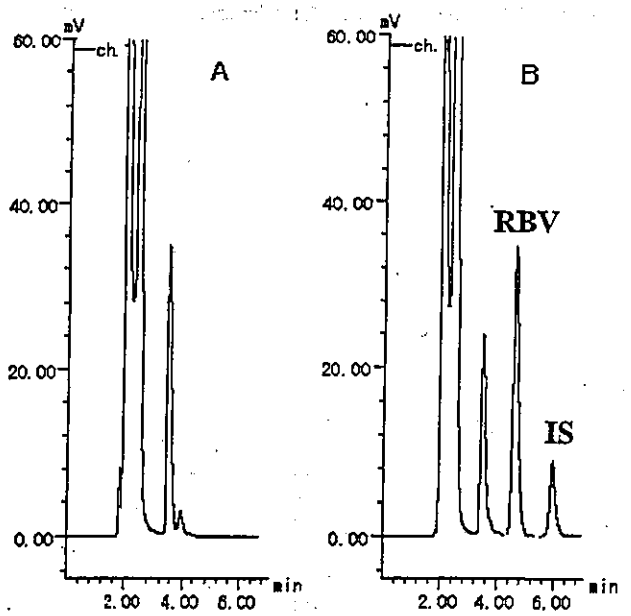


FIG. 1. HPLC chromatograms of blank whole blood (A) and whole blood spiked with 1,024 μM ribavirin (RBV) and an internal standard (IS) (B).

containing ribavirin concentrations of 21, 341, and 1,024 μM. Validation coefficients of variation for intra- and interday assay precision was assessed five times in a single run and five times in separate runs, respectively (Table 1). Coefficients of variation for intra- and interday assays were 2.9 to 5.8% and 4.3 to 8.3%, respectively.

We tested our new method by using it to determine ribavirin cellular levels for two HCV patients. No interfering peaks were observed on the chromatograms despite various medications that were administrated concomitantly, such as an analgesic (acetaminophen), an antacid (Sucralfate), and a cholagogue (ursodesoxycholic acid). A decrease in hemoglobin levels was observed for both patients within the first 3 weeks of combination treatment, as the concentration of cellular ribavirin increased (Fig. 2). Cellular ribavirin reached steady-state levels within 3 to 4 weeks after the combination therapy was begun. Steady-state levels of cellular ribavirin were 146 times higher than plasma ribavirin levels for patient 1 and 150 times higher than plasma ribavirin levels for patient 2 (Table 2). Around 90% of cellular ribavirin consisted of phosphorylated metabolites, which were not detected in plasma (Table 2). The difference in hemoglobin drop between the two patients appeared to

TABLE 1. Validation coefficients of variation for intra- and interday assay precision

| Concn (μM) | Observed concn (μM) | | | |
|---|---|---|---|---|
| | Intraassay ($n = 5$) | | Interassay ($n = 5$) | |
| | Mean ± SD | CV[a] | Mean ± SD | CV |
| 21 | 22.8 ± 1.3 | 5.8 | 20.1 ± 1.7 | 8.3 |
| 341 | 344 ± 10 | 2.9 | 308 ± 16 | 5.3 |
| 1,024 | 1027 ± 34 | 3.4 | 1040 ± 44 | 4.3 |

[a] CV, coefficient of variation.

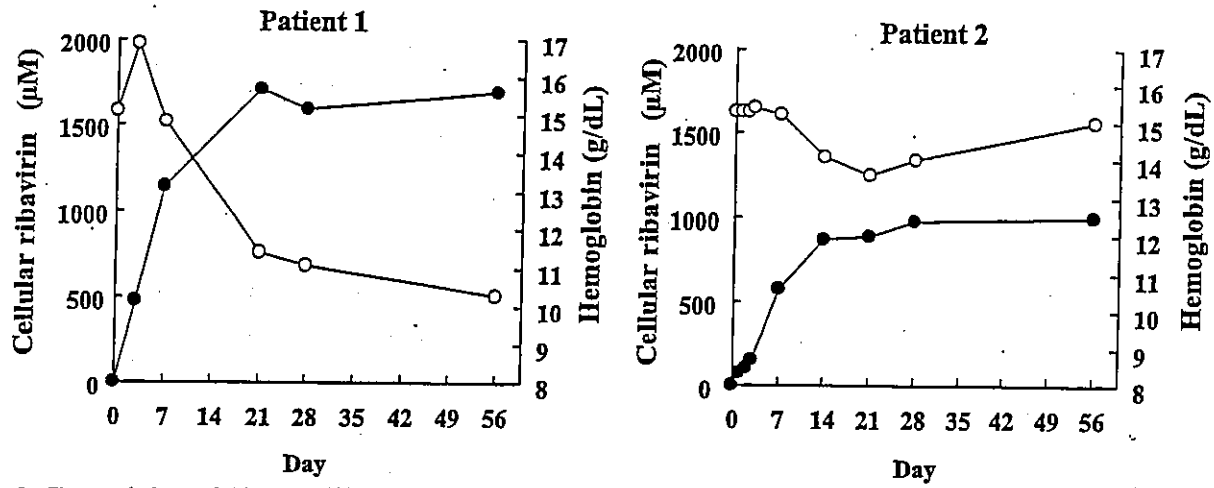


FIG. 2. Changes in hemoglobin levels (○) and ribavirin concentrations in blood cells (●) during combination treatment of hepatitis C patients with alpha interferon 2b and ribavirin.

correspond to their cellular ribavirin levels. That is, patient 1, who showed marked hemoglobin reduction, had a higher cellular ribavirin concentration (1,662 μM) than did patient 2 (929 μM).

## DISCUSSION

In this study, we succeeded in simplifying the quantification of ribavirin in whole-blood samples. The advantage of the present method is the elimination of the handling required for precipitation of whole blood and the tedious pH adjustment prior to enzyme digestion (both of which were required with the previous method). Since ribavirin concentrations in whole blood were extremely high (>1,000 μM at steady state), we could decrease the sample volume, which enabled us to carry out hemolysis with ice-cold water instead of by acid precipitation. Another modification was the use of a TSK-Gel ODS-80Ts column (4.6 [inner diameter] by 150 mm) instead of a Novapak column (3.9 [inner diameter] by 300 mm) as the analytical $C_{18}$ reverse-phase column. The TSK-Gel ODS-80Ts column achieved baseline separation of the ribavirin and internal standard peaks from those of other components more efficiently, even though the detection wavelength of 225 nm was lower and retention times were shorter than in a previously described method (4, 7). The limit of detection was also improved from 2.4 to 1.2 ng in terms of the amounts injected. This corresponds to ribavirin concentrations of 0.2 to 0.1 μM. And the signal-to noise ratio of >3 was higher than by the previous method.

We tested the revised method by determining ribavirin concentrations in plasma and blood cell samples of two HCV patients receiving alpha interferon 2b and ribavirin in combination. Cellular ribavirin concentrations reached steady-state levels 2 to 3 weeks after the patients started combination therapy, when hemoglobin reduction also bottomed out (Fig. 2). Interestingly, changes in cellular ribavirin concentration were inversely related to changes in the levels of hemoglobin. Patient 1, with 1,662 μM cellular ribavirin, showed a marked decrease in hemoglobin ($-4.8$ g $dl^{-1}$) compared with patient 2, whose cellular ribavirin level was only 929 μM. These observations are consistent with the hypothesis that marked accumulation of intracellular ribavirin in erythrocytes accelerates hemolysis by reducing erythrocyte life spans (5, 8). We should mention that interferon can also lead to a decrease in hemoglobin.

Cellular/plasma ratios of ribavirin were 146 and 150 in patients 1 and 2, respectively. The ratios were much higher than that reported for a previous study (i.e., 60) by Lertora et al. (11). This discrepancy is due to differences between the present HPLC assay method and the previous radioimmunoassay method. Since the radioimmunoassay procedure did not include a dephosphorylation step, it could not correctly determine the concentrations of phosphorylated metabolites, leading to underestimation of total cellular ribavirin levels.

Concerning the antiviral effects of ribavirin, normalization of the level of alanine aminotransferase and undetectable amounts of HCV RNA were observed with samples from patient 1 but not with samples from patient 2 (data not shown). Jen et al. (10) suggested that higher plasma ribavirin concentrations in treatment week 4 were associated with a higher virologic response rate. However, discussion of the antiviral effects of ribavirin in the present study would be inappropriate because of the small sample size of two patients. We intend to investigate this hypothesis further by studying a larger number of patients.

In conclusion, our HPLC method can be used for routine monitoring of cellular ribavirin disposition in blood. The impact of measuring cellular ribavirin levels to evaluate the effi-

TABLE 2. Steady-state average of plasma and cellular ribavirin concentrations and maximal drop in hemoglobin

| Patient | Δ Hb[b] (g/dl) | Ribavirin concn (μM)[a] | |
|---|---|---|---|
| | | Plasma | Cellular (metabolites) |
| 1 | 4.8 | 11.4 ± 1.1 | 1,662 ± 50 (1,430 ± 31) |
| 2 | 1.7 | 6.2 ± 0.8 | 929 ± 55 (869 ± 76) |

[a] Values are means ± SD.
[b] Maximum decrease of hemoglobin from baseline during combination treatment with alpha interferon-2b plus ribavirin.

cacy and toxicity of treatment needs to be clarified in future clinical studies with a large number of patients.

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