ニックのデータが非常に重要であることが確認され，中嶋委員が担当することとなった．雌MutaMouseを用い，3および1.5%の混餌で4週間の投与とし，肝臓における変異を調べる．他の臓器も凍結保存しておき，必要に応じて解析する．また，DNAシークエンスの解析が必要な場合には衛研が協力することとした．その他，雌雄を用いるべき，BMDが求められるように多くの用量段階について検討すべき，Comet，肝小核等を組みあわせて行っては，等の意見が出されたが，さらに検討することとした．

4.8．第8回検討会(2003年10月22日)

コウジ酸のSVK14およびHepG2を用いたin vitro小核試験の結果に関する祖父尼委員のレビューが紹介された．

コウジ酸のラット肝RDS試験およびショウジョウバエ試験の中間結果が宇野委員より報告された．RDS試験は強制経口投与と混餌投与で行い，投与3日後に両条件とも最高用量でRDSが誘発された．肝の重量変化および病理組織変化はなかった．体重当たり投与量は混餌投与の方で多かったが，体重増加量は強制経口投与で強く抑制された．甲状腺重量は強制経口投与より混餌投与で著しく増加した．ショウジョウバエのDNA修復および翅毛スポット試験は陰性であった．

コウジ酸のMutaMouseを用いた遺伝子突然試験の計画概要が中嶋委員によって紹介され，内容が協議された．投与用量は1，2および3%とすること，摘出器官に甲状腺を加えることになった．

Kerry L. Dearfield et al.: Genotoxicity risk assessment: a proposed classification strategy. Mutation Res., 521, 121-135(2002)のJETOCによる日本語の解説記事が森田委員より紹介された．

4.9．第9回検討会(2003年11月20日)

コウジ酸の遺伝毒性について，長尾委員より新しいデータが紹介された．3ロットのコウジ酸の各HPLC分画について復帰突然変異誘発能を検討したところ，ロット間に相違は認められず，またNMR分析により活性物質はコウジ酸であることが確認された．このことから，既報告の相反するin vitro試験結果は，各ロットに含有されている可能性のある不純物によるものではないことが明らかとなった．

日本環境変異原学会発表スライドの改訂版が長尾委員より提示された．スライドは若干の変更がかけられた後，最終版とされる予定である．今後，リスク評価に関し必要とされるであろう議論内容について，以下の項目が上げられた：

- 閾値以下のものであれば，いくら加えても問題はないのか？
- 化学物質の使用をやめた場合のリスクは？
- 何に使うかによって許容できるレベルは異なるのでは？
- 発癌性のリスクには閾値があっても，遺伝毒性のリスクとしてはどうか？

4.10．第10回検討会(2003年12月25日)

中嶋委員から，コウジ酸のTG試験(1, 2, 3%混餌)の進捗状況について説明がなされた．12月19日に解剖が終わり，必要な組織(肝臓，胃，結腸，骨髄，甲状腺)を凍結した．肝臓の一部をDNAの酸化的障害(8-OH-dG)を検討するため葛西委員に送付した．マクロでは全処理群で甲状腺の肥大，および最高用量群で子宮の肥大(5倍程度)が観察された．また，最高投与群において軽度な体重増加抑制が認められたが，その他特記すべき事項はない．また，最終日には末梢血のAO超生体染色による小核試験のための標本を作製したので，順次解析予定である．さらに，肝臓の一部を用いてComet試験を実施するための準備を進めている．

森田委員から，Commission of the European CommunitiesのRegulation of the European Parliament and of the Council, concerning the Registration, Evaluation, Authorisation and Restrictions of Chemicals (REACH)に関する紹介がなされた．一般化学物質が対象で，CMR effects(carcinogenicity, mutagenicity and toxicity for reproduction)を中心に考慮し，危険物を規制しようとするものである．生産量により要求される試験項目が変わる点，陰性の場合in vitroの試験がなくても受け入れ可能なin vivo試験結果があれば，それでの評価も行う点等が新しい点であろう．

4.11．第11回検討会(2004年1月16日)

葛西委員からコウジ酸を投与したTGマウス肝における8-OH-dGの測定結果の説明があった．以前の実験では，2%コウジ酸を2週間投与したラットの甲状腺で明らかな減少がみられたが，MutaMouseの肝では3%混餌投与群で有意に増加した．葛西委員の発表について質疑がなされた．主なものとしては，コウジ酸は抗酸化物質なのに今回8-OH-dGが上がった理由に関し，アスコルビン酸でも高用量だと上がることが判っており，コウジ酸の構造から考えて，ラジカルができている可能性が指摘された．その他，ラットでは甲状腺での8-OH-dGの低下がマウスでも再現可能かとの疑問が提示されたが，マウスの甲状腺は非常に小さく，技術的に困難であることが指摘された．また，データのばらつきに関する質問等が出された．また，反応の強さ，再現性に関しての質疑，応答があった．

MutaMouseを用いる試験の進捗状況について中嶋委員から報告がなされた．現時点で，DNA抽出まで終了

しており，これから解析にかかる．また．同じ動物でのComet assay(28日投与の3日後の測定)と末梢血MNは陰性であった．

4.12．第12回拡大検討会(クローズド会議：2004年2月12～13日，国際シンポジウム：2004年2月14日)

本検討会は，海外コンサルタント(M.J. Aardema, D. Benford, D.H. Blakey, S.M. Galloway, D. Kirkland, Y-J. Surh, V. Thybaud, D. Tweats, L. Müller)を招き，拡大検討会として2回に分けて開催した．

1) クローズド会議

第12回拡大検討会議を海外の識者を招いてのコンサルテーション会議として開催するにあたり，本検討会の背景および目的が林委員長より説明された．最終目標は，3年間(2003年4月～2006年3月)の研究期間の最後までの残り2年で，食品関連物質の遺伝毒性に関するリスク評価の戦略を構築し，提言をまとめることにある．

長尾委員より，食品添加物としてのコウジ酸の遺伝毒性リスク評価について説明がなされた．すなわち，日本における食品添加物の規制の歴史，今回のコウジ酸規制の理由，薬事食品審議委員会で検討されたコウジ酸の発癌性ならびに遺伝毒性データの要約，それらを基にしたHERP法やBMD法による発癌リスクの計算結果などが報告され，極めて弱い反応しか示さない化合物の評価をどう扱うかについて問題提起を行った．

宇野委員より，コウジ酸の遺伝毒性に関する新規データについて説明がなされた．本検討会がこの1年間で実施した下記11種の試験/検討の結果を，データをふまえて報告した．

- 培養液のpH：影響なし
- Ames試験における不純物の影響：コウジ酸に起因
- Ames試験(再確認など)：陽性
- 光プラスミド試験：陽性
- TK6/WTK-1細胞による突然変異および小核試験：陽性
- TK6/WTK-1細胞によるComet試験：陽性
- TK6細胞による光遺伝毒性試験：陽性
- ショウジョウバエ翅毛スポット試験：陰性
- 雌MutaMouseによる肝臓遺伝子変異：陰性
- 雌MutaMouseによる肝臓の8-OH-dG形成：陽性
- ラット肝臓RDS試験：陽性

海外コンサルタントを中心として，コウジ酸の発癌性および遺伝毒性データが検証された．一連の議論は「コウジ酸はヒトにも該当する遺伝毒性発癌物質かどうか？」という点に集約された．その結果，結論を導くには現時点ではデータ間のギャップが多く，そのギャップを埋める必要があるとされた．相反する結果が得られた場合には，一般的にはなんらかのクライテリアでその重み付けをすることがあり，その中ではGLP試験か非GLP試験なのか，発表された論文の掲載誌は何か，試験のデザインは適切か，著者の評判はどうなのか，といった点も考慮されることがあるとされた．コウジ酸については，肝発癌性が遺伝毒性メカニズムに起因するものか否かを評価するには，いくつかの不明点，疑問点あるいは問題点すなわちギャップが存在することが指摘された．例えば，発癌性データに関しては，肝腫瘍の質(良性か悪性か)や用いたコウジ酸の質(かび毒の含有率など)が明らかでないこと，ノックアウトマウスを用いた検討では群あたりの匹数が少ないことなど，また遺伝毒性データに関しては，肝臓小核ではマウス陽性/ラット陰性，骨髄/末梢血小核ではラット陽性/マウス陰性のように明確に一致していない点のあること，Comet試験ではtail momentの測定が必要とされたことなどである．これらの事項を解決するには，スライド標本の再評価やADMEデータ収集を含む追加確認試験の実施などが必要と提言された．

2) 国際シンポジウム

第12回拡大検討会議「その2」を，「食品関連物質等のリスクアセスメント戦略」と題した国際シンポジウムとして開催するにあたり，まず，その趣旨が長尾委員より説明された．以下，海外シンポジスト7名および国内シンポジスト2名による発表ならびに総合討論が行われた．その演題の中で，クローズド会議をふまえ，海外コンサルタントの提言が報告された．本シンポジウムの開催により，遺伝毒性を中心とした食品関連化学物質の安全性に関心のある多くの参加者に，遺伝毒性発癌物質であっても閾値を設定できる可能性のあることが認識されたものと思われる．今後は，どのようなケースに閾値の設定が可能なのか，また，その設定はどのように行うのが適切で人々に受け入れられるものなのかを明らかにする必要がある．

4.13．第13回検討会(2004年3月15日)

2004年2月の鎌倉，東京会議について，今回の会議の成果については，Dr. Tweatsより提出される報告書の内容が重要であり，報告書を検討した上で今後の方向性を検討する．会議ではコウジ酸の発がん性，特に肝臓に対する発がん性に疑問が集中した．コウジ酸に遺伝毒性であったとしても甲状腺がんとの直接作用の関係はないであろう．また，肝がんを引き起こすとしても，それが遺伝毒性によるものがはっきりしない．従って，肝臓に対する遺伝毒性の有無を明らかに必要があるのではないか？　肝臓での小核試験を実施する際の問題点(ラットorマウス，加齢による影響，肝部分切除の影響)が話し合われた．

次年度コウジ酸に関する試験としては以下の試験を追

加試験として行うことが提案された：肝小核，肝臓でのDNAアダクトの検出，光毒性を考慮したMLA，ラット肝UDS，TK試験．試験データの信頼性を向上させるため，GLPでの試験，もしくは試験プロトコール等の精査の必要性である．今年度でコウジ酸に関する試験を終了させ，試験結果については来年度中に論文にまとめることを目指す．

次回のモデル化合物としてはアマランス，もしくは他のアゾ化合物を候補とする．化合物が決まり次第，手分けしてこれまでの試験データをレビューする．具体的な進め方に関しては，Dr. Tweatsの報告書を精査した上で，もう一度話し合う．

5．おわりに

「食品および食品添加物に関する遺伝毒性の検出・評価・解釈」に関する臨時委員会の目指しているところを軸に，活動中間報告をまとめた．コウジ酸1つを取り上げても，そのリスク評価は容易なものではなく，検討すべき事項は山積している．検討を重ね，残りの1年半で，JEMSの会員からも理解の得られる遺伝毒性の「検出・評価・解釈」についての戦略を構築したい．

リスクアセスメントの現状と展望
—食品添加物の立場から—

長尾美奈子[1*]，日本環境変異原学会臨時委員会[2]

[1]共立薬科大学　〒105-8512 東京都港区芝公園1-5-30

Present situation and perspective of risk assessment: From the view point of food additives

Minako Nagao[1*] and The Ad hoc Committee of JEMS[2]

[1]Kyoritsu University of Pharmacy, 1-5-30, Shibakoen, Minato-ku, Tokyo 105-8512, Japan
[2]Japanese Environmental Mutagen Society

Summary

Kojic acid (KA), belonging to existing food additives for which compositions or usages are not clarified, had been used for prevention of enzymatic browning. In 1995, the food sanitation law was largely revised to harmonize with JECFA, OECD and FDA. Under the new law, reevaluation of existing food additives was required. In 1998, it was found that KA induced tumors in the thyroid and liver of mice. KA also showed genotoxicities; gene mutations in *S. typhimurium*, chromosome aberrations in CHO-K1 and CHL/IU cells in vitro, and micronuclei in the liver of mice and hematopoietic cells in rats. Although it has not been clarified whether liver or thyroid tumors were induced by genotoxic effects of KA or not, use of KA as a food additive was banned in 2003, based on the fact that KA was not used in any country at that time. The ad hoc committee which was set-up for a three-year task from 2003-2005 considered that KA was an appropriate model compound to re-evaluate the strategies presently used to detect genotoxicity in vitro and in vivo, and to re-evaluate the regulatory rules (use of genotoxic carcinogens as food additives should be totally avoided; genotoxic non-carcinogens in rodents can be used as food additives). First of all, we confirmed the genotoxicity of KA; we demonstrated that genotoxicity in *S. typhimurium* was due to KA itself, but not due to contaminants, KA induced TK mutations, micronuclei and DNA damage (Comet) in human lymphoblastoid cells, TK6 and WTK-1. These results support the finding that KA is genotoxic in vivo, although it is not clear yet whether KA induces tumors by its genotoxicity or not. Speculating that liver tumors induced by KA were due to its genotoxicity, human risks to KA to which humans are exposed by taking fermented food products was calculated to be 2×10^{-7} by the linearized multistage model.

Keywords: kojic acid, genotoxicity, tumorigenicity, risk, regulatory rule

* E-mail: mnagao@m8.dion.ne.jp
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背　景

食品添加物については，遺伝毒性の検出は規制の点から極めて重要である．遺伝毒性は単純，明確なマーカーのように思われるが，これをリスク評価に使おうとすると多くの問題がある．林　　真 日本環境変異原学会前会長（2002～2003年）の提案で，「食品および食品添加物に関する遺伝毒性の検出・評価・解釈」を検討することを目的とし，環境変異原学会の中に臨時委員会が設けられた．この臨時委員会は委員長・林　　真，副委員長・長尾美奈子，委員・宇野芳文，太田敏博，祖父尼俊雄，布柴達男，能美健彦，本間正充，森田　健氏からなる．実験的裏づけに基づいて問題点を整理したいという考えから，林　　真を班長とする厚生労働科学研究補助金（食品安全確保研究事業）「既存添加物の遺伝毒性検出の戦略に関する研究」の班員，田中憲穂，太田敏博，中鳩　圓，葛西　宏，長尾美奈子，佐々木　有，本間正充と合同で，毎月1回会合を持っている．研究班としては，最近使用禁止になった既存添加物・コウジ酸を取り上げて，遺伝毒性検出に関わる補充実験を行っている．この臨時委員会―厚労省研究班合同会議での，遺伝毒性検出および評価に関する取り組みについて，また，現在得られている結果に基づくリスクアセスメントについて報告する．

食品添加物の規制

わが国の食品添加物の規制は，厚生労働省（旧厚生省）の管轄になってから50年以上が経過する．現在使用が許可されている食品添加物は指定添加物，既存添加物，天然香料，その他に分類される．平成13年1月現在でそれぞれ338，489，612および72品目ある．コウジ酸は既存添加物，いわゆる天然添加物に属し，1995年の法改正の時点で既に使われていたものであり，成分規格・使用基準が設定されていなかった．しかし現在の法律では，新規食品添加物は，化学合成品か天然由来かを問わず，全て規格・基準の設定が要求されており，また，既存添加物についても規格・基準の設定が順次行なわれている．そのような過程で，コウジ酸が遺伝毒性発がん物質である可能性が示され，2002年12月に薬事・食品衛生審議会毒性・食品添加物合同部会，食品安全委員会に於いてその可能性が認められ，また，2002年には流通実態がなかったこともあり，パブリックコメントを求め，WTO通報した後，2003年10月に添加物として使用しないよう告示された（厚生労働省，2003）．

ここでわが国の食品添加物の規制について簡単に紹介する．国際的な食品添加物規制に関わる機関としては，FAO（Food and Agriculture Organization）とWHOが合同で設立したコーデックス国際食品規格委員会（CAC）があるが，日本もコーデックス加盟国である．コーデックス食品添加物・汚染物質規格部会に科学的裏づけを提供しているのがJECFA（Joint FAO/WHO Expert Committee on Food Additives; FAO/WHO合同食品添加物専門家委員会）である．

わが国の食品添加物の毒性に関する試験として要求されているものは，①28日反復投与毒性試験，②90日反復投与毒性試験，③1年間反復投与毒性試験，④繁殖試験，⑤催奇性試験，⑥発がん性試験，⑦1年間反復投与毒性・発がん性併合試験，⑧抗原性試験，⑨変異原性試験，⑩一般薬理試験である（厚生労働省行政情報）．

変異原性試験としてはⅠ．微生物を用いる復帰変異試験，Ⅱ．哺乳類培養細胞を用いる染色体異常試験，Ⅲ．げっ歯類を用いる小核試験を実施し，これらの試験結果を補足する必要がある場合には，哺乳類培養細胞，ショウジョウバエ，げっ歯類を用いる遺伝子突然変異試験，げっ歯類の骨髄細胞，生殖細胞を用いる染色体異常試験，げっ歯類を用いる優性致死試験，微生物を用いるDNA修復試験，哺乳類細胞を用いるUDS試験，哺乳類細胞を用いるSCE試験を追加実施する，となっている（厚生労働省行政情報）．

遺伝毒性の評価にはin vivoのデータが採用されるが，それを裏づけるin vitroのデータが重要である．つまり，in vivoではじめて陽性になる遺伝毒性物質は，in vivoではじめて代謝活性化が起こることを示す必要がある．また，微生物を用いる変異原性試験で陽性ということは，その物質または代謝物がDNAと反応する確率が極めて高い点で，重要な意味をもっている．

毒性試験の結果の評価については，わが国でもJECFAでも，発がん性を有し，in vivoで遺伝毒性が示された物質は，食品添加物としての使用は認められていない．遺伝毒性発がん物質を使用禁止している理由を説明しているJECFAの文章をそのまま下に引用する（Food Standards Agency, UK, 2002）．

All studied neoplasms contain mutations of one type or another; there is a single copy of DNA in every cell, therefore, it is reasoned, there can be no threshold of damage below which DNA damage has no consequence, hence, there can be no safe exposure level to a carcinogen that is genotoxic.

JECFAでは，エピジェネティックな変化が発がんに関与すること，遺伝毒性があっても実験動物で発がん性を示さない物質が多々あること，遺伝毒性を示す発がん物質が非遺伝毒性のメカニズムでがんを誘発する場合があることを述べているが，これらを如何に取り扱うべきかについてまでは，言及していない（Food Standards Agency, UK, 2002）．われわれは，げっ歯類のin vivoで

遺伝毒性を示すが，げっ歯類で発がん性を示さない物質がどの位あるのかは重要な問題であると考えている．

in vivo遺伝毒性の試験には，骨髄における造血系細胞を標的としたものが一般的に評価に用いられているが，化合物によっては骨髄に分布し難いものがある．例えばMeIQ—DNA付加体レベルは，骨髄では肝臓の1/70であり（Nagao et al., 2001），*N*-NO-dipropylamineは肝小核では陽性であるが骨髄小核では陰性である（Noguchi et al., 1994）．また，直接変異原で，肝臓で不活性化されるものは，口腔や胃で遺伝毒性を示していないかについては調べられていない．

以上のように，コウジ酸の評価をしながら，現在行なわれている評価法の問題点を考え，一歩ずつ問題解決に向けて本委員会は活動している．

コウジ酸

コウジ酸は麹菌（*Aspergillus oryzae*）の産生する化合物で抗菌作用およびメラニン合成阻害作用を有する．チロシナーゼの阻害作用によるものである．食品添加物（蟹やエビの黒変を防ぐための製造用剤）および医薬部外品（化粧品）として使用されていた．冷凍保存法の普及により，現在では，食品添加物としては使われていない．1998年にマウスにおいて，甲状腺腫瘍および肝腫瘍誘発作用が検出された．発がん性を，Fujimotoら（1998）の論文に基づいて纏めたものをTable1に示す．甲状腺腫瘍に関してはコウジ酸のプロモーション作用によることが，マウスおよびラットで示唆されている（Fig. 1）（Fujimoto et al., 1998; 1999; Tamura et al., 2001）．

一方，コウジ酸にin vivoで遺伝毒性が検出され，特にマウス肝で小核が誘発されたこともあり，遺伝毒性発がん物質の可能性が示唆された．さらに，p53$^{+/-}$ヘテロマウスを用いた発がん実験で，コウジ酸の肝発がん性が遺伝毒性によることを否定できなかった（Takizawa et al., 2003）．

また，薬事・食品衛生審議会に提出された遺伝毒性の成績をTable 2およびTable 3に纏めた．

コウジ酸の遺伝毒性は，サルモネラ菌突然変異，哺乳動物培養細胞染色体異常で陽性，ラット（幼若）骨髄および末梢血における小核，マウス肝（成熟，再生肝）における小核で陽性であったが，マウス骨髄（成熟）およびラット肝（幼若）小核試験は陰性であった．そして，このラットとマウスにおける遺伝毒性発現の差を説明できる状態にはなかったが，2002年の時点ではコウジ酸

Table 1 Carcinogenicty of kojic acid[1)]

	Kojic Acid	Tumor incidence (%)	
	%	Thyroid tumor	Liver tumor
Male	0	2	48
	1.5	65**[2)]	69
	3	87**	47
Female	0	2	0
	1.5	8	4
	3	80**	10*

[1)] Fujimoto et al., 1998. Kojic acid was administered to B6C3F1 mice in diet for 20 months. Effective numbers of animals were between 48-53.

[2)] * and **; significantly different from the control value at $P < 0.05$ and $P < 0.01$, respectively

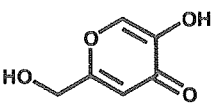

Fig. 1 Kojic acid

Table 2 Genotoxicity of kojic acid in bacteria and mammalian cells in vitro[1)]

Marker for DNA damage	Cell	S9	No. of experiments	Result
Bacteria				
SOS repair	*E. coli*	with and without	1	Negative
Rec assay	*B. subtilis*	without	1	Positive
Gene mutation	*S. typhimurium*	with and without	6	Positive 5; Negative 1 (5 mg/mL)[2)]
Mammalian cells				
Hprt mutation	V79	with and without	1	Negative (3 mg/mL)
Hprt mutation	L5178Y	with and without	1	Negative (1.4 mg/mL)
SCE	CHO-K1	with and without	1	Positive
Chromosome aberration	CHO-K1	with and without	1	Positive
Chromosome aberration	CHL/IU	with and without	2	Positive and Negative (5 mg/mL)
Chromosome aberration	V79	with and without	1	Positive due to toxicity
Micronucleus	HepG2	with and without	1	Negative (8 mg/mL)
Micronucleus	CHL/IU	with and without	1	Inconclusive (2 mg/mL)

[1)] December 2002. Consultation meeting for dugs and foods, MHLW

[2)] The maximum concentration used

Table 3 Genotoxicities of kojic acid in vivo[1)]

	Tissue, cell	Marker for DNA damage	Lowest effective dose (g/kg×times)	Maximum dose (g/kg×times)	Number of experiments	Result
Mice						
	Bone marrow (adult)	Micronuclei		1×2	3	Negative
	Hepatocytes (adult, PH)	Micronuclei	1×1		1	Positive
	Hepatocytes (adult)	Comet	1×1	1×1	2	Positive and negative
	Liver (adult)	Lac Z		1.6×28	1	Negative
	Thyroid (adult)	Comet		0.75×1	1	Negative
Rats						
	Bone marrow (young)	Micronuclei	2×2		1	Positive
	Peripheral blood (young)	Micronuclei	2×2		2	Positive
	Hepatocytes (young)	Micronuclei		2×1	2	Negative
	Hepatocytes (adult)	Comet	1×1		1	Positive
	Hepatocytes (adult)	UDS		1.5×1	1	Negative

[1)] December 2002. Consultation meeting for drug and foods, MHLW

Table 4 Genotoxicity of kojic acid in human lymphoblastoid cells *

Marker	Cells	Result	Effective concentrations of kojic acid (mg/mL)
Mutation (TK)	TK6	Positive	1～4
	WTK-1	Positive	2～4
DNA damage (Comet)	TK6	Positive	2.5～5
	WTK-1	Positive	2.5～5
Micronuclei	TK6	Positive	2～3
	WTK-1	Positive	1～2

* Genotoxicities were examined without S9 mix.

が食品添加物としては使用されていなかったこともあり，使用禁止の措置がとられたわけである．

コウジ酸の遺伝毒性の再評価

以上の結果を踏まえて，本委員会で遺伝毒性検出法の問題点を討議し，試験物質のロットの違いによる可能性に配慮すると共に，さらにデータの補充を行なうことからはじめた．

1. サルモネラ菌，大腸菌における復帰突然変異

3種の異なるロットのコウジ酸，食品添加物用（5312），医薬部外品用（2Y181），および試薬（052K2516）を用いた．サルモネラ菌TA100，TA98，TA102，大腸菌WP2*uvrA*/pKM101に対し，いずれのロットもほぼ同程度の変異原性を示した．S9 mix存在下では活性はやや低い傾向にあった．比活性はTA100，−S9 mixで，～100変異コロニー/mgであった．さらにコウジ酸をHPLCで分離し，＋S9 mixおよび−S9 mixで検出される変異原性がコウジ酸自身によることを明らかにした．

2. ヒト培養細胞における遺伝毒性

哺乳類の細胞としては，近い将来にヒトの細胞を使うことが望まれる．また，コウジ酸の培養細胞における変異原性は*Hprt*を標的とした解析が行なわれていたが，いずれも陰性であったことから，TKを標的とした解析を行なった．用いた細胞は，ヒトリンパ芽球様細胞TK-6（$TK^{+/-}$）およびWTK-1（$TK^{+/-}p53^{mut/-}$）である．TK変異，小核，DNA傷害（コメット）いずれも陽性であった．突然変異頻度を自然突然変異頻度の2倍に増加させる濃度は両細胞とも2 mg/mLであった．

食品添加物の指針には，遺伝毒性の試験に用いられる試験物質の濃度は最高 10 mMと定められているが（その理由はDNAと直接反応しない，いわゆる閾値のある遺伝毒性物質を拾う確率が高いためである，Scott et al., 1991），サルモネラ菌に対する変異原性から，ヒトリンパ芽球様細胞における染色体異常は遺伝毒性により誘発されたと考えられる．

3. In vivo遺伝毒性

Table 4に示すようにコウジ酸は*LacZ*トランスジェニックマウスで陰性との報告がある（Nohynek et al., 2004）．しかし，肝発がん性が観察されたのは雌マウスであること，また，強制経口投与であることから，雌マウスに混餌投与することにより確認実験を行なってい

Table 5 Risk evaluation of kojic acid naturally present in fermented foods

Human exposure	
0.6 μg/kg/day	
HERP index	
Based on male mice thyroid tumor	$TD_{50} = 1.4 \times 10^3$ mg/kg/day
Based on feale mice liver tumor	$TD_{50} = 1.4 \times 10^4$ mg/kg/day
HERP index = 4.2×10^{-7} (thyroid)	
HERP index = 4.2×10^{-8} (liver)	
Linearized multistage model for genotoxic carcinogen	
Mouse BMD_{10} = 29,600 ppm	
Mouse LED_{10} = 18,600 ppm	
Mouse kojic acid intake at $LED_{10} = 2.4 \times 10^6$ μg/day	
Human tumor risk	$0.6/2.4 \times 10^{-7} \times (50/0.025)^{0.75} = 1.67 \times 10^{-7}$

る．また薬事・食品衛生審議会に提出された遺伝毒性の成績のうちマウスおよびラットの，それぞれ肝および骨髄における小核試験は，当委員会委員の実験結果であり，実験の詳細についての議論が可能であった．用いた動物の週齢，部分肝切除の適用などの点を統一した上で，なおラットとマウス間で遺伝毒性の発現に差がある場合は代謝の差に由来するのかを追求する方針である．

食品中のコウジ酸のリスク評価

薬事・食品衛生審議会では，醗酵食品中におけるコウジ酸の濃度を調べた結果，味噌41検体，醤油32検体，酒29検体のうち，味噌2検体から各々0.5 ppmおよび1 ppm濃度で，醤油1検体から1 ppm濃度で検出されたと報告されている．国民栄養調査に基づき，1日摂取量を味噌3g，醤油27 mLとし，味噌および醤油には1 ppmのコウジ酸が含まれているとすると，コウジ酸の摂取量は0.6 μg/kg/dayと見積もられる．

ヒトのコウジ酸に対する暴露量を0.6 μg/kg/dayとして，コウジ酸による発がんリスクの算出を試みた．一般に最もよく用いられている，HERP index（Human exposure to rodent potency index）および，米国EPAが遺伝毒性発がん物質のリスク評価に用いている線形多段階モデル（Linearized multistage model）法に基づいて算出した．結果をTable 5に示す．

HERPは，発がん物質が遺伝毒性であるか否かを問わず，動物発がんにおいて用量相関が直線であり，動物の単位体重量当たりの暴露量に基づくデータを，そのままヒトに適用する方法である（Gold et al., 1991）．B6C3F1マウス雄甲状腺腫瘍およびB6C3F1マウス雌肝腫瘍誘発率に基づくHERPはそれぞれVSD（virtually safety dose；10^{-6}の発がん率）の1/10～1/100であった．なお，Tamuraら（2001）は甲状腺腫瘍に関してはコウジ酸のNOAEL（no-observed-adverse effect for thyroid tumor-promoting effect）を0.03％（15.5 mg/kg/day）としている．一般にはNOALEに安全係数100を用いて1日許容摂取量が算出される．其の値は155 μg/kg/dayとなり，食品からのヒト暴露量はその1/260となる．

線形多段階モデルではBMD_{10}およびLED_{10}を用いる（関沢ら，2001）．ベンチマーク量（BMD, Benchmark Dose）は観察された領域内での用量―反応曲線の下限値を決定する一つの代替法であり，発がん実験では用いる動物の匹数からいって，10％の発がん率の増加（BMD_{10}）は検出できる下限値である．発がん実験のデータよりMultistage model（Armitage and Doll, 1961）に従ってBMD_{10}を求め，低用量側95％信頼限界の値LED_{10}（Lower 95% confidence limit for the dose giving the animals an increased tumor incidence of 10%）を求める．それより低濃度側における用量相関は，0点を通る直線を示すとしてリスクを評価する方法である．この方法では，薬物代謝は体表面積に比例する，すなわち体重の0.75乗（$W^{0.75}$）に比例するとしている．この評価法に従っても肝発がんリスクはVSD値より低い1.65×10^{-7}であった．

終わりに

コウジ酸が遺伝毒性発がん物質であるか否かまだ結論は出ていない．コウジ酸は，医薬部外品として化粧品に用いられている．現在は，「新しくはコウジ酸入りの化粧品は作らない」という暫定措置が取られている状態であり，マウス肝発がんが遺伝毒性により誘発されるのか，ラットに発がん性を示すのか，光との相互作用はどうか等が検討されている．

たとえコウジ酸が遺伝毒性発がん物質であるとしても，遺伝毒性も発がん性も極めて弱い．コウジ酸自身のリスクは極めて低いといって過言では無いであろう．しかし，ヒトのがんは多数の遺伝子変異の結果であり，その全ての過程が一つの因子によって起こるのは，職業がんの場合位であろう．多くのヒトのがんは，いろいろな因子により惹き起こされた遺伝子変異の結果と考えられので，そういうもののリスクを如何ように評価すべきな

のか実はよく判らないのである．科学的によく判らない時には，安全性を重視し，代替品を含めて総合的に，社会的良識を加味して判断していくことになる．

いずれにしても，in vivoにおける遺伝毒性の検出，つまり調べたいと思う臓器での遺伝毒性を測定できるようにすることが急務であろう．

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Simultaneous HPLC analysis of 8-hydroxydeoxyguanosine and 7-methylguanine in urine from humans and rodents

Peter Svoboda, Hiroshi Kasai*

Department of Environmental Oncology, Institute of Industrial Ecological Sciences, University of Occupational Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan

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Abstract

With a recently developed high-performance liquid chromatography (HPLC) method based on anion exchange chromatography, precise fraction collection, and reversed-phase chromatography, the oxidative DNA damage marker 8-hydroxydeoxyguanosine (8-OH-dG) was measured in human urine samples. The HPLC analysis was further modified to measure 8-OH-dG in rat and mouse urine samples. In addition, the urinary RNA degradation product 7-methylguanine (m^7Gua) was analyzed simultaneously. The correlation coefficient (r) for the correlation between urinary creatinine and m^7Gua was 0.9 for rats and 0.8 for humans and mice. Levels of 8-OH-dG in relation to urinary creatinine were compared and found to be similar for humans and rats and twice as high for mice. Urinary levels of m^7Gua, as normalized to creatinine, were several-fold higher in rodents as compared with human levels, thereby correlating with the higher resting metabolic rate of rodents. The presented results show that 8-OH-dG and m^7Gua can be analyzed simultaneously and reliably in urine from humans and rodents. In addition, m^7Gua may be used as a reliable marker instead of creatinine for the normalization of 8-OH-dG in urine from rats and mice and also may be used in addition to normalization with creatinine in measurements of 8-OH-dG in human urine samples.

Keywords: 7-Methylguanine; 8-Hydroxydeoxyguanosine; Oxidative DNA damage

Oxidative DNA damage occurs intracellularly in response to the endogenous formation of oxygen radicals and as a result of attacks from exogenous sources such as ionizing radiation and certain mutagenic compounds [1]. The types of damage produced may be strand breaks in DNA or different types of base damage such as 8-hydroxydeoxyguanosine (8-OH-dG)[1] [2]. This potentially mutagenic product is repaired by the process of base and nucleotide excision, released from the cell, and eventually excreted through the urine [3]. It has been estimated that several hundred molecules of 8-OH-dG/cell/day are formed, as well as similar levels of the base 8-OH-guanine, as measured in excreted urine [1,4].

Recently, the reliability and speed of 8-OH-dG analysis in human urine have been further improved using an automated high-performance liquid chromatography system coupled to an electrochemical detector (HPLC-EC) [5]. This system is based on anion exchange chromatography in the first chromatography step (HPLC-1), precise fraction collection, and reversed-phase chromatography in the second chromatography step (HPLC-2). There is no need to prepurify the urinary samples; thus, the sensitivity and reproducibility are enhanced. Currently, this new system is being used to measure the human urinary excretion of 8-OH-dG in response to

* Corresponding author. Fax: +81 93 601 2199.
E-mail address: h-kasai@med.uoeh-u.ac.jp (H. Kasai).

[1] *Abbreviations used:* 8-OH-dG, 8-hydroxydeoxyguanosine; HPLC-EC, high-performance liquid chromatography system coupled to an electrochemical detector; m^7Gua, 7-methylguanine; 8-OH-G, 8-hydroxyguanosine; RMR, resting metabolic rate.

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various lifestyle factors, such as diet and smoking, and exposure to toxic agents, such as mercury and polycyclic aromatic hydrocarbons.

In this article, we present a modification of this method to measure the urinary excretion of 8-OH-dG from rats and mice. In addition, with anion exchange chromatography (HPLC-1), we identified the human and rodent urinary RNA degradation product 7-methylguanine (m^7Gua). This urinary product was found to be a suitable marker for the normalization of 8-OH-dG values between samples from different individuals or between different collection times. Currently, the metabolic product creatinine, from skeletal muscle metabolism [6,7], is measured to normalize urinary 8-OH-dG values. However, creatinine values may differ between individuals due to age and sex differences in the ratio between skeletal muscle and total body lean mass [8], and they are also affected by exercise and diet [9–12]. The degradation and urinary excretion of specific RNA metabolites, such as 5,6-dihydrouridine, pseudouridine, m^7Gua, and N2-dimethylguanosine, have been shown to correlate with the resting metabolic rate (RMR) in humans and rats [8,13,14]. Topp et al. [15] suggested that the excretion values of 8-OH-dG should be normalized to the metabolic rate to account for the effects of prooxidants, antioxidants, and individual differences in DNA repair [15]. Thus, we evaluated the normalization of urinary 8-OH-dG to the urinary m^7Gua, as well as to creatinine, in humans, rats, and mice. The benefits of this analysis would be that a single urine sample may be analyzed for both 8-OH-dG and m^7Gua in the same sample run, thereby eliminating the need for creatinine measurements with variations due to different amounts and aliquots of the sample analyzed. In addition, a sample may be assayed for 8-OH-dG and m^7Gua rapidly, that is, in approximately 1 h. Urinary levels of m^7Gua increase in response to methylating agents such as those from tobacco smoke [16]. Thus, measuring urinary m^7Gua would also be useful for detecting changes in DNA and RNA methylation levels due to exposure to exogenous methylating agents such as *N*-nitroso compounds or to the endogenous *S*-adenosylmethionine activity [17].

For studies of the various effects of agents or conditions on homogeneous groups, such as rats and mice, normalizing urinary 8-OH-dG to urinary m^7Gua would be expected to be a more rapid and comparably accurate method than would normalizing levels to creatinine.

Materials and methods

Materials

The 8-OH-dG and m^7Gua used for standards were obtained from Sigma Chemical (USA). 8-Hydroxyguanosine (8-OH-G), used as a marker for fraction collection, was prepared as described previously [5,18]. The anion exchange resin MCI GEL CA08F (7 μm, Cl^- form) was purchased from Mitsubishi Chemical (Japan) and was prepared as described previously [5] before it was manually packed in a guard (1.5 × 50-mm) column and a main (1.5 × 150-mm) column for use in HPLC-1. The reversed-phase column (Capcell Pak C18, 5 μm, 4.6 × 250 mm) used in HPLC-2 for the analysis of the 8-OH-dG fraction was purchased from Shiseido (Japan). The same type of reversed-phase column was used for the separation of m^7Gua and its subsequent detection with a photo diode array UV detector. HPLC-grade methanol and acetonitrile were purchased from Wako Pure Chemical (Japan) and Kanto Chemical (Japan), respectively.

Collection of urine samples

From a stock of frozen (−80 °C) human urine samples from a previous study [19], a subset of 44 urine samples from nonsmokers was used in the current study. Male Wistar rat urine samples were collected individually from 36 rats, ages 10–11 weeks, during a period of 24 h by spontaneous excretion in metabolic cages. Female C3H/He mouse urine samples were collected individually from 22 mice, ages 13–24 weeks, during a period of 4 or 24 h by spontaneous excretion in metabolic cages. Rats and mice were furnished with a standard diet and drinking water ad libitum. At the end of the collection period, the urine from rats or mice was transferred to Eppendorf tubes and frozen (−80 °C).

Measurement of urinary creatinine levels

Creatinine in urine samples (1 ml) from humans and rats was measured by a commercial laboratory (BML, Japan) using a colorimetric method. Because the amount of available urine from the mice was small (500–1000 μl), samples were diluted with an equal amount of water before the creatinine analysis by the commercial laboratory. A few mouse urine samples that were very small (100 μl) were measured for creatinine with a colorimetric assay kit (Jaffe's method) at our university.

Analyses of 8-OH-dG and m^7Gua in human urine

Human urine samples were defrosted and mixed with an equal volume of a 4% acetonitrile solution containing the ribonucleoside marker 8-OH-G (120 μg/ml), 130 mM NaOAc, and 0.6 mM H_2SO_4 [5]. The Eppendorf tubes containing this solution were then stored at 5 °C for a minimum of 4 h before they were centrifuged at 13,000 rpm for 5 min. Samples were transferred to plastic HPLC injector vials, and 20-μl aliquots were analyzed for 8-OH-dG by the use of an automated HPLC system,

as described in detail previously [5]. In essence, the system was composed of a sampling injector (Gilson 231XL), a pump (Shiseido Nanospace SI-2) for the anion exchange guard and main column in HPLC-1 (the flow rate was 37 μl/min and the column oven was set at 65 °C), a UV detector (Toso UV-8020 with a micro cell), a second pump (Shimadzu LC-10AD) for the analysis of the 8-OH-dG fraction with a reversed-phase column in HPLC-2 (the flow rate was 1 ml/min and the column oven was set at 40 °C) connected with an EC detector (ESA Coulochem II), and two switch valves. A third pump (Shiseido Nanospace SI-2) was used to back-wash the guard column (flow rate 37 μl/min) for 32 min after valve switching at approximately 13 min after each sample injection. For HPLC-1, the solvent was composed of 2% acetonitrile in 0.3 mM sulfuric acid. For HPLC-2, the solvent was composed of 10 mM phosphate buffer, pH 6.7, 5% methanol, and an antiseptic Reagent MB (100 μl/L), and it was recycled for a time period of 1 week. The guard column was back-washed with 0.5 M ammonium sulfate:acetonitrile (7:3 v/v). For the detection of the m^7Gua peak in HPLC-1, the UV detector was set to 305 nm instead of 254 nm, as described previously [5], to minimize interference from neighboring peaks. The 8-OH-G marker peak used for automatic peak detection [5] of 8-OH-dG was detected at 305 nm. After automatic peak detection at two-thirds of the height of the marker peak, the 8-OH-dG fraction was precisely collected by valve switching, injected on HPLC-2, and detected by a Coulochem II EC detector (ESA) with a guard cell (5020) and an analytical cell (5011). The applied potentials were as follows: guard cell = 400 mV, E1 = 280 mV, and E2 = 350 mV. The total time between the analyses of consecutive samples was 60 min. The automatic peak detection was controlled by software from Gilson, and the chromatograms were recorded (PowerChrom EiCOM EPC-300 Data Processor) and integrated with computer software (PowerChrom 2.1).

Analyses of 8-OH-dG and m^7Gua in rodent urine

Urine samples from rats and mice were prepared in the same way as the human samples and were stored at 5 °C overnight before they were centrifuged at 13,000 rpm for 5 min. Samples (20 μl) were analyzed for 8-OH-dG by the use of a similar automated HPLC system as described for the human samples above. The system was composed of a sampling injector (ESA 542), a pump (ESA 582) for the anion exchange guard and main column in HPLC-1 (the flow rate was 45 μl/min and the column oven was set at 65 °C), an experimental model of a UV detector (FLOM, Japan) with integrated hardware peak recognition set at a single wavelength (254 nm), an additional UV detector (Tosoh UV-8020 with micro cell) for the detection of m^7Gua at 305 nm, a second pump (ESA 542) for the reversed-phase HPLC-2 column (for rat urine analysis, the flow rate was 0.67 ml/min and the column oven was set at 48 °C; for mouse urine analysis, the flow rate was 0.33 ml/min and the column oven was set at 60 °C) connected with an EC detector (ESA Coulochem III), and two switch valves. A third pump (ESA 582) was used to back-wash the guard column (the flow rate was 45 μl/min) for 32 min after valve switching at approximately 13 min after each sample injection. The solvents used for the HPLC-1 and guard columns were the same as those described above for the analysis of human urine. The solvent used in HPLC-2 was composed of 10 mM phosphate buffer, pH 6.0, 2% methanol, and an antiseptic Reagent MB (100 μl/L), and it was recycled for a time period of 1 week. After automatic peak detection at one-half the height of the marker peak, the 8-OH-dG fraction was collected and then injected on HPLC-2, for detection by a Coulochem III EC detector (ESA) with a guard cell (5020) and an analytical cell (5011). The applied potentials were as follows: guard cell = 350 mV, E1 = 170 mV, and E2 = 300 mV. The total time between the analyses of consecutive samples was 80 min. Chromatograms were recorded (Dionex UCI-100) and integrated with computer software (Chromeleon 6.30).

Verification of urinary 8-OH-dG and m^7Gua with standards

The specificity of the 8-OH-dG peak in HPLC-2 for the human, rat, and mouse samples was verified using three different methods. The first test was to calculate the peak ratio between the lower potential setting on the EC detector (E1 = 170 mV) and the higher setting (E2 = 300 mV) for a random sample of mouse urine (prepared as described above) using two different flow rate and column temperature conditions in HPLC-2 (0.67 ml/min and 48 °C or 0.33 ml/min and 60 °C). The calculated ratios were compared with that of an 8-OH-dG standard analyzed using the same conditions. In the second test, three random urine samples from human, rat, and mouse, respectively, were pooled, prepared as described above, and then spiked with known concentrations of an 8-OH-dG standard (0, 1, 2.5, and 4 ng/ml). For the spiked samples, the recovery of 8-OH-dG in HPLC-2 was calculated. The applied potentials on the EC detector were as follows: guard cell = 420 mV, E1 = 200 mV, and E2 = 370 mV. Finally, in the pooled samples from human, rat, and mouse, the specificity of the 8-OH-dG peak detected in HPLC-2 was verified with that of an 8-OH-dG standard by comparing the electrochemical voltammograms. A pooled urine sample or an 8-OH-dG standard (5 ng/ml) was analyzed at the following applied potentials on the EC detector: guard cell = 450 mV, E1 = 0 mV, and E2 = 175–400 mV. For these tests, the HPLC equipment described above for the analysis of rodent urine was used. The solvent for

HPLC-2 was that used for the analyses of human and rodent urine. The peak detection for automatic fraction collection in HPLC-1 was set at one-half the height of the marker peak (254 nm). The volume of the injected sample or standard was always 20 μl (after mixing with an equal volume of a 4% acetonitrile solution containing the ribonucleoside marker). Settings for the applied potentials on the Coulochem III EC detector were adjusted according to the sensitivity of each different analytical cell (5011) used. The concentration of the 8-OH-dG standard was determined using a molar absorption extinction coefficient (ε) of 12,300 $M^{-1} cm^{-1}$ at 245 nm in H_2O [20].

The m^7Gua was identified from rat urine after repeated collections of 20-μl fractions of the eluted peak at 12 min. From these pooled fractions, a 150-μl aliquot was injected on an HPLC system connected with a photo diode array UV detector (Hewlett–Packard, series 1100), and after separation with a reversed-phase column (the mobile phase used was a gradient of 0.1–20% acetonitrile) the absorbance spectrum for the eluted peak at 12 min was recorded and compared with that of a 150-μl aliquot of the m^7Gua standard (28.4 mg/L). The concentration of the m^7Gua standard was determined using a molar absorption extinction coefficient of 7300 $M^{-1} cm^{-1}$ at 283 nm in 100 mM sodium phosphate, pH 7.0.

Summary of various electrode settings used

The settings for the applied potentials on the Coulochem II/III EC detectors had to be manually adjusted for each different analytical cell (5011) used in HPLC-2. This is due to the inherent different sensitivity of each analytical cell. The guard cell (5020), used to remove traces of impurities from the recycled solvent, was always set at a 50-mV higher setting than the highest setting used for E2 of the analytical cell. Thus, for measurement of human urine samples, the applied potentials of the specific analytical cell used were E1 = 280 mV and E2 = 350 mV (guard cell = 400 mV). For rodent urine samples, another analytical cell was used with the applied potentials of E1 = 170 mV and E2 = 300 mV (guard cell = 350 mV). Because of deterioration of the previously used analytical cells, a new analytical cell was used with human and rodent urine to obtain electrochemical voltammograms. In this case, the analytical cell was set to E1 = 0 mV and E2 = 175, 200, 225, 250, 275, 300, 325, 350, 375, and 400 mV (guard cell = 450 mV). After the same analytical cell was washed with acetone, it was used for the analysis of urine samples spiked with known concentrations of an 8-OH-dG standard. For that purpose, the analytical cell was set to E1 = 200 mV and E2 = 370 mV (guard cell = 420 mV). With the use of an in-line graphite filter (ESA) before the analytical cell, the cell may be used for several months without washing or replacement.

Calculation of results and statistics

The HPLC chromatograms for 8-OH-dG and m^7Gua were recorded and integrated with a computer, as specified above, and were quantified by comparing the peak areas with those obtained from external standards analyzed on a daily basis. The yields were recalculated to 8-OH-dG/creatinine (μg/g creatinine) and nanograms of 8-OH-dG collected during 24 h normalized to body weight (ng/24 h/kg BW). Yields of m^7Gua are presented after normalization to creatinine (mg/g creatinine) or as micrograms of m^7Gua collected during 24 h normalized to body weight (μg/24 h/kg BW). To calculate the correlation between creatinine and m^7Gua, yields were recalculated to grams per liter of urine (g/L).

To test for significant differences between the mean values, the Student's t test was used. Results were considered as significantly different at $P < 0.05$.

Results

Analysis of 8-OH-dG in human and rodent urine

In addition to the electrochemical analysis of human urinary 8-OH-dG after automatic peak recognition and fraction collection [5], we detected 8-OH-dG in rat and mouse urine. In HPLC-2, at a flow rate of 1 ml/min (eluent pH 6.7 and 5% methanol) and with the column oven set at 40 °C, the human urinary 8-OH-dG peak eluted at around 20 min (Fig. 1B). With the flow rate changed to 0.67 ml/min (eluent pH 6.0 and 2% methanol) and the column oven set at 48 °C, the rat urinary 8-OH-dG peak eluted at around 37 min (Fig. 2B). For the detection of mouse urinary 8-OH-dG, the flow rate was set to 0.33 ml/min (eluent pH 6.0 and 2% methanol) and the column oven was set at 60 °C. Under these conditions, all interfering peaks were separated and the mouse urinary 8-OH-dG peak eluted at around 39 min (Fig. 3B). As shown in Figs. 1B, 2B, and 3B, the 8-OH-dG peaks are completely separated from the neighboring peaks. The peak purity was further verified by calculating the ratio between the E1 peak areas (lower lines in Figs. 1B, 2B, and 3B) and the E2 peak areas (upper lines in those figures). These peak ratios were equal to those of the analyzed 8-OH-dG standards, thereby confirming the peak purity for 8-OH-dG in each individual sample analyzed (chromatograms are not shown for the 8-OH-dG standards).

Evaluation of the 8-OH-dG detection reliability in human and rodent urine

It is important to confirm that the 8-OH-dG peak in HPLC-2 is not contaminated by impurities. In Fig. 4B, we show an example of the detection of a falsely positive

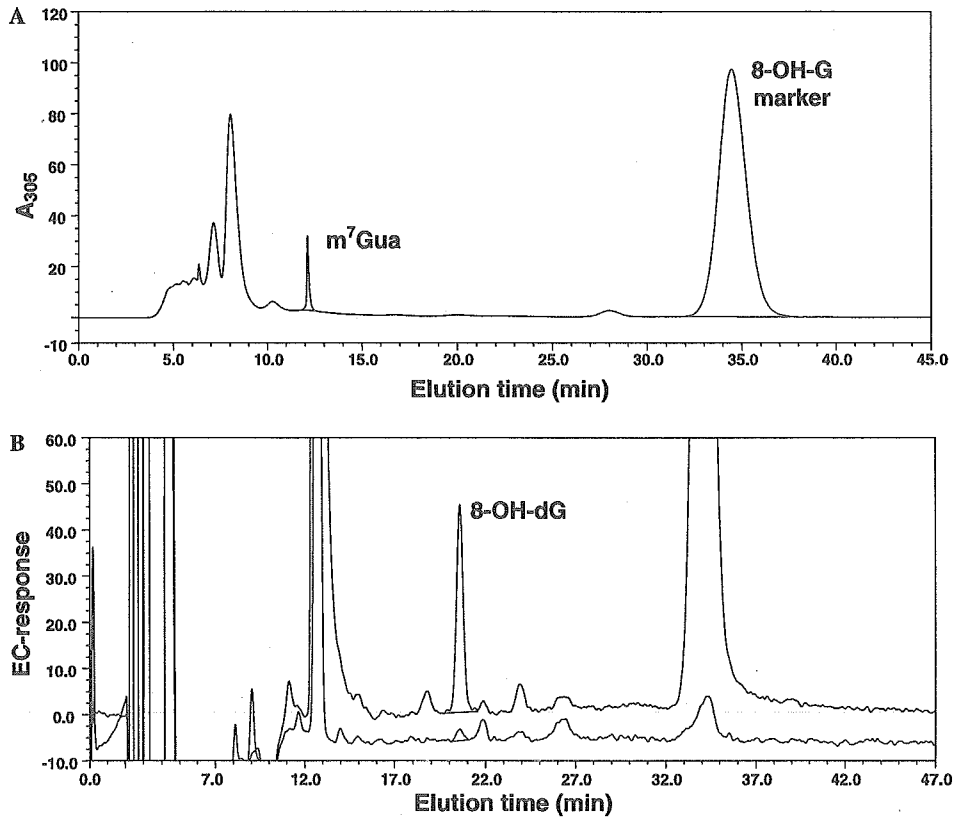

Fig. 1. Chromatograms of a human urine sample (male nonsmoker): (A) HPLC-1 UV detection of m^7Gua and 8-OH-G marker peaks and (B) HPLC-2 electrochemical detection of 8-OH-dG at applied potentials of E1 = 280 mV (lower line) and E2 = 350 mV (upper line).

8-OH-dG peak. A random sample of mouse urine was analyzed using two different flow rate and column temperature conditions in HPLC-2. In the first chromatogram (Fig. 4A), where the flow rate was 0.67 ml/min and the column temperature was 48 °C, the 8-OH-dG peak detected at an applied potential of E1 = 170 mV (lower line in Fig. 4A) had a peak area that was 55% of the peak area detected at an applied potential of E2 = 300 mV (upper line in Fig. 4A). However, using the same conditions, the mean peak area of the 8-OH-dG standards ($n = 4$), as detected at E1, was 22% of the peak area detected at E2 (chromatograms not shown). This means that the mouse sample peak area, as detected at E1 (lower line in Fig. 4A), was more than twice as high as expected and, thus, would be classified as a false positive due to the presence of contamination. Changing the conditions for HPLC-2, to a flow rate of 0.33 ml/min and a column temperature of 60 °C, resolved the interfering peak/peaks in the same mouse urine sample (Fig. 4B). The 8-OH-dG peak detected at E1 (lower line in Fig. 4B) had a peak area that was 26% of the peak area detected at E2 (upper line in Fig. 4B). Under these conditions, the mean peak area of the 8-OH-dG standards ($n = 4$), as detected at E1, was 24% of the peak area detected at E2 (chromatograms not shown). This means that the ratio between the E1 and E2 peak areas (cf. lower and upper lines in Fig. 4B) was nearly equal to the expected ratio. Thus, each urine sample analyzed can be tested for a false 8-OH-dG positive if an interfering compound has significantly different electrochemical properties than does genuine 8-OH-dG. To check for urine sample matrix effects on the 8-OH-dG detection, pooled urine samples from human, rat, and mouse were spiked with 0, 1, 2.5, and 4 ng/ml of an 8-OH-dG standard (Table 1). All spiked samples had 8-OH-dG recoveries of nearly 100% and showed a linear increase in the amount of 8-OH-dG detected (Table 1). Thus, we concluded that the detected 8-OH-dG peak was not affected by any urine sample matrix effects. Also, the linear increase in the amount of 8-OH-dG detected in the spiked urine samples shows that there is no binding of the added 8-OH-dG to the vial surface or coprecipitation of 8-OH-dG with urine components. In Fig. 5, unspiked pooled urine from humans (lower line) can be compared with the same urine that was spiked with 4 ng/ml of an 8-OH-dG standard (upper line). As expected, the peak area increased after spiking the sample (cf. lower and upper lines in Fig. 5), corresponding to an increase in the amount of 8-OH-dG from 7.2 ng/ml (unspiked sample) to 11.2 ng/ml (spiked sample). The urine samples and standards (5 ng/ml) were also analyzed at different potentials set on the electrochemical detector to obtain voltammograms. Figs. 6–8 show that

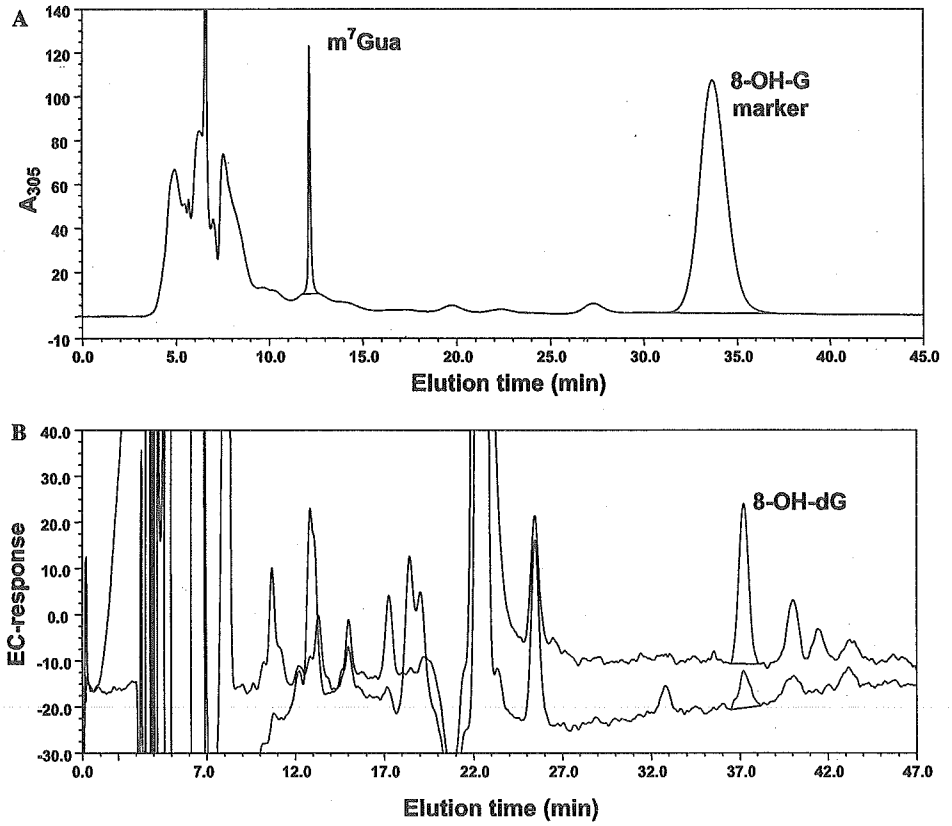

Fig. 2. Chromatograms of a rat urine sample (male Wistar rat): (A) HPLC-1 UV detection of m^7Gua and 8-OH-G marker peaks and (B) HPLC-2 electrochemical detection of 8-OH-dG at applied potentials of E1 = 170 mV (lower line) and E2 = 300 mV (upper line).

the voltammograms of 8-OH-dG from the pooled urine samples from humans, rats, and mice, respectively, conform well with the voltammograms of the corresponding 8-OH-dG standards under the different conditions used (see Materials and methods).

Analysis of m^7Gua in human and rodent urine

In addition to the detection of urinary 8-OH-dG, a sharp peak appeared at 12 min in every HPLC-1 chromatogram (Figs. 1A, 2A, and 3A). Based on its UV spectrum (see Materials and methods), it was identified as m^7Gua, an RNA degradation product. The absorbance spectrum of m^7Gua in rat urine is identical to that of a pure m^7Gua standard (cf. Figs. 9A and B). For the detection of m^7Gua, a 305-nm wavelength was used to reduce the interference from the neighboring peaks in human or mouse urine. For rat urine, it is also possible to use a 254-nm wavelength for the detection of m^7Gua (chromatogram not shown).

Correlation of urinary levels of m^7Gua with creatinine

The linear regression lines for the correlations between the concentrations of urinary creatinine and m^7Gua for humans, rats, and mice are presented in Figs. 10–12, respectively.

For the human data from 44 male nonsmokers, the linear relationship for the concentrations [g/L] of urinary creatinine and m^7Gua is $[m^7\text{Gua}] = 0.0067x[\text{creatinine}] + 0.0023$ and the correlation coefficient (r) is 0.79 ($r^2 = 0.62$) (Fig. 10). For the data from 36 rats, the linear relationship is $[m^7\text{Gua}] = 0.0302x$ [creatinine] + 0.0026 with a good correlation coefficient (r) equal to 0.90 ($r^2 = 0.81$) (Fig. 11). For the data from 22 mice, the linear relationship is $[m^7\text{Gua}] = 0.0284x[\text{creatinine}] + 0.0065$ with a correlation coefficient equal to 0.77 ($r^2 = 0.59$) (Fig. 12).

The levels of urinary m^7Gua are also presented as milligrams per gram creatinine (Table 2). All of these levels are significantly different ($P < 0.01$) among humans, rats, and mice. The level of human m^7Gua is four times lower than that of rats and five times lower than that of mice (Table 2). The level in mice is similar to that in rats (16% higher in mice). For a convenient comparison with the values found in the literature, the yields of m^7Gua from a subset of the samples from rats ($n = 10$) and mice ($n = 10$) were also calculated as micrograms of m^7Gua collected during 24 h normalized to body weight (μg/24 h/kg BW) (Table 2).

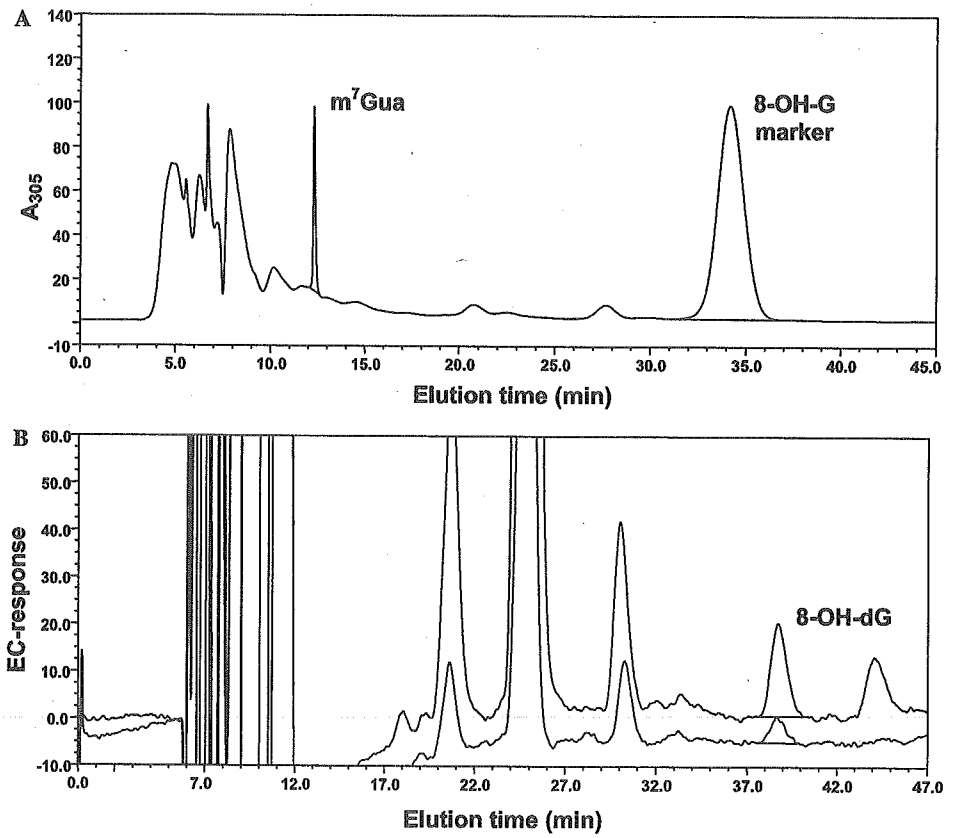

Fig. 3. Chromatograms of a mouse urine sample (female C3H/He mouse): (A) HPLC-1 UV detection of m^7Gua and 8-OH-G marker peaks and (B) HPLC-2 electrochemical detection of 8-OH-dG at applied potentials of E1 = 170 mV (lower line) and E2 = 300 mV (upper line).

8-OH-dG levels in human and rodent urine

The levels of 8-OH-dG normalized to creatinine (μg/g creatinine) all are significantly different ($P < 0.05$) among humans, rats, and mice (Table 2). The level of human 8-OH-dG is similar (15% higher) to that of rats. The ratios of 8-OH-dG/creatinine in mice are approximately twice as high as those in humans and rats. For comparison with other published values, the yields of 8-OH-dG from a subset of the samples from rats ($n = 10$) and mice ($n = 10$) were also calculated as nanograms of 8-OH-dG collected during 24 h normalized to body weight (ng/24 h/kg BW) (Table 2).

Discussion

The measurement of urinary 8-OH-dG is difficult due to the large amounts of other compounds present. Also, the amount of urine excreted, and thus the concentration of 8-OH-dG, differs among sampling times and individuals. Thus, a reliable method to distinguish only 8-OH-dG among all of the other compounds is needed. At the same time, the level of 8-OH-dG must be corrected for the degree of dilution between different samples. This correction is usually made by normalizing the 8-OH-dG values from a 24-h urine collection to the body weight or by normalizing the 8-OH-dG values to the amount of urinary creatinine. A new method based on HPLC-EC was developed to improve the speed and reliability of the urinary 8-OH-dG measurements [5]. The main features are the use of an 8-OH-G marker that is added to the urine sample, anion exchange chromatography (HPLC-1), and automatic peak detection of the marker peak to precisely collect the subsequent fraction of 8-OH-dG [5]. The 8-OH-dG is then detected with an electrochemical detector after reversed-phase chromatography in HPLC-2 [5]. The main advantages are that the fraction collected is not sensitive to daily variations in elution time and that the collection of neighboring interfering peaks is avoided [5]. For human samples, this system is currently used routinely to detect 8-OH-dG. However, urine samples from animal experiments (rats and mice) could not be analyzed reliably using the same conditions as for the detection of human 8-OH-dG in HPLC-2 (see Materials and methods). This is probably due to differences in the compositions and concentrations of the compounds found in urine samples from different species. Also, regarding the collection of urine from animals in metabolic cages, some contamination would be expected from feces and food [21]. Thus, keeping the conditions for the first anion exchange column constant,

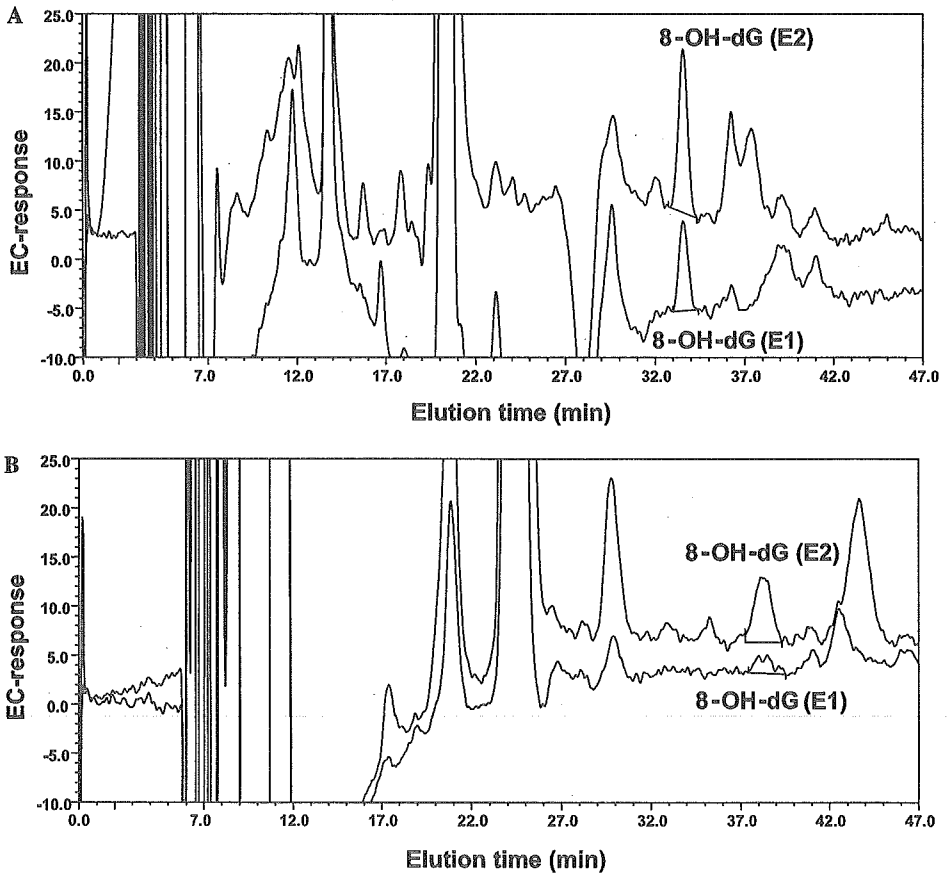

Fig. 4. Chromatograms of a mouse urine sample (female C3H/He mouse) showing the detection of false positives for 8-OH-dG. (A) HPLC-2 electrochemical detection of 8-OH-dG using the same conditions as in the rat urine analysis (the flow rate was 0.67 ml/min and the column oven was set at 48 °C) was unfavorable due to contamination of the 8-OH-dG peak detected at an applied potential of E2 = 300 mV (upper line), as seen from the large peak area ratio (55%) between the peak area at an applied potential of E1 = 170 mV (lower line) and the peak area of E2 (upper line). (B) Changing the HPLC-2 conditions for electrochemical detection of 8-OH-dG to resolve the interfering peaks in mouse urine (the flow rate was 0.33 ml/min and the column oven was set at 60 °C) decreased the ratio (24%) between the peak area at an applied potential of E1 = 170 mV (lower line) and the peak area at an applied potential of E2 = 300 mV (upper line). The decreased peak area ratio for E1/E2 in B, as compared with that in A, shows that the contaminating compounds have been removed from the 8-OH-dG peak by changing the HPLC conditions.

Table 1
Recovery of 8-OH-dG from spiked urine of humans, rats, and mice

Spike	Urine from humans[a]		Urine from rats[b]		Urine from mice[c]	
Added 8-OH-dG (ng/ml)	Detected 8-OH-dG (ng/ml)	Recovery 8-OH-dG (%)	Detected 8-OH-dG (ng/ml)	Recovery 8-OH-dG (%)	Detected 8-OH-dG (ng/ml)	Recovery 8-OH-dG (%)
0.0	7.4 ± 0.2		5.7 ± 0.2		4.9 ± 0.2	
1.0	8.5 ± 0.2	108 ± 10	6.8 ± 0.3	101 ± 23	5.9 ± 0.3	108 ± 14
2.5	10.0 ± 0.2	102 ± 2	8.4 ± 0.2	105 ± 11	7.6 ± 0.1	111 ± 7
4.0	11.5 ± 0.4	102 ± 8	10.0 ± 0.3	106 ± 7	9.1 ± 0.1	106 ± 6

Note. Mean values ± standard deviations are presented.

[a] Each value is based on four repeated analyses of pooled urine samples from humans ($n = 3$). The linear correlation between added and detected 8-OH-dG in human urine is $r = 0.99$.

[b] Each value is based on three repeated analyses of pooled urine samples from rats ($n = 3$). The linear correlation between added and detected 8-OH-dG in rat urine is $r = 0.99$.

[c] Each value is based on three repeated analyses of pooled urine samples from mice ($n = 3$). The linear correlation between added and detected 8-OH-dG in mouse urine is $r = 0.99$.

we modified some conditions for the reversed-phase column in HPLC-2 by reducing the mobile phase pH and the methanol content, reducing the flow rate, and increasing the column temperature (see Materials and methods). However, measuring rat samples typically means that the anion exchange column in HPLC-1 lasts

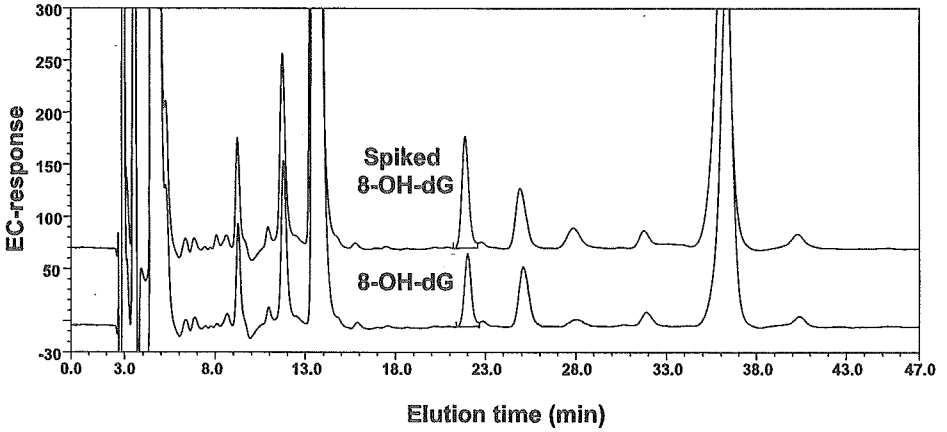

Fig. 5. Chromatograms of pooled urine from three humans (two male nonsmokers and one female nonsmoker), unspiked or spiked with the 8-OH-dG standard: (lower line) HPLC-2 electrochemical detection (E2) of 8-OH-dG from pooled urine with a peak area corresponding to 7.2 ng/ml of 8-OH-dG and (upper line) pooled urine spiked with 4.0 ng/ml of the 8-OH-dG standard with a peak area corresponding to 11.2 ng/ml of 8-OH-dG. Applied potentials were E1 = 200 mV and E2 = 370 mV.

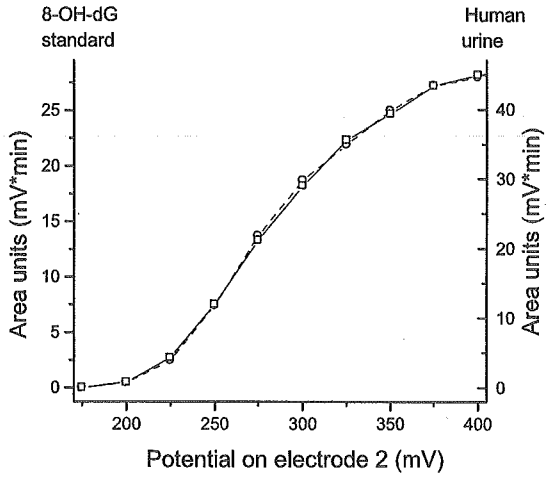

Fig. 6. Voltammograms of (□) 8-OH-dG standard and (○) pooled urine from humans at applied potentials on electrode 2 (E2) of 175–400 mV (E1 = 0 mV and guard cell = 450 mV).

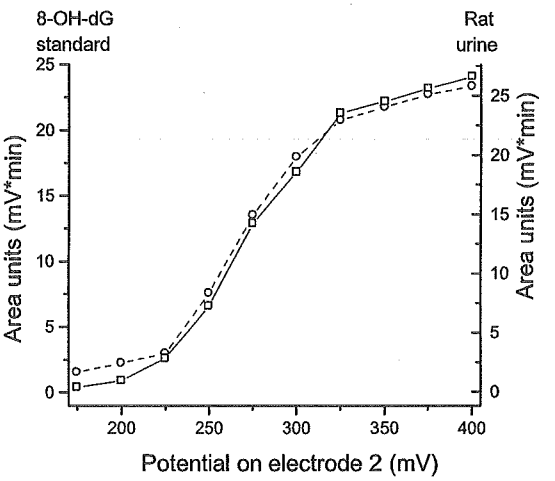

Fig. 7. Voltammograms of (□) 8-OH-dG standard and (○) pooled urine from rats at applied potentials on electrode 2 (E2) of 175–400 mV (E1 = 0 mV and guard cell = 450 mV).

for a shorter time and also needs the column filter replaced due to a pressure increase after 100–200 samples. When analyzing mouse urine, the filter might need to be replaced after every 10–20 samples injected. Also, the reversed-phase column in HPLC-2 needs to be washed more frequently with methanol and might last a shorter time due to the increased column oven temperatures used. In comparison, up to 1000 human urine samples can be analyzed continuously without any replacements [5]. Currently, these modified system settings allow the reliable and continuous automated analysis of 10–200 mouse or rat samples.

The human whole-body lean mass degradation rates of mRNA, tRNA, and rRNA all have been correlated with their specific degradation products in urine and with the RMR [8]. For example, the urinary 5,6-dihydrouridine levels were 4.7 times higher in rats than in humans, reflecting the difference between their RMRs [13]. Another such product is m^7Gua, which is derived from all three RNA classes [8]. Thus, it would be expected that the amount of m^7Gua detected in urine would reflect the RMR and be a suitable marker for the normalization of urinary 8-OH-dG. No significant variation in the urinary excretion of m^7Gua was found in terms of different types of diet [8]. However, some variations in the expected amount of human m^7Gua between individuals would be expected due to the fact that approximately 30–40% of the m^7Gua is converted to 8-hydroxy-7-methylguanine by human xanthine oxidase [8,22]. We may also find some deviations in the amounts of excreted m^7Gua due to exposure to methylating agents such as *N*-nitroso compounds from tobacco smoke and the endogenous *S*-adenosylmethionine activity [16,17].

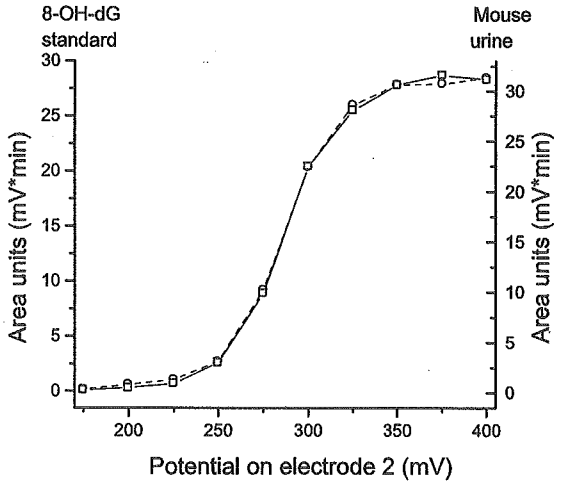

Fig. 8. Voltammograms of (□) 8-OH-dG standard and (○) pooled urine from mice at applied potentials on electrode 2 (E2) of 175–400 mV (E1 = 0 mV and guard cell = 450 mV).

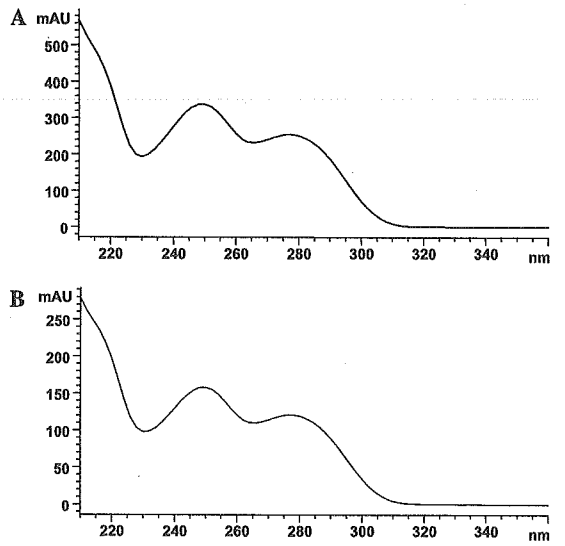

Fig. 9. UV absorbance spectrum of (A) m^7Gua standard and (B) a rat urine HPLC-1 fraction (0.5 min) eluted at 12 min.

In this article, we have presented a good correlation between urinary creatinine and m^7Gua in humans and those in mice ($r = 0.80$) and rats ($r = 0.90$) (Figs. 10–12). For human samples, the correlation was lower (Fig. 10), probably due to larger variations in the excreted creatinine and m^7Gua in the human population. For the mouse samples, the lower correlation would be explained by the small deviations in the m^7Gua and creatinine content among the individual urine samples collected in this study (Fig. 12). The urinary level of human m^7Gua presented in Table 2, 8.6 mg/g creatinine, correlates well with the previously published value of 7.0 mg/g creatinine (as recalculated from 4.80 nmol/μmol creatinine) [23]. For rats, the value for m^7Gua excreted during 24 h and normalized to body weight, 1100 μg/24 h/kg

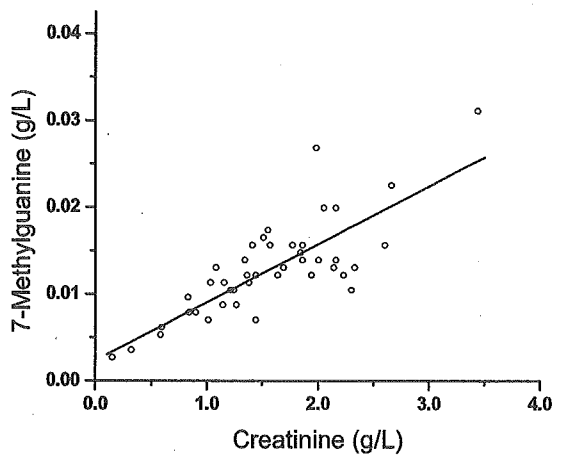

Fig. 10. Regression line for the correlation ($r = 0.79$) between human urinary creatinine and m^7Gua content. Urinary samples were measured in 44 individuals.

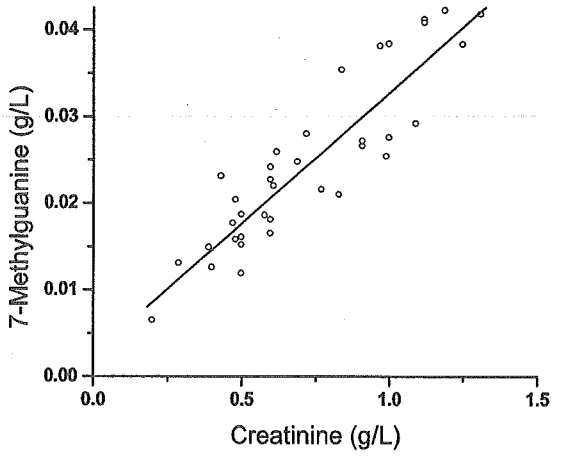

Fig. 11. Regression line for the correlation ($r = 0.90$) between rat urinary creatinine and m^7Gua content. Urinary samples were measured in 36 individual rats.

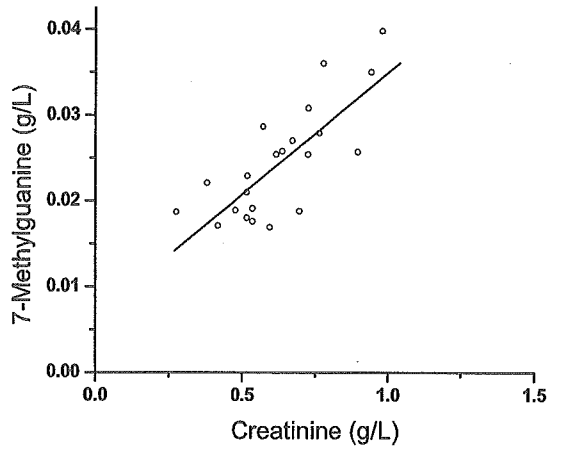

Fig. 12. Regression line for the correlation ($r = 0.77$) between mouse urinary creatinine and m^7Gua content. Urinary samples were measured in 22 individual mice.

Table 2
Urinary excretion of 8-OH-dG and m^7Gua in humans and rodents

Species	8-OH-dG		m^7Gua	
	Creatinine[a] (μg/g)	BW[b] (ng/24 h/kg)	Creatinine[a] (mg/g)	BW[b] (μg/24 h/kg)
Human	4.2 ± 1.2		8.6 ± 2.3	
Rat	3.7 ± 0.6	120 ± 39	34.3 ± 6.4	1100 ± 300
Mouse	8.2 ± 1.2	159 ± 55	39.9 ± 9.2	720 ± 110

Note. Mean values ± standard deviations are presented.

[a] Values are based on urine samples from humans ($n = 44$), rats ($n = 36$), and mice ($n = 22$). Mean values of 8-OH-dG, as normalized to creatinine, are significantly different between humans and rats ($0.01 < P < 0.05$), between humans and mice ($P < 0.001$), and between rats and mice ($P < 0.001$). Mean values of m^7Gua, as normalized to creatinine, are significantly different between humans and rats ($P < 0.001$), between humans and mice ($P < 0.001$), and between rats and mice ($0.001 < P < 0.01$).

[b] Values are based on urine samples from rats ($n = 10$) and mice ($n = 10$). Urine was collected during 24 h, and the average volumes were 13.7 ml/rat and 0.9 ml/mouse. The average weights of the animals were 363 g/rat and 23.6 g/mouse.

BW, is only 11% higher than the previously published value of 983 μg/24 h/kg BW for female LAC:P rats (recalculated from 172 μg/24 h and an average BW of 175 g/rat) [24]. For mice, assuming similar weights of animals, our value for m^7Gua excreted during 24 h, 16.9 μg/24 h (recalculated from 720.0 μg/24 h/kg BW), is within the range of the previously presented value, 12.6 μg/24 h, for 17-week-old male C57B1/J6 mice (recalculated from 76.0 nmol/24 h) [25] (Table 2).

The mean level of human 8-OH-dG in Table 2, 4.2 μg/g creatinine, correlates well with our findings using a previous version of our HPLC-EC method (4.1 μg/g creatinine) [19] and data from others using the HPLC-EC or GC-MS methods (3.3–4.0 μg/g creatinine) [21,26]. For rats, our value of 3.7 μg 8-OH-dG/g creatinine is approximately half of the previously presented value (7.8 μg/g creatinine) [21,27]. However, a recently published value for the excretion of urinary 8-OH-dG in male Wistar rats is 94.4 μg/24 h/kg BW (recalculated from 333.2 pmol/24 h/kg BW) [28], which is comparable to our value of 120 μg/24 h/kg BW. Differences in the amounts of urinary 8-OH-dG could be due to various factors such as contamination of urine with feces and food, differences in rat strains used, and ages of rats. In the same report [28], the values expressed as picomoles of 8-OH-dG excreted in urine per day and kilograms body weight were similar for humans (281.7 ± 179.1) and rats (333.2 ± 47.4), a comparison in agreement with our values for 8-OH-dG/creatinine, where the human levels were only 15% higher than those in rats. In addition, in this report [28], the RMR difference was calculated as 4.2 times higher for rats (420 kJ/day/kg BW) than for humans (100 kJ/day/kg BW), similar to the ratio of 3.4 calculated previously (humans = 107 and rats = 364 kJ/day/kg BW) [29]. Interestingly, this is in good agreement with our value for human m^7Gua as normalized to creatinine, which is four times lower than that for rats. Thus, for the comparison between humans and rats, the urinary m^7Gua content agrees relatively well with the RMR level, although no correlation exists between RMR and urinary 8-OH-dG/creatinine (Table 2) or between RMR and 8-OH-dG excreted in urine per day and kilograms body weight [28]. For mice, the level of urinary 8-OH-dG (8.2 μg/g creatinine) (Table 2) is close to the previously estimated value of 6.9 μg/g creatinine [21,30]. This means that the level of 8-OH-dG excreted per gram of creatinine is 2.2 times higher in mice than in rats, which correlates well with the twofold higher RMR for mice than in rats (mice = 760 and rats = 364 kJ/day/kg BW) [29]. However, the amount of m^7Gua excreted in urine, when normalized to creatinine, is only 16% higher in mice than in rats (Table 2). Thus, RMR and the amount of excreted m^7Gua might not show a linear relationship among the various species studied. Also, the relatively large differences in RMR among humans, rats, and mice do not correlate with the similar differences among the levels of excreted 8-OH-dG/creatinine. Thus, when considering the RMR differences, the repair capacity for 8-OH-dG is similar in rats and mice and also is considerably lower than the repair capacity for 8-OH-dG in humans.

The presented results show that urinary 8-OH-dG and m^7Gua in humans, rats, and mice can be analyzed rapidly and reliably in the same sample run. For rats and mice, m^7Gua may be used as a reliable marker instead of creatinine for the normalization of 8-OH-dG content in urine. In human urine samples, m^7Gua may be analyzed in addition to 8-OH-dG normalized with creatinine.

Acknowledgments

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