

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 5
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
MAURICIO JARAMILLO CAMPUZANO

Asunto: Radicación: 12-41745- -4
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 10373
 Folios: 5

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2010/046211				
Fecha de Presentación Internacional	20/08/2010				

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado No. 12-041745-00000-0000	09/03/2012
Reivindicaciones:	Radicado No. 12-041745-00000-0000	09/03/2012
Prioridad:	US 61/247,875	21/08/2009
	US 61/280,860	09/11/2009
	US 61/283,524	04/12/2009
	US 12/777,238	10/05/2010

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuandola solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. Para el caso de la presente solicitud se estudiaran las reivindicaciones 1 a 33 (Radicado No. 12-041745-00000-0000).

Título de la solicitud: “COMPOSICIONES ALCOHÓLICAS QUE TIENEN UN RIESGO REDUCIDO DE ACETALDEHIDEMIA”.

El problema planteado en esta solicitud consiste en la necesidad de proveer una composición y métodos para incrementar el periodo de tiempo entre el consumo de una bebida alcohólica y llegar a niveles pico en sangre de etanol, y métodos para retardar el inicio de los síntomas inducidos por etanol asociados con el consumo de bebidas alcohólicas.

Para resolver este problema técnico la solicitud en estudio proporciona una bebida alcohólica que comprende agua y etanol, en donde al menos 5 moles por ciento del etanol es un alcohol deuterizado.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12C11/00.

4. Excepción a la Patentabilidad (Art 20 D 486 literales (a) – (d))

El objeto de las reivindicaciones 11, 12 y 28 no es patentable de acuerdo con el Art citado.

5. De la solicitud de patente

Titulo

El título no corresponde con el objeto de la solicitud porque no hace referencia al objeto reclamado que corresponde a bebidas alcohólicas, composiciones farmacéuticas y métodos para elaborarlas que contienen alcohol deuterizado para disminuir los efectos del etanol, por lo cual se sugiere al solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1 y 18 carecen de concisión debido a que abarcan un indefinido número de composiciones y no se limita la estructura del etanol deuterizado.

6. Unidad de invención (Art 25 D 486)

La solicitud no cumple con el requisito de unidad de invención porque se refiere a 3 ... grupos inventivos.

Los grupos inventivos están definidos por las siguientes características esenciales:

- 1 Una bebida alcohólica que comprende agua y etanol donde al menos 5 moles por ciento de etanol es un alcohol deuterizado.
2. Método para elaborar una bebida alcohólica que comprende el paso de adicionar a una bebida un alcohol deuterizado.
3. Un acomposición farmacéutica que comprende un ingrediente farmacéuticamente activo y etanol, al menos 5 moles por ciento de etanol es un alcohol deuterizado.
4. Un método para elaborar una composición famacéutica que comprende la etapa de combinar un ingrediente farmacéutico activo con un alcohol deuterizado.

Se sugiere presentar las solicitudes divisionales que considere necesarias, o limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 21 de agosto de 2009, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	Bengsh, Corval, Martin. Organic Magnetic Resonance Vol. 6, Issue 4, pages 195-199, Abril 1974	Analyse isotopique de mélanges d'éthanol deuté riés C ₂ H _{5-<i>n</i>} D _{<i>n</i>} OH	1974
D2	Biochem J. 1985 Jul 15; 229(2):315-22	“Hydrogen transfer between ethanol molecules during oxidoreduction in vivo”	1985
D3	Taizo Nagano, Tatsunori Takayasu and Hitoshi Maeda. Kanazawa University School of Medicine	“A New Method for the Determination of Ethanol in the Blood and Urine by Pulse Heating”	22/05/1989

D1^[1] discute análisis cualitativos y cuantitativos de mezclas de isómeros de etanol (C₂H_{5-*n*}D_{*n*}OH), donde el porcentaje de etanol deuterizado se encuentra en el rango de 0.9 a 94% lo que se encuentra dentro del rango de 5 moles % a 95 moles% (resumen)

D2^[2] divulga soluciones de etanol [1,1- ²H] y [2,2,2-²H₃] (resumen, pág. 316).

D3^[3] enseña etanol-d6 para administración oral en donde entre 5 moles% a 95 moles %. Corresponden a etanol deuterizado (documento completo).

8. Examen de Patentabilidad (Art 14 D486)

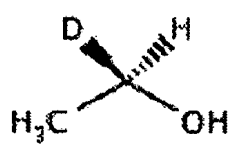
Para efectos de la presente solicitud se evaluará el nivel inventivo del grupo 1.

Novedad (Art 16 D486)

La reivindicación 1 consiste en una bebida alcohólica que comprende agua y etanol donde al menos 5 moles por ciento de etanol es un alcohol deuterizado

Comparación de las características técnicas esenciales, con las del estado de la técnica:

1[] URL: <http://onlinelibrary.wiley.com/doi/10.1002/mrc.1270060403/abstract>
 2[] URL: <http://www.ncbi.nlm.nih.gov/pubmed/4038269>
 3[] URL: <http://www.ncbi.nlm.nih.gov/pubmed/2585819>

Características esenciales	D1	D3
Una bebida alcohólica que comprende: Agua y etanol, en donde al menos 5 moles% del etanol es un alcohol deuterizado que tiene la fórmula:  En donde cada H puede ser independientemente hidrógeno o deuterio.	Mezclas que comprenden: 1 Isómeros nucleares de etanol (C2H5-nDnOH). Las mezclas deuterizadas de etanol. El porcentaje de etanol deuterizado se encuentra en el rango de 0.9 a 94% lo que se encuentra dentro del rango de 5 moles% a 95 moles% (resumen)	Solución acuosa que comprende: Etanol-d6 (25% v/v%) que se encuentra dentro del rango de 5 moles% a 95 moles% de alcohol deuterizado, el cual se administra a bebidas como cerveza y sake (resumen).

Las características esenciales de las bebidas alcohólicas de la reivindicación 1 habían sido divulgadas previamente en los documentos D1, D2 y D3, como se evidencia en la tabla comparativa.

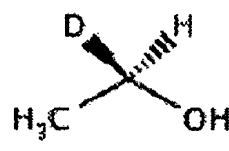
En consecuencia, la reivindicación 1 no es nueva, según lo divulgado en los documentos D1 y D2.

Las reivindicaciones dependientes 2 –10 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en una bebida alcohólica que comprende agua y etanol donde al menos 5 moles por ciento de etanol es un alcohol deuterizado

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2	D3
Una bebida alcohólica que comprende: Agua y etanol, en donde al menos 5 moles% del etanol es un alcohol deuterizado que tiene la fórmula:  En donde cada H puede ser independientemente hidrógeno o deuterio.	Mezclas que comprenden: 1 Isómeros nucleares de etanol (C2H5-nDnOH). Las mezclas deuterizadas de etanol. El porcentaje de etanol deuterizado se encuentra en el rango de 0.9 a 94% lo que se encuentra dentro del rango de 5 moles% a 95 moles% (resumen)	Solución salina que comprende: etanol de [1,1- 2H] y [2,2,2-2H3] (resumen). Los derivados 2H etanol fueron andministrados mediante inyección intraperitoneal de soluciones al 20%w/v en solución salina al 0.9% estando en el rango de 5moles% a 95moles% de etanol.	Solución acuosa que comprende: Etanol-d6 (25% v/v%) que se encuentra dentro del rango de 5 moles% a 95 moles% de alcohol deuterizado, el cual se administra a bebidas como cerveza y sake (resumen).

El documento D3 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga la administración de etanol-d6 (25% v/v% de solución acuosa de etanol-d6 que se encuentra dentro del rango de 5moles% a 95

moles% de etanol deuterizado) a voluntarios mediante la ingesta de bebidas alcoholicas como cerveza o sake, y a quienes se les evaluó la concentración plasmática (documento completo).

La diferencia entre la invención, definida en la reivindicación 1 y el producto de D3 consiste en que la anterioridad no divulga alcohol deuterizado que esté sustituido específicamente por deuterio sobre los carbonos C1 o C2.

El efecto que logra el incluir esta sustitución de los hidrógenos directamente unidos a C1 o C2 por deuterio consiste en obtener formulaciones alternativas para la disminución de los niveles de metabolitos del etanol.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: ¿cómo modificar las formulaciones conocidas en D3 para conseguir bebidas alternativas?.

Sin embargo, la utilización de isómeros nucleares de etanol para preparar soluciones, ya está divulgada en el documento D1 que se refiere a análisis isotópico cuantitativo y cualitativo de mezclas de isómeros nucleares de etanol (C₂H_{5-n}D_nOH) que incluyen CH₂DCH₂OH; CHD₂ CHD₂OH; CHD₂ CH₂OH; CH₂D CHDOH; CH₃CD₂OH; CD₃CH₂OH; CHD₂CHDOH; CHD₂CD₂OH y CD₃CD₂OH. El porcentaje de etanol deuterizado dentro de las mezclas esa en el rango de 09 a 94% (resumen).

En consecuencia, el la persona del oficio normalmente versada en la materia, incluiría isómeros nucleares de etanol como los divulgados en el documento D1 en las bebidas con alcohol deuterizado divulgadas en el documento D3 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Conclusión:
La reivindicación 1 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

9. Conclusión

Los requisitos de patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Bengsh, Corval, Martin. Organic Magnetic Resonance Vol. 6, Issue 4, pages 195-199, Abril 1974	1-10	Novedad Nivel inventivo
D2	Biochem J. 1985 Jul 15; 229(2):315-22	1-10	Novedad
D3	Taizo Nagano, Tatsunori Takayasu and Hitoshi Maeda. Kanazawa University School of Medicine	1-10	Novedad Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-06-18

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Hydrogen transfer between ethanol molecules during oxidoreduction *in vivo*

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(Received 5 November 1984/25 March 1985; accepted 3 April 1985)

Rates of exchange catalysed by alcohol dehydrogenase were determined *in vivo* in order to find rate-limiting steps in ethanol metabolism. Mixtures of [1,1- $^2\text{H}_2$]- and [2,2,2- $^2\text{H}_3$]ethanol were injected in rats with bile fistulas. The concentrations in bile of ethanols having different numbers of ^2H atoms were determined by g.l.c.-m.s. after the addition of [$^2\text{H}_6$]ethanol as internal standard and formation of the 3,5-dinitrobenzoates. Extensive formation of [$^2\text{H}_4$]ethanol indicated that acetaldehyde formed from [2,2,2- $^2\text{H}_3$]ethanol was reduced to ethanol and that NADH used in this reduction was partly derived from oxidation of [1,1- $^2\text{H}_2$]ethanol. The rate of acetaldehyde reduction, the degree of labelling of bound NADH and the isotope effect on ethanol oxidation were calculated by fitting models to the found concentrations of ethanols labelled with 1–4 ^2H atoms. Control experiments with only [2,2,2- $^2\text{H}_3$]ethanol showed that there was no loss of the C-2 hydrogens by exchange. The isotope effect on ethanol oxidation appeared to be about 3. Experiments with (1S)-[1- ^2H]- and [2,2,2- $^2\text{H}_3$]ethanol indicated that the isotope effect on acetaldehyde oxidation was much smaller. The results indicated that both the rate of reduction of acetaldehyde and the rate of association of NADH with alcohol dehydrogenase were nearly as high as or higher than the net ethanol oxidation. Thus, the rate of ethanol oxidation *in vivo* is determined by the rates of acetaldehyde oxidation, the rate of dissociation of NADH from alcohol dehydrogenase, and by the rate of reoxidation of cytosolic NADH. In cyanamide-treated rats, the elimination of ethanol was slow but the rates in the oxidoreduction were high, indicating more complete rate-limitation by the oxidation of acetaldehyde.

Several metabolic effects of ethanol are considered to be due to the increased ratio between the concentrations of free NADH and free NAD^+ in the cytosol of the liver cell (Reitz, 1979). The mechanism behind this increase is not known, but the production of NADH in the first, cytosolic, step in ethanol metabolism makes it likely that the mechanism is related to this reaction. Thus the regulation of this reaction has been intensely debated (Dawson, 1983a; Kacser, 1983; Crow, 1983). The rate was first suggested to be determined by the rate of dissociation of the alcohol dehydrogenase–NADH complex (Theorell & Bonnichsen, 1951). The redox effects observed by Forsander *et al.* (1958) led to the idea that the reaction was limited by the rate of reoxidation of cytosolic NADH (Videla & Israel, 1970; Hillbom, 1971). It has also been suggested that acetaldehyde oxidation is the rate-limiting reaction, with the rate being determined by the concentration of

acetaldehyde, and with this concentration being set by an equilibrium in the alcohol dehydrogenase system (Krebs, 1969).

If the dissociation of the enzyme–NADH complex were rate-limiting, the rate of association would be slow relative to the rate of ethanol elimination. Indications that this might be the case have come from measurements of ^2H excess in compounds formed during metabolism of [1,1- $^2\text{H}_2$]ethanol. The ^2H excess was much higher in a reduction catalyzed by alcohol dehydrogenase than in other reductions (Cronholm, 1974; Curstedt, 1982), indicating incomplete equilibration of free and bound NADH.

If instead the oxidation of acetaldehyde were rate-limiting, the rate of acetaldehyde reduction would be high relative to the rate of ethanol elimination. This reduction has been observed *in vivo* in isotope experiments. Thus Gershman (1975) found that [^{14}C]acetaldehyde was reduced during

ethanol metabolism *in vivo*, and the rate of reduction was at least 2% of the rate of ethanol oxidation.

In the present study an attempt was made to identify the rate-limiting step under physiological conditions by simultaneously measuring the rates of NADH dissociation and acetaldehyde reduction in relation to the rate of ethanol elimination. This could be done by measuring the intermolecular hydrogen transfer at C-1 of suitably deuterated ethanol.

Materials and methods

Materials

[1,1-²H₂]Ethanol (99.6 atom % ²H) and [2,2,2-²H₃]ethanol (99.0 atom % ²H) were obtained from Rohstoff-Einfuhr G.m.b.H., Düsseldorf, Germany. The [2,2,2-²H₃]ethanol used to study the retention of ²H was a gift from Dr. A. L. Burlingame, San Francisco, CA, U.S.A. [²H₆]Ethanol (more than 98 atom% ²H) was obtained from Merck AG, Darmstadt, Germany. (1*S*)-[1-²H]Ethanol was prepared from [1,1-²H₂]ethanol by exchange with yeast cells essentially as described by Günther *et al.* (1973). The isolated ethanol and the acetaldehyde formed in a reaction catalyzed by yeast alcohol dehydrogenase were analysed as previously described (Cronholm & Fors, 1976). The results showed that 6.7% was unlabelled, 2.0% was labelled only in the 1-*pro-R* position, 85.8% was labelled only in the 1-*pro-S* position, and 5.5% was [1,1-²H₂]ethanol.

[1-¹⁴C]Ethanol (6.9 mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.).

Calcium cyanamide was obtained from American Cyanamide Company (Wayne, NJ, U.S.A.).

Animal experiments

Female Sprague-Dawley rats weighing about 220g were anaesthetized with diethyl ether and provided with bile fistulas and a tube into the peritoneal cavity to simplify injections. They were kept in restraining cages with free access to food pellets and a 0.5% (w/v) NaCl solution. After about 18h the deuterated ethanol were administered intraperitoneally as 20% (w/v) solutions in saline. Bile was collected in 0.5h fractions starting at the time of injection. The fractions were weighed and [²H₆]ethanol was added as an internal standard. Bile (10 μl) was taken for quantification of ethanol by g.l.c. with propan-1-ol as internal standard on a column of 0.2% Carbowax 1500 on Carbowax C (Supelco, Inc., Bellefonte, PA, U.S.A.).

Radioactivity was determined in a scintillation counter with Instagel (Packard Instrument Co., Downers Grove, IL, U.S.A.) as scintillator.

Analysis of deuterated ethanol

The 3,5-dinitrobenzoate of ethanol was prepared by a modification of the method described by Holley & Holley (1952). Pyridine (5 μl), benzene (150 μl) and (in an ice bath) 0.55g of anhydrous K₂CO₃ were added to the bile sample (less than 0.5ml). Finally 25mg of 3,5-dinitrobenzoyl chloride (Merck AG) and 2ml of dichloromethane were added, and the mixture was stirred for 18h at room temperature. The dichloromethane phase was removed and the water phase was extracted twice with 2ml of dichloromethane. The dichloromethane phases were pooled and washed with 1 ml of water, dried with anhydrous Na₂SO₄ and concentrated by evaporation of the solvent.

The ethyl 3,5-dinitrobenzoate was purified on an 8.2ml column of Lipidex 5000 (Packard Instrument Co.) in heptane. The 15–30ml eluate was concentrated by evaporation of the solvent and analysed by g.l.c.–m.s. in an LKB 9000 instrument equipped with a quartz capillary column (CP Sil 5 CB; Chrompack, Middelburg, The Netherlands) and a falling-needle injection system. The column temperature was 145°C and that of the ion source was 310°C. The electron energy was 22.5eV. Spectra were recorded on magnetic tape by repetitive accelerating voltage scanning over the region *m/z* 239–*m/z* 246 in 0.5s. The values were bunched (Axelson *et al.*, 1974) and the area of the central 30% of each mass spectrometric peak was used in the calculation of isotope excess in the molecular ion (Biemann, 1962).

Results

Evaluation of the analytical method

Aqueous 20mM-ethanol test samples (0.5ml) containing [1-¹⁴C]ethanol were treated by the method. The recovery of ¹⁴C was about 10%. In order to study if there were isotope effects in the method and if the mass spectrometric response was the same for ethanol labelled with ²H in different ways, the method was applied to 20mM samples of the deuterated ethanol which were used in the biological experiments. The response was essentially the same for the different ethanol. Thus, the ratio between the relative responses (m.s. response/amount added) for [²H₁]- and [²H₆]ethanol was 1.05. It was therefore considered possible to use the method for quantitative analysis in spite of the low recovery.

Retention of ²H in [2,2,2-²H₃]ethanol

Two rats were given [2,2,2-²H₃]ethanol (1g/kg) intraperitoneally, and bile was collected. The ratio between dideuterated and trideuterated molecules was about 0.05 as in the injected ethanol and with

no tendency to increase. There were no molecules having one or four ^2H atoms. It was concluded that the acetaldehyde formed as intermediate in the reversible oxidoreduction of ethanol did not exchange ^2H for ^1H of the medium at a rate that was sufficient to affect the interpretation of the hydrogen transfer in experiments where $[1,1-^2\text{H}_2]\text{ethanol}$ and $[2,2,2-^2\text{H}_3]\text{ethanol}$ were simultaneously administered.

Rate of metabolism of (1S)-[1- $^2\text{H}_1$]ethanol relative to that of [2,2,2- $^2\text{H}_3$]ethanol

In order to study the isotope effect on the oxidation of acetaldehyde, two rats were given an intraperitoneal injection of (1S)-[1- $^2\text{H}_1$]ethanol (1 g/kg) together with [2,2,2- $^2\text{H}_3$]ethanol (1 g/kg) as reference. Quantification of differently labelled ethanol in bile was performed by g.l.c.-m.s. after addition of $[^2\text{H}_6]\text{ethanol}$ as internal standard. The ethanol in bile was mainly monodeuterated and trideuterated (Fig. 1). The small and about equal concentration of $[^2\text{H}_2]\text{ethanol}$ and unlabelled ethanol were ascribed to the presence of these in the (1S)-[1- $^2\text{H}_1$]ethanol used. The fraction of the excreted ethanol that was monodeuterated increased during the experiment, indicating a primary isotope effect on the oxidation of acetaldehyde and reduction of a fraction of the acetaldehyde. The isotope effect ($1/u$) was evaluated by comparisons with models. It was assumed that there was no secondary isotope effect on the oxidation of [2,2,2- $^2\text{H}_3$]ethanol, (1S)-[1- $^2\text{H}_1$]ethanol or [2,2,2- $^2\text{H}_3$]acetaldehyde. The rate (v) of decrease in concentration of the sum of mono- and trideuterated ethanol was assumed to be constant, and was evaluated by linear regression analysis. The rates of decrease with time (t) of the concentrations of $[^2\text{H}_1]\text{ethanol}$ (d_1) and $[^2\text{H}_3]\text{ethanol}$ (d_3) are then given by the following equations:

$$\frac{dd_1}{dt} = \frac{v}{1 + (d_3/ud_1)}$$

$$\frac{dd_3}{dt} = \frac{v}{1 + (ud_1/d_3)}$$

The calculations of models were performed by making the approximation:

$$\frac{\Delta d_n}{\Delta t} = \frac{dd_n}{dt}$$

using a Δt (0.01 h) that was sufficiently small to give similar results when smaller values were used. Models were calculated with u values differing by 0.01 and compared with the observed results by calculation of the sum of the squared deviations. The best fit was obtained with isotope effects of 1.37 and 1.16, respectively (Fig. 1).

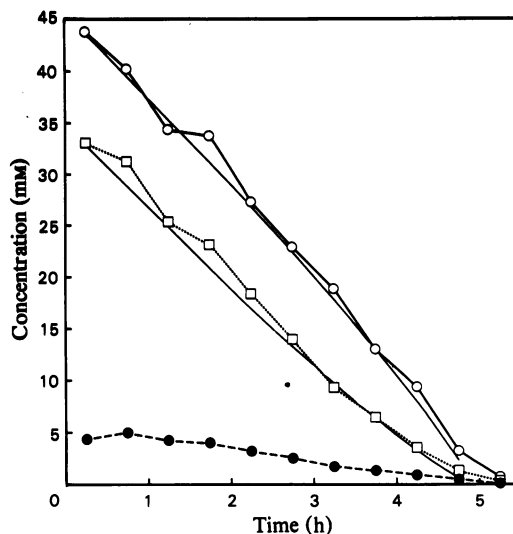


Fig. 1. Detection of an isotope effect on the oxidation of acetaldehyde during ethanol metabolism *in vivo*. A mixture of (1S)-[1- $^2\text{H}_1$]ethanol (1 g/kg) and [2,2,2- $^2\text{H}_3$]ethanol (1 g/kg) was administered to a rat, and the concentrations in 0.5 h bile fractions of $[^2\text{H}_1]\text{ethanol}$ (○), $[^2\text{H}_2]\text{ethanol}$ (●) and $[^2\text{H}_3]\text{ethanol}$ (□) were measured. The $[^2\text{H}_2]\text{ethanol}$ was present as a contaminant in the (1S)-[1- $^2\text{H}_1$]ethanol. The curves give the calculated concentrations assuming an isotope effect of 1.37.

Exchange between [1,1- $^2\text{H}_2$]- and [2,2,2- $^2\text{H}_3$]ethanol

A mixture of [1,1- $^2\text{H}_2$]ethanol (0.5 g/kg) and [2,2,2- $^2\text{H}_3$]ethanol (0.5 g/kg) was administered intraperitoneally to two rats, and the ethanol excreted in bile was quantified by g.l.c. and analysed with regard to isotopic composition. Molecules having one and four ^2H atoms appeared during the elimination of the ethanol, but no molecules having five ^2H atoms were detected. $[^2\text{H}_6]\text{Ethanol}$ could therefore be used as an internal standard, and was added to the bile in the other experiments. Three rats were given the approx. 1:1 mixture of the labelled ethanol in a total dose of 2 g/kg. The concentrations of $[^2\text{H}_1]\text{ethanol}$ and $[^2\text{H}_4]\text{ethanol}$ increased rapidly during the first 1 h after the administration, and at the same time the concentrations of $[^2\text{H}_2]\text{ethanol}$ and $[^2\text{H}_3]\text{ethanol}$ decreased rapidly (Fig. 2). This indicates extensive oxidoreduction of the ethanol with transfer of ^2H between molecules. During the last hours, the concentrations of $[^2\text{H}_1]\text{ethanol}$ and $[^2\text{H}_2]\text{ethanol}$ were essentially equal and the same was true for the concentrations of $[^2\text{H}_3]\text{ethanol}$ and $[^2\text{H}_4]\text{ethanol}$. Apparently the molecules had equilibrated to a large extent, with the ^2H content of the hydrogen that was introduced during reduction of acetaldehyde being about 50%. This is close to the fraction

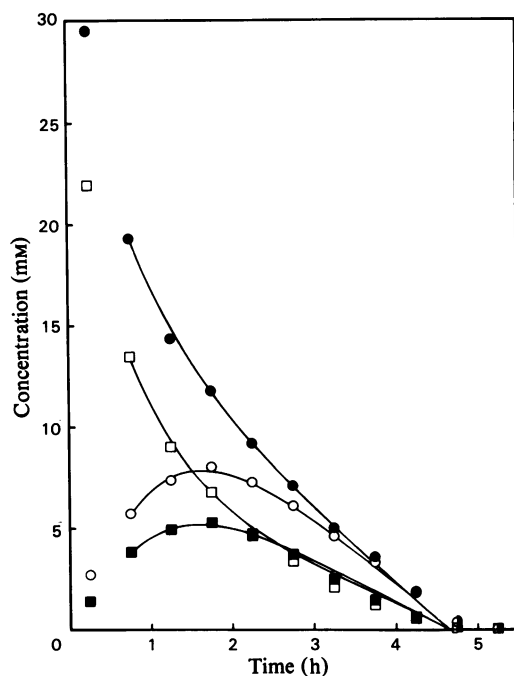


Fig. 2. Intermolecular hydrogen transfer and oxidoreduction during ethanol metabolism *in vivo*

A mixture of [1,1- $^2\text{H}_2$]ethanol (1 g/kg) and [2,2,2- $^2\text{H}_3$]ethanol (1 g/kg) was administered to a rat, and the concentrations in 0.5 h bile fractions of [$^2\text{H}_1$]ethanol (○), [$^2\text{H}_2$]ethanol (●), [$^2\text{H}_3$]ethanol (□) and [$^2\text{H}_4$]ethanol (■) were measured. The curves give the calculated concentrations assuming the values given in Table 2 (expt. no. 5).

of molecules having four or two ^2H atoms, that should have been equal to the fraction of labelled coenzyme if the primary isotope effect on ethanol oxidation was small. This indicates that ^2H was lost only to a small extent by exchange of NADH bound to alcohol dehydrogenase with free, less labelled NADH.

Formation of [$^2\text{H}_1$]ethanol and [$^2\text{H}_4$]ethanol took place in all the experiments, but to different extents (Table 1). The mean concentration of [$^2\text{H}_4$]ethanol was about half that of [$^2\text{H}_1$]ethanol, consistent with a high degree of retention of ^2H during the oxidoreduction.

The decrease in the total ethanol concentration was essentially linear, and the rate (v) was calculated by linear regression analysis (Table 2). In the rats given 2 g/kg, the first value was higher than expected from a linear decrease, and this value was not used in the calculation of the rate. It appears possible that there was some direct transfer of ethanol from the peritoneal cavity into the bile.

The quantitative interpretation in terms of extent of oxidoreduction and transfer of hydrogen

Table 1. Areas under time-curves for concentrations of different deuterated ethanols in bile of rats given a 1:1 mixture of [1,1- $^2\text{H}_2$]- and [2,2,2- $^2\text{H}_3$]ethanol, expressed as percentages of the sum of these areas

Expt. no.	Ethanol dose (g/kg)	Labelled ethanol in bile (%)			
		$^2\text{H}_1$	$^2\text{H}_2$	$^2\text{H}_3$	$^2\text{H}_4$
1	1	7.1	58.1	31.7	3.1
2	1	10.1	56.8	29.6	3.6
3	2	14.0	51.3	28.7	6.0
4	2	8.5	56.5	31.1	4.0
5	2	19.7	42.4	26.2	11.8

Table 2. Results on ethanol oxidation and NADH bound to alcohol dehydrogenase, obtained by fitting models to concentrations in bile of deuterated ethanols after administration of a 1:1 mixture of [1,1- $^2\text{H}_2$]- and [2,2,2- $^2\text{H}_3$]ethanol to rats

The dose was 1 g/kg in expts. 1 and 2, and 2 g/kg in expts. 3–5. For details on the models, see the text.

Expt. no.	Net rate (mM/h)	Total rate divided by net rate	NADH labelling (fraction of maximum)	
			(fraction of maximum)	Isotope effect
1	12.8	1.7	0.64	2.6
2	12.9	2.1	0.65	2.7
3	12.3	4.2	0.57	4.6
4	14.0	2.1	0.50	3.2
5	10.8	7.4	0.75	4.2

was done by comparisons with models. These were calculated using the net rate (v) and the concentrations of the different deuterated ethanols at the beginning of the linear decrease in total concentration, and assuming different ratios between the total rate of ethanol oxidation and the net rate of ethanol elimination (f), different degrees of labelling of NADH, relative to the theoretical maximum when no exchange with free NADH takes place (y), and different isotope effects in the removal of the 1-*pro-R* hydrogen of ethanol ($1/q$). It was assumed that the rates of oxidation of the different molecular species were proportional to their concentrations, divided by the isotope effect in case of molecules having a ^2H in the 1-*pro-R* position, and that the rates of reduction of acetaldehyde molecules were proportional to their concentrations. The isotope effect in the removal of the 4-*pro-R* hydrogen of NADH (Bush *et al.*, 1973) was neglected in the models. The primary isotope effect in the oxidation of acetaldehyde (see above) was neglected in the first approximation. With d_n being the concentration of [$^2\text{H}_n$]ethanol, the theoretical maximum of NADH labelling is:

$$\frac{q(d_2 + d_4)}{d_1 + d_3 + q(d_2 + d_4)}$$

and multiplication by y gives the probability (a) for deuteration of an acetaldehyde molecule during reduction. The rates of increase with time (t) of the different molecular species are then given by the following equations, where $c = d_1 + d_3 + q(d_2 + d_4)$:

$$\frac{dd_1}{dt} = v \cdot \frac{(1-a)(f-1)(d_1 + qd_2) - fd_1}{c}$$

$$\frac{dd_2}{dt} = v \cdot \frac{a(f-1)(d_1 + qd_2) - fqd_2}{c}$$

$$\frac{dd_3}{dt} = v \cdot \frac{(1-a)(f-1)(d_3 + qd_4) - fd_3}{c}$$

$$\frac{dd_4}{dt} = v \cdot \frac{a(f-1)(d_3 + qd_4) - fqd_4}{c}$$

The calculation of models was performed as described above with the steps being 0.1 for f and 0.01 for y and q . The models with the best fit were in good agreement with the observed values (Fig. 2). The total rate of ethanol oxidation was 1.7–7.4 times higher than the net rate of ethanol elimination (Table 2), indicating significant reduction of acetaldehyde. The labelling of bound NADH was 0.62 ± 0.09 (S.D.) of the theoretical labelling in the absence of exchange with free NADH. In the rats with a high rate of acetaldehyde reduction, the labelling of bound NADH was also high. The primary isotope effect on ethanol oxidation was 3.4 ± 0.9 (S.D.).

Models were also calculated with account taken to the primary isotope effect on acetaldehyde oxidation ($1/u$). The rate of oxidation of acetaldehyde derived from $[^2\text{H}_1]$ - and $[^2\text{H}_2]$ ethanol as a fraction (g) of the total rate of acetaldehyde oxidation is then:

$$g = \frac{u(d_1 + qd_2)}{u(d_1 + qd_2) + d_3 + qd_4}$$

The rates of the changes in concentrations of deuterated ethanols could then be calculated as described above, if $f(d_1 + qd_2) - cg$ was substituted for $(f-1)(d_1 + qd_2)$ and $f(d_3 + qd_4) - c(1-g)$ was substituted for $(f-1)(d_3 + qd_4)$. Tests with this model in the manner described above yielded values for the isotope effect which were close to 1 (1.00–1.18), and values for y which were slightly higher than in the other model.

Effects of cyanamide

Two rats were given calcium cyanamide (30 mg/kg) intraperitoneally in 2 ml of saline, and after 2 h a mixture of $[1,1\text{-}^2\text{H}_2]$ ethanol (1 g/kg) and $[2,2,2\text{-}^2\text{H}_3]$ ethanol (1 g/kg) was given intraperiton-

eally. By linear regression analysis, the rate of decrease in total ethanol concentration was found to be 5.5 and 2.6 mm/h, respectively, in the two rats. $[^2\text{H}_1]$ - and $[^2\text{H}_4]$ ethanol appeared rapidly in bile, and after the first 1 or 2 h the concentrations of all four labelled ethanols decreased with approximately the same rates (Fig. 3). The results indicate rapid interconversion of ethanol and acetaldehyde. With d_n being the concentration of $[^2\text{H}_n]$ ethanol, the labelling of the hydrogen incorporated during acetaldehyde reduction in this steady state would be $d_4/(d_3 + d_4)$ or $d_2/(d_1 + d_2)$. This labelling relative to the maximal labelling ($d_2 + d_4$) was essentially constant after the first 2 h, the mean $\pm$ S.D. being 0.95 ± 0.01 and 1.03 ± 0.01 , respectively, in one rat (Fig. 3) and 0.93 ± 0.02 and 1.03 ± 0.01 , respectively, in the other rat. The values are close to 1, indicating that the loss by exchange of ^2H from the enzyme-bound NADH was slow. This makes it possible to calculate the rate of reduction of acetaldehyde from the estimated initial rates of appearance of $[^2\text{H}_4]$ - or $[^2\text{H}_1]$ ethanol and the maximal labelling of NADH ($d_2 + d_4$). The rate of acetaldehyde reduction (divided by the distribution volume for ethanol) was 120 and 70 mm/h in the two rats, respectively, which is about 25 times the net rate of ethanol elimination.

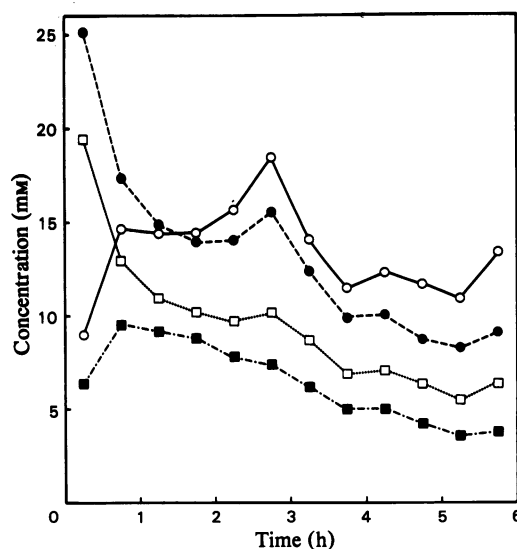


Fig. 3. Effect of cyanamide on the intermolecular hydrogen transfer and oxidoreduction during ethanol metabolism *in vivo*

Cyanamide (30 mg/kg) was given to a rat 2 h before a mixture of $[1,1\text{-}^2\text{H}_2]$ ethanol (1 g/kg) and $[2,2,2\text{-}^2\text{H}_3]$ ethanol (1 g/kg) was administered. The concentrations in 0.5 h bile fractions of $[^2\text{H}_1]$ ethanol (○), $[^2\text{H}_2]$ ethanol (●), $[^2\text{H}_3]$ ethanol (□) and $[^2\text{H}_4]$ ethanol (■) were measured.

Although the loss of ^2H by exchange of enzyme-bound NADH was slow, as discussed above, it was not absent. The rate (divided by the distribution volume for ethanol) was calculated from the decrease in the sum of the concentrations of $[^2\text{H}_2]$ - and $[^2\text{H}_4]$ ethanol (determined by linear regression analysis) by subtracting the decrease due to ethanol elimination, and it was then found to be 0.6 and 0.5 mm/h, respectively, in the two rats.

Discussion

The method

Intermolecular hydrogen transfer during oxidation *in vivo* has been previously demonstrated for C_{19} -steroid sulphates by a method similar to the one used in the present study (Cronholm & Rudqvist, 1982). Oxidoreduction may also be detected by the decrease in $^3\text{H}/^{14}\text{C}$ ratio of a substrate labelled with ^{14}C and ^3H at the hydroxylated carbon. However, the degree of oxidoreduction is underestimated in such experiments, since the extent of intermolecular hydrogen transfer is not determined. The back-reaction with incorporation of labelled and unlabelled hydrogen atoms also have to be taken into account in studies on isotope effects, which otherwise might be underestimated. On the other hand, the changes in apparent isotope effect might be used to calculate the extent of the back-reaction (Damgaard, 1983; Lundquist & Iversen, 1984).

A disadvantage of using ^2H instead of a carbon isotope to label the molecule is that exchange reactions might take place with loss of ^2H . The absence of this type of exchange was demonstrated in the present study with the use of $[2,2,2\text{-}^2\text{H}_3]$ ethanol. There might also be secondary isotope effects when deuterated compounds are used, but these are usually small and were therefore neglected. The primary isotope effect on oxidation of acetaldehyde was found to be small and it was considered that the values obtained were more accurate when it was not determined and corrected for.

Deuterium was transferred from C-1 of ethanol to the acetaldehyde that was being reduced. In the quantitative evaluation it was assumed that the rate of ethanol elimination was linear, although it is now believed to obey Michaelis-Menten kinetics (Lundquist & Wolthers, 1958; Wilkinson, 1980). In view of the high ethanol concentrations used this would not introduce any serious error in the calculations, and it also seems quite possible that the elimination was in fact linear since factors other than saturation of alcohol dehydrogenase may determine the rate, as discussed below. Oxidation of ethanol catalysed by systems other than alcohol dehydrogenase would not seriously

affect the results if the reactions are irreversible, as is probably the case with both catalase and the microsomal ethanol-oxidizing system.

Hydrogen transfer between ethanol molecules

During metabolism of a mixture of $[1,1\text{-}^2\text{H}_2]$ - and $[2,2,2\text{-}^2\text{H}_3]$ ethanol, the calculated labelling of the NADH bound to alcohol dehydrogenase was about 62% of the labelling at C-1 in the ethanol (Table 2). This is in good agreement with the labelling of the bound NADH measured as the ^2H excess in the cyclohexanol formed from cyclohexanone in similar experiments, which was 67% of that in the $[1,1\text{-}^2\text{H}_2]$ ethanol (Cronholm, 1974).

With knowledge of the labelling of the free NADH pool used in binding to alcohol dehydrogenase it would be possible to calculate the rate of association of NADH to the enzyme in relation to the total rate of oxidation of ethanol. However, different values (18–37% of that in the $[1,1\text{-}^2\text{H}_2]$ ethanol) have been obtained for the labelling of the NADH used in different cytosolic reductions (Cronholm *et al.*, 1974; Cronholm & Fors, 1976; Curstedt, 1982). The highest value (37%) was observed for the hydrogen at C-2 of the glycerol moiety in newly formed phosphatidylcholines (Curstedt, 1982). Using this value and the labelling of bound NADH relative to that at C-1 in ethanol (y , Table 2) the rate of association of NADH in the five rats could be calculated from the expression $(1-y)/(y-0.37)$ to be 0.7–3.9 times the total rate of ethanol oxidation, or 2.3–9.0 times the net rate of ethanol elimination. The rate of association of NADH divided by the distribution volume for ethanol was then 29–113 mm/h.

The difference in ^2H excess of different pools of NADH makes it possible that the free NADH close to alcohol dehydrogenase had a higher ^2H excess than assumed above and that the rate of association of NADH thus was higher than calculated. On the other hand, the isotope effect on the reduction of acetaldehyde with (4R)-[4- ^2H]NADH causes underestimation of the ^2H content of bound NADH. With this isotope effect being 2.6 (Bush *et al.*, 1973) the ^2H excess of bound NADH would have been 72–89% instead of 50–75% (Table 2) of that in the ethanol, and the rate of association of NADH would have been 0.7–2.3 times the net rate of ethanol elimination, or, after division by the distribution volume, 9–28 mm/h. According to deJuan & Taylor (1976), there is no isotope effect on the binding of (4R)-[4- ^2H]NADH, which otherwise could have influenced the results.

The loss of ^2H by exchange of bound and free NADH was inhibited when aldehyde dehydrogenase was inhibited by cyanamide, as seen when the rate was calculated assuming absence of isotope

effect on the reduction of acetaldehyde and assuming that the labelling of free NADH was unaffected by cyanamide (Cronholm, 1985). Thus, the rate of association of NADH divided by the distribution volume for ethanol was 1.1 and 1.6 mm/h in two rats. This effect was probably due to a lower concentration of free NADH in the presence of the inhibitor, caused by the inhibition of the net elimination of ethanol.

Backward reaction during elimination of ethanol

The oxidation of ethanol was found to be accompanied by a rapid backward reaction. Reduction of administered [^{14}C]acetaldehyde during ethanol metabolism has previously been observed to occur *in vivo* to a limited extent (Gershman, 1975). Lundquist & Iversen (1983, 1984) have observed the reduction in incubations with isolated hepatocytes and concluded that it also occurs *in vivo*. In the present study, the backward rate was 0.7–6.4 times the net rate of ethanol elimination. This very rapid rate of acetaldehyde reduction was not expected from the concentration of acetaldehyde in the liver of rats given ethanol (10 μM) and the kinetic characteristics of purified rat liver alcohol dehydrogenase determined by Crabb *et al.* (1983). Thus, the data and equations in the paper by Crabb *et al.* (1983) could be used to calculate that the backward rate should be about 0.2% of the forward rate. The rate of ethanol elimination *in vivo* has been found to proceed at the rate expected from the activity of the isolated enzyme, but since the backward reaction has not been considered in these calculations, the present results support the opinion that the activity *in vitro* cannot readily be used to calculate the rate of elimination *in vivo* (Higgins, 1979).

Inhibition of ethanol elimination by addition or accumulation of acetaldehyde has previously been observed *in vitro* and *in vivo* (Lindros *et al.*, 1972; Tottmar & Marchner, 1976; Crow *et al.*, 1977; Cederbaum & Dicker, 1979, 1981; Dawson, 1981). The present results strongly suggest that the inhibition is due to reversal of the alcohol dehydrogenase reaction.

Administration of the aldehyde dehydrogenase inhibitor cyanamide further increased the rate in the backward reaction, in spite of the decreased concentration of free NADH (see above). This was probably due to an increase of the acetaldehyde concentration, indicating that the concentration is not normally high enough to saturate the alcohol dehydrogenase, and this is in agreement with the K_m value (37 μM) and concentration of acetaldehyde in the absence of inhibitor (10 μM) reported by Crabb *et al.* (1983). The net rate of ethanol elimination was much lower than in the absence of

the inhibitor, as previously observed by Tottmar & Marchner (1976). The total rate of ethanol oxidation was actually higher than in the absence of the inhibitor, indicating that the effect on the net rate was due to the backward reaction and not to inhibition of alcohol dehydrogenase by acetaldehyde (Dawson, 1983b) or cyanamide (Sharkawi, 1980). The increase in the total rate of ethanol oxidation might have been due to an elevated concentration of the binary complex containing NAD^+ , resulting from a rapid rate of reduction of acetaldehyde, and to the lower concentration of free NADH, caused by the lower net rate of ethanol elimination.

Rate-limiting of ethanol elimination

The rate of dissociation of NADH from alcohol dehydrogenase was at least 1.7–3.3 times the rate of net ethanol elimination. This is not in agreement with the contention that the rate of elimination is determined only by the rate of dissociation of the binary complex, as it often is *in vitro* (Theorell & Bonnichsen, 1951) and as it has been calculated to be *in vivo* from kinetic data obtained *in vitro* (Crabb *et al.*, 1983). It indicates an additional role for the concentrations of NADH and NAD^+ since these would determine the extent to which the free enzyme would bind the coenzymes. Thus, the rate of reoxidation of NADH would be of importance in determining the rate of ethanol elimination (Videla & Israel, 1970; Hillbom, 1971). On the other hand, results from studies with the redox couple cyclohexanol/cyclohexanone as indicator of the redox state of the coenzyme bound to alcohol dehydrogenase have shown that ethanol causes a greater change of the redox state of the bound than of the free coenzyme, indicating that the dissociation of NADH from, or association of NAD^+ to, alcohol dehydrogenase might be slow and contribute to the rate-limitation of ethanol elimination (Cronholm, 1983). In agreement with this, the present study has shown that the equilibration of free and bound NADH during ethanol elimination is incomplete.

The observed reduction of acetaldehyde could only occur if oxidation of acetaldehyde was at least partly rate-limiting for the net elimination of ethanol. Since the oxidation of ethanol appears to be rapid and reversible, the reoxidation of NADH could determine the concentration of acetaldehyde and thus its oxidation, as suggested by Krebs (1969). In cyanamide-treated rats, the ethanol–acetaldehyde interconversion was very rapid and the net rate of ethanol elimination was slow. Thus the oxidation of acetaldehyde became more completely rate-limiting for the elimination of ethanol in these rats.

It may be concluded that the oxidation of acetaldehyde, the oxidation of cytosolic NADH, and the dissociation of the alcohol dehydrogenase-NADH complex all contribute to the rate-limitation of ethanol elimination.

Isotope effects

Several of the reactions involved in hydrogen transfer between ethanol molecules during ethanol elimination may be influenced by isotope effects. In the present experiments there was competition between labelled and unlabelled molecules and they were thus influenced by apparent isotope effects on the ratio between $V_{\max}$ and K_m [$^D(V/K)$; Northrop, 1977]. The isotope effect on the removal of the 1-*pro-R* hydrogen of ethanol (about 3.4, Table 2) is in good agreement with that seen *in vitro* (3.0; Damgaard, 1981). The observation of smaller isotope effects *in vivo* (Cronholm, 1974; Damgaard, 1981) was probably due to lack of correction for oxidoreduction of ethanol with partial hydrogen retention (Damgaard, 1983).

The oxidation of [$1\text{-}^2\text{H}$]acetaldehyde was found to be subject to an isotope effect [$^D(V/K)$] of 1.2 and 1.4, respectively, in two rats. This is much less than the $^D(V/K)$ of about 3 found *in vitro* with the cytosolic enzyme from horse liver (Eckfeldt & Yonetani, 1976) or human liver (Vallari & Pietruszko, 1981). This could be due to the differences in kinetics of the cytosolic enzyme and the mitochondrial enzyme, since the latter is considered to be most important for acetaldehyde oxidation (Weiner, 1979).

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A New Method for the Determination of Ethanol in the Blood and Urine by Pulse Heating

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Abstract. We have established a new method for the determination of ethanol and other gaseous substances in even minute amounts of biological material by utilizing a Curie-point pyrolyzer (Model JHP 3, Japan Analytical Industry) connected to GC or GC-MS. No pretreatment of the biological material is needed and the procedure is based on the principle that volatile or gaseous substances can be evaporated from biological materials by pulse heating and introduced for analysis directly to a GC or GC-MS.

The blood from rats that had been administered ethanol-d6 orally, as well as blood and urine collected from healthy volunteers that had ingested alcoholic drinks and blood and tissue specimens taken from autopsied cadavers were examined for ethanol concentrations by both this new method and by the usual head space-GC method.

To test this new method, one microliter of the blood or urine was injected into a tubularly folded ferromagnetic alloy (Pyrofoil[®]) and heated pulsatively five times at 160°C (pulse heating). The vapor that evaporated from the sample then was directly introduced to the GC or GC-MS, and the time required to complete the analysis took approximately 5 min. In parallel, for comparison purposes, portions of the same samples (0.2-0.5g) were examined by means of the head space method as well.

The measured values of ethanol-d6 and ethanol in the rat blood and in the human whole blood, blood plasma, and urine that were obtained by this pulse heating procedure correlated almost perfectly with those obtained by head space method. These results show the accuracy and reproducibility of this new method. Further, "pulse heating" makes it possible to determine the presence of ethanol very quickly from a very small amount of the specimen.

We have termed this new investigative method for determining volatile and gaseous substances the "pulse heating method". Informatively, nitrous oxide (N₂O, laughing gas) was identified by this pulse heating GC-MS procedure from the blood and organ specimens of a victim that had succumbed to gas asphyxia.

Key words : Toxicology, Ethyl alcohol, Mass fragmentography

Introduction

The analysis of ethanol in biological materials is a routine procedure in the medico-legal practice and it has a great significance when investigating traffic incidents. For determining the presence of ethanol, several methods are available, though they are relatively complicated and time-consuming. Procedures that use gas chromatography for the detection of ethanol and other volatile poisons in biological materials have been reported¹⁾⁻⁷⁾.

Among them, a head space method¹⁾⁻⁴⁾ is now widely utilized because of its many advantages. This method, however, also has some disadvantages that will be mentioned in the Discussion portion of this paper. Further, Ueda and Fukui⁸⁾ and Ishizawa et al.^{9),10)} have reported on a possible method for determining the presence of ethanol by a process of evaporation that requires only a very small amount of the specimen to be tested and uses an electric furnace-type pyrolyzer. While their methods are theoretically appropriate, they

have not been practically utilized because of the reasons that also will be mentioned in the Discussion.

Recently, a Curie-point pyrolyzer has been developed that uses ferromagnetic alloys which very quickly generate heat by electromagnetic induction and can be warmed to an accurately defined Curie temperature^{11/12}). Thus, we have made an attempt to establish a new method for determining the presence of ethanol and other volatile and gaseous substances by pulse heating, utilizing this Curie-point pyrolyzer and connecting it to a gas chromatograph (GC) apparatus or a gas chromatograph-mass spectrometer (GC-MS).

In this study, we have carried out a fundamental animal experiment using ethanol-d6 and were able to determine the presence of alcohol in human blood and urine by this method, which we have termed the "pulse heating method", and have compared our results with the results achieved by the head space method.

Materials and Methods

1. Standard solution.

Deuterium-labelled ethanol-d6 (MSD Isotopes, Canada; 0.2-2.0 mg/ml) and natural ethanol (0.2-5.0 mg/ml) were added to distilled water.

2. Ethanol-d6 containing samples.

Ethanol-d6 (25 v/v% aqueous solution) was administered orally to rats (Wister strain, ca. 200 g in weight) at a dose of 1 g per kg body weight. The blood was extracted from 15 to 90 min after this administration, and stored in small glass vials at 4°C.

3. Human blood and urine samples.

Healthy adult volunteers were given alcoholic drinks: beer in the amount of 750 and 1,300 ml; and, Japanese sake in the amount of 700 and 950 ml. Blood and urine samples then were collected at about 30 min after ingestion. The whole blood, plasma, and urine were stored at 4°C until use.

Blood samples also were collected from 12 autopsy cases and examined as well.

4. Equipments and analytical conditions.

A Curie-point pyrolyzer (Japan Analytical Industry, Model JHP-3) was connected to a GC (JEOL, Model GCG-06, FID) or a GC-MS system (JEOL, Model JMS-DX303 & DA5100 Data System). Fig. 1 is a schematic diagram that shows

GC-probe

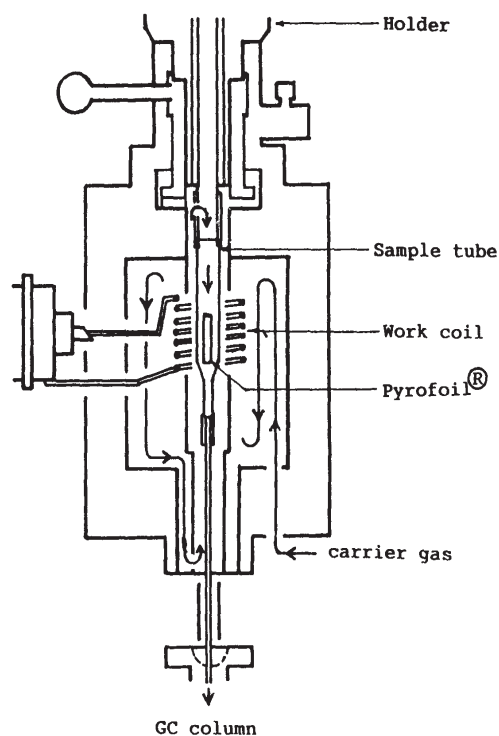


Fig. 1. Schematic illustration of GC-probe of Curie point pyrolyzer Model JHP-3.

the construction of the "GC-probe" of the pyrolyzer. Pulse heating (abbreviated as Py)-GC or -GC-MS was carried out under the following conditions:

Pyrolyzer (JHP-3)

frequency 450 kHz, power 225W

Pyrofoil®, of Curie-point 160°C

needle temperature 200°C

GC (GCG-06, FID)

column: Porapak Q (80-100), 2.6 mm × 1 m

temperature: column oven 160°C

injection 180°C

detector 222°C

carrier gas flow (N₂) 50 ml/min

calculator Shimadzu Chromatopac C-R3A

GC-MS (JMS-DX303 & DA5100 Data Systems)

GC column: Porapak Q (80-100),

2.6 mm × 1 m

temperature: column oven 170°C

injection 180°C

separator 180°C

carrier gas flow (He)	45 ml/min
MS EI (positive) mode	
accel volt	3 kV
ionization volt	70 eV
ionization current	0.3 mA
ionization chamber temperature	150°C

5. Analytical procedure.

For calibration curves, 0.5, 1.0, 2.0, 3.0, and 5.0 μ l of standard solutions of ethanol-d6 and ethanol were used. Ethanol standard solutions of 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mg/ml were used to determine within-run and day-to-day precision. Each sample was divided into two portions and analyzed in parallel by both the head space method and the "pulse heating" method.

Pulse heating method : One microliter of each sample was extracted with a microsyringe and injected into a Pyrofoil® which was folded in a tubular form. The foil was immediately inserted into a "sample tube" which was fixed on a holder and set on the "GC-prove" of the pyrolyzer (Fig. 1). The sample then was heated at 160°C for 4 sec five times pulsatively at intervals of 20 sec in order to completely evaporate the volatile substances. The vapor was analyzed by the Py-GC or -GC-MS.

Head space method : According to the method of Nanikawa and Kotoku⁴⁾, samples (0.5 ml) pipetted into vials were incubated with an internal standard (0.2 mg/ml tert-butanol) at 55°C for 20-25 min. The head space gas (about 1 ml) then was analyzed by GC or GC-MS.

6. A case of determination of gaseous anesthetics in human blood and tissue specimens.

During our investigations, we also had the opportunity to analyze the amount of gaseous anesthetics present in the heart blood and the brain, and also in the heart muscle, lung, liver, and kidney by means of both methods. The above materials were obtained from a cadaver of a 9-year-old, who had died in an anesthetic accident. A small amount of each material was sealed into a vial, and frozen at -30°C immediately after being taken from the cadaver. Two days later the materials then were examined by the two methods described above.

Results

1. Mass chromatographic analysis of ethanol-d6

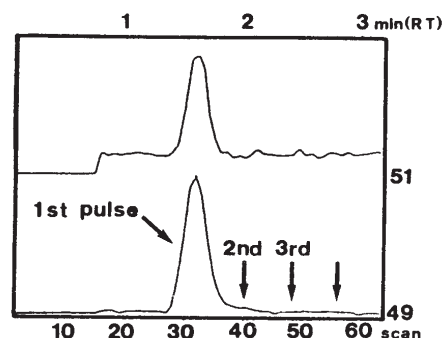


Fig 2 Mass chromatogram of ethanol d6 (d5) extracted by a computer data process from TIC Rat heart blood (1 μ l) was analyzed by pulse heating at 160°C for 4 sec, five times at intervals of 20 sec No peaks appeared after the second pulse heating

from animal blood by the pulse heating method.

Rat blood containing ethanol-d5, into which ethanol-d6 immediately changed in water, were analyzed by the pulse heating method. Mass chromatogram of ethanol-d5 (ion current profiles at m/z 49 and 51) that was extracted by a computer-data process from a series of consecutively recorded mass spectra is shown in Fig. 2. When the blood samples were examined by 5 pulse heatings, only one peak was noted at a retention time (1.6 min) of ethanol-d6 (-d5) in the mass chromatogram (Fig. 2). The EI mass spectrum of the peak showed predominant ions of ethanol-d5 at m/z 33, 49 and 51. Additionally, very small ion peaks at m/z 50, 46 and 48 were observed, which were also detected in standard solutions. No other ions, including natural ethanol (m/z 31 and 45), were noted.

2. Calibration Curves.

Using 0.2-2.0 mg/ml of an ethanol-d6 solution as the standard solution, calibration curves were drawn from an estimation of the peak area and peak height of their mass-chromatograms. Each line was linear through the zero-point as shown in Fig. 3. By means of the peak area, the following equation was obtained : $Y=4.40 \times 10^6 X$, where Y is the peak area per 1 μ l and X is the concentration of ethanol-d6. Similar results were obtained when natural ethanol (0.2-5.0 mg/dl) was used instead of ethanol-d6. When 0.5, 2.0, 3.0, and 5.0 μ l of each standard solution was applied, the calibration curves were almost identical to that seen in the

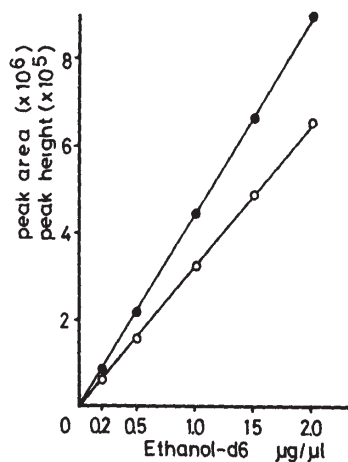


Fig 3 Calibration curves for ethanol d6 (d5) measured at m/z 49 Standard solution in the distilled water ●—● peak area ○—○ peak height

Table 1 Measured values of ethanol d6 in the rat heart blood

sample No	Measured by	
	head space method	pulse heating method
1	0 7611 mg/ml	0 7500 mg/ml
2	0 6841	0 6921
3	0 9799	0 9667
4	0 6690	0 8269
5	0 8282	0 8924
6	0 8894	0 9990
7	0 5120	0 5227
8	1 1531	1 2860

use of 1 µl of the standards.

For within-run precision, the coefficients of variation (CVs) ranged from 2.7 to 5.1% at 0.2-5.0 mg/ml level. For day-to-day precision, CVs ranged from 5.5 to 8.6%.

3. Measured values of ethanol d6 concentrations in the animal blood.

Ethanol-d6 (changed into -d5) concentrations in the rat heart blood measured by the pulse heating and the head space methods are given in Table 1. The measured values by both methods very closely approximated each other. The correlation between the values by both methods is shown in Fig. 4 and the regression curve was estimated per the following formula: $Y=0.03 + 1.03 X$ (Y, the

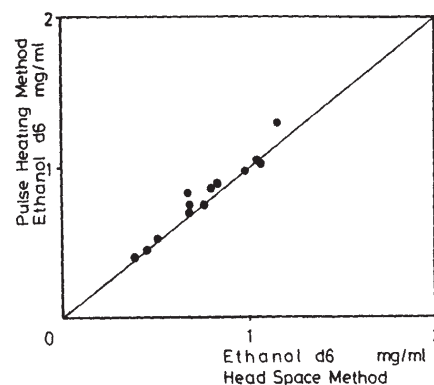


Fig 4 Comparison of ethanol d6 (d5) concentrations in the rat heart blood measured by the head space method and by the pulse heating method

Table 2 Measured values of the ethanol concentration in human blood and urine after ingestion by volunteers

sample No	Measured by	
	head space method	pulse heating method
whole blood		
1	0 475 mg/ml	0 489 mg/ml
2	0 619	0 665
3	2 474	2 485
4	2 790	2 873
plasma		
1	0 539	0 556
2	0 691	0 712
3	2 654	2 713
4	3 057	3 226
urine		
1	0 624	0 679
2	0 721	0 756
3	2 446	2 767
4	3 993	4 323

measured value by the pulse heating method; X, the measured value by the head space method) with a correlation coefficient (r) 0.965 (sample size n=15).

4. Measured values of ethanol concentration in human whole blood, plasma, and urine.

Ethanol concentrations of the whole blood, plasma, and urine collected from healthy volunteers were measured by both the pulse heating and the head space methods. The data obtained are shown in Table 2. The correlation between the

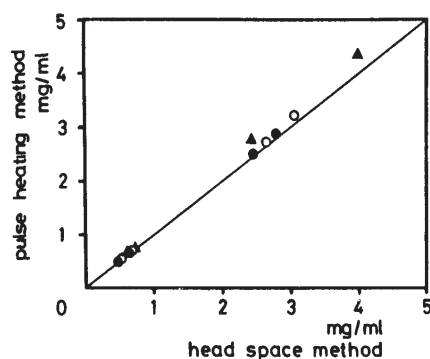


Fig 5 Comparison of ethanol concentrations in the volunteers blood and urine measured by the head space method and by the pulse heating method
● whole blood ○ plasma ▲ urine

Table 3 Measured values of the blood ethanol concentration in autopsy cases

sample No	Measured by	
	head space method	pulse heating method
1	2 323 mg/ml	2 147 mg/ml
2	3 977	3 611
3	0 787	0 772
4	1 486	1 315
5	1 056	1 068
6	2 980	2 985
7	3 330	3 370
8	1 560	1 512
9	<0 001	<0 001
10	1 537	1 535
11	<0 001	<0 001
12	0 580	0 592

measured values by both methods is shown in Fig. 5 and the regression curve was estimated per the following formula: $Y=0.017 + 1.03X$ ($r=0.998$, $n=12$).

5. Measured values of ethanol concentration in whole blood in autopsy cases.

In autopsy cases as well, the measured values of the blood ethanol concentrations by means of the pulse heating were almost identical with the head space methods (Table 3). For the correlation between both methods, the regression curve was estimated as follows : $Y=0.020 + 0.952X$ ($r=0.997$, $n=12$).

All processes to complete the analysis required

about 5 min.

6. Determination of gaseous anesthetics in the human blood and tissue specimens.

By both the pulse heating GC-MS method and the head space-GC-MS method, nitrous oxide (N₂O, laughing gas) was qualitatively identified in the all tissue specimens tested.

Discussion

Because of its many advantages, the head space GC method is widely used internationally. There are many reasons for this ; the method has a high reliability and shows a fairly good reproducibility, and the head space gas can be directly applied to a GC-apparatus for measurement. However, particularly when the ethanol concentrations are very low or abnormally high or when the amount of the materials to be tested is very minute, there are also some disadvantages in utilizing this method. Contrary it is theoretically appropriate to determine ethanol and other volatile or gaseous substances which were evaporated out from the sample specimens Attempts⁽⁸⁻¹⁰⁾ have been made to use an electric furnace type pyrolyzer to evaporate ethanol from minute samples as a method of gas content analysis. Using this approach Ueda and Fukui⁽⁸⁾ have tried to analyze the ethanol content by heating samples sealed in polyethylene tubes that were melted by heat. Although, as they have described, the interference from the pyrolysis products of the polyethylene containers was negligible, some small peaks appeared that resulted from polyethylene, with a longer retention time than that of the ethanol. Therefore, the next sample could not be introduced for about 22 min until all the pyrolysis product of the polyethylene had left the column. Similarly, the gas chromatographic method reported by Ishizawa et al.^(9) 10) requires more than 30 sec to reach the necessary temperature, by which time the ethanol will have completely evaporated from the specimens.

In contrast the Curie-point pyrolyzer, which we employed, possesses a valuable heating capacity that utilizes a self-heating capability of ferromagnetic alloys. In other words, by means of electromagnetic induction, hysteresis generates heat in the metal due to friction caused by the changing polarity. At the Curie-point, the metal then suddenly loses its magnetism owing to a

drastic change in the magnetic permeability. Informatively, the ferromagnetic metal, Pyrofoils, that was used in this study reaches the Curie-point within 0.2 sec, whereas the temperature in a furnace pyrolyzer rises far more slowly, taking longer than 5 sec. Further, Pyrofoil® cools rapidly due to the interruption of the electromagnetic induction. Therefore, samples injected or wrapped in a tubularly folded foil can be instantaneously and repeatedly heated to a given Curie temperature, and the heating duration can be appropriately controlled.

As shown in Fig. 2, by using a Curie-point pyrolyzer that was connected to a GC or GC-MS, it was possible to determine the ethanol content very quickly from a very small sample of the material (ca. 0.5-5 μ l), whereas the head space method required ca 0.5 ml of the sample. Further, the pulse heating method could be repeated at short intervals until the ethanol-d5 was completely released from the specimens (Fig. 2). The blood samples gave only one peak (retention time, 1.6 min) in spite of 5 pulse heatings, and all the necessary processes for one analysis were completed within approximately 5 min. Informatively, the peak width of the ethanol (or -d5) was about 20 sec. For a comparison the furnace pyrolyzer method used by Ueda and Fukui⁸⁾ and by Ishizawa et al.^{9),10)} gave an ethanol peak width that was about 45 sec and required approximately 22 and 25 min for one analysis, respectively.

This shows that a Py-GC (or GC-MS) by means of a Curie point pyrolyzer can be performed more rapidly than the methods that use a furnace pyrolyzer⁸⁾⁻¹⁰⁾ and that the peaks are remarkably sharper. The Py-GC-MS analyses of ethanol-d6 (-d5) in blood samples showed no other peak than ethanol-d6. This indicates that there occurred no artifacts, such as the degradation of ethanol-d5 or pyrolysis production of interfering substances from the blood.

Further, all the calibration curves of ethanol (or -d6) obtained by repeated examinations were almost identical, and the stability of the calibration curves is reflected in the within-run and day-to-day precision.

From these data, it can be said that the pulse heating-GC or -GC-MS method has good accuracy and sufficient reproducibility for practical assays

of ethanol in the blood and urine. A series of examinations to prove the reliability of this method showed that the measured values from the blood and urine (liquid samples) very closely approximated those obtained by the head space method (Figs. 4 & 5).

In the rat whole blood and in human whole blood, as well as in the plasma and urine from volunteers, the measured values obtained by the pulse heating method had a tendency to be slightly higher than the values obtained by the head space method (Tables 1 & 2 and Figs. 4 & 5).

Thus, the use of a Curie point pyrolyzer is suitable for determining the ethanol content of biological materials. Employing this new method, the analysis can be very quickly performed and the materials to be tested require no pretreatment. Additionally, the quantitative accuracy and reproducibility are comparable to those obtained by the head space method.

Further, our preliminary study has shown that this method can be applied to the analysis of other volatile and gaseous substances, such as petrolic components and gaseous anesthetics, and the details of our results will be published separately.

Informatively, we had an opportunity to determine gaseous anesthetics in the heart blood and in other tissue specimens of a 9-year-old who unexpectedly died while under anesthesia. Using our pulse heating GC-MS method, laughing gas was determined in all the specimens tested. This indicates the possibility of determining the presence of volatile and gaseous drugs and poisons in all kinds of biological materials whether liquid or solid by the pulse heating method.

We are now seeking to establish a reliable and rapid screening system for determining volatile and gaseous substances in biological materials.

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