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Determination of the absolute configuration of 2-hydroxyglutaric acid and 5-oxoproline in urine samples by high-resolution NMR spectroscopy in the presence of chiral lanthanide complexes

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Abstract

Determination of the absolute configuration of some metabolites in body fluids is important for the diagnosis of some inborn errors of metabolism. Presently available methods of such determinations are tedious and usually require highly specialized instrumentation. In this work, an alternative method, based on high-resolution nuclear magnetic resonance spectroscopy in the presence of the chiral lanthanide shift reagent as an auxiliary additive, has been proposed (NMR/LSR). The method involves the lineshape analysis of a chosen multiplet of the one-dimensional ^{1}H NMR spectrum or application of the two-dimensional ^{1}H-^{13}C correlation spectroscopy (HSQC). In order to confirm the resonance assignments and to boost the signal–noise ratio, the addition of an amount of racemic analyte to the urine sample is recommended. The entire procedure is simple in application and demands minimal or no preprocessing of urine samples. The effectiveness of the method has been confirmed by finding the expected forms of 2-hydroxyglutaric acid and 5-oxoproline in the urine samples of an independently diagnosed patient with 2-D-hydroxyglutaric aciduria and 5-L-oxoprolinuria, respectively.

Keywords: Inborn error of metabolism; Metabolite absolute configuration; 2-Hydroxyglutaric aciduria; 5-Oxoprolinuria; Enantioselective NMR; Chiral lanthanide shift reagent

1. Introduction

Enzyme catalyzed reactions are highly stereospecific and usually provide a given product in one enantiomeric form. As a result, practically all living matter is built of amino acids of the L-series and sugar units of the D-series. Both absolute configurations do appear occasionally in living systems, but as a rule, the biochemical roles of enantiomeric counterparts and their biological origin are different. It is obvious that stereochemical considerations, including the problem of the optical isomerism of biomolecules, are of high

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importance for understanding biochemical and physiological mechanisms. Stereochemical problems have implications for diagnosis too [1,2]. In conventional procedures, the diagnostic indications are based on the abnormal concentrations of specific metabolites found in body fluids [3,4]. These marker molecules are most frequently chiral and the determination of their absolute configuration may also be crucial for diagnosis. Such a situation is encountered, for example, in the case of 2-L-hydroxyglutaric and 2-D-hydroxyglutaric acidurias, which manifest themselves by the excretion of excessive amounts of one of the optical isomers of 2-hydroxyglutaric acid [5–8]. Similar questions also arise when abnormal levels of 5-oxoproline [9], glyceric [2] or lactic [2] acid are observed in urine, cerebrospinal fluid or plasma. Naturally, more examples are known [10,11] and there is no doubt that many remain unknown.

The validity of the problems mentioned above is widely recognized and many authors recommend determining the absolute configuration of the marker metabolites besides the routine analysis of physiological fluids performed for diagnostic or scientific purposes [1–11]. Unfortunately, the laboratory methods enabling such determinations are tedious and/or require highly specialized equipment. The oldest method based on the measurement of the optical rotation of the given metabolite is now probably never used, as it demands separation and exact purification of the investigated species. Another classical method based on diastereomeric derivatization followed by chromatography [6], though more practicable, is again very laborious due to the necessity of at least partial isolation and chemical processing of the investigated marker. The use of biochemical methods based on the selective enzymatic degradation of one enantiomer [9] is limited by the accessibility of the adequate enzyme or biosystem. Moreover, the results of those methods are sensitive to various disturbing factors connected with the a priori unknown composition of the investigated sample. Probably the most convenient methods, though not limitation-free, are chromatographic or electrophoretic determinations, in which the conventional fractionalization is followed by separation of enantiomers on a chiral phase [1,8]. Recently, enantioselective chromatography has been combined with mass spectrometry [8,10,11].

After a long initiation period, high-resolution nuclear magnetic resonance spectroscopy has been introduced to the 'in vivo' as well as 'in vitro' medical assays [12–18]. Usually, the method provides a possibility of overcoming sample pre-processing, at least in the case of urine analysis and seems to be much simpler in practical use than other instrumental methods. In this paper, the application of high-resolution NMR spectroscopy in the presence of a chiral lanthanide shift reagent (NMR/LSR) for enantiomeric recognition is proposed. This alternative method has been shown to be effective and convenient, especially when the absolute configuration of a dominating enantiomer is of the only interest.

2. Materials and methods

The europium compounds $EuCl_3 \cdot 6H_2O$ and Eu_2O_3, chloroacetic acid, 2-(chloromethyl)pyridine hydrochloride, (*R*)-1,2-diaminopropane dihydrochloride, racemic and L forms of 5-oxoproline (Aldrich) and glutamic acid (Sigma) were obtained commercially.

The optically pure ligands (*R*)-1,2-propylenediaminetetraacetic acid (H_4PDTA) [19] and (*R*)-*N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)-1,2-propylenediamine (TPPN) [20], as well as appropriate europium complexes $Na[Eu(PDTA)(H_2O)_3] \cdot 2H_2O$ **1** [21] and $[EuCl_2(TPPN)]ClO_4$ **2** [22], were prepared using literature procedures.

The racemic form and L isomer of 2-hydroxyglutaric acid were prepared by the diazotization reaction, with preservation of the configuration at C-2, of the appropriate form of glutamic acid [23]. Purity was checked by ^{1}H NMR. Two urine samples with 2-hydroxyglutaric aciduria and two others with 5-oxoprolinuria from independently diagnosed patients were supplied from The Children's Memorial Health Institute in Warsaw.

The one-dimensional ^1H NMR measurements of urine were performed for samples composed of 0.50 cm^3 of unprocessed urine, 0.05 cm^3 of D_2O (lock signal) and an appropriate amount of 3-trimethylsilyl-1-propanesulfonic acid sodium salt (Merck). The signal of the latter compound was used as the chemical shift reference and the intensity standard. The samples were prepared directly in 5-mm o/d NMR tubes. NMR spectra were recorded also for reference solutions containing ≈ 0.03 mol/dm^3 of the investigated metabolite and ≈ 0.003 mol/dm^3 of LSR (**1** or **2**) in H_2O, or for mixtures of this solution with the urine. LSR containing samples were centrifuged before measurement, if necessary. The sample pH was controlled directly in the NMR tubes, using a pH electrode (Cole-Parmer NMR tube P-05990-30) and pH-meter (Cole-Parmer P-59002-00). This measuring system was standardized using pH-4.01, pH-7.00, pH-10.01 buffers. The pH of the solutions was adjusted by adding small amounts of 0.1 M HCl or 0.05 M NaOH H_2O solutions.

The proton NMR spectra were recorded using Varian Mercury 400 or GEMINI 2000 spectrometers operating at 9.4 and 4.7 T magnetic fields, respectively. The water signal was saturated prior to the observing pulse for 4 s. The standard measurement parameter sets were pulse width (pw) = 5 μs (pw90 = 18 μs), acquisition time (at) = 5 s, spectral width (sw) = 15 ppm and pw = 7 μs (pw90 = 23 μs), at = 5 s, sw = 16 ppm when using 400 and 200 MHz spectrometer, respectively. At least 256 scans were accumulated and, after zero-filling to 64 K, FID signals were subjected to Fourier transformation. In some cases, in order to achieve a satisfactory signal–noise ratio, the accumulation time had to be as long as several hours. The measurement temperature 25–40 °C was set and stabilized using the spectrometer variable temperature unit and was not additionally calibrated.

The two-dimensional z-gradient assisted ^1H-^{13}C HSQC spectra were recorded five times for concentrated urine samples using a Bruker DRX500 Avance spectrometer. The resulting linewidths and digital resolutions allowed signals separated by 2 Hz and 0.15 ppm in the ^1H and ^{13}C NMR dimensions, respectively, to be distinguished. The spectral windows were adjusted in both dimensions to cover all the proton signals and all the aliphatic carbon signals. After the appropriate zero-filling, the data matrices were of 2×2 K dimension. Owing to the relatively high solute concentration only ≈ 1 h data accumulation time per spectrum was needed.

The lineshape fitting program, LSFIT, written in Fortran, optimized parameter values based on the least squares sum of theoretical to experimental spectrum deviations using the Gauss–Newton method. It was assumed that spectral lines were of the Lorentzian shape and that the contribution of the dispersion mode was constant throughout all the spectral fragments analyzed. Linewidths were assumed to be the same within each of the multiplets, but they were allowed to be different in multiplets originating from different enantiomers.

Moreover, it was assumed that the multiplet patterns of both enantiomers were identical in terms of the number of lines (not fitted), line-to-line separations along the frequency axis and their relative intensities. The base line was described by a third order polynomial. All calculations were performed using a PENTIUM2 120 MHz personal computer.

3. Results and discussion

3.1. The idea

Enantiomeric structures are, by definition, mirror images of each other and their high-resolution NMR spectra recorded in achiral media are identical. For a long time, however, various ways of overcoming this difficulty have been known [24]. Apparently, the most efficient approach allowing the NMR spectra of enantiomers to be distinguished is the use of chiral paramagnetic lanthanide compounds as auxiliary additives [25]. These compounds are known as lanthanide shift reagents (LSR), as they cause changes of the chemical shifts of other species present in the investigated solution. LSR have the ability of forming complexes of moderate or low stability with molecules possessing amino, hydroxy, carbonyl or some other electron-rich groups. In the

investigated solution, the species of interest exchange rapidly on the NMR timescale between complexed and uncomplexed forms. As a result, the averaged spectrum is observed rather than the spectra of the complexed and uncomplexed forms. Spectra of these two forms contribute to the resultant spectrum proportionally to their populations. The differences between the spectra of enantiomers arise because: (i) spectra of the complexed and uncomplexed forms are, in general, different; (ii) complexes of the enantiomeric objects with the chiral LSR are diastereomeric (not mirror images) and so have different spectra; (iii) the complexation equilibrium constants for diastereomeric complexes can be different, leading to different population factors.

In the last years, several chiral LSR suitable for investigating aqueous solutions were invented. At least two: $Na[Eu(PDTA)(H_2O)_3]\cdot 2H_2O$ (**1**) and $[EuCl_2(TPPN)]ClO_4$ (**2**) (see Fig. 1), seemed to be appropriate for investigating urine samples. Complex **1** was proved to split some signals in ^1H NMR spectra of anions of amino acids and 2-hydroxyacids including lactate and glycerinate [26–28]. Complex **2** was reported to be effective towards amino acids [22]. In this study, the effectiveness of both complexes has been examined toward anions of 2-hydroxyglutaric acid (**3**) and

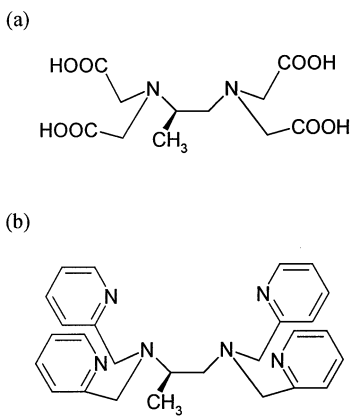


Fig. 1. (a) (*R*)-1,2-Propylenediaminetetraacetic acid (H_4PDTA) and (b) (*R*)-*N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)-1,2-propylenediamine (TPPN), the chiral ligands of the europium(III) cation in the shift reagents $Na[Eu(PDTA)(H_2O)_3]\cdot 2H_2O$ (**1**) and $[EuCl_2(TPPN)]ClO_4$ (**2**) used in this work.

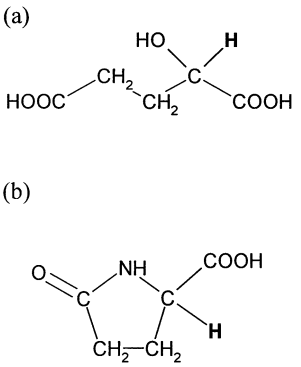


Fig. 2. (a) 2-Hydroxyglutaric acid (**3**) and (b) 5-oxoproline (**4**); 2-H protons marked with bold phase letters.

5-oxoproline (**4**) (Fig. 2), two other metabolites the absolute configuration of which has been of diagnostic validity.

The influence of complex **1** on the H-2 proton resonance of **3**, chosen as the diagnostic signal in this study, is illustrated in Fig. 3. In this figure, the appropriate spectral fragments are shown for the solution of the racemic metabolite in the absence of LSR (a) and in the presence of LSR (b), as well as for the latter solution artificially enriched with the metabolite L-enantiomer (c). It is to be stressed that all the spectra were recorded for H_2O (rather than D_2O) solutions and for low solute concentrations. Such measurement conditions were similar to those encountered in urine samples and far from those optimal for detecting enantiomeric recognition. Nevertheless, differences between 'a' and 'b' and then 'b' and 'c' spectra were evident. The signal patterns 'b' and 'c' were not as clear due to the extensive line broadening normal for LSR-containing samples. Still, even visual examination of 'b' signal showed that it is composed of two convoluted multiplets of the 'a' type. When passing from 'b' to 'c', the overall change of the signal shape could be described as the relative intensity enhancement of the up-field multiplet (in the case of **3**). Similar results were obtained for the system composed of the complex **2** and metabolite **4**. In this case, the 'c' type spectrum showed that the low-field signal originated from the L-enantiomer, in variance to most hitherto reported cases of the enantiomeric recognition of 2-hydroxyacids and 2-aminoacids

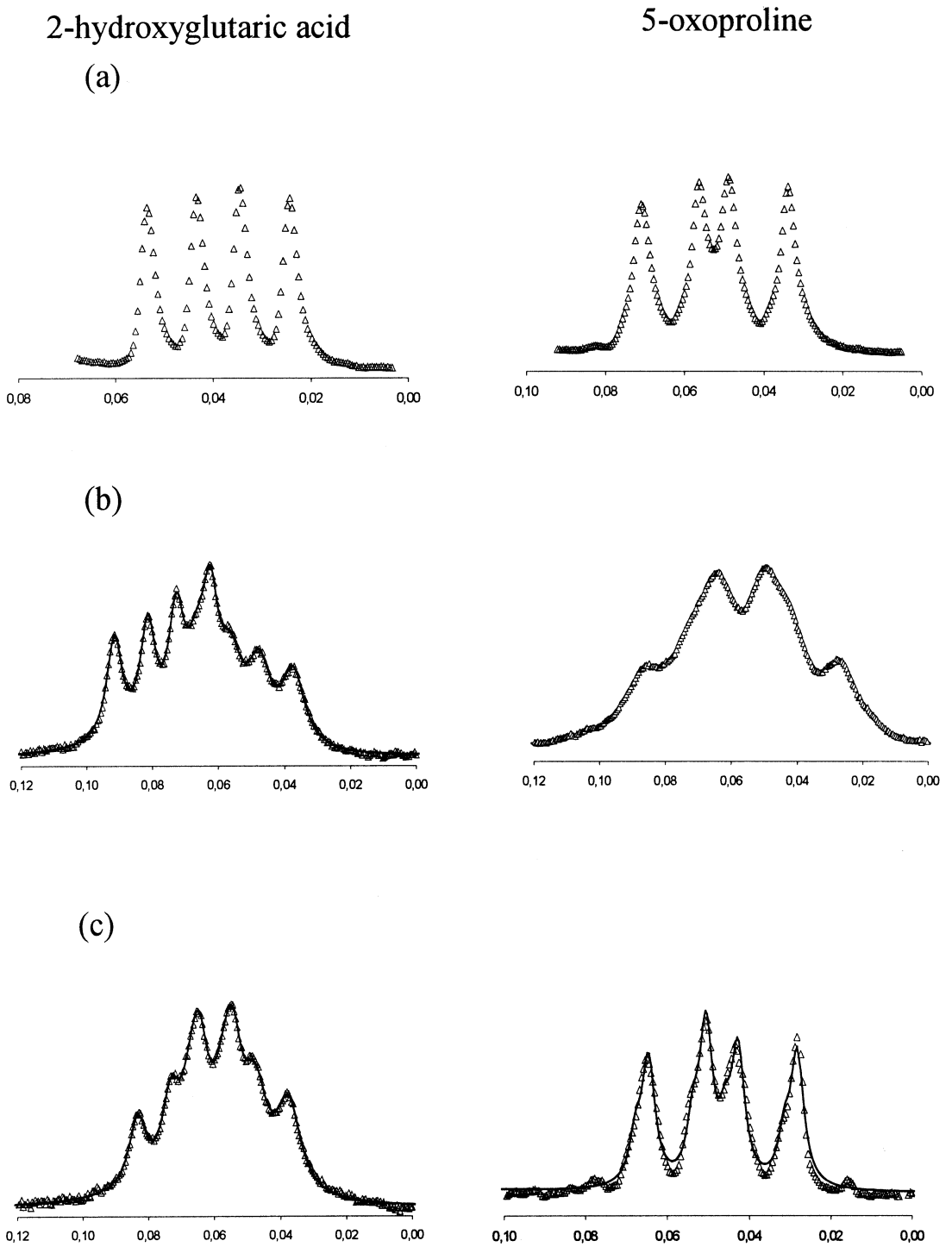


Fig. 3. The regions of the H-2 signals in the ^{1}H NMR spectra of 2-hydroxyglutaric acid (**3**, left) and 5-oxoproline (**4**, right); (a) racemic metabolite; (b) racemic metabolite in the presence of LSR (0.1 mol **1**/mol of **3** or 0.04 mol **2**/mol of **4**); (c) metabolite enriched in L enantiomer in the presence of LSR; (0.03 M, H_2O, pH 5.0 for **3** and 6.4 for **4**, temperature 30 °C, 400 MHz); △, experimental spectrum; —, theoretical spectrum (see text).

by complexes **1** and **2** [22,25]. The correctness of these assignments was evidenced by the lineshape analysis (see below; Fig. 3, continuous line).

3.2. Some practical recommendations

As a first step in any analysis, the recording of the standard urine spectrum is advisable. Examination of such a spectrum prior to the analysis enables checking whether the crucial metabolite signal is sufficiently separated from other signals. If this precondition is not fulfilled for a given urine sample, the analysis is severely complicated, though not unfeasible (see below).

It might be argued that the differences demonstrated above between the signals of the L and D enantiomers of **3** caused by LSR are too small for reliable distinction of the enantiomers in urine samples, taking into account the variability and complexity of their composition. This difficulty can be, however, overcome with a simple trick of internal referencing, well known in analytical and physical chemistry practice. Namely, beside the direct examination of a urine sample, it is recommended to perform measurements for the sample containing an addition of the racemic marker metabolite (solution of the 'c' type, Fig. 3). Such a procedure has at least two crucial advantages: (i) the presence of the signals of both enantiomers in the spectrum allows checking if the given sample composition really ensures the enantiomer distinction and finding out which enantiomer occurs in excess; (ii) the intensity enhancement of the marker signals compared with the signals of other urine components, practically precludes misassignments.

As outlined above, the enantiomeric excess is determined for samples of the analyzed urine enriched in the racemic marker metabolite. When selecting the proportion of the two components, one has to take into account that reliable determination of the integral intensity differences $< 10\%$ can be difficult. On the other hand, for the sake of the determination accuracy, concentrations of the solution components have to be selected so that the intensities of the signals to be compared would not be very different.

General information concerning other measurement conditions, such as relative concentration of LSR, solution pH, measurement temperature etc., being optimal from the point of view of the enantiomer spectra differentiation, can occasionally be found in the literature [28]. The conditions appropriate for a given analysis depend on the lanthanide complex as well as on the metabolite type. Actually, due to much lower solute concentrations in biological samples than in those used in typical physicochemical studies, it may be justifiable to look for conditions more appropriate for the problem in hand. Moreover, urine always contains various solutes, which compete with the metabolite of interest for LSR. Using elevated LSR concentration in analytical solutions is suggested, as it can partially compensate for this effect. The detailed conditions used in this study are given in the figure captions.

It is well known that the presence of LSR in the investigated solution besides producing the desired changes in the chemical shifts, also causes line broadening, which sometimes makes the interpretation of the spectral effects difficult. The reliability of the final result depends critically on the sample composition and the measurement conditions. Line broadening may have various origins [29]. After the addition of LSR, the precipitation of solids is frequently observed, which can usually be removed by centrifuging the sample. More troublesome are relaxation effects due to the paramagnetic additive and residual dynamic effects caused by incomplete averaging of the free and complexed metabolite spectra. The enantiomer signal separations on the frequency scale are proportional to the magnetic field used, whereas the dynamic broadenings are proportional to the square of this field. Thus, the case in hand is quite unusual, as use of a higher magnetic field may increase the signal overlap. Increasing the measurement temperature is expected to shift backward the complexation equilibrium but, on the other hand, diminish the dynamic broadenings. Even the most cautious selection of the measurement conditions, however, does not always ensure a fully satisfactory spectrum is obtained. A deeper physicochemical discussion of this complicated system exceeds the scope of this article.

When enantiomer signals strongly overlap each other or when both optical isomers are present in the analyzed sample, a numerical lineshape analysis of the spectra is recommended. The computational analysis, though more laborious, does deliver the unequivocal answers and enables the quantitative assessment of the enantiomeric composition of a given marker metabolite, including the error estimations. The method takes the advantage of the close proportionality of the integral intensity of ^{1}H NMR signals and solute concentrations obeyed when certain measurement conditions are fulfilled. It is important that the reliability of the result obtained in that way can be well verified and the determination error diminished. Namely, for a given sample being analyzed, a series of spectra can be obtained changing measurement temperature, solution composition or using spectrometers working at different magnetic fields. The lineshape analysis of such spectra actually provides independent estimates of the enantiomeric ratio, which can then be averaged. Finally, it is to be stressed that the results of the NMR/LSR method are free of artifacts, which can be introduced by sample preprocessing.

3.3. Analyses of urine samples

The applicability of the proposed NMR/LSR method to real problems was illustrated by the results of analyses of urine samples of two independently diagnosed patients, one suffering from 2-hydroxyglutaric aciduria and the second from 5-oxoprolinuria. Examination of the standard ^{1}H NMR spectra of these two urine samples (Fig. 4) showed that, from the analytical point of view, they represented quite different cases, demanding application of different techniques from the rich arsenal of modern NMR spectroscopy [30]. 2-Hydroxyglutaric acid occurred in the first of the investigated samples in relatively low amounts (0.8 mol/mol creatinine) and its H-2 signal was overlapped by signals of other metabolites. In the second case, 5-oxoproline was the metabolite dominating the NMR spectrum (0.09 mol/dm^3, $\approx$40 mol/mol creatinine) and no interfering signals were observed.

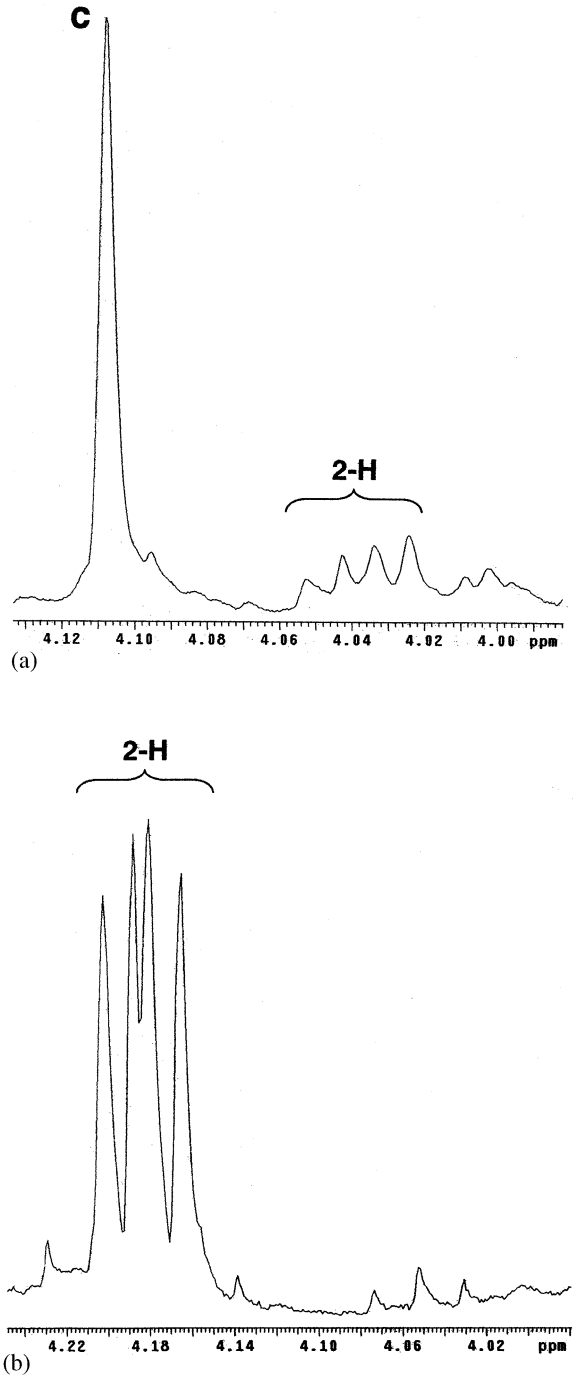
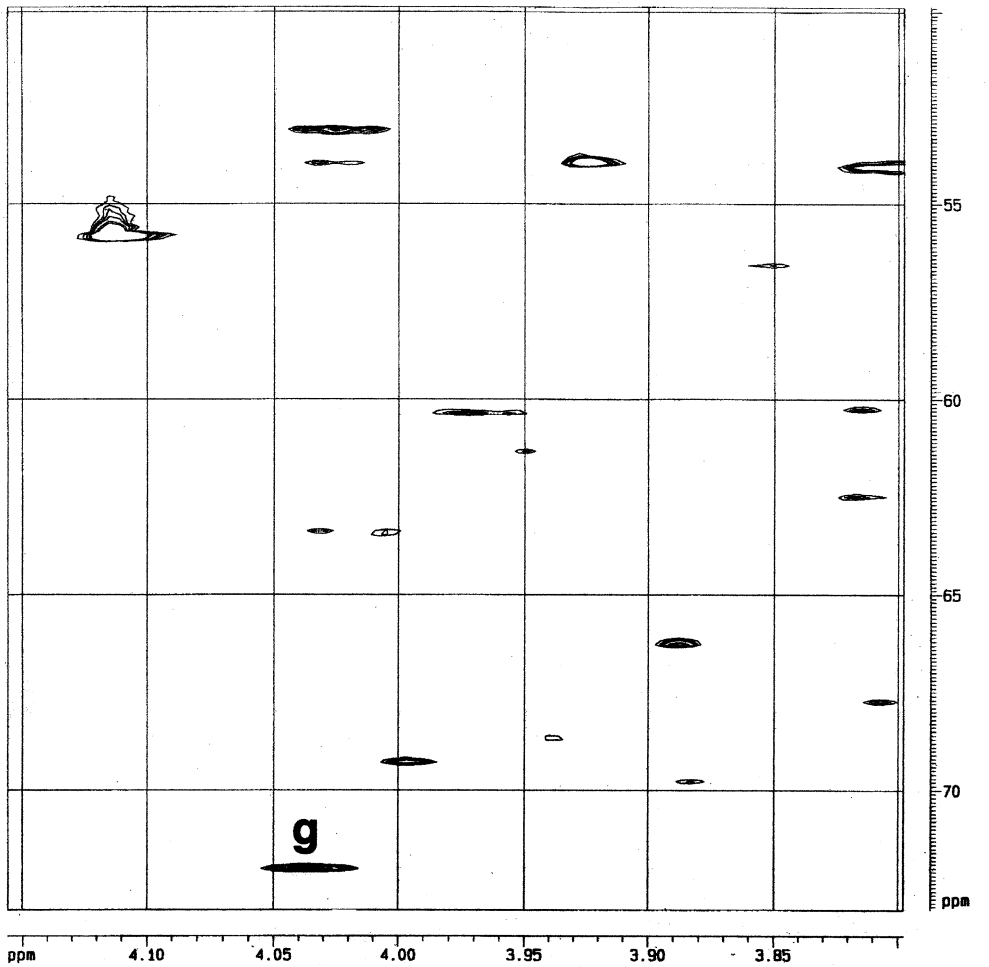


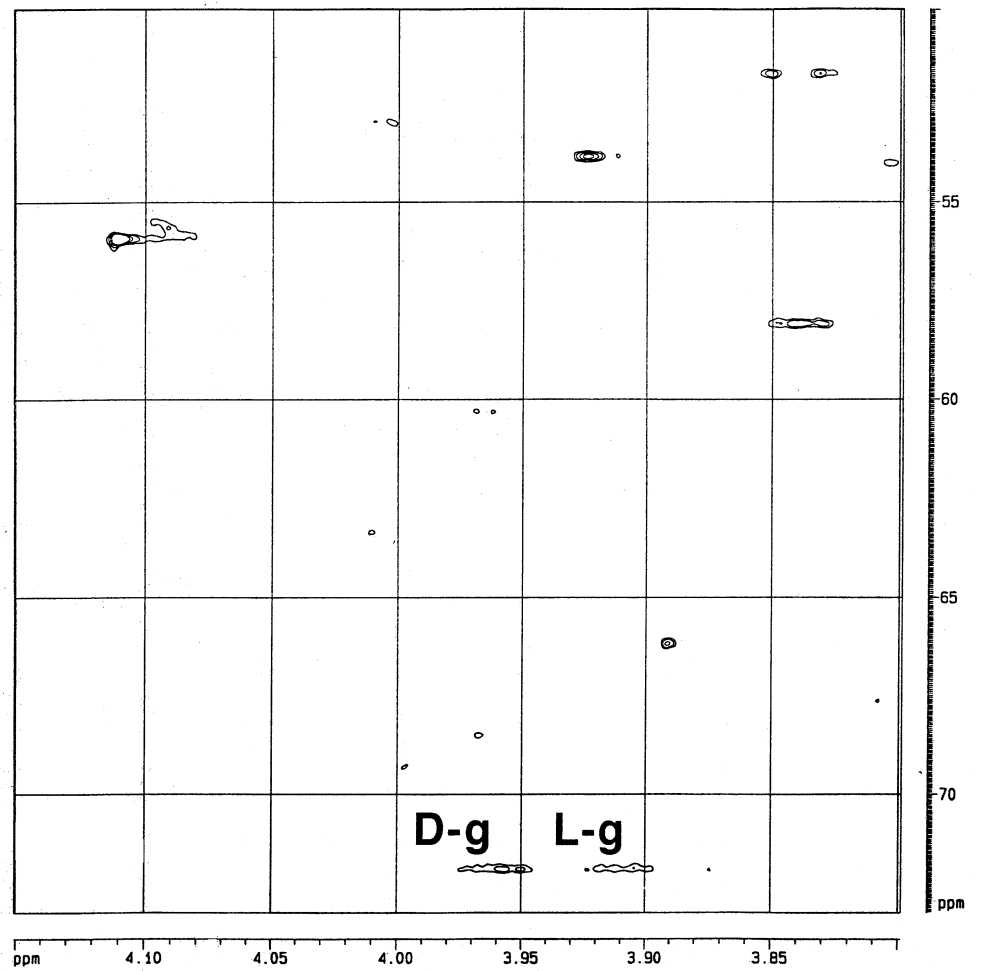
Fig. 4. Partial ^{1}H NMR spectra of urine samples covering regions of H-2 signals of 2-hydroxyglutaric acid (a) and 5-oxoproline (b), respectively. (Room temperature, 400 MHz; **c**-CH_2 signal of creatinine.)



(a)

Fig. 5. Partial 2D HSQC spectra showing ^{1}H-^{13}C correlation for the H-2 proton signal of 2-hydroxyglutaric acid **g** and for other interfering proton signals; (a) urine; (b) urine enriched in racemic **3**, in the presence of LSR.

In the standard ^{1}H NMR spectra of the 2-hydroxyglutaric aciduria samples (Fig. 4a), the form of the H-2 proton signal of **3** was complicated by overlap with signals from other metabolites. Actually, in the two-dimensional HSQC spectrum [30], this proton signal gave four correlation peaks to directly bonded carbons (Fig. 5a). The ^{13}C cross-peak at 71.9 ppm originated from C-2 of **3**, whereas the remaining ones are from other, at the moment unknown, metabolites. After the addition of LSR and some amounts of the racemic **3**, the 71.9 ppm peak was shifted up-field in the ^{1}H dimension and split into two peaks (Fig. 5b), the up-field one being of higher intensity. This meant that D enantiomer of **3** predominated in the investigated urine sample. Thus, even this rather difficult case could be analyzed owing to the robustness of the two-dimensional, proton detected ^{1}H-^{13}C correlation spectroscopy (HSQC), the technique being commonly accessible by means of modern NMR spectrometers. In order to reduce spectrometer time to 1 h per spectrum, however, we found it necessary in the above analysis to concentrate five times the investigated urine.



(b)

Fig. 5. (*Continued*)

As mentioned above, in the case of 5-oxoprolinuria samples the relative concentration of **4** was high and its H-2 signal was not disturbed by any other signal of significant intensity. In such a situation, pointing out the dominating enantiomer can be occasionally possible by the visual examination of standard one-dimensional spectra. One has to be cautious, however, as the incomplete separation of the enantiomer signals and their linewidth difference can be misleading, as it was in the case of our 5-oxoprolinuria sample. Nevertheless, finding the absolute configuration of the predominating enantiomer, as well as quantitation of the enantiomeric excess, could be carried out by performing the numerical lineshape analysis of the spectra. It was found that in the analyzed sample only the L-isomer of **4** was present. Results of the lineshape analyses are illustrated in Figs. 3 and 6. Overall agreement between experimental and theoretical spectra was in each case satisfying. The multiplet patterns found were always close to those obtained for the spectrum of metabolite in the absence of LSR. Moreover, the test lineshape analyses of the spectra of the solution containing racemic **4** and LSR yielded equal (within the estimation error) molar fractions of

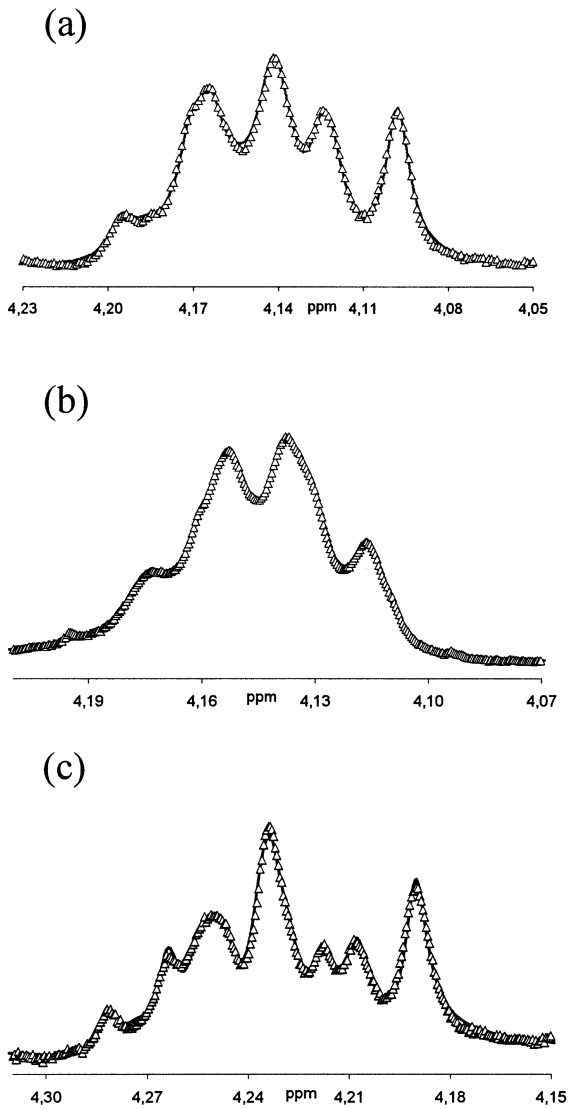


Fig. 6. The lineshape analysis of the region of H-2 proton signal of 5-oxoproline **4** in 1D ^{1}H NMR spectra of the urine sample enriched in the racemic marker metabolite, in the presence of LSR; (a) temperature 30 °C, spectrometer frequency 200 MHz, molar ratio L-**4**/D-**4** 0.62; (b) 30 °C, 400 MHz, 0.66; (c) 40 °C, 200 MHz, 0.63; △, experimental spectrum; —, theoretical spectrum (see text).

the L and D enantiomers. Finally, the absolute stereochemistries of both the marker metabolites determined during the urine analysis were as expected according to the known clinical and GCMS diagnoses of patients' diseases and biochemical pathways in which they are involved. Thus, all these results proved the practicability and reliability of the adopted approach.

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