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Journal of Chromatography A, 778 (1997) 389–396

JOURNAL OF CHROMATOGRAPHY A

# Separation and quantitation of debrisoquine and 4-hydroxydebrisoquine in human urine by capillary electrophoresis and high-performance liquid chromatography

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**Abstract**

A comparative study on the use of reversed-phase high-performance liquid chromatography (RP-HPLC) and capillary electrophoresis (CE) for the determination of debrisoquine (D) and its metabolite, 4-hydroxydebrisoquine (4-HD), in human urine is presented. Four different urine pre-treatments are compared for purification of samples prior to their injection in HPLC and CE. The use of a solid-phase extraction with a $C_{18}$ cartridge provides the best results for the urine sample treatment, with good recoveries, i.e., 94.5% for D and 93.4% for 4-HD, and high reproducibility, i.e., R.S.D.$_{n=10}$ values of 1.7% and 1.2%, respectively. Under our separation conditions it is shown that CE is twice as fast and provides slightly better analysis time reproducibility than HPLC for this type of sample. Both the sensitivity and peak area reproducibility are better when HPLC is used. The two techniques show good agreement when employed for determination of phenotypes for hydroxylation, which seems to corroborate the usefulness of CE for this type of study. © 1997 Elsevier Science B.V.

*Keywords:* Sample handling; Drugs; Phenotype; Debrisoquine; Hydroxydebrisoquine

## 1. Introduction

Debrisoquine (D) [3,4-dihydro-2-(1H)-isoquinoline-carboxamidine] is an antihypertensive drug which acts as an adrenergic antagonist. This drug is mainly metabolized to 4-hydroxydebrisoquine (4-HD) in man [1–3]. This extensive hydroxylation has been shown to be genetically controlled with two phenotypes present in the population, i.e., extensive metabolizers and poor metabolizers [4]. The latter group, which represents a small percentage, shows a genetically transmitted enzymatic defect which leads to reduced hydroxylation of D to 4-HD [5]. It has been shown that several other drugs, which undergo oxidation, exhibit similar genetic polymorphism which can be diagnosed by using debrisoquine as a test or marker [5,6]. The oxidation status of a subject is determined by measuring the concentrations of D and its main metabolite 4-HD in urine, usually over an 8 h period, after a single oral dose of D.

Determination of this metabolic ratio is also a useful method to identify persons who could be subject to an increased incidence of drug side-effects as well as the distribution of this characteristic in the population [4], e.g., population studies have revealed large inter-ethnic differences in the occurrence of the different phenotypes. Moreover, it seems that extensive metabolizers of D could be subject to increased risks of developing some types of cancer [7].

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*PII* S0021-9673(97)00285-9

Several methods have been developed to determine D and 4-HD in urine. Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) have been used after derivatization of compounds with acetylacetone [8–10]. These methods involve lengthy sample preparation and time-consuming derivatization procedures. High-performance liquid chromatography (HPLC) has been also applied in the analysis of D and 4-HD [3,11–20], providing better reproducibility than that obtained with GC. Similar to GC, these methods involve in general extensive sample preparation and derivatization. A procedure which simplifies the sample preparation step in HPLC has been reported [14], i.e., direct injection of diluted urine was used, but it required fluorescence detection. In general, however, although resolution is normally adequate, HPLC run times are relatively long, and interfering peaks can appear [19].

Capillary electrophoresis (CE) has been shown to be a fast, powerful and efficient separation technique for a variety of compounds. These characteristics are related to the use of high separation voltages and efficient dissipation of Joule heat in a narrow capillary (typically 25–100 μm I.D.). Clinical applications of CE have grown tremendously during the last 2 years [21–29], showing the potential of CE to monitor drugs and metabolites in clinical samples, e.g., plasma and urine.

Recently, CE has been shown to be well suited for metabolic phenotyping in man via analysis of urine. Thus, compounds such as dextromethorphan and dextrorphan [29,30], 5-acetylamino-6-formylamino-3-methyluracil, 1-methylxanthine, 1-methyl uric acid and 5-acetylamino-6-amino-3-methyluracil [25,31] have been analyzed in urine to determine the oxidative or acetylator status in man.

The goal of this work is to provide a comparative evaluation on the use of reversed-phase HPLC and CE for the determination of D and its metabolite 4-HD in human urine. The comparison was undertaken to determine the advantages and drawbacks of each technique when used for routine analysis with emphasis on reproducibility, selectivity and sensitivity. Also, a comparative study on different sample preparation procedures, i.e., solid-phase extraction with different sorbents and liquid–liquid extraction is presented, leading to a single, rapid and specific solid-phase clean-up procedure for the analysis of these substances.

## 2. Experimental

### 2.1. Chemical and reagents

All chemicals were of analytical grade, pharmaceutical quality or better and were used as purchased without further purification. Declinax tablets, debrisoquine sulfate and 4-hydroxydebrisoquine sulfate were gifts from Hoffmann-La Roche (Basel, Switzerland). Acetonitrile and methanol were of HPLC grade (Montplet and Esteban, Barcelona, Spain). 2-[N-Morpholino]ethanesulfonic acid (MES) was obtained from Aldrich (Milwaukee, WI, USA). All other reagents: potassium dihydrogenphosphate ($KH_2PO_4$), potassium hydroxide and hydrochloric acid were from Merck (Darmstadt, Germany). Water was purified by using a Milli-Q system (Millipore, Bedford, MA, USA).

Stock solutions containing 100 mg/l of D and 4-HD were prepared in water and stored at 4°C. By conveniently mixing these solutions with water and by injecting them into the HPLC system, a calibration plot of peak area vs. concentration was obtained for the two substances. Urine standard solutions containing known concentrations of D and 4-HD were prepared by appropriately diluting the stock solutions with drug-free urine. The standards in urine were employed for studying the different sample preparation procedures, i.e., recovery calculation and for calibration plots. Recoveries were measured by HPLC, using the calibration plot obtained with aqueous solutions of D and 4-HD.

D and 4-HD concentrations were determined in urine collected 8 h following a 10 mg oral dose of Declinax (debrisoquine sulfate) in nine normal volunteers. Real urine samples were extracted in the same way as the urine standards. Concentration of D and 4-HD in samples were calculated from calibration plots obtained with the urine standard solutions.

The mobile phase in the RP-HPLC method was prepared daily by mixing acetonitrile with a solution of 8 m*M* $KH_2PO_4$ adjusted to pH 5 with 2 *M* KOH

(70:30, v/v). This mobile phase was filtered and degassed through a 0.22 μm polyamide filter.

CE separation buffer was prepared by dissolving the appropriate amount of MES in Milli-Q water to obtain a solution 50 m*M*, which was adjusted to pH 7 with 0.1 *M* NaOH.

### 2.2. HPLC procedure

The analysis were carried out on a Model SCL liquid chromatograph (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan) equipped with an autoinjector and a SPD-6AV variable-wavelength spectrophotometric detector operating at 208 nm. Data were collected and analyzed using a PICOS software version 1.0 (Department of Pharmacology, University of La Laguna, Spain) running on a 80386 personal computer. The analytical column was a Spherisorb $C_8$ (Phase Separations, Norwalk, CT, USA) with dimensions of 250×4.6 mm I.D. and a particle diameter of 5 μm. A Spherisorb $C_8$ precolumn of 20×4.6 mm I.D. was employed to protect the analytical column. Separations were done at room temperature at a flow-rate of 1.5 ml/min.

### 2.3. CE procedure

The analyses were carried out in a P/ACE 5510 (Beckman Instruments, Fullerton, CA, USA) CE apparatus, equipped with a diode array detector. The fused-silica capillary used was 57 cm total length(50 cm effective length)×75 μm I.D. (Beckman Instruments). Injections were made using $N_2$ pressure of 0.5 p.s.i. for 2 s (1 p.s.i.=6894.76 Pa). The capillary was thermostatted at 30°C. Detection took place at 208 nm. Data were collected and analyzed using a System Gold software from Beckman running on a 486DX2-66 Mhz computer. The running buffer of separation was 50 m*M* MES pH 7. For separation, a voltage of 16 kV was used. In order to increase migration time reproducibility, the capillary was rinsed between injections with 0.1 *M* NaOH and buffer for 2 min with each solution.

### 2.4. Sample preparation

All samples were filtered through a Swinny Stainless, 13 mm (Millipore) equipped with 0.45 μm filter of cellulose acetate (Millipore). D and 4-HD were extracted from urine by solid-phase extraction using Accell Plus CM cartridges (1.1 ml), or $C_{18}$ cartridges (0.8 ml) (both from Millipore), or by liquid–liquid extraction. Solid-phase extractions were performed using a Millipore vacuum manifold. The following procedures were used:

#### 2.4.1. Solid-phase extraction using Accell Plus CM cartridges

The sorbent was first conditioned with 1 ml of a mixture acetonitrile–0.1 *M* hydrochloric acid (40:60, v/v) followed by 1 ml of water. 1 ml of urine was aspirated through the solid-phase, then the cartridge was washed with 1 ml of water, 1 ml of acetonitrile–water (50:50, v/v), 1 ml of water and 0.5 ml of 0.1 *M* hydrochloric acid. The compounds adsorbed on the cation exchange phase were eluted with 1 ml of acetonitrile–0.1 *M* hydrochloric acid (40:60, v/v). An aliquot of this extract was then injected into the HPLC or CE system.

#### 2.4.2. Solid-phase extraction using $C_{18}$ cartridges

The sorbent was first conditioned with 4 ml of methanol followed by 4 ml of water. 1 ml of urine, previously adjusted to pH 5 with 0.1 *M* hydrochloric acid, was aspirated through the solid-phase, then the cartridge was washed with 3 ml of water, 1 ml of methanol–water (10:90, v/v). The compounds adsorbed on the $C_{18}$ phase were eluted with 1 ml of methanol–water (90:10, v/v). An aliquot of this extract was then injected into the HPLC or CE system.

#### 2.4.3. Liquid–liquid extraction

To 1 ml of urine 1 ml of 1 *M* sodium hydroxide and 2 ml of 1-butanol or 1-pentanol were added and the tubes shaken for 15 min. After centrifugation at 4000 rpm. for 5 min, 1 ml of the upper organic layer was taken and evaporated to dryness under a $N_2$ stream. The dry residue was solved in 1 ml of acetonitrile–water (90:10, v/v) and an aliquot of this extract was then injected into the HPLC or CE system.

## 3. Results and discussion

### 3.1. Optimization of sample pre-treatment

As a preliminary step, we first carried out an optimization of the separation conditions for D and 4-HD employing aqueous solutions of the analytes. The final conditions for HPLC, as indicated in Section 2.2, were slightly different from those obtained by other authors using similar type of column [3,15]. That is, the mobile phase and flow-rate employed in the present work allowed us to obtain good separations between the system peak (or front), D and 4-HD in a relatively short time. For the optimization of the CE conditions no previous works dealing with these analytes were found in the literature, therefore we tested different buffers and conditions. The best results in terms of resolution of the separation of D and 4-HD were obtained with a 50 m*M* MES buffer at pH 7.

Under the above conditions we tested the determination of the analytes by simply injecting filtered urine without further treatment, as done by other authors [14]. However, we had to abandon this attractive procedure since no peaks were detected by any of these techniques. This was probably due to the high background originated by interfering urine components. Therefore, more sophisticated urine treatments were tested.

Four different sample pre-treatment procedures were studied, i.e., solid-phase extraction with reversed-phase ($C_{18}$) or ion-exchange (Accell Plus CM) cartridges and liquid–liquid extraction using 1-pentanol or 1-butanol. The recoveries for the different treatments were calculated using drug free urine spiked with known quantities of D and 4-HD as explained in Section 2.

When the liquid–liquid extraction was employed using either 1-pentanol or 1-butanol recoveries lower than 11% were obtained for both analytes. Besides, it was observed that the reproducibility of this treatment was very poor, with R.S.D.$_{n=10}$ values up to 25%. Examples of these low recoveries can be seen in Fig. 1A and B, and clearly indicate that under these conditions it was not possible to carry out the analysis of D and 4-HD.

The average recovery values obtained using the solid-phase extraction employing the Accell Plus CM cartridges were 76.5% for D and 72.3% for 4-HD with R.S.D.$_{n=10}$ values of 1.9% and 2.1%, respectively. An example of the electropherograms obtained using the Accell Plus CM cartridges is shown in Fig. 1C. In spite of the great improvement in the clearness of the electropherogram observed by using this method, the still low recovery pushed us to test another sample preparation procedure.

The use of reversed-phase extraction with a $C_{18}$ cartridge provided, indeed, the best results, with average recoveries of 94.5% for D and 93.4% for 4-HD with R.S.D.$_{n=10}$ values of 1.7% and 1.2%, respectively. Moreover, it was observed that the $C_{18}$ cartridges, after cleaning, could be reused without noticeable carry over nor recovery loss. As can be seen in Fig. 1D, the use of this cartridge for the urine treatment, allowed us to obtain a good separation of both analytes in less than 4 min. The peak labelled with an asterisk is an unidentified compound. It can also be deduced from Fig. 1D that the used CE conditions gave efficiencies higher than 500 000 plates per meter for these compounds.

A comparison of the separations obtained by HPLC for a sample of urine before and after the solid-phase extraction with a $C_{18}$ cartridge is shown in Fig. 2. As can be seen, when no treatment was applied (Fig. 2A) it was not possible to perform the separation of D and 4-HD, since the large front of urine compounds interfered with the peaks of interest. In Fig. 2B, it can be observed that the treatment allows the analysis of D and 4-HD reducing dramatically the interferences.

By comparing Figs. 1D and 2B the differences in selectivity between the two technique are evident. In HPLC the first peak, 4-HD, is the most polar and consequently is less retained onto the $C_8$ column, while in CE the first peak is D, with higher electrophoretic mobility, because of a smaller molecular mass with a charge identical to 4-HD. Besides, it can be observed that CE (Fig. 1D) allows the separation of both compounds in a shorter time, ca. 4 min, than HPLC, ca. 8 min (Fig. 2B).

In Fig. 3 the electropherograms obtained from a drug free urine sample and a urine containing D (4 mg/l) and 4-HD (7 mg/l) are shown. Under these conditions no endogenous components interfere at the detection wavelength chosen.

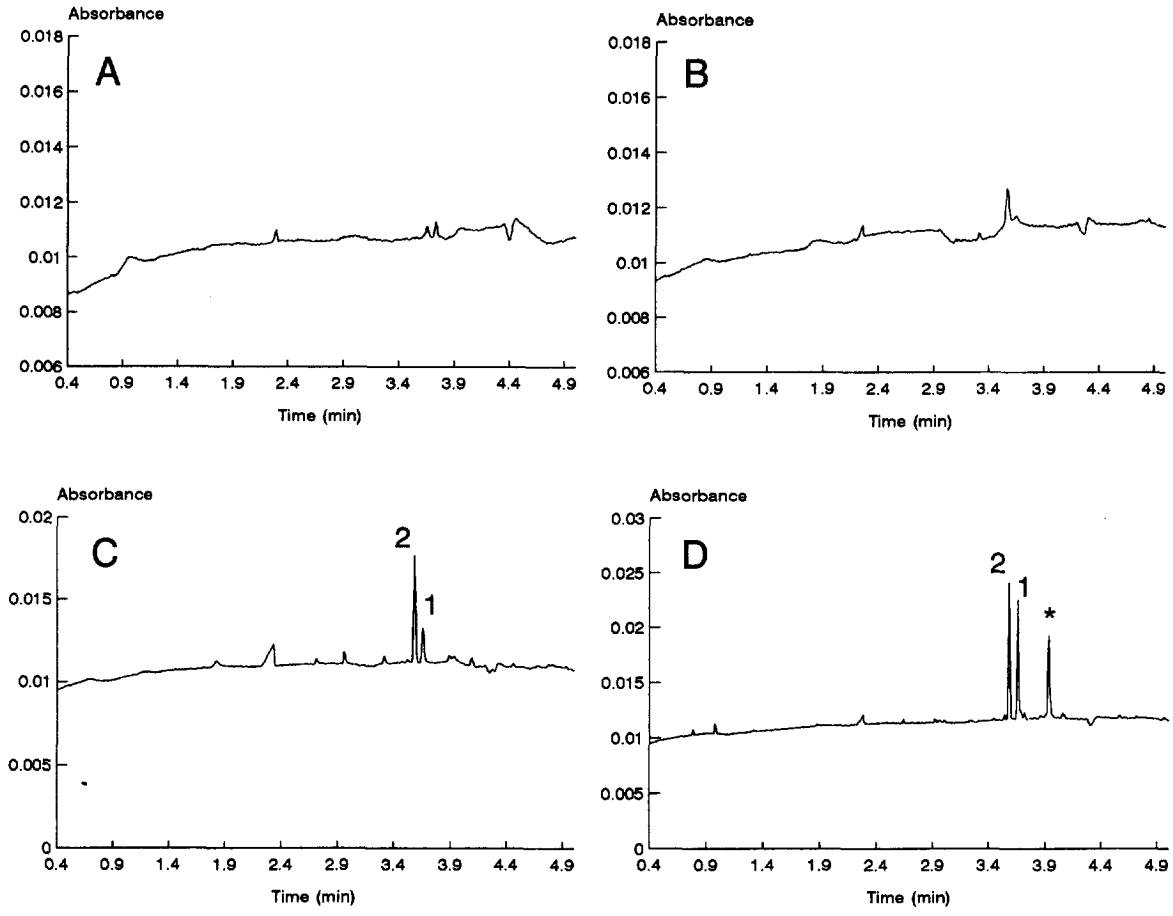


Fig. 1. Effect of the urine treatment on the electropherograms of D and 4-HD. Urine containing 18 mg/l of D (peak 2) and 13 mg/l of 4-HD (peak 1) subjected to: (A) liquid–liquid extraction using 1-butanol, (B) liquid–liquid extraction using 1-pentanol, (C) solid-phase extraction using an Accell Plus CM cartridge and (D) solid-phase extraction using a $C_{18}$ cartridge. Separation conditions: fused-silica capillary 57 cm total length (50 cm effective length)×75 μm I.D.. Injections using $N_2$ pressure (0.5 p.s.i.) for 2 s. Detection at 208 nm. Separation buffer: 50 m*M* MES pH 7. Run voltage: 16 kV. Peak labelled with an asterisk is an unidentified impurity.

### *3.2. Validation*

In Table 1, intra-day and inter-day reproducibility values for analysis time and peak area obtained with both techniques are shown. The precision of the method was determined from replicate assays of drug-free urine spiked with known concentrations of D and 4-HD. As can be seen, slightly better time reproducibility was obtained by using CE compared with that obtained from HPLC in both inter- and intra-day analyses. These better reproducibility values can be related to the harsh washing conditions used in CE which could bring about a regeneration of the capillary wall between injections, cleaning it of any urine compound adsorbed during the separation. In HPLC, on the other hand, this undesired adsorption is probably behind the relatively high %R.S.D. values obtained in our experimental results.

Also, in Table 1, when area reproducibilities are compared it is observed that HPLC provides better results than CE, mainly for inter-day assays in which the R.S.D. values obtained with HPLC were 0.75%, while those obtained with CE were 3.5% for D and 6.6% for 4-HD. This lack of reproducibility in terms of injected quantity observed for CE has been already mentioned by several authors [32,33], con-

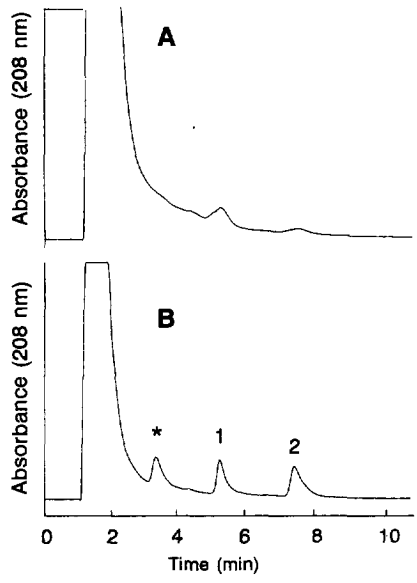


Fig. 2. Effect of the sample treatment on the HPLC separations. Urine containing 14 mg/l of D (peak 2) and 12 mg/l of 4-HD (peak 1) subjected to: (A) no treatment, (B) solid-phase extraction using a $C_{18}$ cartridge. HPLC separation conditions are indicated in Section 2.2. Peak labelled with an asterisk is an unidentified impurity.

cluding that this technique, to approach the level of reproducibility normally obtained for HPLC, seems to require the use of an internal standard [34,35]. The results shown in this work seem to support that theory.

By comparing the sensitivity of both techniques, it was observed that HPLC is more sensitive than CE. The detection limit (signal-to-noise ratio=2) using HPLC was 0.2 and 0.15 mg/l for D and 4-HD, respectively. By using CE these values were 0.85 and 1 mg/l, respectively. The lower sensitivity observed when CE is used can be mainly due to the small optical path length used in this technique. Besides, another probable reason for the difference in sensitivity is related to the different detection schemes employed, i.e., traditional spectrophotometry in HPLC and diode-array in CE, which is inherently less sensitive.

The assay was linear over the concentration range 0.3–20 mg/l for 4-HD [$y=1.127(\pm0.037)x-0.090(\pm0.003)$, $r^2=0.996$] and 0.4–20 mg/l for D [$y=1.172(\pm0.036)x-0.127(\pm0.039)$, $r^2=0.998$] in urine when HPLC was employed. By using CE the assay was linear over the range 2–40 mg/l for both compounds [$y=103.96(\pm0.39)x-1.42(\pm7.61)$, $r^2=0.999$ for D and $y=113.75(\pm2.93)x+89.06(\pm57.91)$, $r^2=0.994$ for 4-HD, number of points = 6]. These concentration ranges seem to be suitable for carrying out most of the analyses of D and 4-HD in human urine, according to the values reported in the literature [3,18].

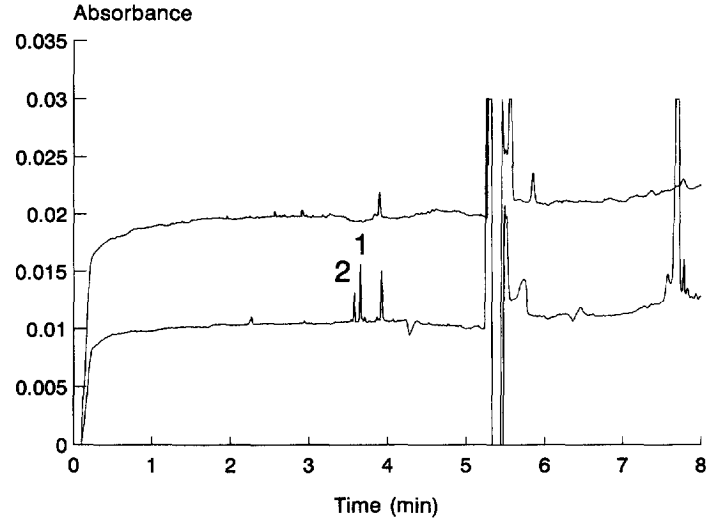


Fig. 3. Comparison of the electropherograms obtained from a urine blank (top) and urine spiked with 4 mg/l of D and 7 mg/l of 4-HD (bottom) after solid-phase extraction using a $C_{18}$ cartridge. Analytical conditions as in Fig. 1.

Table 1
Analysis time and peak area reproducibility for intra- and inter-day analysis of D and 4-HD in urine samples using HPLC and CE

| | Intra-day ($n$=5) | | | | Inter-day ($n$=3 days) | | | |
|---|---|---|---|---|---|---|---|---|
| | D | | 4-HD | | D | | 4-HD | |
| | $t^a$ | $A^b$ | $t$ | $A$ | $t$ | $A$ | $t$ | $A$ |
| CE | 3.63 | 260 | 3.71 | 284 | 3.64 | 264 | 3.72 | 293 |
| | (0.61%)[c] | (1.21%) | (0.63%) | (1.38%) | (1.14%) | (3.52%) | (1.09%) | (6.66%) |
| HPLC | 7.56 | 778 | 5.30 | 576 | 7.67 | 780 | 5.33 | 574 |
| | (1.22%) | (0.50%) | (1.10%) | (0.73%) | (2.06%) | (0.75%) | (1.31%) | (0.75%) |

[a] Analysis time values given in minutes.
[b] Peak area values given in arbitrary units.
[c] R.S.D. values in parentheses.

### 3.3. Application to hydroxylation-phenotype determination

For calculation of the hydroxylation index (HI), the ratio between the concentrations of D/4-HD measured in an 8 h collection of urine following a 10 mg oral dose of Declinax is employed. Fig. 4 shows electropherograms obtained after injection of a $C_{18}$ cleaned urine from three subjects. As can be seen, Fig. 4A–C show the profiles for three "extensive" metabolizers (i.e., HI<12.1). This technique could be useful to distinguish the different groups among the extensive metabolizers. Individual 3 (Fig. 4C) could be a heterozygote for the CITP450 IID6 enzyme, whereas individual 1 (Fig. 4A) is possibly a homozygote for this enzyme [36]. The only way of confirming this, is by using genotyping techniques, which is not in the scope of this paper.

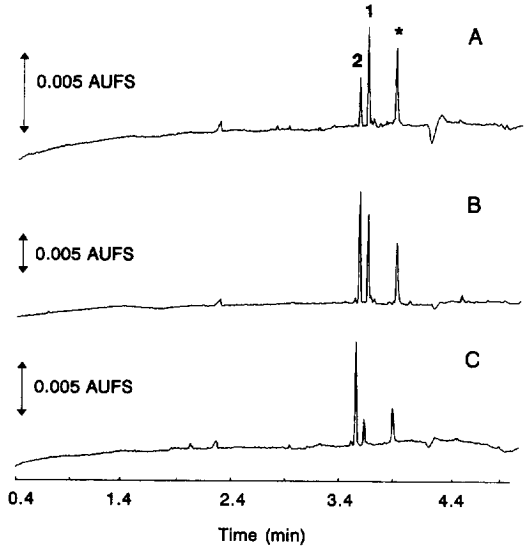


Fig. 4. Electropherograms of treated urine from three different type of metabolizers. (A) test person 1, (B) test person 2 and (C) test person 3. Analytical conditions as in Fig. 3. For more details see Section 2.3.

A comparison between HPLC and CE, regarding the concentrations of D and 4-HD determined in the three urine samples, gave the results presented in Table 2. In general, a good agreement was obtained between the two techniques, except for the values of D and 4-HD calculated for test person 2 which resulted in higher CE values. However, ratios were very similar, as can be seen comparing the hydroxylation index obtained in all the cases studied.

## 4. Conclusions

From the present work it is deduced that the use of a solid-phase extraction with $C_{18}$ cartridges provides

Table 2
Comparison of the quantitative analysis of D and 4-HD and their ratio indicated as hydroxylation index (HI)

| | HPLC | | | CE | | |
|---|---|---|---|---|---|---|
| | D[a] | 4-HD[a] | HI[b] | D | 4-HD | HI |
| Person 1 | 3.09 | 7.36 | 0.42 | 3.83 | 6.96 | 0.55 |
| Person 2 | 14.1 | 11.97 | 1.18 | 20.29 | 15.0 | 1.35 |
| Person 3 | 12.4 | 3.06 | 4.05 | 11.63 | 3.12 | 3.73 |

The measurements were carried out by CE and HPLC in urine from the same test persons indicated in Fig. 4.
[a] Concentrations in mg/l.
[b] Hydroxylation index (HI).

urine purification suitable for HPLC and CE analysis. This pre-treatment allows the determination of D and 4-HD found in human urine. The comparison between the two separation techniques shows that CE is twice as fast as HPLC for this type of analysis, while HPLC provides better sensitivity and peak area reproducibility. The good agreement obtained between CE and HPLC results and the fact that the latter technique has been extensively employed for determining phenotypes for hydroxilation, corroborate the utility of CE for this type of study.

## Acknowledgments

Authors thank Dr. J.L. Gonzalez Mora for the development of the PICOS software. Authors acknowledge financial support of this work to the Local Government of the Canary Islands.

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