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Characterization of the genetic polymorphism of dihydrocodeine O-demethylation in man via analysis of urinary dihydrocodeine and dihydromorphine by micellar electrokinetic capillary chromatography

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Abstract

The genetic polymorphism of dihydrocodeine O-demethylation in man via analysis of urinary dihydrocodeine (DHC) and dihydromorphine (DHM) by micellar electrokinetic capillary chromatography is described. Ten healthy subjects which are known to be extensive metabolizers for debrisoquine ingested 60 mg of DHC and collected their 0–12 h urines. In these samples, about 1% of the administered DHC equivalents are shown to be excreted as DHM. Premedication of 50 mg quinidine sulfate to the same subjects is demonstrated to significantly reduce (3–4 fold) the amount of O-demethylation of DHC, a metabolic step which is thereby demonstrated to co-segregate with the hydroxylation of debrisoquine. Thus, in analogy to codeine and other substrates, extensive and poor metabolizer phenotypes for DHC can be distinguished. Using the urinary DHC/DHM metabolic ratio to characterize the extent of O-demethylation, the metabolic ratio ranges of extensive and poor metabolizers in a frequency histogram are shown to partially overlap. Thus, classification of borderline values is not unequivocal and DHC should therefore not be employed for routine pharmacogenetic screening purposes. Nevertheless, the method is valuable for metabolic research and preliminary data demonstrate that the same assay could also be used to explore the metabolism of codeine.

Keywords: Dihydrocodeine; Dihydromorphine; Debrisoquine

1. Introduction

For certain hydroxylation and acetylation reactions, inherited variations of human drug metabolism are known. The occurrence of such polymorphisms has been shown for many classes of drugs and has been linked to the activity of specific enzymes. Enzymatic deficiencies can lead to unusually high concentrations of drugs in blood, or in the case of a prodrug, to insufficient blood levels of the pharmacologic active substance. Phenotyping of individuals can be carried out using a suitable probe drug followed by chemical analysis of its excretion and/or its

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urinary metabolites. Typical probe drugs for the determination of the hydroxylation phenotype status associated with the enzyme CYP2D6 are debrisoquine, sparteine and dextromethorphan. Mephenytoin is typically employed for hydroxylation phenotyping in regard to CYP2C19 and caffeine, isoniazid or dapsone are used as substrates to investigate the actylator polymorphism related to CYP1A2 [1–5]. Alternatively, phenotypes can also be predicted by genotyping methods [6].

For phenotyping with drug substrates, mostly chromatographic assays have been employed. Recently, capillary zone electrophoresis and micellar electrokinetic capillary chromatography (MECC) have been shown to be rapid, inexpensive and highly efficient analytical methods for phenotyping in man via analysis of urinary dextromethorphan and dextrorphan [7], metabolites of caffeine [8,9] and mephenytoin and 4-hydroxymephenytoin [10,11]. Screening for hydroxylation and acetylation polymorphisms via simultaneous analysis of urinary metabolites of mephenytoin, dextromethorphan and caffeine has also been discussed [10]. Furthermore, the stereoselectivity of the 4-hydroxylation of mephenytoin has been investigated [11].

The O-demethylation of codeine (COD) to morphine (MOR) (Fig. 1A) is known to cosegregate with that of dextromethorphan to dextrorphan and thereby with the hydroxylation of debrisoquine [12–14]. The therapeutic impact of this polymorphism, however, remains contradictive [15–19]. Furthermore, dihydrocodeine (DHC) exhibits a similar metabolic pathway as COD (Fig. 1B; Refs. [20–23]), which is caused by the same genetic polymorphism. For such oxidation polymorphisms, humans can be classified either as poor metabolizer phenotypes (PM, exhibiting the genetic drug metabolizing deficiency) or as extensive metabolizer phenotypes (EM). It is further known, that the oxidation of debrisoquine [24] and the O-demethylation of COD [17,19] can be inhibited by quinidine (QUI). Preliminary data from our laboratory suggest that for DHC a PM status can also be induced using QUI [22]. The aims of this work were (i) to investigate the use of MECC for phenotyping with DHC, (ii) to confirm the possibility of inducing a PM status for DHC with QUI, and (iii) to examine the employment of the same MECC assay for the exploration of the COD metabolism.

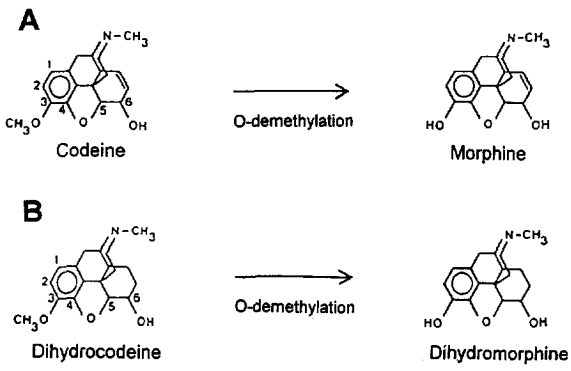

Fig. 1. Chemical structures for O-demethylation of (A) COD and (B) DHC.

2. Experimental

2.1. Drugs, chemicals and standard solutions

All chemicals were of analytical or research grade. COD, codeine-6-glucuronide (CODG) and MOR were a kind gift of the Institute of Pharmacy (University of Berne, Berne, Switzerland). DHC and its synthesized metabolites dihydrocodeine-6-glucuronide (DHCG), nordihydrocodeine (NDHC), dihydromorphine (DHM) and nordihydromorphine (NDHM) were received from Mundipharma (Basle, Switzerland). Sodium dodecyl sulfate (SDS) was from Sigma (St. Louis, MO, USA) and methanol (p.a.), ammonia (p.a., 25%), acetic acid, KH_2PO_4, Na_2HPO_4, $Na_2B_4O_7$, NaOH and KOH were from Merck (Darmstadt, Germany). Standard solutions of drugs and metabolites were prepared at concentrations of 1 to 2 mg/ml in water (glucuronides) or methanol (all other compounds) and stored at −20°C. Spiking of urines occurred by addition of known aliquots of the standard solutions prior to sample injection, sample hydrolysis or extraction.

2.2. Test subjects, drug administration and urine collection

A total of 17 healthy volunteers participated in this investigation. They gave their informed consent to the study. With the exception of one individual being a PM for debrisoquine, all subjects were determined to be EMs for both debrisoquine and mephenytoin (for procedure see below). For the determination of DHC phenotype status, 0–12 h urines of 10 male Caucasians (all EM for debrisoquine; age range: 25–35) were collected after oral administration of 60 mg DHC in a slow release form (DHC Continus tablets containing 90 mg DHC hydrogentartrate, Napp Laboratories, Cambridge, UK). A second set of urines produced by the same subjects on a different day (wash-out period >7 days) was collected after oral administration of 50 mg quinidine sulfate (capsules manufactured by the hospital pharmacy of the Inselspital, Berne, Switzerland) 2 h prior to swallowing the same DHC Continus tablet. Furthermore, the 0–12 h urine of a PM for debrisoquine who ingested 60 mg of DHC (DHC Continus) and the six 0–8 h urines from a previous study [22] were also analyzed. Two 0–8 h urines of an EM for desbrisoquine were collected after ingestion of 14.2 mg COD (60 drops of Resyl Plus, Zyma, Gland, Switzerland). In one case, 50 mg quinidine sulfate was administered 2 h prior to COD. Additionally, two 0–8 h urines of two EMs were collected after administration of 7.1 mg COD (30 drops of Resyl Plus). All urines were stored at −20°C.

2.3. Urine pretreatments

For hydrolysis of conjugates, 5 ml of urine were incubated with 10 μl of internal standard solution containing 2 mg/ml COD and with 1 ml of concentrated hydrochloric acid for 30 min at 120°C using two screw-capped Sovirel test tubes. Thereafter the pH of the hydrolysate was adjusted to pH about 7 through addition of about 1.25 ml of 10 *M* KOH. The sample was then centrifuged for 3 min at ca. 1500 *g*. Extraction was carried out with 5 ml of urine (or 5 ml of hydrolysed urine) as described before [22] using disposable cartridges which contain a proprietary copolymeric material (Bond Elut Certify 1211–3050, sorbent amount: 130 mg, reservoir volume: 10 ml) and a Vac-Elut setup (all from Analytichem International, Harbor City, CA, USA). Elution of opioids was affected twice with 1 ml of methanol containing 30% of concentrated ammonium hydroxide solution. Throughout the extraction procedure the columns were never dried under full vacuum. Eluates were evaporated to dryness under a gentle stream of nitrogen and if not otherwise stated, the residues were redissolved in 100 μl of MECC running buffer.

2.4. Instrumentation for MECC, running conditions and assay specs

Throughout the work a buffer containing 6 m*M* $Na_2B_4O_7$, 10 m*M* Na_2HPO_4 and 75 m*M* SDS (pH ca. 9.2) was employed. Electrokinetic measurements were made in two instruments featuring 75 μm I.D. fused-silica capillaries of 70 cm effective length; the Europhor Prime Vision IV which features fast scanning polychrome detection for solute identification (Europhor, Toulouse, France) and the automated Model 270A-HT capillary electrophoresis system (Applied Biosystems, San Jose, CA, USA). The applied power was 25 kV (current 68–78 μA) and 30 kV (about 90 μA), respectively. If not otherwise stated, injection was effected with application of vacuum for 0.5 and 0.6 s, respectively. On the ABI 270A-HT, the temperature was set at 35°C, whereas the Europhor instrument was operated at ambient temperature. For data evaluations, the absorbance signal of the 270A-HT was interfaced to a PC Integration Pack (version 3.0, Kontron Instruments, Zürich, Switzerland) and data acquisition, storage and evaluation were executed on a Mandax AT 286 computer system. Quantitation was based on peak-area measurements and employing multi-level internal calibration as described elsewhere [22]. With sample pretreatment as described above, day-to-day and run-to-run variations were determined to be <8% ($n = 5$) and <3% ($n =$

10), respectively. All data were generated by single determinations.

2.5. Routine phenotyping by chromatographic means

Each volunteer was screened for his ability to oxidize drugs via administration of a tablet containing debrisoquine (10 mg, Declinax, Hoffmann-LaRoche, Basel, Switzerland) and mephenytoin (100 mg, Mesantoin, Sandoz, Basel, Switzerland), quantitative collection of the 0–8 h urine and chromatographic determination of the two hydroxylation statuses according to Küpfer and Preisig [2]. Urinary 4-hydroxymephenytoin was determined by HPLC according to Caslavska et al. [10]. Debrisoquine and 4-hydroxydebrisoquine were monitored by GC using a modification of the method of Dick et al. [25]. Briefly, for derivatization, 100 μl of urine to which guanfacine (20 μg/ml) has been added as internal standard, 150 μl of water, 100 μl of 1 *M* $NaHCO_3$, 1 ml of toluene and 50 μl of hexafluoroacetylacetone were pipetted into a screw-capped Sovirel test tube and incubated at 100°C for 60 min. After cooling to room temperature, 5 ml of 3 *M* NaOH and 2 ml of toluene were added, and the closed test tube was vortex-mixed for 10 s and centrifuged for 5 min at 1500 *g*. For analysis, aliquots of 2 μl of the organic phase were injected onto a Model 4100 GC equipped with a temperature programmer and an ECD detector module 251 (Carlo-Erba, Milan, Italy). The detector voltage was 50 V and the pulse width 0.5 μs. An 1.8-m (2 mm I.D.) glass column packed with 3% SE30 on Chromosorb W HP 80/100 mesh (Supelco, Gland, Switzerland) was employed. The temperatures of the column, injector and detector were 170, 250 and 250°C, respectively. The carrier gas used was argon–methane (95:5) and its flow-rate was set to 45 ml/min (about 2.0 kg/cm^2). Peak registration and integration was obtained by a Model 3392A integrator (Hewlett-Packard, Widen, Switzerland). Quantitation of debrisoquine and 4-hydroxydebrisoquine was based on internal calibration using peak areas. Debrisoquine hydroxylation was expressed as the debrisoquine to 4-hydroxydebrisoquine metabolic ratio (MR) as reported by Küpfer and Preisig [2]. An antimode of 12.6 between EM and PM distinction was used. Typical chromatograms are presented in Fig. 2.

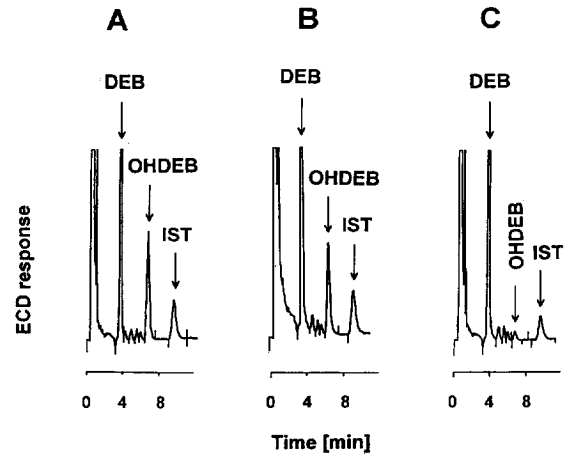

Fig. 2. GC chromatograms obtained for phenotyping with debrisoquine of (A) an EM with an MR of 1.94 (individual A.L.), (B) an EM with an MR of 3.82 (individual S.B.) and (C) a PM with an MR of 25.4 (individual R.E.). DEB, OHDEB and IST refer to debrisoquine, hydroxydebrisoquine and internal standard, respectively.

3. Results and discussion

3.1. DHC O-demethylation in presence and absence of quinidine

To test the polymorphism of DHC O-demethylation (Fig. 1B), the 0–12 h urines of 10 male subjects were analyzed by MECC. Independently, the volunteers were determined to be EMs for debrisoquine. The data presented in Fig. 3 represent electropherograms of the urines of individual A.L. obtained without (panel A) and with QUI as comedication (panel B). Upper graphs represent data which were generated by direct urine injection whereas the lower graphs are data produced after acid hydrolysis and extraction. Direct urine injection revealed the presence of free DHC, DHCG* and NDHC (panel A), as well as the same three compounds together with QUI (panel B). In agreement with

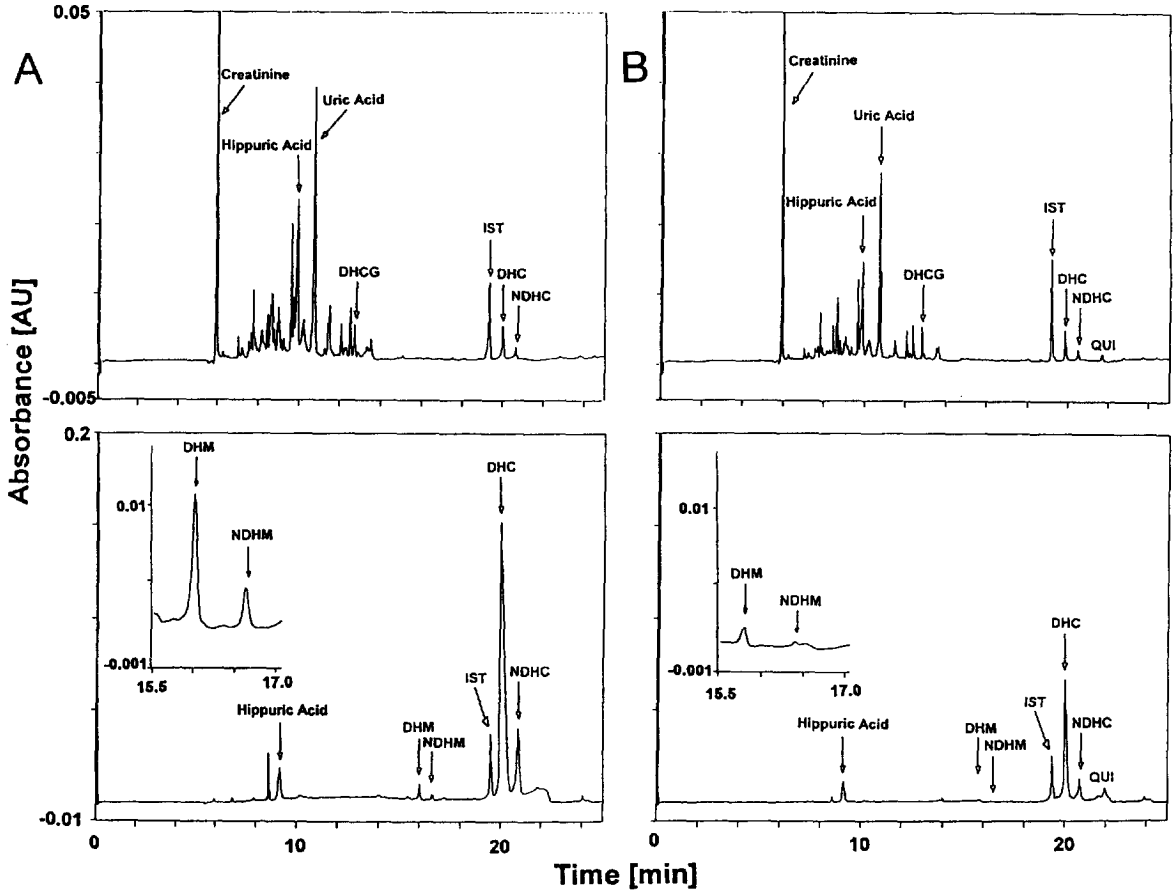

Fig. 3. MECC electropherograms at 210 nm obtained on the ABI apparatus with the urines of an EM (individual A.L.) which were collected (A) without and (B) with QUI premedication. The top and bottom graphs were obtained with direct urine injection and after acid hydrolysis and extraction, respectively. The inserts in the lower graphs depict sections of the data drawn on elongated y-scale.

previously published data [22], DHM and NDHM could not be detected with this assay. After extraction and analysis of the reconstituted extracts, the same was found to be true for more than half of the urines. Thus, the urines were first hydrolized and then extracted. With this procedure, DHM was sufficiently concentrated which permitted its unambiguous detection in all urines (lower graphs of Fig. 3). As is shown with the inserts in the lower graphs of Fig. 3, DHM and NDHM peaks appear to be somewhat smaller when QUI was administered. Comparison of peak heights, however, is not sufficient to determine whether QUI is hampering the O-demethylation of DHC to DHM (Fig. 1B). Thus, concentrations were determined using COD as internal standard and considering urine volumes, the total recovery of DHM was calculated. The statistical evaluations of the DHM recoveries (% of DHC dose) for both sets of 10 samples are presented as box plots in Fig. 4A. DHM recoveries in EMs are larger than those observed under coadministration of QUI. The medians (means) were determined to be 0.91 (1.09) and 0.28 (0.32) % of DHC dose, respectively. The amount DHM formed in presence of QUI was determined to be about 3.4-fold smaller compared to the DHM formed in absence of QUI. According to the Mann–Whitney rank sum test, there is a statistically significant difference be-

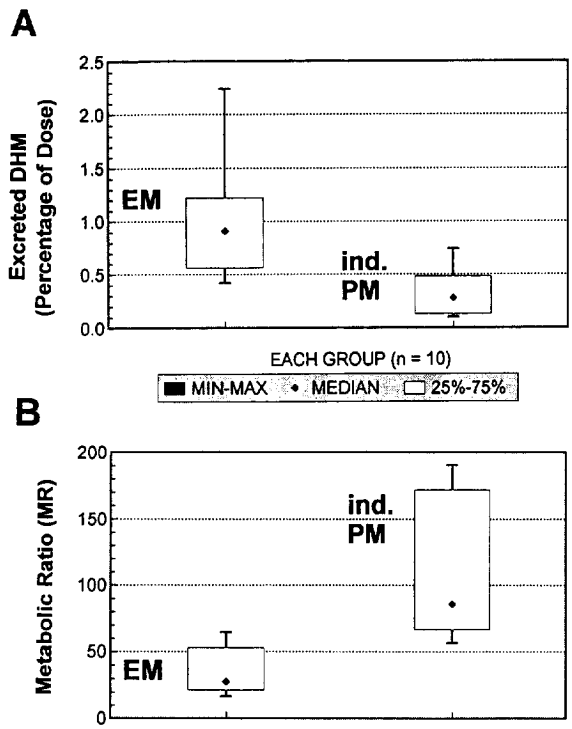

Fig. 4. Box plots of (A) urinary DHM recovery data expressed as percentage of the DHC dose and (B) urinary metabolic ratios of 10 EMs and 10 QUI-induced PMs. The boxes are drawn for 25 and 75% percentiles, the diamonds represent medians and the bars indicate minimum and maximum values.

tween the two groups ($p < 0.001$), this meaning that the difference in the medians values of the two groups is greater than would be expected by chance.

Furthermore, in analogy to previous work with other substrates [1,2], the metabolic ratio (MR) defined as

$$MR = \frac{\text{urinary recovery of DHC (mmol)}}{\text{urinary recovery of DHM (mmol)}} = \frac{[DHC]}{[DHM]} \quad (1)$$

where [X] is the urinary concentration of X, was employed to characterize the magnitude of O-demethylation of DHC to DHM. Statistical analysis (Fig. 4B) again revealed a significant difference between the MR values of the two input groups ($p < 0.001$). The MR values of the QUI-induced PMs are larger than those obtained with EMs. The medians (means) were determined to be 81.3 (98.7) and 26.7 (31.9), respectively. Thus, for these samples obtained from 10 male subjects of 25 to 35 years of age it can be concluded that QUI is clearly reducing O-demethylation. No age or sex dependence was investigated.

3.2. Use of DHC as probe drug for differentiation between EM and PM statuses

The MECC 0–12 h urine data of an individual who was determined to be a PM for debrisoquine (MR for debrisoquine = 25.4, Fig. 2C) are pre-

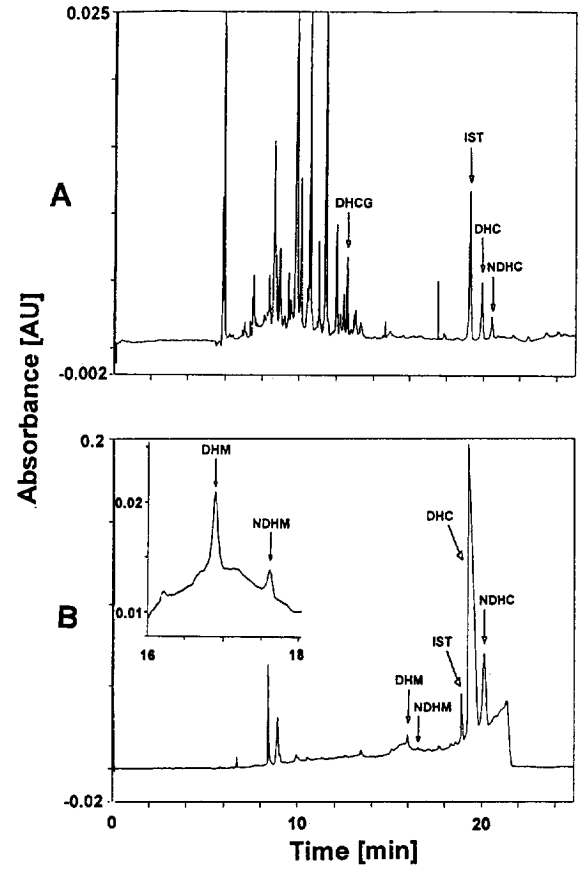

Fig. 5. MECC electropherograms obtained on the ABI instrument with (A) direct urine injection and (B) injection of the acid hydrolysed and extracted urine of a PM. Other conditions as for Fig. 3.

sented in Fig. 5. With direct urine injection, DHC, DHCG and NDHC could be unambiguously monitored (panel A). In the hydrolysed and extracted urine (panel B) small amounts of DHM and NDHM could be monitored. The DHM recovery was determined to be 0.6% of the DHC dose, a value which compares well with those of the QUI-induced PMs (Fig. 4A).

Fig. 6A presents a frequency histogram of the log MR values of the two sets of ten urines discussed above. It represents a bimodal-like distribution with an antimode of about 55 (marked with arrow). EMs were found to have MR values between 16.7 and 64.4 (Fig. 4B). The MR values of the QUI-induced PMs were distributed over a wider range (56.7–190.3). The analysis of the urine of one EM (subject S.B.) resulted in an MR of 64.4 (marked with asterisk), this being within the range of the PMs. It was interesting to realize that the MR for debrisoquine of that individual was also the highest value measured within the screened group of ten subjects (MR of 3.82, chromatogram presented in Fig. 2B). The median and mean of the 10 MR values for debrisoquine were determined to be 0.91 and 1.34, respectively. Linear regression analysis where MR values for DHC and debrisoquine were taken as independent (x-axis) and dependent (y-axis) variables resulted in $y = 0.072x - 0.955$ with a regression coefficient r of 0.899.

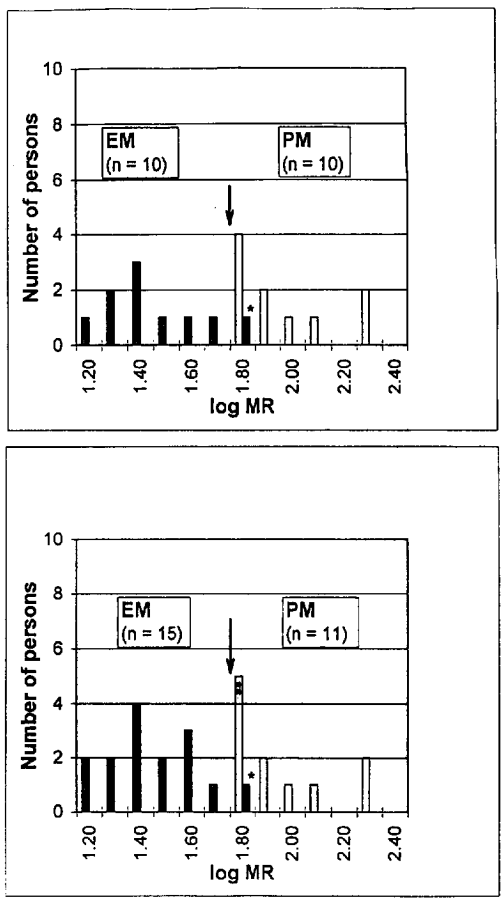

Fig. 6. Frequency histograms of DHC metabolic ratios of (A) 10 EMs and 10 QUI-induced PMs, and (B) the values of panel A supplemented with those obtained with a PM (marked with two asterisks) and 5 urines of EMs collected between 0–8 h after DHC intake. The antimode is marked with an arrow. The data obtained with an EM for debrisoquine with a very high MR for DHC is marked with one asterisk.

On a separate occasion, the urines of five individuals (4 males, 1 female) which tested as EMs for debrisoquine were also analyzed. At that time, 0–8 h urines were collected. Thus, the question arised whether these data could be compared with those obtained after 0–12 h urine collection. First, it was interesting to find that the total DHC dose equivalents excreted in the case of 0–12 h ($29.5 \pm 17.3\%$, $n = 10$) was higher compared to that after 0–8 h urine collection ($24.6 \pm 6.2\%$, $n = 5$), whereas the excreted equivalents for DHM were about equal [$1.01 \pm 0.57\%$ ($n = 10$) and $0.99 \pm 0.77\%$ ($n = 5$), respectively]. Nevertheless the MRs of the 0–8 h urines were found to fit well into the bimodal distribution (Fig. 6B). Thus, it appears that MR values of urines collected between 0–8 or 0–12 h after administration of DHC can be directly compared. This is not surprising in the sense that Yue et al. [12] also described the independence of MR values and urine collection time intervals when COD is employed as substrate.

Also included in Fig. 6B is the MR of a real PM (marked with two asterisks) whose electropherogram is shown in Fig. 5B. For that case, an MR of 55.7 was determined. To investigate the MR of native PMs on a large scale a population study would have to be undertaken. Compared to frequency plots obtained with

debrisoquine [2], the plots presented in Fig. 6 exhibit a partial overlap between the MR ranges of EMs and PMs. Thus, classification of borderline values is not unequivocal and DHC should therefore not be employed for routine pharmacogenetic screening purposes.

3.3. Comparison to codeine

The suitability of employing the MECC assay for the exploration of the COD metabolism was also investigated. The data presented in Fig. 7 were obtained with a 0–8 h urine of an individual who was determined to be an EM for debrisoquine and DHC and who ingested 14.2 mg of COD. Due to the lower COD dose compared to DHC (4.2 times less drug molecules), only codeine-6-glucuronide (CODG) could be detected by direct urine injection (Fig. 7A). This finding is not surprising, because Yue et al. [12] reported the degree of COD glucuronidation to be 93.5% in the 0–8 h urine. In contrast to that, only 52% of DHC was determined to be conjugated in the urine collected during the same time interval [22].

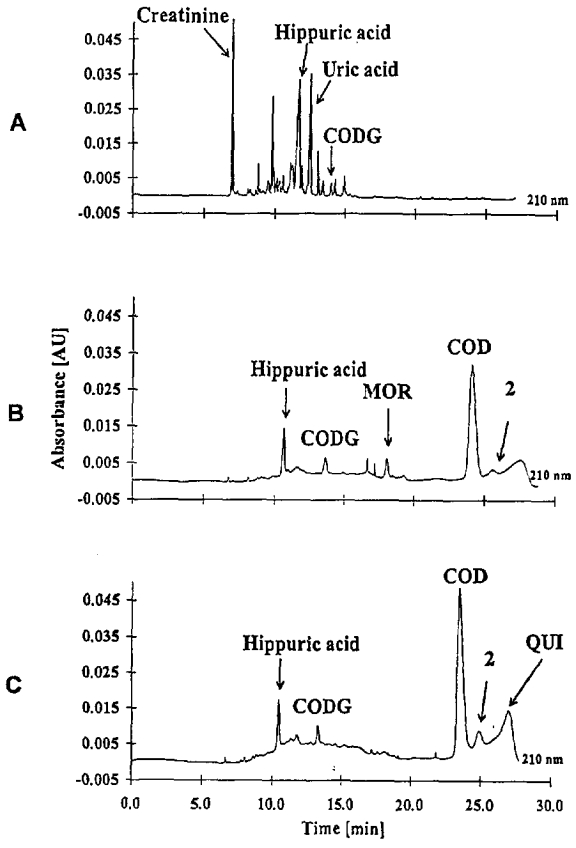

Fig. 7. MECC electropherograms obtained on the Europhor with (A) a directly injected urine which was collected after COD administration, (B) the same urine as in panel A but injected after acid hydrolysis and extraction, and (C) an extract of an acid hydrolysed COD urine of the same person but collected after QUI premedication.

In the acid hydrolysed and extracted urine (Fig. 7B), COD and MOR could easily be detected. Furthermore, peak 2 eluting shortly after COD exhibits a UV spectrum similar to that of COD. Thus, in analogy to the patterns seen with DHC ([22] and Fig. 3) and to the published metabolic pathway of COD [12–14], this peak could be assigned to norcodeine. Unfortunately, no reference substance was available to prove that assumption. Also, CODG was observed in the electropherogram shown as Fig. 7B, this indicating that CODG was incompletely cleaved by acid hydrolysis. Not surprisingly, after QUI premedication, MOR could not be detected (Fig. 7C). Thus, the MECC assay could also be employed with COD as substrate. For the investigation of the extent of O-demethylation, however, a COD dose >14.2 mg should be administered.

4. Conclusions

MECC is shown to be a simple method to investigate the urinary metabolites of DHC and COD. Within 12 h after DHC ingestion of subjects which are EMs for debrisoquine, about 1% of the administered DHC equivalents are shown to be excreted as urinary DHM. This is a lower amount than the 3.2% of MOR reported to be excreted after intake of COD [12]. Thus, it is well possible that these variations might cause differences in the analgesic effects observed after ingestion of DHC and COD. Premedication with QUI is demonstrated to significantly reduce the amount of O-demethylation of DHC, a metabolic step which is thereby confirmed to co-segregate with the hydroxylation of debrisoquine. In

analogy to COD and other substrates, EM and PM phenotypes for DHC can be distinguished. Using the urinary DHC/DHM MR to characterize the extent of O-demethylation, the MR ranges of EMs and PMs in a frequency histogram are shown to partially overlap. Thus, classification of borderline values is not unequivocal and DHC should therefore not be employed for routine pharmacogenetic screening purposes.

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