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Screening for hydroxylation and acetylation polymorphisms in man via simultaneous analysis of urinary metabolites of mephenytoin, dextromethorphan and caffeine by capillary electrophoretic procedures

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Abstract

Phenotypes for hydroxylation can be predicted by using mephenytoin and dextromethorphan as substrates, whereas phenotypes for acetylation can be determined with caffeine as probe drug. After single-dose administration of one of these drugs, of two of them simultaneously, or of the three drugs together, the major urinary metabolites (4-hydroxymephenytoin; dextrorphan, 3-methoxymorphinan, 3-hydroxymorphinan; 5-acetylamino-6-amino-3-methyluracil as decomposition product of 5-acetylamino-6-formylamino-3-methyluracil, 1-methylxanthine, respectively) of these substrates were analyzed by capillary electrophoretic techniques. No sample pretreatment other than enzymatic hydrolysis of the conjugated compounds was applied. Assays based on micellar electrokinetic capillary chromatography are shown to allow simultaneous and unambiguous phenotyping with mephenytoin and dextromethorphan or mephenytoin and caffeine. Simultaneous screening for all three polymorphisms with a single injection of a hydrolyzed urine is shown to be possible via use of multiwavelength absorption detection only. Phenotypes determined by electrokinetic capillary techniques are shown to agree with those obtained by analysis with customary assays based on high-performance liquid chromatography.

1. Introduction

Polymorphisms of hydroxylation and acetylation are common inherited variations of human drug metabolism. The existence and therefore the investigation of such polymorphisms is clinically important, since enzymatic deficiencies can lead to unusually high plasma concentrations of certain drugs and to an increased incidence of side-effects, or, in case of prodrugs, insufficient therapeutic effects. Furthermore, population studies have revealed large interethnic differences in the occurrence of the different phenotypes. Phenotypes for hydroxylation and acetylation are typically determined via use of probe drugs followed by chemical analysis of urinary metabolites [1–3]. They can also be predicted by genotyping methods based on mutation-specific amplification of DNA by the polymerase chain reaction [4]. The latter, newer

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approaches appear to be quite costly and time consuming and it is therefore unlikely that they will replace the *in vivo* methods for the determination of the metabolic capacity in man. Thus, there is still considerable interest in developing new analytical procedures for urinary metabolites of probe drugs, particularly assays which are simple and less time consuming than the commonly employed chromatographic approaches.

Mephenytoin and dextromethorphan are common substrates used for the determination of two different genetic oxidative polymorphisms in man. For that purpose, the hydroxylation of the *S*-isomer of mephenytoin [5,6] and the O-demethylation of dextromethorphan to dextrorphan which occurs under the same genetic control as the 4-hydroxylation of debrisoquin [7,8], are investigated. Humans can be classified either as poor metabolizer phenotypes (PM, exhibiting the genetic drug metabolizing deficiency through lack of an active cytochrome P-450 enzyme, enzymes IIC and IID6 for mephenytoin and dextromethorphan, respectively), or as extensive metabolizer phenotypes (EM). The oxidative metabolism of many drugs is under genetic control, and for the two independent hydroxylation defects, the incidence of PMs in Caucasians is 6–10% each. PMs are characterized by very small or almost undetectable amounts of a particular metabolite in urine. In EMs, large quantities of metabolites can be found and the urinary excretion of the parent drug is typically of negligible importance. Widespread determination of polymorphic oxidative drug metabolism is hampered somewhat by phenotyping costs and labour-intensive phenotyping procedures. While coadministration of mephenytoin and debrisoquin has been employed routinely for the past decade in our departmental screening program, and combination of mephenytoin with dextromethorphan has been studied elsewhere [9,10], no report could be found describing a specific assay suitable for the simultaneous determination of the urinary excretion patterns of two probe drugs in a single aliquot of urine. For each drug, separate chromatographic assays based on thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC) or gas chromatography–mass spectrometry are typically employed.

Caffeine is a probe drug commonly employed for the determination of the acetylator status in man [11,12]. The rate of acetylation of a number of relevant drugs prior to excretion was found to be genetically determined and polymorphic in nature, *i.e.* individuals can be distinguished as either fast or slow acetylators on the basis of their ability to convert certain amine and hydrazine drug substrates to their N-acetyl metabolites [2]. In Caucasians, the incidences of fast and slow acetylator phenotypes are about equal. Many adverse effects of drugs have been associated with acetylator status, this being the clinical rationale for the knowledge of the acetylator phenotype of individuals. Using caffeine for phenotyping, metabolic ratios of different urinary metabolites, including 5-acetylamino-6-formylamino-3-methyluracil (AFMU), 1-methylxanthine (1X) and 1-methyl uric acid (1U), have been investigated [11]. Under neutral and alkaline conditions, AFMU was found to gradually and irreversibly deformylate to 5-acetylamino-6-amino-3-methyluracil (AAMU) [12]. Although the decomposition reaction can be minimized by acidification immediately after urine collection, complete control and exclusion of inaccurate results is only obtained when either both AAMU and AFMU can be monitored or AFMU is completely converted to AAMU prior to analysis of the latter substance.

Recently, capillary zone electrophoresis (CZE) 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 [13], urinary AFMU and 1X [14] and AAMU, AFMU, 1X and 1U [15]. Furthermore, inclusion of β-cyclodextrin as buffer additive in MECC has been shown to easily permit the investigation of the stereoselectivity of the 4-hydroxylation of mephenytoin and of the S:R enantiomeric ratio

of mephenytoin in human urine, thereby also revealing an oxidative metabolizing ability in man [16]. On this basis, CZE and MECC assays for the determination of the major urinary metabolites of mephenytoin (4-hydroxymephenytoin) and dextromethorphan (dextrorphan, 3-methoxymorphinan, 3-hydroxymorphinan) including dextromethorphan itself were developed (for the structures of the compounds see Fig. 1). Major emphasis was focused on the simultaneous analysis of all compounds in an enzymatically hydrolyzed urine specimen which was collected between 0 and 8 h after single-dose coadministration of mephenytoin (100 mg) and dextromethorphan (25 mg). The electrokinetic capillary data were compared with those obtained by HPLC. Furthermore, caffeine (140 mg) was coadministered with the two hydroxylator phenotype probe drugs and the collected urine was analyzed by MECC for the caffeine and mephenytoin metabolites or for the metabolites of all three substrates simultaneously.

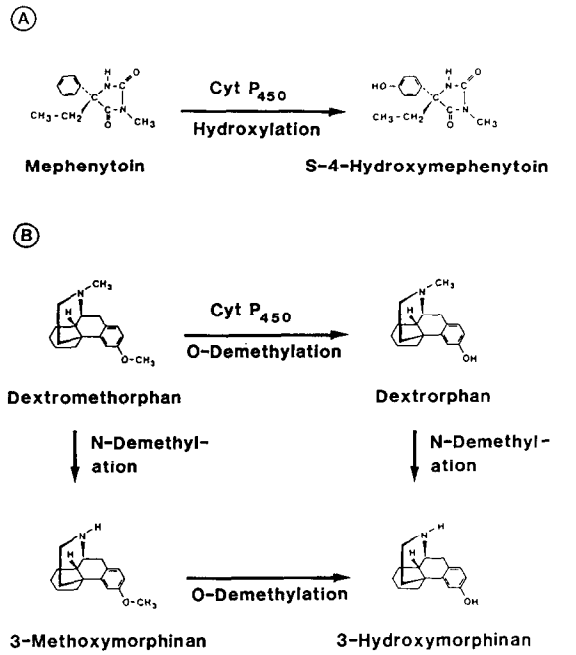

Fig. 1. Chemical structures of (A) mephenytoin and *S*-4-hydroxymephenytoin and (B) dextromethorphan and 3 of its metabolites. For the structures of caffeine and its metabolites see ref. 15.

2. Experimental

2.1. Drugs, chemicals, samples and standards

Dextromethorphan (as hydrobromide), 3-hydroxymorphinan, 3-methoxymorphinan (as hydrobromide), dextrorphan (as tartrate) and levallorphan (as tartrate) were a kind gift of F. Hoffmann-La Roche (Basel, Switzerland). AFMU was a kind gift of Nestlé (Vevey, Switzerland). Mephenytoin was from Siegfried (Zofingen, Switzerland) and 4-hydroxymephenytoin was synthesized in house [17]. 1X and 1U were purchased from Aldrich (Steinheim, Germany) and Fluka (Buchs, Switzerland), respectively. Sodium dodecyl sulfate (SDS) was from Sigma (St. Louis, MO, USA), methanol and acetonitrile (both HPLC grade) were from Rathburn Chemicals (Walkerburn, UK), hexane, acetic acid, KH_2PO_4, Na_2HPO_4, $Na_2B_4O_7$ as well as NaOH and KOH were from Merck (Darmstadt, Germany), isopropanol was from Fluka. β-glucuronidase/arylsulfatase (from *Helix pomatia*) was purchased from Boehringer Mannheim (Rotkreuz, Switzerland) and β-glucuronidase (glucurase, from bovine liver) was from Sigma.

Eight healthy adults, aged 25–45 years, after having been fully informed, voluntarily participated in this study. After single-dose administration of mephenytoin (100 mg, 1 tablet Mesantoin, Sandoz, Basel, Switzerland) and/or dextromethorphan (25 mg, 10 ml of Bexin sirup, Spirig, Egerkingen, Switzerland), the 0–8 h urine was collected. On a separate occasion, two of the volunteers took the two hydroxylator phenotype probe drugs (same dosage as above) together with a cup of coffee containing 140 mg caffeine [15] and their urines were collected between 0–4 and 4–8 h after administration of the drugs. Our own urines were used as blank matrices.

Standard solutions were prepared in methanol (mephenytoin, 4-hydroxymephenytoin), water (morphinans, phenobarbital) or buffer (caffeine metabolites) at concentrations between 500 and 1250 μg/ml and stored at 4°C. The gradual

deformylation of AFMU to AAMU was employed to provide a standard solution of AAMU. Spiking of blank urines occurred through addition of known aliquots of these solutions to a test tube and evaporation to dryness (at 37°C and under a gentle stream of air), followed by reconstitution with urine blank.

2.2. CZE/MECC

Electrokinetic measurements were made in two instruments featuring 75 μm I.D. fused-silica capillaries and multiwavelength absorbance detection, a home-made setup described previously [15], and the Europhor Prime Vision IV (Europhor, Toulouse, France). The effective capillary lengths (distances between anodic capillary end and detector) were 68–70 cm in both instruments and the total lengths 90 and 105 cm, respectively. The two instruments used the same on-column detector, the fast forward scanning UVIS 206 PHD (Linear Instruments, Reno, Nevada, USA). Throughout this work the detector was employed in the high-speed polychrome mode by scanning from 195 to 320 nm at 5 nm intervals (26 wavelengths). Multiwavelength data were read, evaluated and stored employing a Mandax AT 286 computer system and running the 206 detector software package version 2.0 (Linear Instruments, Reno, NV, USA) with Windows 286 version 2.1 (Microsoft, Redmont, WA, USA). In the home-made device, sample application was effected manually via gravity through lifting the capillary end, dipped into the sample vial, some 34 cm for *ca.* 5 s. The applied power was a constant 20 kV. Sample application in the Prime Vision occurred by its vacuum system (typically 1 s) and the applied voltage was between 12 and 20 kV. Prior to each run, capillaries were first rinsed with 0.1 *M* NaOH (3 to 5 min) and then with buffer (5 to 8 min).

Enzymatic hydrolysis of conjugates in urines was effected by mixing equal volumes of urine and of 0.2 *M* sodium acetate buffer (pH 5.4) prior to addition of 20 μl of β-glucuronidase/arylsulfatase per ml urine and overnight incubation at 37°C. Hydrolyzed urine was either directly injected or diluted with water prior to analysis. For MECC, the running buffer, if not stated otherwise, was composed of 75 m*M* SDS, 6 m*M* $Na_2B_4O_7$ and 10 m*M* Na_2HPO_4 (pH 9.2–9.3). Depending on the application, the buffer pH was increased or decreased by addition of NaOH (1 *M*) or phosphoric acid (1 *M*), respectively. In certain cases, isopropanol (6–10%, v/v) or acetonitrile (10%, v/v) were added as organic modifiers. For analysis of caffeine metabolites together with 4-hydroxymephenytoin, a pH 8.6 buffer composed of 70 m*M* SDS, 16.25 m*M* $Na_2B_4O_7$ and 16.25 m*M* NaH_2PO_4 (adjusted to pH 8.6 with NaOH, [15]) was employed. For CZE, a buffer composed of 140 m*M* $Na_2B_4O_7$ (*ca.* pH 9.4) was employed.

2.3. HPLC of 4-hydroxymephenytoin in human urine

HPLC analyses were performed with a modification of the procedure reported by Küpfer *et al.* [18] using a Model M45 solvent delivery system, a WISP 712 autosampler (both from Waters Associated, Milford, MA, USA), a reversed-phase C_8 column (Hibar LiChrosorb RP-8 5 μm, 125 × 4 mm I.D., Merck) and a UV detector Model Spectroflow 757 (Kratos Analytical, Ramsey, NJ, USA). Chromatograms were recorded and integrated by a Model 4290 integrator (Spectra Physics, Allschwil, Switzerland). The mobile phase consisted of water–acetonitrile (5:1, v/v) containing 20 μl of concentrated phosphoric acid per 600 ml. Phenobarbital (250 μg/ml urine) served as internal standard, the flow-rate was 1.0 ml/min, the temperature was ambient and detection wavelength was 230 nm.

To 0.1 ml of urine, spiked with the internal standard (phenobarbital) and diluted to 1 ml with water, 1 ml of concentrated HCl was added prior to incubation for 2 h at 100°C. Liquid–liquid extraction of hydroxymephenytoin and the internal standard was carried out with 6 ml ethylacetate in a 10-ml screw-capped Sovirel test tube. After vigorous shaking for 5 min and centrifugation at 1500 *g* for 10 min, the upper (aqueous) phase was discarded and the organic phase was transferred to a centrifuge glass tube

with short conical bottom and evaporated to dryness under a gentle stream of air at 37°C. The residue was dissolved in 1 ml of methanol. For analysis, 10 μl were injected. Data evaluation was based upon internal calibration employing peak areas. Calibration graphs between 20 and 200 μg/ml urine (4 data points) were constructed. Interpretation of data was performed via calculation of a hydroxylation index (HI, [6]).

2.4. HPLC of dextromethorphan and metabolites in urine

HPLC analyses were performed according to Zysset *et al.* [19] using a Model M45 solvent delivery system, a WISP 712 autosampler (Waters), a C6H5 column (ET 250/8/4 Nucleosil 7 C6H5, Macherey-Nagel, Oensingen, Switzerland) and a fluorescence detector Model LS 30 (Perkin-Elmer, Basel, Switzerland). Chromatograms were recorded and integrated by a Model 4290 integrator (Spectra Physics). The mobile phase consisted of a mixture of an aqueous 10 m*M* phosphate buffer containing 2.5 m*M* of the sodium salt of 1-octanesulfonic acid (adjusted to pH 2.5 with concentrated phosphoric acid), methanol and acetonitrile (70:10:20, v/v). Levallorphan (5 μg/ml urine) served as internal standard, the flow-rate was 1.3 ml/min and the temperature was ambient. For fluorescence detection, excitation and emission wavelengths were set to 270 and 312 nm, respectively. Data evaluation was based upon internal calibration employing peak areas. Calibration graphs between 0.25 and 10 μg/ml of each compound (6 data points) were constructed.

For enzymatic hydrolysis of the conjugated compounds, 0.25 ml urine and 0.25 ml of a 0.14 *M* sodium acetate buffer (pH 5) were mixed prior to addition of 25 μl of β-glucuronidase solution from bovine liver (glucurase, activity 5000 units/ml) and overnight incubation at 37°C. Liquid–liquid extraction of the morphinans was achieved under basic conditions using hexane. The hydrolyzed urine, 1.5 ml of glycine buffer (pH 11.3) and 6 ml hexane containing 10% butanol were added into a 10-ml screw-capped Sovirel test tube. After vigorous shaking for 10 min and centrifugation at *ca.* 1500 *g* for 10 min, the organic phase was transferred to a centrifuge glass tube with a short conical bottom and evaporated to dryness under a gentle stream of air at 37°C. The residue was dissolved in 200 μl of mobile phase. For analysis, 10 μl were injected. Interpretation of data was performed via metabolic ratios, *i.e.* concentration ratios of urinary dextromethorphan divided by urinary dextrorphan (EM: ratio < 0.005, PM: ratio *ca.* 1 [8]).

3. Results and discussion

3.1. Phenotyping with mephenytoin using HPLC, CZE and MECC

In our drug assay laboratory, routine determination of the hydroxylator status with mephenytoin is currently performed using HPLC instead of the GC assay employed for the large population studies [6]. Due to the low urinary concentration of mephenytoin, only 4-hydroxymephenytoin is determined. For classification, calculation of a hydroxylation index (HI) which represents the ratio between the *S*-mephenytoin dose (half of the administered dose of racemic mephenytoin) and the recovery of *S*-hydroxymephenytoin in the 0–8 h urine is employed [6]. An HI of 16.4 serves as antimode with EMs being characterized by HI < 16.4. From a practical point of view, HPLC chromatograms showing a peak for 4-hydroxymephenytoin are typically obtained with urine specimens of EMs, whereas no peak is detected with samples of PMs. Typical chromatograms of a blank urine, a calibrator urine containing 100 μg/ml (427.4 μ*M*) racemic 4-hydroxymephenytoin, a urine of a test person with the genetic drug metabolizing deficiency (PM) and two urines of test persons which are EMs for mephenytoin are depicted in panels A to E, respectively, of Fig. 2. It is apparent that the 4-hydroxymephenytoin peak in the chromatogram of the PM (panel C) is missing, whereas well-defined peaks of the analyte were registered for the EMs. 4-Hydroxymephenytoin

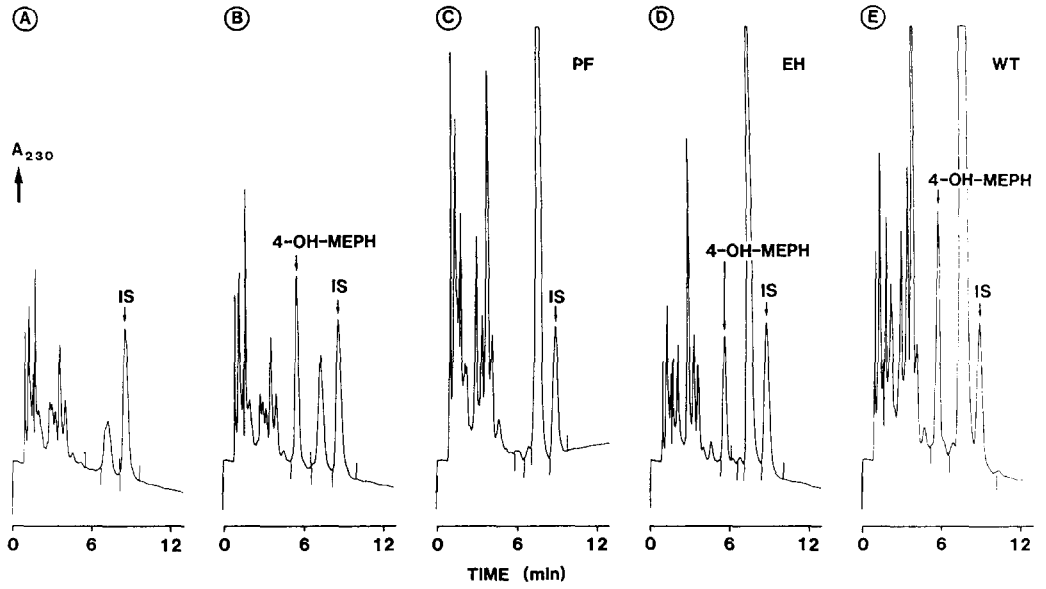

Fig. 2. HPLC of (A) a blank urine spiked with the internal standard (I.S., phenobarbital), (B) a calibrator urine with 100 μg/ml racemic 4-hydroxymephenytoin (4-OH-MEPH), (C) a urine of a PM (test person PF), and (D,E) two urines of EMs for mephenytoin (test persons EH and WT, respectively).

concentrations for the data of panels D and E were determined to be 282.5 and 625.1 μM, respectively. For the two individuals, HI values of 1.73 and 1.50, respectively, were calculated. It is important to note, that generation of such HPLC data requires that urine specimens be hydrolyzed and extracted prior to analysis.

Analysis of urinary 4-hydroxymephenytoin by CZE employing a borate buffer (140 mM) was largely unsatisfactory and no further efforts were undertaken in finding suitable CZE conditions for that compound. However, in analogy to the cyclodextrin modified MECC approach reported elsewhere [16], the determination of urinary 4-hydroxymephenytoin was also studied by MECC using a pH 9.2 phosphate–tetraborate buffer containing 75 mM SDS (*cf.* Experimental). Typical electropherograms obtained with the two instruments are presented in Fig. 3. Using the Europhor, the data of a urine blank and this blank spiked with 4-hydroxymephenytoin are depicted in panels A and B, respectively. Data obtained with the home-made instrument via direct injection of enzymatically hydrolyzed urines of a PM and an EM for mephenytoin are presented in panels C and D, respectively, of Fig. 3. Peak assignment was made by spectral analysis of the eluting peaks and spiking with racemic 4-hydroxymephenytoin as discussed previously [16]. As was the case with HPLC, well-resolved peaks for 4-hydroxymephenytoin were noted, this permitting unambiguous phenotyping using MECC and direct injection of hydrolyzed urine specimens. Thus, using the MECC assay, the extraction step required for HPLC is omitted.

3.2. Phenotyping with dextromethorphan using HPLC, CZE and MECC

The HPLC method employed in our laboratory for the determination of dextromethorphan and its major urinary metabolites is based on enzymatic hydrolysis and extraction prior to analysis. Chromatograms of a blank urine, a blank urine spiked with 5 μg/ml of the morphinans and two urines of test persons are presented in panels A to D of Fig. 4, respectively. From the data of panel B, all four compounds and the internal standard are shown to extract

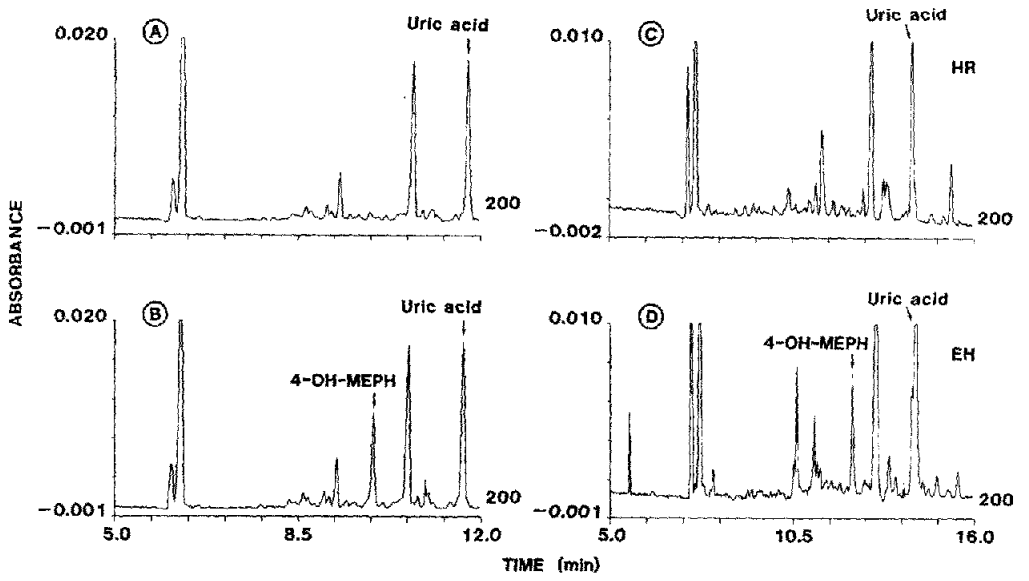

Fig. 3. MECC of urinary mephenytoin obtained with (A,B) the Europhor and (C,D) the home-made instrument. Data of (A) a urine blank, (B) the urine blank spiked with *ca.* 34 μg/ml 4-hydroxymephenytoin (4-OH-MEPH), (C) a urine of a PM (test person: HR), and (D) a urine of an EM for mephenytoin (test person: EH). The applied voltages (currents) were 28 kV (87 μA) and 20 kV (81 μA), respectively.

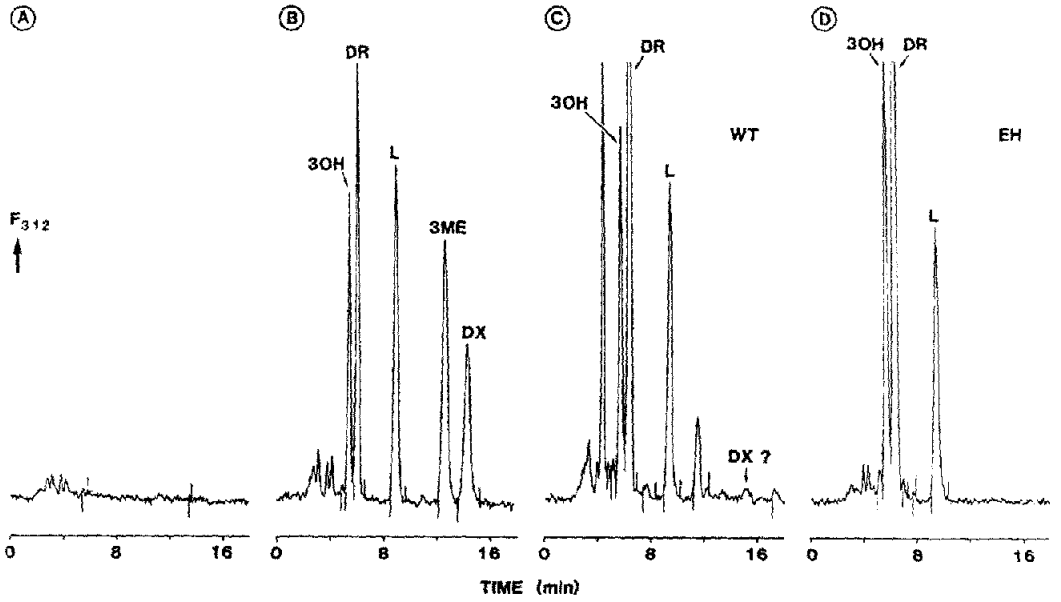

Fig. 4. HPLC of (A) a blank urine, (B) a calibrator urine (5 μg/ml of each compound) containing dextromethorphan (DX), dextrorphan (DR), 3-hydroxymorphinan (3OH), 3-methoxymorphinan (3ME) and the internal standard (L, levallorphan), and (C,D) two urines of EMs for the O-demethylation of dextromethorphan (test persons WT and EH, respectively).

and separate well. In both test specimens (panels C and D, same individuals as for Fig. 2) only dextrorphan and 3-hydroxymorphinan could be determined unambiguously. The total urinary concentrations of these two compounds for the data of panel C were 7.23 and 6.51 μg/ml, respectively. The concentrations of both metabolites in the chromatogram of panel D were found to be 10.65 μg/ml. Furthermore, in some of the investigated urine samples (*e.g.* in Fig. 4C but not in Fig. 4D) indication of the presence of a small amount of dextromethorphan was found. In the analysis of eight urine specimens, 3-methoxymorphinan was never detected. Due to the presence of dextrorphan and the lack of substantial amounts of dextromethorphan in these urines, all individuals could be characterized as having extensive metabolizing capacity for dextromethorphan (metabolic ratio DX/DR < 0.005 [8]).

As previously described by Li *et al.* [13], phenotyping with dextromethorphan can easily be performed by CZE in borate buffers. Electropherograms obtained with urine blank and a urine blank spiked with the morphinans and levallorphan (5 μg/ml each) are depicted in panels A and B of Fig. 5. As in HPLC (Fig. 4B), excellent resolution of all five substances was obtained. Furthermore, no interference from endogenous compounds was noted for dextromethorphan and its three metabolites, whereas levallorphan comigrated with an unknown substance (marked with an asterisk in panel A). The data presented in panels C and D were obtained after injection of an enzymatically hydrolyzed urine of a test person (panel C, same individual as for Fig. 4C) and the same sample spiked with dextromethorphan and the three metabolites (*ca.* 20 μg/ml each), respectively. Spiking and spectral analysis (data not shown) permitted the unambiguous identification of dextrorphan and 3-hydroxymorphinan in that persons urine, this clearly revealing its EM status. Again, there is an indication of the presence of a small amount of dextromethorphan in panel C, an amount which is too small for unambiguous identification. As with HPLC, 3-methoxymorphinan could not be detected. The CZE assay

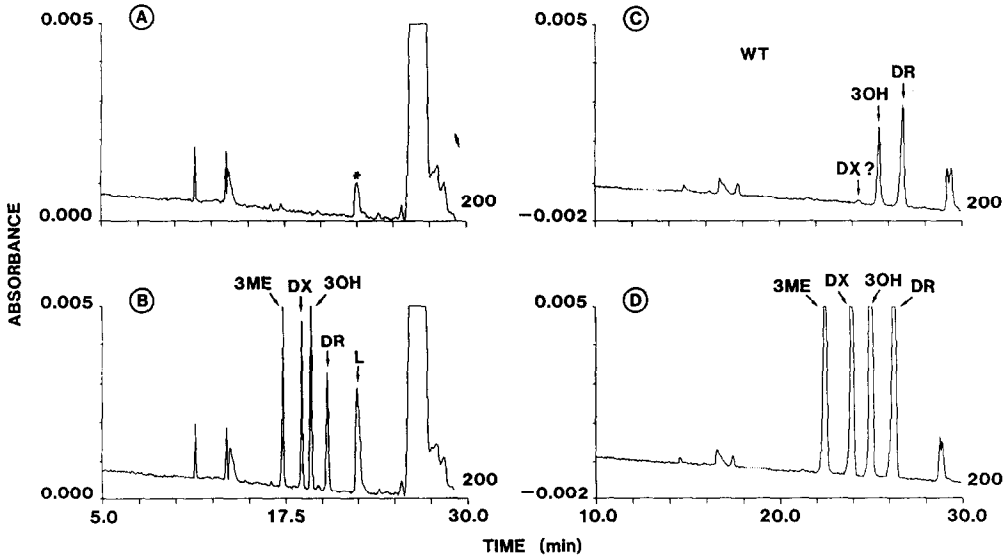

Fig. 5. CZE (200 nm) measured with the Europhor of (A) urine blank, (B) urine blank spiked with 3ME, DX, 3OH, DR and L (*ca.* 5 μg/ml each), (C) a hydrolyzed urine of test person WT, and (D) the hydrolyzed urine spiked with 3ME, DX, 3OH and DR (*ca.* 20 μg/ml each). The applied voltages were 14, 14, 12, 12 kV, respectively. The currents were *ca.* 117 μA (panels A,B) and *ca.* 89 μA (panels C,D). The key for the substances is the same as for Fig. 4. The asterisk marks an endogenous compound.

appears to provide sufficient sensitivity for phenotyping when using UV absorbance instead of fluorescence as in HPLC (Fig. 4). For this CZE determination of morphinans in urine, detection limits of *ca.* 0.5 μg/ml each were found (data not shown).

In another approach, the potential of MECC analysis of dextromethorphan and its urinary metabolites was investigated. Using the pH 9.2 buffer of the hydroxymephenytoin assay (Fig. 3), all four morphinans were found to coelute and appeared as one peak towards the end of the electropherogram (after most endogenous compounds). With the addition of acetonitrile (10%, v/v) 3 peaks were observed, whereas with isopropanol (8–10%, v/v) proper separation of all four compounds could be achieved. This, however, only at the expense of a substantially increased run time (up to two-fold for dextromethorphan when using 10% isopropanol) compared to that without the organic modifier. Using the pH 9.2 micellar buffer containing 8% (v/v) isopropanol, data obtained with a directly injected urine blank and this blank spiked with the four morphinans are presented in panels A and B of Fig. 6, respectively. The electropherogram obtained with an enzymatically hydrolyzed urine specimen of a test person is shown in panel C of Fig. 6. Spiking and spectral analysis of the two peaks which elute between *ca.* 51 and 54 min showed them to be dextrorphan and 3-hydroxymorphinan, respectively (data not shown). Thus, this method revealed this individual's EM status for dextromethorphan. Similar data were generated for hydrolyzed urine samples of some eight volunteers.

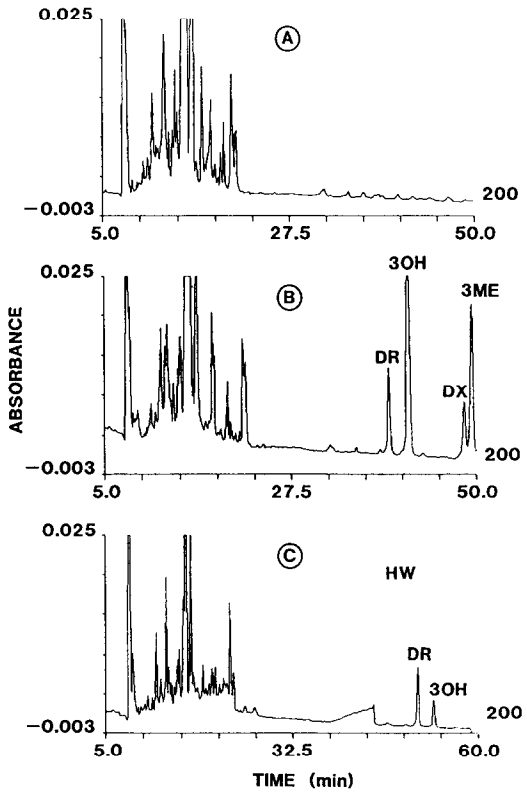

Fig. 6. MECC (200 nm) measured with the Europhor of (A) urine blank, (B) urine blank spiked with 3ME (*ca.* 40 μg/ml), DX (*ca.* 10 μg/ml), 3OH (*ca.* 80 μg/ml) and DR (*ca.* 30 μg/ml), and (C) a hydrolyzed urine of test person HW. The applied voltages were 30 kV (panels A,B) and 28 kV (panel C). The currents were *ca.* 88 μA (A,B) and 77 μA (C). The key for the substances is the same as for Fig. 4.

3.3. Simultaneous phenotyping with mephenytoin and dextromethorphan using MECC

The data presented in Figs. 3 and 6 suggest that it should be possible to monitor 4-hydroxymephenytoin and the morphinans in the same MECC run, via direct injection of hydrolyzed urine specimens. For that purpose, mephenytoin and dextromethorphan were coadministered and the 0–8 h urine was collected. Enzymatically hydrolyzed urine was analyzed by MECC with the buffer containing isopropanol (8%, v/v). Electropherograms obtained with the specimens of two individuals are presented in panels A and B of Fig. 7. With this buffer, 4-hydroxymephenytoin was found to elute earlier than most endogenous compounds (in a similar fashion as shown in Fig. 3), and dextrorphan and 3-hydroxymorphinan were found to elute at the end of the elution range (as in Fig. 6). Panels C and D depict normalized and background corrected absorbance spectra for 4-hydroxymephenytoin and dextrorphan, respectively, which were extracted from the data of Fig. 7A and compared to those of a computer-stored

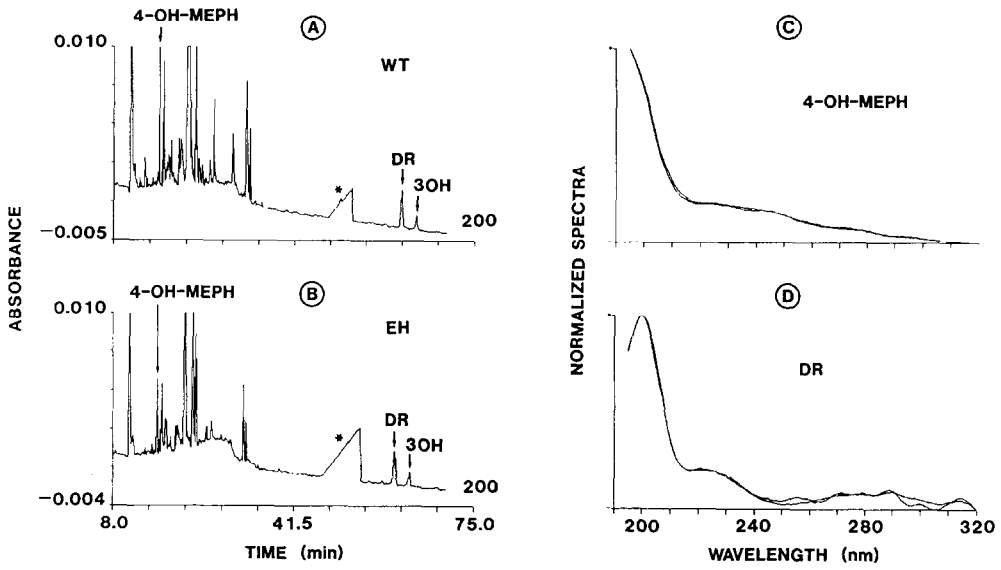

Fig. 7. MECC (200 nm) measured with the home-made setup of two enzymatically hydrolyzed urines [test persons: (A) WT and (B) EH] which were collected after coadministration of mephenytoin and dextromethorphan. The applied voltage was 20 kV and the current *ca.* 62 μA. Panels C and D depict spectral identity proofs for 4-hydroxymephenytoin (4-OH-MEPH) and dextrorphan (DR), respectively. The zones marked by asterisks are detected after enzymatic deglucuronidation only.

model run. There is good agreement between the spectra, thus permitting unambiguous identification of the compounds. Furthermore, detection of 4-hydroxymephenytoin and dextrorphan, as well as absence of detectable dextromethorphan, reveals the EM status for both compounds. The two individuals are the same as those analyzed before by HPLC (Figs. 2D,E and 4C,D), showing good agreement of the two techniques in the assignment of phenotype status for the two test persons. The major difference between phenotyping with MECC and HPLC is that with the former method a hydrolyzed urine specimen can be injected directly and the analytes of interest can be monitored in a single run, while with the latter one two different assays with different extractions have to be employed.

A similar electrophoretic pattern was obtained using the cyclodextrin-modified buffer of Desiderio *et al.* [16]. The electropherogram presented in Fig. 8 was obtained with the same sample as that used for Fig. 7A, but with a buffer composed of 5.6 m*M* sodium tetraborate and 8.4 m*M* disodium hydrogen phosphate (titrated to pH 9.10 with phosphoric acid), 95 m*M* SDS, 40 m*M* β-cyclodextrin and 8% (v/v) 2-propanol. It is important to note that with this buffer the specific detection of the metabolized *S*-4-hydroxymephenytoin is achieved [16]. Due to the stereoselective hydroxylation of mephenytoin, however, both MECC assays (data of Figs. 7 and 8) provide the required information for simultaneous phenotyping with mephenytoin and dextromethorphan.

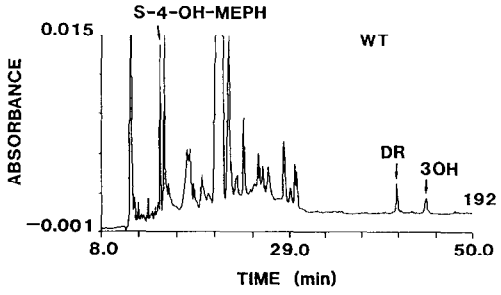

Fig. 8. β-Cyclodextrin modified MECC (192 nm) measured with the Europhor at pH 9.1 of a hydrolyzed urine specimen of test person WT (coadministration of mephenytoin and dextromethorphan). The applied voltage and current were 20 kV and 48 μA, respectively. The key is the same as for Fig. 7.

3.4. Simultaneous phenotyping with caffeine, mephenytoin and dextromethorphan using MECC

According to the procedure of Guo and Thormann [15], four metabolites of caffeine and thereby the acetylation phenotype status can be determined by MECC with direct injection of urine employing a buffer composed of tetraborate and dihydrogenphosphate (16.25 m*M* each) containing 70 m*M* SDS (pH 8.6). This buffer composition was used for the investigation of the urine samples which were collected after administration of all three probe drugs. For simplification, AFMU was converted to AAMU prior to analysis [15]. Panels A to C of Fig. 9 show electropherograms obtained with such a specimen. Simplest determination of the phenotype status is by peak-height ratio of AAMU and 1X. For the data of Fig. 9B a value slightly lower than unity was obtained, thus revealing a slow acetylator phenotype status for this test person which was in agreement with the data obtained previously by MECC and HPLC (test person WT, [15]). Application of the hydrolyzed urine of the same individual provided the data presented in panels D to F of Fig. 9. Again, AAMU and 1X were unambiguously detected together with the prediction of a slow phenotype status (panel F). Furthermore, 4-hydroxymephenytoin was found to elute interference-free between AAMU and 1X. Its presence revealed the EM status which was previously determined by HPLC (Fig. 2E) and MECC (Fig. 7A). Thus, these data suggest that application of hydrolyzed urine permits simultaneous phenotyping with

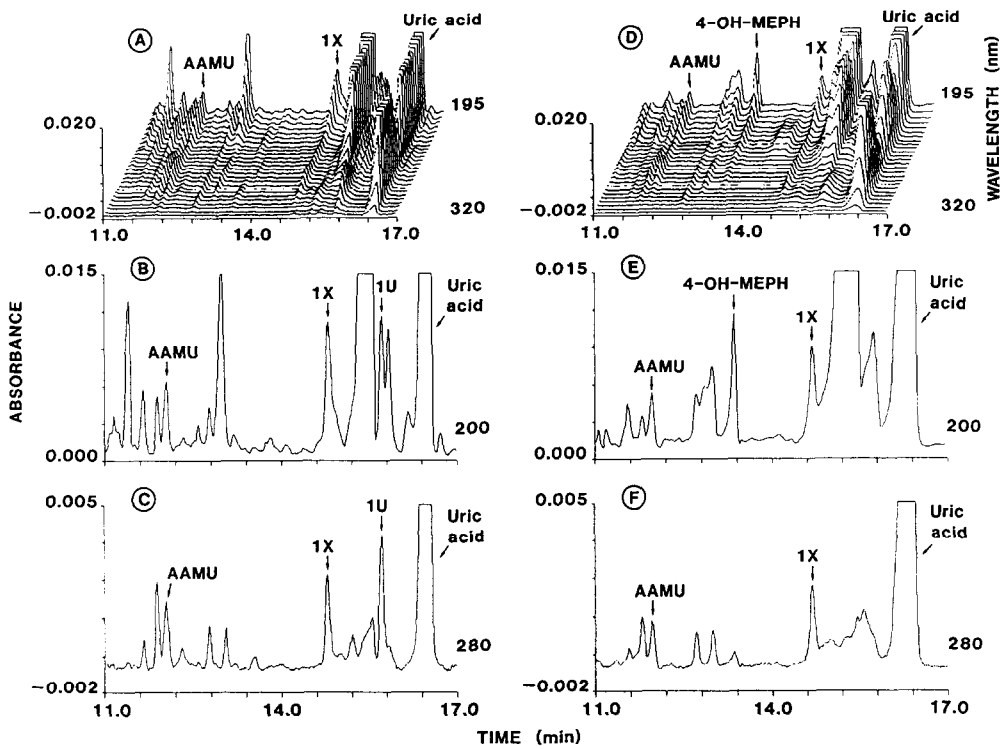

Fig. 9. Simultaneous screening for caffeine and mephenytoin metabolites in the 4–8 h urine collected after coadministration of three probe drugs (test person: WT). Multiwavelength (panels A and D) and single-wavelength MECC (panels B,C,E,F) obtained on the home-made instrument after injection of (A–C) plain urine and (D–F) hydrolyzed urine. A pH 8.6 buffer was used [15]. The applied voltage and current were 20 kV and 96 μA, respectively.

caffeine and mephenytoin. Technically, analysis of dextromethorphan metabolites with such a buffer would also be possible. However, elution times of the morphinans would be unattractively high, *i.e.* much beyond 50 min with the instrumental setup used to produce the data shown in Fig. 9. Therefore, for simultaneous phenotyping with caffeine, mephenytoin and dextromethorphan as test drugs, the suitability of other buffer systems was explored.

For analysis of 4-hydroxymephenytoin, AAMU, 1X and the morphinans in hydrolyzed urine within a shorter time-period, the buffer pH had to be increased. Thus far, best results were obtained with a 75 m*M* SDS buffer of pH 10 containing 6% (v/v) isopropanol. This buffer was prepared by adding NaOH (1 *M*) to the buffer commonly used in our laboratory (*cf.* Experimental). The electropherograms shown in Fig. 10 were obtained with the same urine specimen as that of Fig. 9 (4–8 h urine). As before in other buffers (Figs. 7 and 8), the data obtained allowed unambiguous identification of 4-hydroxymephenytoin at the beginning of the elution pattern and dextrorphan and 3-hydroxymorphinan at the end of the elution range (panel A). Allocation of AAMU and 1X was possible as well, this, however, not without use of spectral analysis of the eluting peaks. For these two compounds, incomplete resolution from other substances was noted, this being illustrated by the data having elongated time scales which are depicted in panels C and D of Fig. 10. On the other hand, the slow acetylation phenotype status of this individual is fully recognizable. Similar data were obtained with the urine specimen collected 0–4 h after drug administration, as well as with those of another test person (data not shown). Thus, with a single injection of a hydrolyzed urine, simultaneous determination of acetylation and hydroxylation polymorphisms in man appears possible. However, the elucidation of the acetylator phenotype status is clearly more difficult than the two hydroxylation polymorphisms. For that task, multiwavelength zone detection appears to be mandatory.

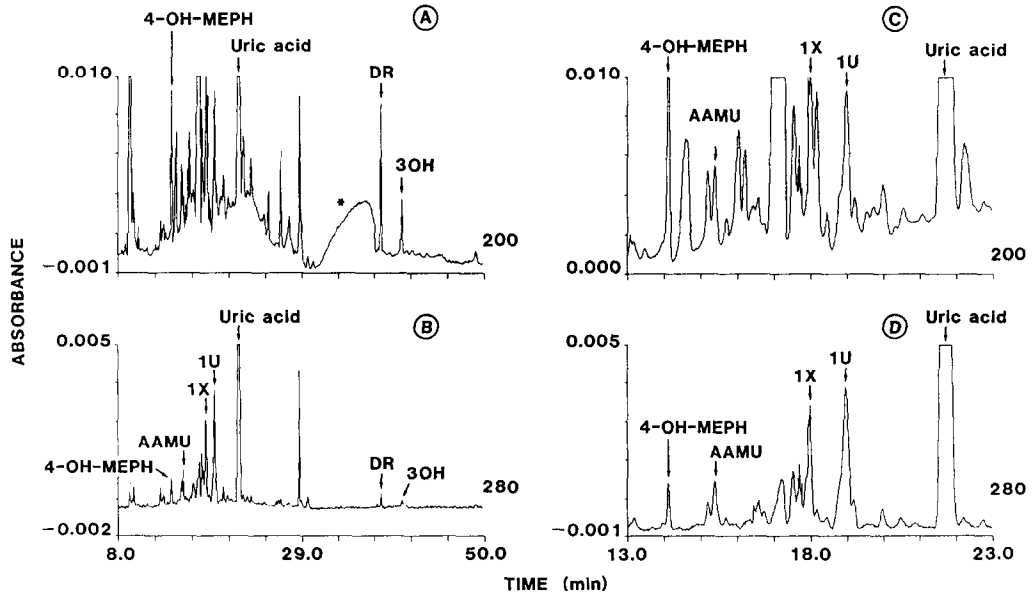

Fig. 10. Simultaneous phenotyping with three probe drugs by MECC at pH 10 (home-made instrument) and application of hydrolyzed urine (4–8 h urine of test person WT). The data shown in panels A and B depict complete electropherograms obtained at 200 and 280 nm, respectively. Panels C and D represent sections with elongated time scales of the data of panels A and B, respectively. The applied voltage and current were 20 kV and 75 μA, respectively. Other conditions as for Fig. 7.

4. Conclusions

The MECC data discussed in this paper show that the compounds required for phenotyping with mephenytoin, dextromethorphan and caffeine can be monitored by MECC with direct injection of enzymatically hydrolyzed urine. Detection times were found to be somewhat matrix dependent with the relative reproducibilities of the detection times being 1–3% ($n = 5$). Thus, an analyte is typically not only identified by its position within the electropherogram, but also by multiwavelength detection and comparison of normalized absorbance spectra. It is important to realize that for MECC analysis no sample extraction or derivatization is required, steps which are customary for chromatographic assays. Depending on the instrumental setup and probe drugs used, MECC analysis for combined phenotyping requires less than 50 min (Figs. 8–10). So far, no optimization has been undertaken to minimize the time for an MECC analysis via use of shorter capillaries and higher applied voltages. Assays based on MECC are shown to allow simultaneous and unambiguous phenotyping with mephenytoin and dextromethorphan or mephenytoin and caffeine. With the data gathered until now, simultaneous determination of all three polymorphisms with a single injection of an enzymatically hydrolyzed urine is possible via use of multiwavelength absorption detection only. Phenotypes determined by electrokinetic capillary techniques are in agreement with those obtained with customary assays based on high-performance liquid chromatography. The MECC assays are simple and when executed on an automated instrument attractive for phenotyping on a large scale.

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6. References

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