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Simultaneous enantioselective determination and quantification of dimethindene and its metabolite *N*-demethyl-dimethindene in human urine using cyclodextrins as chiral additives in capillary electrophoresis

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Abstract: Capillary electrophoresis (CE) has been used for the chiral resolution of the basic racemic drug dimethindene (Fenistil®) and its metabolite *N*-demethyl-dimethindene. After oral administration of 4 mg dimethindene maleate the enantioselective excretion in human urine was determined by capillary electrophoresis using a 50 mM phosphate run buffer (pH 3.3) containing 30 mM hydroxypropyl-β-cyclodextrin. The determination of very low concentrations of dimethindene and *N*-demethyl-dimethindene in human urine was possible using sample stacking conditions to increase the amount of analyte in the capillary with good peak forms and resolutions. The detection limit of the method was approximately 1 ng ml^{-1} urine for each compound.

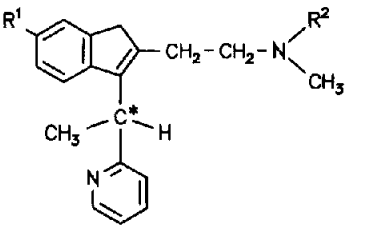
Keywords: *Dimethindene; urine samples; capillary electrophoresis; cyclodextrins; chiral.*

Introduction

Enantiomer resolution is an important field in analytical chemistry, especially in pharmaceutical analysis. About 88% of the synthetic chiral drugs are sold as racemates [1], while often one of the enantiomers is more active than the other or is responsible for inadvertent side effects. For the investigation of the metabolism of racemic therapeutic agents, GC [2] and HPLC [3] are widely used. During the last few years the new technique of capillary electrophoresis (CE) [4] has become a powerful tool, including applications for chiral analysis [5]. Enantiomeric separations can be achieved using chiral buffer additives such as cyclodextrins (CD) [6, 7].

The metabolism of dimethindene maleate, *N*,*N*-dimethyl-3-[1-(2-pyridinyl)ethyl]-1-H-indene-2-ethanamine maleate, has been studied by Radler and Blaschke [8] and Prien and Blaschke [9] using protein and cellulose based chiral stationary phases for enantiomeric separations by HPLC.

In order to avoid the time-consuming achiral HPLC separation of *N*-demethyl-dimethindene (**1**) and dimethindene (**2**; Fig. 1) with a reversed-phase column followed by chiral analysis of the collected samples of **1** and **2** with an α_1-acid glycoprotein column, a sensitive and rapid method has been developed to monitor the stereoselective metabolism of dimethindene in human urine samples by CE [10, 11]. The cumulative excretion of **2** and **1** in human urine will be given as well as the determination of the enantiomeric ratios of **1** and **2**.



Substance	No.	R_1	R_2
N-demethyl-dimethindene	1	H	H
dimethindene	2	H	CH_3
6-methoxy-dimethindene	3	OCH_3	CH_3

Figure 1
Structure of dimethindene (**2**), *N*-demethyl-dimethindene (**1**) and the internal standard 6-methoxy-dimethindene (**3**).

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To separate **2**, its metabolite, **1**, and the internal standard (IS) 6-methoxy-dimethindene (**3**) a run buffer containing 30 mM hydroxypropyl-β-cyclodextrin was used. A number of parameters like cyclodextrin type and concentration, buffer pH, applied voltage and temperature of the capillary have been investigated toward their influence upon selectivity and resolution of the compounds. Those results have been reported elsewhere [12, 13] together with a number of different chiral drugs separated into the enantiomers.

Experimental

Chemicals and reagents

Dimethindene maleate was obtained from Zyma (Nyon, Switzerland). *N*-Demethyldimethindene, 6-methoxydimethindene and the enantiomers of dimethindene (*S*-(+)-**2**/*R*-(−)-**2**) were prepared by Radler [8]. Hydroxypropyl-β-cyclodextrin (HP-β-CD, molar substitution (MS) = 0.9) was a kind gift of Wacker-Chemie GmbH (München, Germany). KH_2PO_4, Na_2HPO_4, H_3PO_4, NaOH (all analytical grade) were purchased from Merck (Darmstadt, Germany).

Apparatus for CE

A Beckman P/ACE 2100 CE system (Beckman Instruments GmbH, München, Germany) was used with an untreated fused silica capillary (Grom, Herrenberg, Germany) of 60 cm effective length and 50 μm i.d.

Standard operating conditions: constant voltage, 26.8 kV (400 V cm^{-1}); temperature, 20°C; sample introduction, low pressure, 35 s; detection, UV 200 nm; anode and cathode buffers, 100 mM phosphate buffer pH 3.3; run buffer, 50 mM phosphate buffer pH 3.3 containing 30 mM HP-β-CD.

A Grom CE system 100 (Grom, Herrenberg, Germany), equipped with a HP 3396 A integrator (Hewlett-Packard Co., Avondale, USA) and a Linear UVIS 200 detector (Linear Instruments Co., Reno, USA) was used with an untreated fused silica capillary of 40 cm effective length, 50 μm i.d. Standard operating conditions: constant voltage, 400 V cm^{-1}; temperature, ambient temperature, 21 ± 3°C; sample introduction, hydrostatic 10 cm, 30 s; detection, UV 205 nm; anode and cathode buffers, 50 mM phosphate buffer pH 3.3; run buffer, 50 mM phosphate buffer pH 3.3 containing 30 mM HP-β-CD.

Buffer and sample preparation

Buffers. A 500 mM KH_2PO_4 buffer stock solution was prepared in double distilled, deionized water, filtered and degassed in an ultrasonic bath. Run and electrode buffers were prepared by dilution to the appropriate molarity and the pH 3.3 was adjusted with H_3PO_4 or NaOH of the same molarity. Electrode buffers were used without CDs, the run buffers contained 30 mM HP-β-CD and were filtered after solution through a 0.46 μm millipore filter.

Standard solutions. Stock solutions of the racemic drugs were prepared in 5 mM phosphate buffer (pH 3.3), stored at 4°C and were diluted with 5 mM phosphate buffer to the appropriate concentration before use.

Blank urine. A blank pooled urine sample collected from human volunteers known to drink coffee or tea, and to smoke, but not taking any drugs, was taken to prepare spiked urine samples.

Urine collection after drug administration. Four milligrams of dimethindene maleate were administrated on an empty stomach in the form of four Fenistil® dragées (*ca* 30 ng dimethindene/kg). To increase the excretion of the basic drug, the urine was acidified by oral administration of a total of 3.0 g NH_4Cl divided into six doses over the period of excretion. Urine was collected during the following intervals: 0 h = blank urine, 0–1, 1–2, 2–3, 3–4, 4–5, 5–10, 10–13 and 13–24 h. The samples were stored frozen at −20°C until analysed.

Extraction of urine samples

Ten millilitres of urine were adjusted to approximately pH 9–10 by the addition of 500 μl NH_3. Fifty microlitres of a solution of **3**; (11.25 μg racemate/ml in 5 mM phosphate buffer pH 3.3) were added to each urine sample, resulting in a final concentration of 28 ng enantiomer/ml urine. The samples were extracted twice using 4 ml *n*-hexane by shaking for 25 min. The organic phases were separated by centrifugation (20 min at 3000*g*), removed, combined and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 μl 5 mM phosphate buffer (pH 3.3), 20 μl were transferred into microvials for pressure injection.

Calibration curve, assay precision and recovery from urine

Standard solutions of **1** and **2** were combined and diluted to reach a final concentration of 0.5–15 μg enantiomer/ml in 5 mM phosphate buffer pH 3.3. These solutions were taken to investigate the linearity of the detector signal of the Grom and the Beckman System as well as to compare the repeatability of subsequent sample introductions. In this case no internal standard was used.

To 10 ml of blank urine 46, 93, 187, 375 and 750 ng of **1**, 44, 88, 177, 355 and 711 ng of **2** and 562 ng of **3** were added and extracted as described above. Each of the concentrations were prepared four or five times. The calibration curve for each enantiomer was obtained by plotting the ratios of the peak areas of the enantiomers of **1** or **2** to the area of the first migrating enantiomer of the IS (**3**) against the concentration. The recovery of **1** and **2** was measured as individual percentages of these data against the ratios of area **1** or **2**/area IS obtained from pure solvent.

Results and Discussion

To decrease the detection limit we used sample stacking conditions. Buffers of higher ionic strength were used as electrode (100 mM) and run (50 mM) buffers, and buffers of low ionic strength were taken to prepare the sample solutions (5 mM). Under an applied voltage the analytes, introduced with the buffer of low ionic strength into the capillary, will migrate fast until they reach the higher concentrated run buffer. In this way the analytes are compressed. Better resolutions and an enhanced selectivity are obtained (Fig. 2) by using this technique [14].

For the quantitative determination of **2** and **1** the P/ACE 2100 was used. Table 1 shows the intra-day repeatability for subsequent injections of (+)-**1** in a concentration range of 0.7–14 μg ml^{-1}. The samples were injected under pressure for 35 s (P/ACE 2100) or by hydrodynamic flow, 10 cm, 30 s (Grom). The relative standard deviations varied from 7.4 to 16.6% in the case of the Grom system. This

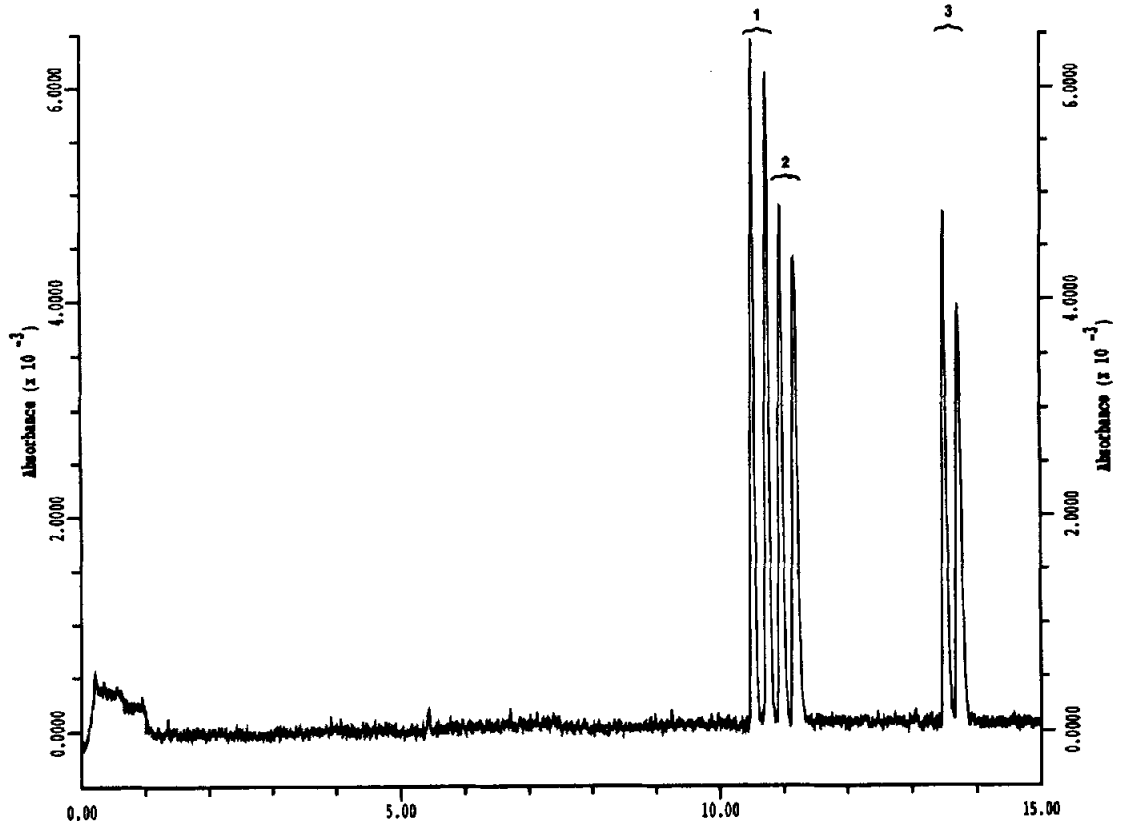

Figure 2
Electropherogram of a standard solution of *N*-demethyl-dimethindene (**1**), dimethindene (**2**), and the internal standard 6-methoxy-dimethindene (**3**). Concentrations: **1**, 15 μg ml^{-1}; **2**, 14 μg ml^{-1}; and **3**, 11 μg ml^{-1}.

Table 1
Intra-day repeatability of subsequent sample introductions using two different capillary electrophoresis systems

Grom system				P/ACE 2100			
Concentration (+)-3 (μg ml^{-1})	AVG*	SD	RSD	Concentration (+)-3 (μg ml^{-1})	AVG*	SD	RSD
13.23	140918	14342	10.18	11.34	0.50329	0.0124	2.46
6.61	59912	4420	7.38	5.67	0.25916	0.0017	0.64
3.31	31494	3506	11.13	2.83	0.13184	0.0029	2.84
1.65	16424	2360	14.37	1.42	0.06534	0.0020	3.10
0.83	8400	1401	16.68	0.71	0.03341	0.0011	3.36

*n = 5.

Table 2
Intra-day and inter-day precision of the method in case of *N*-demethyl-dimethindene (**1**)

Effective concentration μg ml^{-1}	Mean ratio of peak areas		SD		RSD (%)	
	S-(+)-2	*R*-(−)-2	*S*-(+)-2	*R*-(−)-2	*S*-(+)-2	*R*-(−)-2
Within-run (n = 5)						
14.29	2.139	2.184	0.031	0.039	1.77	1.80
7.15	1.181	1.213	0.040	0.048	3.38	3.96
3.57	0.562	0.563	0.018	0.017	3.14	3.04
1.79	0.308	0.308	0.022	0.020	7.09	6.46
0.89	0.154	0.151	0.010	0.009	6.56	6.21
Between-day (n = 10)						
14.29	2.178	2.226	0.076	0.080	3.50	3.58
7.15	1.158	1.192	0.056	0.067	4.86	5.60
3.57	0.569	0.573	0.027	0.025	4.66	4.43
1.79	0.295	0.297	0.021	0.020	7.24	6.80
0.89	0.144	0.149	0.013	0.009	9.15	6.33

might be caused by changes in the ambient air temperature, which is used for capillary cooling, as well as in many manual steps during the sample introduction to the capillary. The repeatability of the automatic sample introduction in the case of the P/ACE 2100 (0.6–3.4%) can be considered to be satisfactory.

Linearity against internal standard

An internal standard can be used for quantitative analysis to minimize the errors during the extraction procedure. A suitable one was found in the closely related **3**, showing similar extraction behaviour and migration times like **1** and **2**. The resolution from the enantiomers of **1** and **2** was complete as well as the enantioseparation of **3** could not be detected as a metabolite in urine [15]. Therefore it was possible to use the first migrating enantiomer of **3** as IS (Fig. 2).

Linearity against the IS is given in the range of 0.5–15 μg ml^{-1} for **1** and **2**. Table 2 shows the intra-day and inter-day precision of the method.

Blank urine samples were spiked with **1** and **2** in a concentration range of 4–75 ng enantiomer/ml and the IS **3** (56 ng ml^{-1}). Each sample was prepared four times and analysed after extraction on different days. The extraction with *n*-hexane resulted in samples free of interferences (Fig. 3). The results are listed in Table 3 together with the data of recovery. The calibration curves for the quantitative analysis of each enantiomer of **1** and **2** in urine are given together with the correlation coefficients in Table 4 and indicate good linearity of these data.

Detection limit

The detection limits for the drugs were calculated from electropherograms using the extracts from human blank urine samples spiked with **1** and **2**. The detection limits of the method were approximately 1–2 ng ml^{-1} urine for all compounds with a signal to noise ratio of 3. For detection at 200 nm, the concentration of the drugs in the sample solution therefore had to be in the range of 0.3–0.4 μg ml^{-1}.

Enantiomeric composition after extraction

In contrast to chromatography, in CE different analytes do not pass the detection window with the same velocity. Thus the slower compound will give a longer detector response

Table 3
Linearity of the signal after extraction from urine and recovery of the enantiomers of *N*-demethyl-dimethindene (**1**) and dimethindene (**2**)

Amount added (ng ml^{-1})	Mean ratio of areas*	SEM	Recovery (%)	Mean ratio of areas*	SEM (ng ml^{-1})	Recovery (%)
	S-(+)-Dimethindene			*R*-(−)-dimethindene		
71.1	2.108	0.019	72.90	2.141	0.020	72.92
35.6	1.042	0.008	72.10	1.068	0.012	72.79
17.8	0.547	0.002	75.67	0.559	0.004	76.11
8.9	0.282	0.006	77.98	0.273	0.011	74.78
4.5	0.124	0.009	68.86	0.135	0.012	73.55
	S-(+)-*N*-demethyl-dimethindene			*R*-(−)-*N*-demethyl-dimethindene		
75.0	1.683	0.033	53.17	1.715	0.032	55.00
37.5	0.780	0.037	49.26	0.785	0.075	50.34
18.8	0.493	0.040	62.27	0.489	0.040	62.73
9.4	0.222	0.005	56.10	0.221	0.016	56.69
4.7	0.125	0.014	63.00	0.142	0.010	72.67

*$n = 4$.

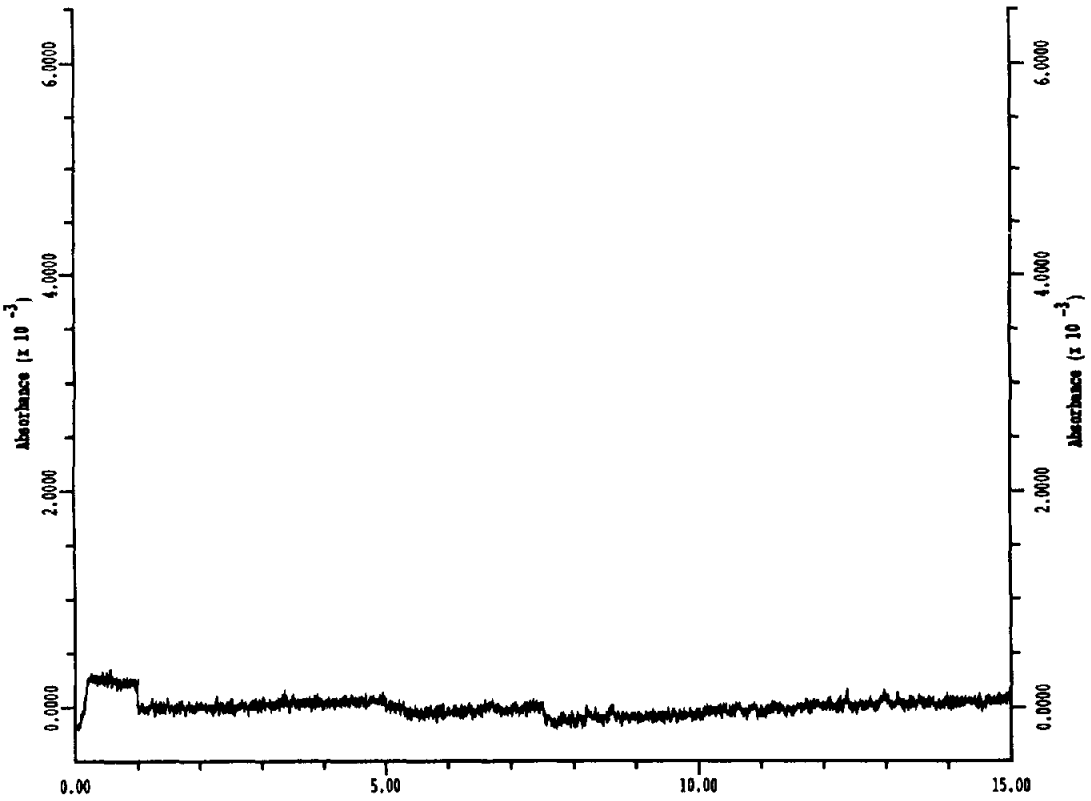

Figure 3
Blank urine sample after the extraction procedure with *n*-hexane.

resulting in larger peak areas. For validation, pure enantiomers of **2** [8] were mixed in a range of 20:80 up to 80:20. As Table 5 shows there is no significant difference between the enantiomeric composition before and after extraction of **2** from urine. So we can state that there are no matrix effects with influence to the enantiomeric composition of the urine samples. The elution order was found to be *S*-(+)-**2** before *R*-(−)-**2**.

Cumulative excretion curves and enantiomeric ratio

The cumulative excretion curves of the enantiomers of **1** and **2** in urine after oral administration of 4 mg dimethindene maleate

Table 4
Calibration curves in the range of 5–75 ng enantiomer/ml urine for the determination of *N*-demethyl-dimethindene (**1**) and dimethindene (**2**) in urine. Analysis was performed using the first migrating enantiomer of 6-methoxy-dimethindene (**3**) as IS. The concentrations of **1** and **2** are the same as in Table 3. The equation is $y = ax + b$, where a is the slope and b is the intercept on the y-axis, r the correlation coefficient

Compound	a	b	r
(+)-**3**	0.02184	0.02570	0.99342
(−)-**3**	0.02219	0.02530	0.99349
(+)-**4**	0.02948	0.00825	0.99953
(−)-**4**	0.02997	0.00924	0.99947

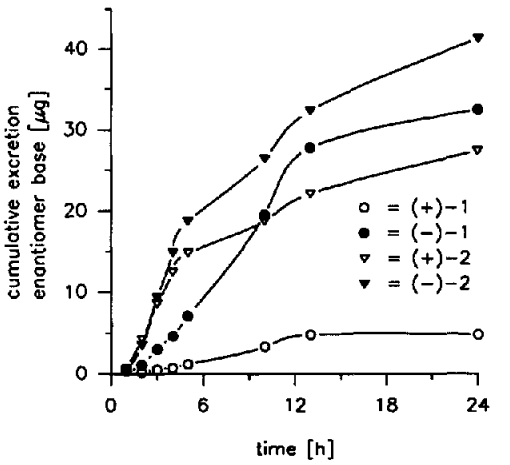

Figure 4
Cumulative excretion of the enantiomers of dimethindene (**2**) and *N*-demethyl-dimethindene (**1**) in human urine over a period of 24 h.

Table 5
Enantiomeric composition of pseudoracemates before and after extraction from urine

(+)-**2**-Debit (%)	(+)-**2**-Before extraction (%)		(+)-**2**-After extraction (%)	
	AVG*	SD	AVG*	SD
80	79.94	1.098	79.77	0.760
70	70.12	0.129	70.41	0.645
60	60.12	0.660	61.75	0.392
50	50.65	0.155	51.77	0.415
40	41.72	0.706	42.57	0.306
30	29.37	0.635	29.02	0.324
20	19.70	0.660	20.06	0.122

*$n = 4$.

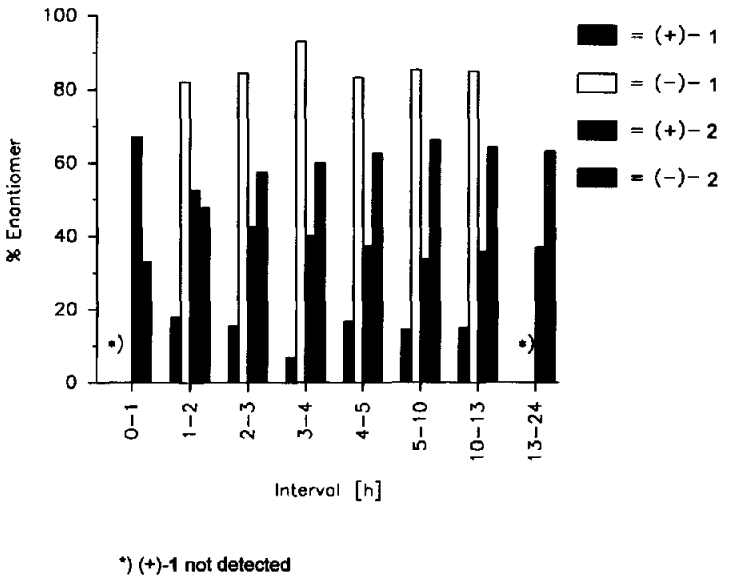

Figure 5
Enantiomeric composition of dimethindene (**2**) and *N*-demethyl-dimethindene (**1**) in human urine over a period of 24 h in % enantiomer.

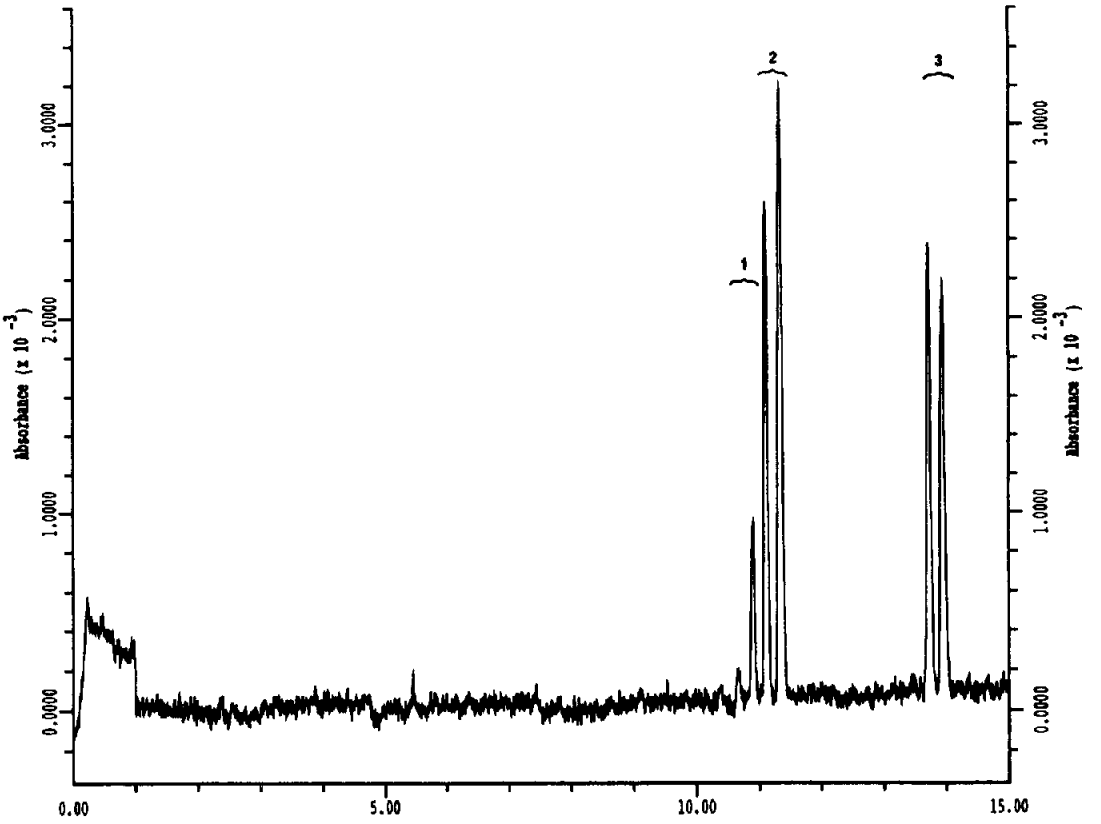

Figure 6
Urine sample 3 h after oral application of 4 mg dimethindene maleate.

to a healthy volunteer are shown in Fig. 4. After a period of 24 h approximately 1.3% of the applied dose was excreted as **1**, about 2.4% as unmetabolized **2**. The enantiomeric composition of **1** and **2** in urine is presented in Fig. 5. The excretion of *S*-(+)-**2** dominates during the first 2 h, followed by an increase of the concentration of *R*-(−)-**1** reaching a nearly constant ratio of (+):(−) 40:60 after 5 h. Figure 6 shows a urine sample 3 h after application. The obtained values, both the enantiomeric ratios and the absolute concentrations, are comparable to those determined by HPLC [8, 9].

Conclusion

Capillary electrophoresis is a valuable alternative to liquid chromatography for the determination of basic drugs in human urine. The described assay is sensitive, reproducible and suitable for the rapid simultaneous quantification and determination of the enantiomeric ratios of dimethindene and *N*-demethyldimethindene in human urine. Baseline resolutions are obtained in <13 min with high efficiency. In comparison to the HPLC methods [8, 9] nearly the same limit of detection was achieved by this CE method. However, the limit of detection probably could be improved by, for example, a preconcentration electrophoretic step or Z-shaped detection cells.

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