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Determination of phase II drug metabolites in equine urine by micellar electrokinetic capillary chromatography

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Abstract

Micellar electrokinetic capillary chromatography (MECC) using diode array detection has been investigated for the determination of phase I and phase II metabolites of drugs in biofluids. Methods were optimised for the determination of morphine, morphine-3-glucuronide, morphine-6-glucuronide, normorphine, meclofenamic acid and its metabolites in equine urine. Solid-phase extraction procedures were developed to concentrate and purify the analytes from spiked and post administration urines for MECC analysis. A simple on-line procedure for monitoring the kinetics of hydrolysis of morphine-glucuronide conjugates by β-glucuronidase was demonstrated.

Keywords: Morphine; Meclofenamic acid; Glucuronides; Non-steroidal anti-inflammatory drugs

1. Introduction

Drug metabolites are most often studied by HPLC which is excellent for identifying, quantifying and isolating the parent drug and some types of metabolites. However, phase II metabolites, e.g. glucuronide and sulphate conjugates, are acidic and highly polar and frontally elute with little resolution in the reversed-phase systems most commonly used for such separations. Unlike pharmaceutical studies, for forensic purposes it can often be more appropriate to identify metabolites rather than the parent drug, since the additional information yielded by the full metabolic profile of a drug may be important in ascertaining the route of administration. One of the major advantages of CE over alternative separation techniques such as HPLC or GC is its ability to readily separate charged compounds with high efficiency. Because of their inherent polarity, phase II metabolites should be ideal for direct analysis by CE without prior need for derivatisation or hydrolysis, thus simplifying sample preparation.

In addition drug conjugates may be pharmacologically active themselves: for example there has been considerable recent interest in the glucuronide conjugates of the opiate, morphine (M) [1–15]. It is reported [6,8] that morphine-6-glucuronide (M6G) is up to 200 times more potent than M and that morphine-3-glucuronide (M3G) and M6G may be responsible for the different pharmacological effects induced by M [6]. HPLC has been widely used in the analysis of these conjugates [1–6,8–13]. Differential RIA [14], radiometry [7] and GC–MS before/after hydrolysis [15] have also been used for their assay. Recently CE has been used to analyse M3G in human urine [16] and to study the human metabolism of the closely related opioid dihydrocodeine [17]. In both cases, solid-phase extraction (SPE) was

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required to concentrate and purify the sample prior to MECC analysis. The non-steroidal anti-inflammatory drugs (NSAIDs), ibuprofen and flurbiprofen and their phase I hydroxy metabolites, have also been shown by CE–MS [18] to be extensively glucuronidated in man. We have observed, using MECC, that another NSAID, meclofenamic acid, is also phase II metabolised in the thoroughbred racehorse.

When techniques such as HPLC and GC–MS are used to determine phase II metabolites, hydrolysis procedures during sample preparation are unavoidable [15,19]. Drug glucuronides may be derivatised after extraction [20] to reduced polarity, but the derivatisation procedure is complicated and time consuming. Ether conjugates may be cleaved by acid or enzyme catalysed hydrolysis with the latter being the method of choice for acid and heat labile drugs, e.g. morphine [20,21]. Because of the small sample size in CE, it should be possible to monitor enzyme activity on line in instruments with a temperature controlled sample injector.

In this paper, we present general approaches to using MECC to separate phase II metabolites and monitor the enzymatic hydrolysis of the metabolites. We illustrate the approach with methods for morphine and its active metabolites, M3G, M6G and normorphine (NM) and meclofenamic acid and its metabolites.

2. Experimental

2.1. Materials

Bond Elut cartridges, C_2 (500 mg), PRS (100 mg), Certify (300 mg), C_8 (500 mg), C_{18} (100 mg) and SCX (500 mg) were obtained from Anachem (Luton, UK). Isolute cartridges, HAX (300 μg) were obtained from Jones Chromatography (Hengoed, UK).

Morphine (M), morphine-3-β-D-glucuronide (M3G), morphine-6-β-D-glucuronide (M6G), normorphine (N), nalorphine (NAL), and meclofenamic acid (MA) were obtained from Sigma (Poole, UK). Reduced flunixin glucuronide (2[(2-methyl-3-trifluoromethyl)phenyl]amino-3-hydroxyethylpyridine-O-glucuronide) (RFG) was synthesised by Dr. A. Nedderman at the Horseracing Forensic Laboratory Borax (decahydrate), citric acid, sodium citrate, trizma base, ammonium acetate, sodium dodecyl sulfate (SDS) and trifluoracetic acid (TFA) were purchased from Sigma. Acetonitrile, hexane, ethyl acetate and methanol were purchased from Rathburn (Walkerburn, UK). Sodium hydroxide, hydrochloric acid and ammonia solution (specific gravity 0.880) were purchased from BDH (Lutterworth, UK). Arquel granules (5% meclofenamic acid) were obtained from Parke–Davis Veterinary (Pontypool, UK). All reagents were of AnalaR or HPLC grade, drugs were of the highest purity available from the suppliers. β-glucuronidase (type HP2 from Helix pomatia, crude solution) was obtained from Sigma.

Blank urine was collected from thoroughbred racehorses stabled at the Horseracing Forensic Laboratory. Administrations of morphine 0.05 mg kg^{-1} (i.v. as sulfate) and meclofenamic acid 2.4 mg kg^{-1} (orally with food) to 6 and 8 year old geldings, respectively, were performed. Naturally voided urine samples were collected at 96 min post dose (morphine) and 11 h 20 min post dose (meclofenamic acid) and stored at −20°C prior to use.

Standard solutions (1 mg ml^{-1}) of M, NM and NAL (1 mg ml^{-1}) were prepared in methanol and stored at 4°C until used. Standard solutions (1 mg ml^{-1}) of M3G, M6G and RFG were prepared in deionised water and stored at 4°C until used.

2.2. Capillary electrophoresis

The CE apparatus was a ^{3D}CE system with diode array detector (Hewlett–Packard Bracknell, UK). A monitoring wavelength of 195 nm (2 nm bandwidth) was used in studies related to morphine and enzyme kinetics. A monitoring wavelength of 240 nm (2 nm bandwidth) was used in studies related to meclofenamic acid. A reference wavelength of 450 nm (80 nm bandwidth) was used throughout. Spectral data acquisition was performed over the wavelength range 200–400 nm (1 nm step, 100 ms sampling interval). Injection was by pressure, 50 mbar being applied to the sample vial for 3 s and to the run buffer for 1 s prior to application of electric field (470 V cm^{-1}).

Separations were performed in either Polymicro Technologies fused silica capillaries (Composite Metal Services, Hallow, UK) and extended light path

capillaries from Hewlett–Packard. Capillary dimensions were 48.5 cm (40 cm to detector) and 64.5 cm (56 cm to detector) with 50 μm internal diameter, the extended light path capillaries had quoted internal diameter of 150 μm at the detection window. New capillaries were flushed sequentially with NaOH (1.0 *M*), NaOH (0.1 *M*) and run buffer under pressure (900 mbar) for 10, 5 and 5 min, respectively, prior to use and with run buffer (1–4 min) between runs. Capillaries were thermostatted at 25°C. Run buffer was normally 70 m*M* borate pH 9.3 plus 70 m*M* SDS. To improve resolution in separation of post administration urine extracts organic modifiers, acetonitrile and methanol were added (5–10%, v/v). Details are provided in legends to figures.

2.3. Kinetic measurements

Sodium citrate buffer (20 m*M*, pH 5.6) was spiked with standard substrates (M3G, M6G, 50 μg ml^{-1}), placed in a sealed polypropylene vial and incubated in the CE system autosampler which was maintained at 37°C using a circulating waterbath (Radiometer, Crawley, UK). A 1-h temperature equilibration step was performed prior to adding β-glucuronidase (5150 Sigma units ml^{-1}). One Sigma unit liberates 1 μg phenolpthalein from phenolpthalein glucuronide per hour at 37°C at the optimum pH. After enzyme addition, the reaction was monitored by sequential MECC runs at 15 min intervals.

The corrected peak areas (peak area/retention time, a_c) were measured using the Chemstation software. The logarithm of corrected peak area (y axis) with reaction time point (x axis) were subjected to linear regression analysis, and the rate constant (k) and half life of the reaction ($t_{1/2}$) estimated from the slope.

2.4. Solid-phase extraction

All extractions were performed using an IST Vacmaster 20 place manifold (Jones Chromatography), with vacuum set at 10 kPa.

2.4.1. Morphine and metabolites

NAL (1 μg ml^{-1}) was added to all samples as an internal standard. RFG was added to spiked samples as an additional glucuronide internal standard. Urine samples (5 ml) were mixed with Tris–HCl buffer (50 m*M*, pH 7.5, 5 ml) and the pH adjusted to 7.5 using NaOH or HCl, prior to passing through a Bond Elut C_2 column, which had been sequentially conditioned with methanol (2 ml) and Tris–HCl buffer (2 ml). The column was washed with Tris–HCl buffer (2 ml) prior to elution with acetonitrile–water–trifluoroacetic acid (TFA) (50:50:0.1, 2 ml). The eluates were diluted with 0.1% aqueous TFA (8 ml) prior to passing through a Bond Elut PRS column, which had been conditioned sequentially with methanol (0.5 ml), water (0.5 ml) and 0.1% aqueous TFA (0.5 ml). The column was washed sequentially with 0.1% aqueous TFA (0.5 ml) and methanol (2 ml) prior to elution with 3% ammoniacal methanol (1 ml). The eluates were evaporated to dryness in a SF50 centrifugal vacuum evaporator (Genevac, Ipswich, UK), and reconstituted in acetonitrile–water: (10:90, 50 μl) prior to MECC analysis.

2.4.2. Meclofenamic acid and metabolites

Urine (1 ml) was diluted with water (5 ml), acidified to pH 3.0 using HCl, then passed through Isolute HAX columns under vacuum, which had been sequentially conditioned with methanol (2 ml) and 0.1% aqueous TFA (2 ml). The columns were washed sequentially with 0.1% aqueous TFA (2 ml), ammonium acetate buffer (10 m*M*, pH 8, 2 ml) and methanol (5 ml). Elution was performed with 0.1% TFA in acetonitrile (2 ml). The eluates were evaporated to dryness (as above) and reconstituted in acetronitrile–MECC run buffer–water (1:1:10, 50 μl) prior to MECC analysis.

Base hydrolysis was performed by adding NaOH (10 *M*, 20 μl) to urine (1 ml) and incubating in a microwave oven, 650 W, model ER-661E (Toshiba, Tokyo, Japan) for 15 s at maximum power level, prior to extraction as above.

3. Results and discussion

3.1. Capillary electrophoresis

It is our experience that MECC methods are generally more robust than CZE procedures for

analysis of equine urine and urine extracts. We have also observed migration time drift when using phosphate buffers [22]. For the analysis of phase II metabolites, we found that a sodium borate buffer (70 m*M* borate) containing SDS (70 m*M*) provided a rapid and efficient separation of M, MA and their metabolites. Migration time stability between runs was improved when the capillary was flushed with run buffer only for 4 min. Reproducibility and linearity of the MECC method were excellent (Table 1). The MECC method described above was developed with standard mixtures and did not always provide adequate resolution of the analytes from co-extracted compounds from equine urine. The addition of acetonitrile (5%, v/v) improved the resolution of NAL from an interfering peak (Fig. 1C) and the addition of methanol (10%, v/v) to the run buffer resolved a co-eluting metabolite of MA (Fig. 2C).

3.2. Extraction procedures

3.2.1. Morphine and metabolites

Published SPE methods for extraction of M and metabolites from human plasma were evaluated using spiked equine urine. Methods which utilised a hydrophobic retention mechanism on C_{18} or C_8 phases [2,3,5,9,13,16] were found to produce very dirty MECC chromatograms at the wavelength needed to detect the analytes (195 nm). Using 230 nm improved selectivity slightly, but reduced overall sensitivity.

We could not reproduce the SPE method of Chari et al. [4] for M and metabolites in which SCX cartridges were used. This finding agreed with that of Wernly et al. [16]. It is our experience that direct ion exchange mechanisms are of little utility when dealing with a sample matrix of high and variable ionic strength such as equine urine. The method using mixed mode (C_8/SCX) phase gave improved selectivity when compared to procedures based on C_{18} and C_8 phases. This was presumably due to the ability to use a more vigorous washing regime, however, the recovery of the glucuronide conjugates of morphine was low (<20%).

The method of Venn and Mickalkiewicz [1], which utilises a C_2 phase, provided a good recovery (>80%) of M and metabolites. However, the eluent, 50% aqueous acetonitrile containing 0.1% TFA (2 ml), required a long evaporation or freeze drying stage to achieve the necessary concentration of the sample. By first diluting the C_2 eluate with 0.1% aqueous TFA, followed by extraction through a cation-exchange (PRS) phase and eluting with ammoniacal methanol (1 ml), the evaporation time was reduced 10-fold. An added bonus was the additional clean-up of the samples achieved by methanol washing the PRS phase. A similar procedure was attempted by Wernly et al. [16] who applied the SCX method of Chari et al. [4] to extracts from C_8 cartridges. However, problems with irreversible adsorption on the SCX phase were found. This was not a problem with the PRS phase. Precision and recovery of the assay were measured (Table 2) using spiked equine urine (1 μg ml^{-1}, n=5). The assay was more suited to an external standard approach to calibration, as NAL was prone to interference from co-extracted endogenous material. It is possible that recovery may be improved if a larger bed mass PRS column was used. Sensitivity and selectivity may also be improved using fluorescence [3,13] or electrochemical [8] detection rather than UV detection.

The two stage C_2-PRS extraction was used to prepare both spiked and post administration equine urine samples for MECC analysis (Fig. 1). The estimated LOD (S/N=3), established by analysis of extracts of spiked blank urine samples, was 150 ng ml^{-1} for all analytes. This is significantly lower than

Table 1
Precision (25 μg ml^{-1}) and linearity (5–50 μg ml^{-1}, n=5). Data for separation of morphine (M), morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) standards by MECC

Analyte	M	M3G	M6G
Precision (% R.S.D.)			
Retention Time	0.62	0.21	0.22
a_c	5.21	0.01	0.01
Linearity			
Slope ($\times 10^3$)	4.30	4.65	4.24
Intercept ($\times 10^3$)	2.11	4.89	5.10
Residual S.D. ($\times 10^3$)	3.70	6.35	5.30
Correlation	0.999	0.998	0.998

MECC performed in HP fused silica capillary (48.5 cm×50 μm I.D., 150 μm I.D. detection window light path). Buffer=borate (70 m*M*, pH 9.3); SDS (70 m*M*). Injection by pressure (150 mbar s). Field=470 V cm^{-1}. Detection by UV (195 nm). Temperature=25°C.

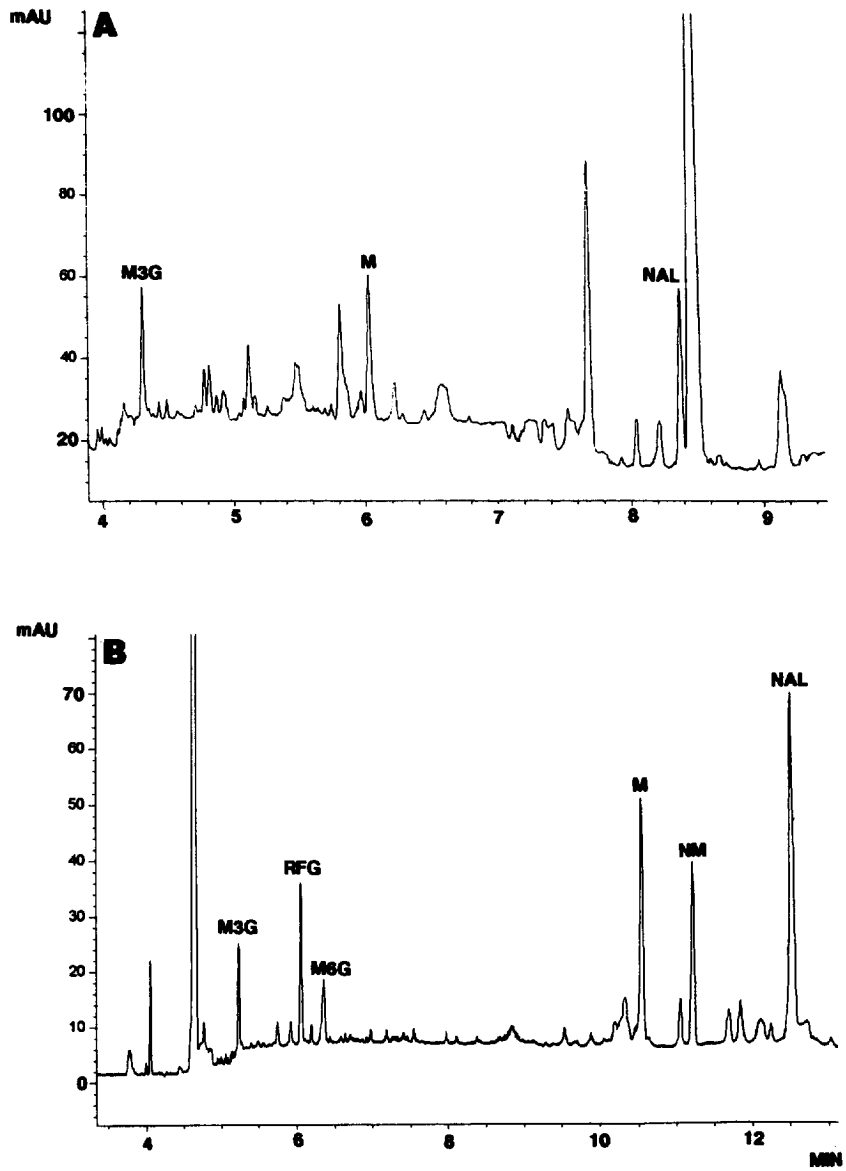

Fig. 1. Chromatograms from MECC analysis of equine urine samples extracted by SPE. A=post administration urine, B=spiked urine (1 μg ml^{-1}). MECC conditions as for Table 1 except A: acetronitrile (5%, v/v) added to run buffer and B: capillary=64.5 cm.

the previously published LOD (1 μg ml^{-1}) for M3G in human urine using MECC with SPE [16]. M3G was detected as the major metabolite of M in a post administration equine urine. Comparison with data from extracts of spiked samples gave an estimated concentration of 1.3 μg ml^{-1}. Unconjugated M was also detected and the concentration was estimated to be 600 ng ml^{-1}. M6G and NM were not detected. Diode array spectra of separated M and M3G were matched to those from a reference standard as an aid to peak identification.

3.2.2. Meclofenamic acid and metabolites

The SPE method of Ashcroft et al. [18], for extraction of NSAIDs and their glucuronide metabolites from human urine prior to CE–MS, was applied

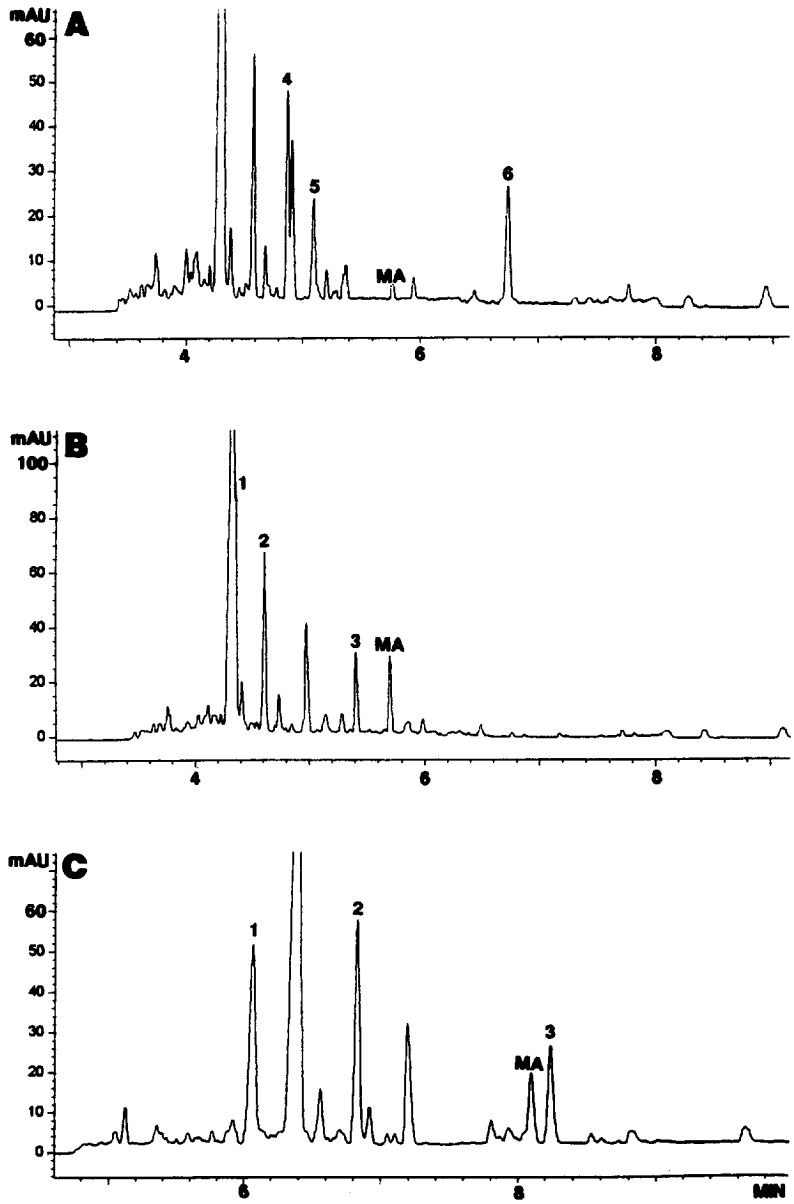

Fig. 2. Chromatograms from MECC analysis of meclofenamic equine urine samples extracted by SPE. A=non-hydrolysed urine and B, C=base hydrolysed urine. MECC conditions as in Table 1 except C=methanol (10%, v/v) added to run buffer: 1, 2, 3=phase I metabolites of MA; 4, 5, 6=Phase II metabolites of MA.

to equine urine post administration samples in an attempt to extract meclofenamic acid and metabolites. The extracts could not be analysed by MECC as they contained a large amount of particulate matter, which could not be reconstituted in a sufficiently small volume or effectively removed by filtration.

The method reported here was developed using mixed mode Isolute HAX cartridges. A 100% methanol wash enabled the removal of interferences prior

Table 2
Precision and recovery (n=9) of the SPE–MECC assay of spiked equine urine (1 μg ml^{-1})

Analyte	Assay 1			Assay 2			Recovery (%)
	X	S.D.	R.S.D. (%)	X	S.D.	R.S.D. (%)	
M3G	0.55	0.05	9.09	0.49	0.06	12.2	39.6
M	1.26	0.15	11.9	1.29	0.09	6.98	78.7
NM	0.66	0.06	9.09	0.67	0.09	13.4	55.5
NAL	1.38	0.22	15.9	1.18	0.09	7.63	54.9

Assay 1 (n=5) and assay 2 (n=4) were performed as described in the text using freshly prepared reagents and samples. X=the mean corrected peak area (a_c, area/retention time, mAU s^{-1}×10), S.D.=standard deviation and R.S.D.= relative standard deviation. The recovery was calculated by comparison of mean peak area (n=9) against that from non-extracted standards (n=2).
MECC conditions as detailed for Table 1.

to elution of the analytes. No further clean-up was found to be necessary. Estimated recovery of meclofenamic acid was only 40% (n=2), but the selectivity was very good allowing adequate detection of the minor metabolites (Fig. 2). The recovery of metabolites was not quantitated since no

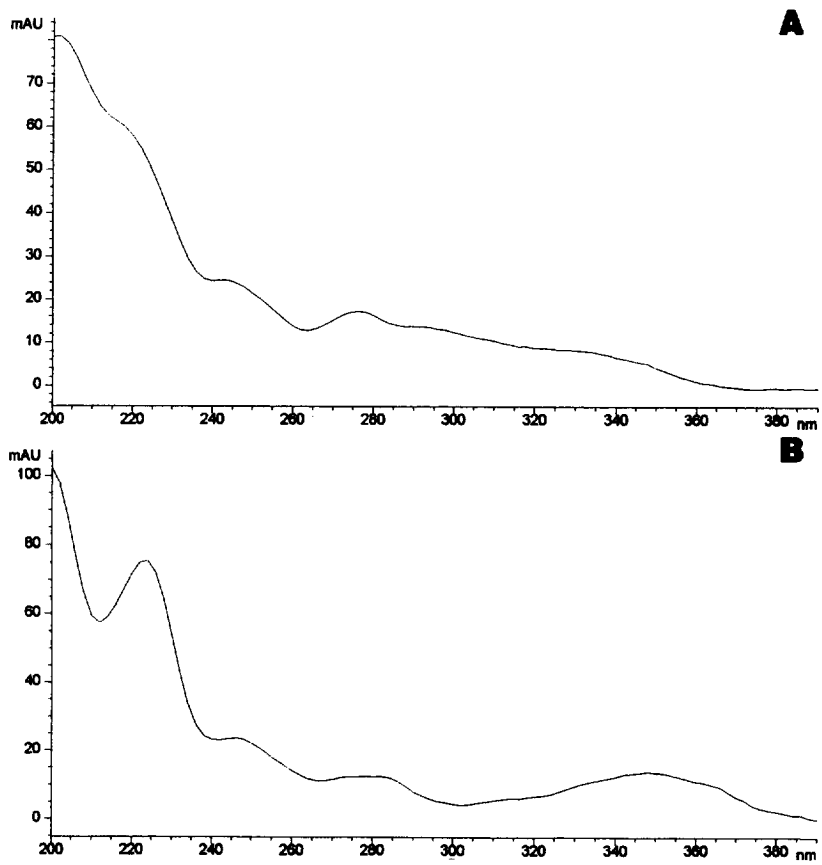

Fig. 3. UV spectra recorded by diode array detector during MECC analysis of MA post administration urine. A=spectrum of unconjugated MA, B=spectrum of conjugated metabolite. MECC conditions as in Table 1.

reference materials were available. A simple base hydrolysis stage using a microwave oven was used to hydrolyse the ester conjugate bonds. Two major phase I metabolites were identified as hydroxy-meclofenamic acid and dihydroxymeclofenamic acid using a published GC–MS procedure [23] after a base hydrolysis step. It is likely that the phase II metabolites are glucuronide conjugates of both free MA and phase I (hydroxy) metabolites, but this has not yet been established. The phase II metabolites of MA were easily identifiable by their UV spectra, which is markedly different from that of the free drug and its phase I metabolites (Fig. 3). The spectra were of good diagnostic value as they were quite unlike the spectra of all co-extracted endogenous compounds.

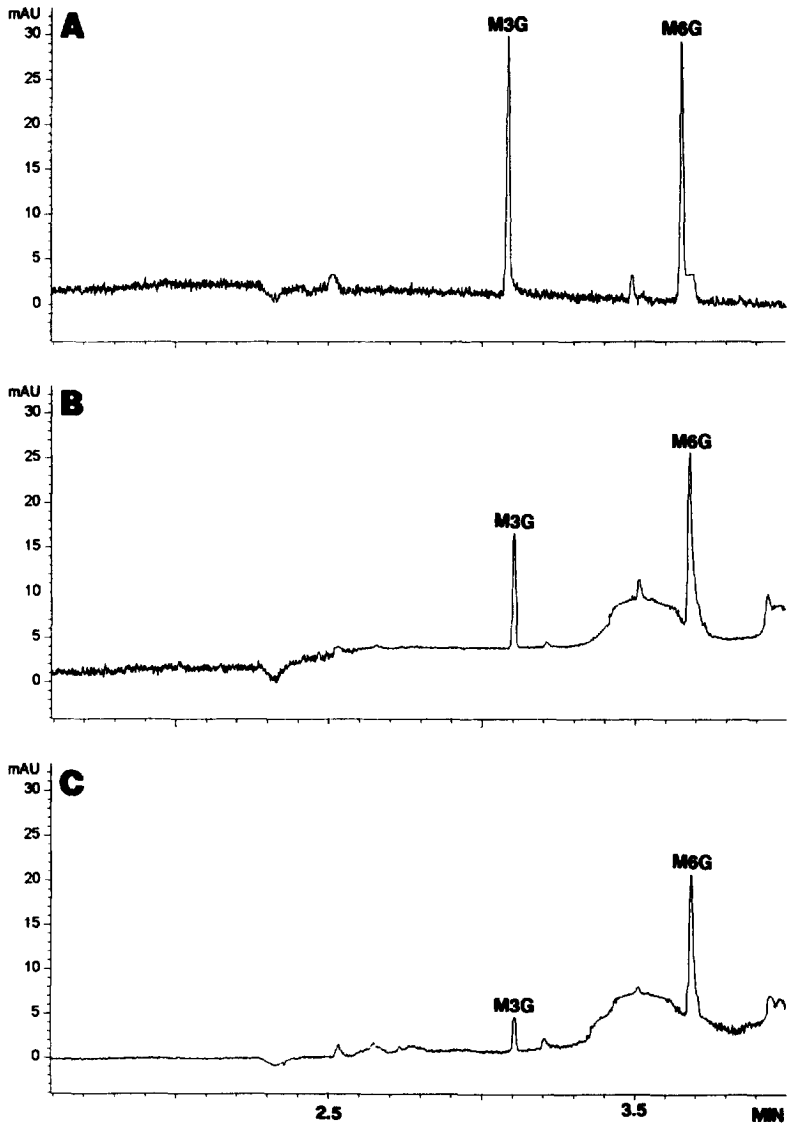

Fig. 4. MECC chromatograms from the hydrolysis of M3G and M6G (initial concentration=50 μg ml^{-1}) catalysed by β-glucuronidase from *Helix pomatia* (5150 Sigma units ml^{-1}). A=standard prior to enzyme addition, B=after 15 min incubation with enzyme at 37°C, C=after 60 min incubation. MECC conditions as in Table 1.

3.2.3. Hydrolysis kinetic measurements

The use of a thermostatted autosampler permitted the simultaneous and on-line monitoring of the hydrolysis of M3G and M6G catalysed by β-glucuronidase enzyme from *Helix pomatia* (Fig. 4). The system was rapid, simple to use and could be operated for long periods of time unattended. Full details of this procedure will be reported elsewhere.

4. Conclusion

We have demonstrated the use of MECC for the analysis of drug conjugates, which have often proved difficult by HPLC. MECC provides a rapid and low cost method for measuring the full metabolic profile of a drug. The low concentration sensitivity of MECC in comparison to techniques such as HPLC and GC–MS may be seen as a disadvantage, but can be overcome by the use of suitable sample preparation procedures, in particular off-line SPE.

In this paper we also demonstrate a simple procedure for the on-line kinetic monitoring of the catalytic activity of β-glucuronidase enzyme derived from Helix pomatia acting upon two conjugated substrates using MECC. This procedure is useful for optimising time consuming sample preparation procedures for a variety of analytical techniques in which enzyme incubation steps are a necessity.

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