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Journal of Chromatography A, 768 (1997) 67–71

JOURNAL OF CHROMATOGRAPHY A

Direct monitoring of enzyme reactions using micellar electrokinetic capillary chromatography
Optimisation of drug glucuronide and sulfate conjugate hydrolysis

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Abstract

This paper demonstrates the use of micellar electrokinetic capillary chromatography (MECC) to monitor enzyme reaction conditions. The hydrolysis reactions of model conjugated substrates (morphine and reduced flunixin glucuronides, napthyl sulfate), by proprietary β-glucuronidase preparations, were studied under varied experimental conditions. Reactions were carried out in autosampler vials with incubation in a thermostatted CE autosampler tray. MECC was performed using borax buffer (17.5 m*M*, pH 9.3) modified with sodium dodecyl sulfate (70 m*M*). Repetitive injections were made from the sample vial throughout the course of the reactions at a frequency of up to 10 h^{-1}. MECC provided a rapid and reproducible assay for the model substrates. Baseline interference from the enzymes prevented measurement of product increase, therefore substrate decrease was measured from the peak areas. Monitoring of reactions in this way has proved valuable in the optimisation of hydrolysis conditions used in sample preparation for drug analysis. β-Glucuronidase preparations from *Helix pomatia* were found to give the best performance of those evaluated in terms of deconjugation efficiency.

Keywords: Glucuronides; Sulfates; Enzymes; Glucuronidase; Morphine; Naphthyl sulfate; Flunixin glucuronide

1. Introduction

One of the major advantages of capillary electrophoresis (CE) over chromatographic separation techniques for drug metabolism studies is that phase II drug metabolites, such as glucuronide and sulfate conjugates, may be analysed directly and simultaneously with the parent drug [1]. For forensic purposes the determination of long lived conjugates in urine could be advantageous. The high acidity and polarity of these compounds causes frontal elution in reversed-phase HPLC systems and complicates analysis in the gas phase. Drug glucuronides may be derivatised after extraction [2] to reduce polarity but the derivatisation procedure is complicated and time consuming. Hydrolysis procedures during sample preparation are unavoidable if techniques such as HPLC and GC–MS are used for drug analysis in biological samples [3,4]. Conjugates may be cleaved by acid, base or enzyme-catalysed hydrolysis with the latter being the method of choice for its applicability to both ester and ether conjugates. Enzyme hydrolysis is also preferable for acid/base or heat labile substrates such as morphine [5].

The optimisation of hydrolysis procedures has obvious benefits. Various authors have shown CE to be a convenient method for studying enzyme catalysed reactions. Chloramphenicol transferase activity has been studied using an off-line method [6]. Reported on-line approaches to study of enzyme activity using CE include the monitoring of the rapid

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PII S0021-9673(96)00941-7

deaminase-catalysed deamination of adenosine in a capillary using multi-point detection [7] and the Michaelis–Menten analysis of immobilised enzymes by affinity CE [8]. In addition, because CE uses so little sample it is possible to make repeated measurements of such reactions on-line.

In this paper we present results from on-line monitoring of the hydrolysis of glucuronide and sulfate conjugated substrates under varied reaction conditions catalysed by proprietary β-glucuronidase enzyme preparations. Reactions were carried out directly in the autosampler vials and substrate decrease was monitored by sequential micellar electrokinetic capillary chromatography (MECC) runs.

2. Experimental

2.1. Materials

Morphine (M), morphine-3-β-D-glucuronide (M3G), morphine-6-β-D-glucuronide (M6G) and α-napthyl sulfate (NS), 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 and sodium dodecyl sulfate (SDS) were purchased from Sigma.

Blank urine was collected from thoroughbred racehorses stabled at the Horseracing Forensic Laboratory. Standard substrate solutions (1 mg ml^{-1}) were prepared in deionised water and stored at 4°C until used. β-Glucuronidase enzymes from *Helix pomatia* (crude solution and GPC purified lyophilised powder), from *Helix aspersa*, from *Patella vulgata*, from abalone entrails and from *Escherichia coli* were obtained from Sigma.

2.2. MECC

The CE apparatus consisted of a ^{3D}CE system with diode array detector controlled by Chemstation software running on a Vectra 486/66xm computer (Hewlett-Packard, Stockport, UK). A monitoring wavelength of 195 nm (2 nm bandwidth) was used with reference wavelength of 450 nm (80 nm bandwidth). Injection was by pressure (50 mbar) applied to the sample vial for 3 s and run buffer for 1 s prior to application of voltage (23 kV).

Separations were performed in Polymicro Technologies fused-silica capillaries (Composite Metal Services, Hallow, UK). Capillary dimensions were 48.5 cm (40 cm to detector)×50 μm I.D.×375 μm O.D. 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. Run buffer was borax (17.5 m*M*, pH 9.3), SDS (70 m*M*). Capillaries were thermostatted at 25°C. Between runs the capillary was flushed with run buffer for 4 min although this was reduced to 1 min for the study of faster enzyme reactions.

2.3. Reaction monitoring

Citrate buffer (20 m*M*, pH 4.1–6.2), spiked with the standard substrates at known concentrations, was placed in a polypropylene vial and incubated in the CE system autosampler which was heated using a recirculating water bath (Radiometer, Crawley, UK). A 1 h temperature equilibration step was performed prior to adding enzyme. Lyophilised β-glucuronidase enzymes were dissolved in deionised water (103 000 Sigma units ml^{-1}) and stored at 4°C prior to use. (One Sigma unit liberates 1 μg phenolphthalein from phenolphthalein glucuronide per hour at 37°C and at the optimum pH. Since the purpose of this work was to evaluate MECC, not to study enzyme kinetics in detail, the enzyme activity was not separately checked using phenolphthalein glucuronide. The amount received from the supplier was taken at face value). After enzyme addition, the autosampler vial was capped and the reaction was monitored by sequential MECC runs. The corrected peak areas (peak area/migration time) were measured using Chemstation software. The natural logarithm of substrate corrected peak area (y) was plotted against reaction time point (x) and the rate constant (k) and half life of the reaction ($t_{1/2}$) calculated from the slope of the resultant line.

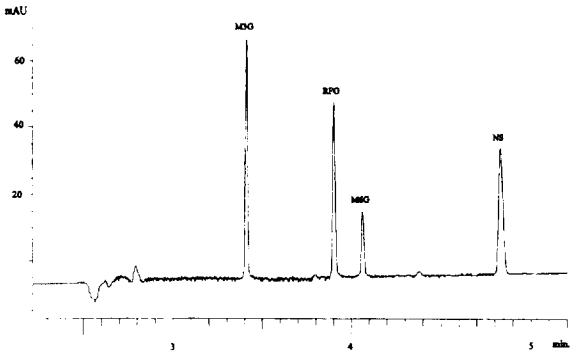

Fig. 1. MECC chromatogram (195 nm) of a standard substrate mixture (M3G, RFG, NS, 50 μg ml^{-1}, M6G, 20 μg ml^{-1}). MECC performed in fused-silica capillary (48.5 cm×50 μm I.D.). Buffer=borax (17.5 m*M*, pH 9.3); SDS (70 m*M*). Injection by pressure (150 mbar). Field=470 V cm^{-1}. Temperature=25°C.

3. Results and discussion

MECC using a borax buffer modified with SDS provided a rapid and well resolved separation of the model substrates (Fig. 1). Reproducibility and linearity of the MECC method were excellent [1]. Using the heated autosampler tray the degradation of drug glucuronide and sulfate hydrolysis by β-glucuronidase preparations was readily monitored using the above MECC method. MECC allows the simultaneous separation of charged and neutral analytes. Reactions could be monitored over long periods of time, despite the high enzyme concentrations, without experiencing reproducibility problems which may be encountered with alternative CE modes such as capillary zone electrophoresis (CZE) or capillary gel electrophoresis (CGE).

Under certain conditions some enzyme reactions obey first order kinetics [7,9,10]. This was observed for most of the β-glucuronidase reactions we studied. Five to ten injections of the enzyme-substrate mixtures were made and plots of ln (substrate area/migration time) versus reaction time were found to be linear. The rate constant, k, was estimated from the slopes of the lines and the substrate half lives, $t_{1/2}$, calculated using the equation $t_{1/2}=(\ln 2)/k$.

The effect of enzyme concentration on the hydrolysis of M3G using β-glucuronidase from *Helix pomatia* (crude solution) was investigated. Due to limitations in sensitivity the initial substrate concentration was higher than would generally be expected in a post-administration urine sample. The dependence of reaction rate on enzyme concentration relative to substrate concentration can clearly be seen (Table 1). The investigation of higher enzyme concentrations was not possible due to baseline interference by UV absorbing components in the *Helix pomatia* crude solution. Such interference prevented accurate measurement of product increase with all enzymes and the MECC assay was therefore limited to monitoring substrate decay (Fig. 2).

Six β-glucuronidase enzyme preparations from different sources were studied using the on-line MECC assay (Table 2). The substrate half lives were found to be enzyme-specific. Some β-glucuronidase preparations derived from molluscs are known to also contain sulfatase and phosphatase activity [11]. β-Glucuronidase from *Helix pomatia* demonstrated the greatest sulfatase activity although the activity towards M3G was greatest for β-glucuronidase from *Patella vulgata*. The bacterial β-glucuronidase from *E. coli* demonstrated no sulfatase activity towards NS under the applied reaction conditions.

RFG was hydrolysed rapidly by all of the enzymes under the conditions described for Table 2. The reactions proceeded to completion in less than 10 min. Since the MECC run time was typically 6 min only one data point was obtained and thus a meaningful rate measurement could not be made using

Table 1
Hydrolysis data for M3G using three different concentrations of β-glucuronidase from *Helix pomatia* (crude solution)

Enzyme concentration (Sigma Units ml^{-1})	k (min^{-1})	$t_{1/2}$ (min)	r^2	Standard error	Residual S.D.	n
258	0.0032	216	0.997	0.008	0.707	4
2575	0.0153	45.5	0.974	0.134	0.116	5
5150	0.0285	24.5	0.991	0.059	0.054	7

Hydrolysis carried out at 37°C with initial substrate concentration of 50 μg ml^{-1}. Reactions monitored for 1.5 h. MECC performed in fused-silica capillary [48.5 cm (40 cm to detector)×50 μm I.D.]. Buffer=borax (17.5 m*M*, pH 9.3); SDS (70 m*M*). Injection by pressure (150 mbar). Field=470 V cm^{-1}. Detection by UV (195 nm). Temperature=25°C.

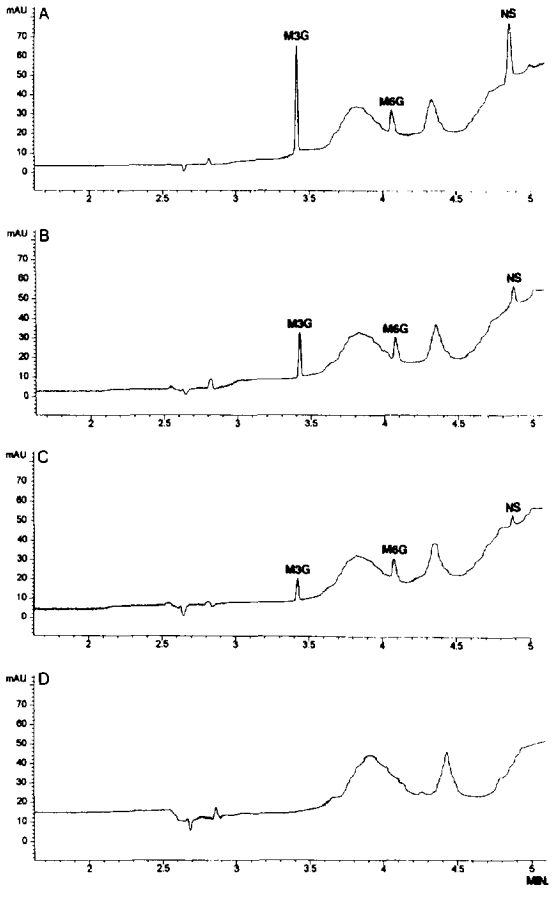

Fig. 2. MECC Chromatograms (195 nm) recorded during the hydrolysis of a substrate mixture (initial concentration M3G, RFG, NS=50 μg ml^{-1}; M6G=20 μg ml^{-1}) in citrate buffer (pH 5.6, 20 m*M*) using β-glucuronidase from *Helix pomatia* (GPC purified, 5150 Sigma units ml^{-1}) at 37°C. A=after 7.1 min hydrolysis, B=after 32.2 min hydrolysis, C=after 58.5 min hydrolysis and D=after 24 h hydrolysis. MECC conditions as for Fig. 1.

this procedure. However, it is the slower reactions that are of most interest in the optimisation of hydrolysis procedures for sample preparation.

M6G was not readily hydrolysed by any of the enzymes within the 1.5 h reaction time period. The greatest rate of hydrolysis of M6G was achieved using β-glucuronidase from *E. coli*. This finding is in agreement with those of Delbeke and Debackere [4]. M6G was not detected in an equine urine sample following administration of M. M3G was detected as

Table 2
Substrate half lives ($t_{1/2}$) during hydrolysis with β-glucuronidase preparations from different sources

Enzyme source	Substrate, $t_{1/2}$ (min)		
	M3G	M6G	NS
Helix pomatia (crude solution)	25.1	134	15.2
Helix pomatia (GPC purified)	22.4	104	13.0
Patella vulgata	16.0	165	–
Helix aspersa	21.1	134	208
Escherichia coli	32.0	96.0	–
Abalone entrails	37.0	121	134

Substrates (50 μg ml^{-1}) in sodium citrate buffer [pH 5.6 (pH 6.8 for *E. coli*), 20 m*M*]. Enzymes (5150 Sigma units ml^{-1}) added after a 1 h temperature equilibration at 37°C. Sample temperature maintained at 37°C during the course of the reaction. MECC conditions as for Table 1. Reactions monitored for 1.5 h at 6–10 min intervals.

the major metabolite of M using MECC with significant amounts of unchanged M remaining [1].

A detailed optimisation using *Helix pomatia* β-glucuronidase (crude solution) was made. Inter-assay precision (n=3) for the hydrolysis of M3G under the reaction conditions given for Table 2 was 6.1%. The effects of pH and incubation temperature on the half life of M3G are presented in Table 3. The rate increased with the incubation temperature. The observed change in reaction rate reduced as the reaction temperature was increased. It was not possible to determine the maximum working temperature of the enzyme as the manufacturers recommended maximum temperature for the autosampler was 50°C. It is expected that higher temperatures would denature the enzyme resulting in a reduced reaction rate.

To evaluate matrix effects equine urine was adjusted to pH 5.6, spiked with M3G then diluted 1:1, 1:4 and 1:10 (v/v) with water prior to adding enzyme and monitoring the reaction. We saw no significant matrix effect although β-glucuronidase catalysed reactions have been reported [4] to suffer interference from the urine matrix.

Enzyme reactions are usually monitored by addition of a stopping reagent at various time points prior to analysis. In MECC there is no need to remove proteins (enzymes) prior to analysis. A major advantage of the on-line MECC approach demonstrated here is that it is not necessary to add stopping reagents since the time cycle between injections is

Table 3
The effect of reaction pH and temperature on the half life ($t_{1/2}$) during hydrolysis of M3G with β-glucuronidase from *Helix pomatia* (crude solution)

pH	$t_{1/2}$ (min), temperature=37°C	Temperature (°C)	$t_{1/2}$ (min), pH=5.6
4.1	64.8	23.5	86.8
4.5	43.0	37	26.2
5.1	29.0	40	24.8
5.6	26.2	45	22.1
6.2	47.4	54	16.2

Substrates (50 μg ml^{-1}) in sodium citrate buffer (20 m*M*). β-glucuronidase from *Helix pomatia* (crude solution, 5150 Sigma units ml^{-1}) added after a 1 h temperature equilibration step. Sample temperature maintained during the course of the reaction. MECC conditions as for Table 1. Reactions monitored for up to 3 h at 6–20 min intervals.

constant. We consider it unlikely that reactions would proceed in the MECC run buffer. Changes in reaction rate will also be evident as the reactions may be monitored throughout the course of the reaction.

The rate of reaction which can be measured by this on-line approach is limited by the number of runs which can be made within the time taken to hydrolyse the substrate. The run time of the MECC procedure used in hydrolysis measurements was 6 min, including a 1 min flush cycle. The speed of the MECC method could be increased, to allow faster reactions to be monitored, using a shorter narrower capillary and higher voltage although Joule heating effects would be a limitation.

4. Conclusion

A simple and rapid method for monitoring hydrolysis reactions catalysed by β-glucuronidase enzymes using MECC has been demonstrated. The procedure has been valuable for optimising time consuming sample preparation procedures for drug analysis and could be applied to a variety of biochemical reactions.

Acknowledgments

The authors would like to thank Dr Angus Nedderman (Synthetic Chemist, Horseracing Forensic Laboratory) for Synthesis of RFG and Mr Brian Heslop (Head Groom, Horseracing Forensic laboratory) for collection of blank equine urine.

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